Molecular Basis of CYP2B2 Induction

by

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Being a thesis presented in accordance with the regulations governing the award of the degree of Doctor of Philosophy at the University of Nottingham

October 1999

ABSTRACT

Many structurally unrelated chemicals can induce members of the cytochrome P450 superfamily with phenobarbital (PB) being a typical example. PB induces CYP2B1/2, which are most highly expressed in the liver. Their mechanism of activation has not yet been elucidated, with advances hampered by the absence of a suitable cell culture system to mimic the *in vivo* PB-mediated induction.

During this thesis a primary rat hepatocyte culture system has been developed which is highly responsive to PB at both RNA and protein levels. A sensitive and specific RNAse protection assay (RPA) has been used to demonstrate that CYP2B2 mRNA is highly inducible *in vitro* by PB. This response occurs in a time and dose-dependent manner. The use of RPA and Western blotting has demonstrated that this primary rat hepatocyte culture system supports the induction of CYP2B2 mRNA and protein levels by PB.

Sequencing -1.4kb of the 5' flanking region of the CYP2B2 gene identified genomic regulatory elements and highlighted the location of the phenobarbital response element (PBRE). The PBRE was sub-cloned into various reporter constructs and transfection technology was used to determine its PB-mediated induction.

A comparative study of the constructs generated in this thesis to that of a construct provided by Anderson's group (Trottier *et al.*, 1995) was undertaken and no differences were found in their PB-responsiveness. The Anderson construct containing the PBRE was shown here to confer a 3.3-fold PB-mediated induction of the CYP2B2 gene by CAT reporter assays. This induction was shown to be both dose and time dependent. The induction is lower than that obtained by other workers due primarily to assay conditions which were not yet optimal. However, the effects of androstane on the constitutively active receptor (CAR) may also play a role in the small inductive response of the phenobarbital response element to PB.

Acknowledgment

I would like to acknowledge Dr. David Bell, my supervisor, for his guidance and irrepressible enthusiasm during this project, his recent cyberscience thesis input (with the help of Britney Spears) was also much appreciated. Special thanks is also due to my industrial supervisor Dr. Maurice Dickins of Glaxo-Wellcome for his invaluable scientific input and support.

I must also acknowledge Dr. Simon (its fixed now) Tomlinson for his advice throughout and of course for keeping OS2 going. I would like to thank all other present members of the Bell research group, especially my shotgun on the hepatocyte preps Munim (Glamour Kid) Choudhury "MumRAH!!!!", Brett (NetBoy-Rottweiler) Jefferies, Wing Commander Sonny (Spawnmeister) Chahal, Chris (the player) Mee, Paul (Captain Cartoon) Clarke and Fan (96-preps) Ming Qi. However I also want to especially mention some of the 'ole school' members, i.e. Alex (Sorted for life) Bell, Yee (you know you wouldn't mind) Heng, Sharon (the story so far is) Kuo and Lousie (where's she gone) Oram. Declan (I drank so much this weekend) Brady deserves a special thanks for drinking so much at weekends and putting his labcoat on occasionally.

Ecnarelot boys Ecnarelot !!!!

I also want to thank the members of Glaxo-Wellcome who made my time there highly useful and enjoyable. Those who deserve a personal mention include Dr. Amanda Woodroffe who supervised my practical work at Glaxo-Wellcome and managed not to strangle me, as well as Steve, Dawn and Rosemary.

I would like to thank my family and friends who have lent their support over the last couple of years and everyone else that I have forgot to mention. Cheers!!!!!!!!!

Lastly I would like to thank the most important person to me over the last four years my Missus Barbara. She has sorted my life out while I've been a PhD. student and has supported me through the good and bad experiments. Sorry I moaned so much and left you to do everything. Babs has had her life on hold for the past four years and I don't know how to thank her. Except perhaps with an apple pie and a cup of tea.

Let's go find a house!!!!!!!!!!!

I'm still sleeping

Dedication

This thesis is dedicated to Barbara, You've sacraficed so much and I love you for it.

Just a few words from the wise and the not so wise.

"If I had a pound for every luciferase experiment that I have done.

And I got dancing lessons for all the times this PhD hasn't been fun.

I'd be a millionaire

I'd be a Fred Astaire." Martin Fry of ABC fame.

"At least once a day make someone laugh.

You're a long time dead!" William B. Horley.

"We humans are inquisitive beings.

Put us in an unfamiliar environment and we will learn about it.

.....If life is a learning curve let's keep it vertical." Shaun Kent. Scrap merchant.

Abbreviations

A	Adenine
AA	Arachidonic Acid
ACO	Acyl-CoA oxidase
AhR	Arylhydrocarbon
Amp	Ampicilin antibiotic
Arnt	AhR-nuclear translocator protein
ATP	Adenosine triphosphate
bHLH	Basic helic-loop-helix
βGAL	β–Galatosidase
BSA	Bovine Serum Albumin
bp	base pair
C	Cytosine
CAR	Constitutively activated receptor
CAT	Chloramphenicol acetyl transferase
CEBP	CCAAT/enhancer binding protein
CFA	Clofibric acid

CoA	Coenzyme
CTP	Cytosine triphosphate
CYP (CYP2)	Cytochrome P450 (P450 2B subfamily)
DEHA	Di(2-ethylhexyl)adipate
DEHP	Di(2-ethyhexyl)phthalate
DEX	Dexamethasome
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
cDNA	Complementary DNA
DLRA	Dual luciferase reporter assay
DOPE	Dioleoylphosphatidylethanolamine
DOTMA	1,2-dioleyloxypropyl-3-trimethyl ammonium bromide
DMF	Dimethylformamide
DMSO	Dimethyl sulphoxide
DMEM	Dulbecco's modified Eagle's medium
DR1	Direct Repeat (of AGGTCA) spaced by 1 nucleotide
DTT	Dithiothreitol

EBSS	Earle's balanced salt solution
EDTA	Ethylene diamine tetra acetic acid (disodium salt)
EET	Epoxyeicosatetraenoic acid
EHS	Engelbreth-Holm-Swarm
EMSA	Electromobility shift assay
G	Guanine
GRE	Glucocorticoid receptor element
GTP	Guanosine triphosphate
GW	Glaxo-Wellcome
hsp90	heat shock protein 90
HNF-4	Hepatic Nuclear Factor-4
IAA	isoamyl alcohol
IPTG	Isopropyl-β-D-thiogalactopyranoside
kb	kilobase
kDa	kiloDalton
KRHB	Krebs ringer hydrocarbonate buffer
KRPB	Krebs ringer Phosphate buffer

L15	Leibovitz medium
LB	Luria-Bertani (medium)
LDH	Lactate dehydrogenase
MCP	Methylclofenapate
MFO	Mono-oxygenase system
MCM	Modified Chee's medium
NF1	Nuclear factor 1
NR	Nuclear receptor binding motifs
ONPG	o-nitrophenyl-β-D-galatopyranoside
PAGE	Polyacrylamide Gel Electrophoresis
PAH	Polycyclic aromatic hydrocarbon
PAS	Per, Arnt, Sim regulatory proteins
PB	Phenobarbital
PBRE	PB response element
PBREM	PB response enhancer module
PBS	Phosphate buffered saline
PBRU	PB response unit

PCR	Polymerase Chain Reaction
PCN	Pregnenolone-16α-carbonitrile
PLB	Passive lysis buffer
PP	Peroxisome Proliferator
PPAR	Peroxisome Proliferator Activated Receptor
PPRE	Peroxisome Proliferator Response Element
PXR	Pregnane-X-receptor
RLB	Reporter lysis buffer
RNA	Ribonucleic acid
mRNA	messenger RNA
RXR	Retinoid X receptor
SAP	Shrimp alkaline phosphatase
SDS	Sodium Dodecyl Sulphate
SRC-1	Steriod receptor co-activator-1
SRS	Substrate Recognition Sites
T	Thymine
TAE	Tris/EDTA/Glacial acetic acid buffer

TBE Tris-Boric acid-EDTA buffer

TCDD 2, 3, 7, 8-tetrachlorodibenzeno-p-dioxin

TCPOBOP 1,4-bis[2-(3,5-dichloropyridyloxy)] benzene

TBS Tris-buffered slaine

TTBS Tween-TBS

TEMED N,N,N',N',-tetramethylethylenediamine

TESS Transcription element search software

Tet Tetracyclin antibiotics

TK Thymidine kinase

TTP Thymidine triphosphate

UHP Ultra High Purity

WWW World Wide Web

WEM William's E medium

X-Gal 5-bromo-4-chloro-3-indolyl β-D-galactophranoside

XRE Xenobiotic response element

All amino acids indicated by their standard single letter or three letter-abbreviations where appropriate.

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Chapter 1 Introduction

Section 1.1 The cytochrome P450 gene superfamily

Section 1.1.1 Xenobiotic biotransformations

All forms of life are exposed to external stimuli, both of a physical and chemical nature. An organism is exposed to foreign xenobiotics every time that it eats, drinks or breathes. These xenobiotics may take the form of both naturally occurring or synthetic compounds. Some of these xenobiotics will prove beneficial to the organism and can be utilised in normal biological processes to provide energy or act as precursors in the synthesis of macromolecules. Other xenobiotics are harmful to the organism and may be chemically reactive with the basic cellular constituents such as proteins, RNA and DNA, and it is these chemical interactions which lead to the disruption of normal cellular function (Lake, 1995). These toxic chemicals are often resistant to metabolism and due to their lipophilic nature, bioaccumulate in the cells. It is this accumulation that leads to cell damage and eventual death.

The xenobiotic induction of cytochrome P450 enhances the detoxification of these compounds. Eventually the xenobiotic will be chemically altered to enhance the likelihood of excretion from the organism. These processes are collectively referred to as biotransformations and generally result in the conversion of a poorly excretable lipophilic xenobiotic to a readily excretable water-soluble compound (Caldwell and Paulson, 1983; Porter and Coon, 1991).

Drug metabolising enzymes present in organisms are responsible for the biotransformation and subsequent excretion of many xenobiotics (Gonzalez *et al.*, 1988). The major organ and primary site at which most chemical biotransformations occur is the endoplasmic reticulum of the liver (Paine, 1981). The liver is thus available to detoxify chemicals arriving though the gastointestinal tract. However, low levels of some cytochrome P450 forms are found in extrahepatic tissues such as, the lungs, kidney, skin and the gastrointestinal tract (Gonzalez and Lee, 1996).

Most of these compounds are lipophilic in nature and are thus difficult to purge from the organism. Their continual presence and accumulation may prove toxic to the organism involved. The metabolism of these xenobiotics is necessary so as to prevent accumulation. The metabolism of these compounds can sometimes lead to a more reactive compound than the parent compound being generated, or the compound can be detoxified and excreted from the system.

To prevent any harmful accumulation of toxic substances, organisms have evolved a complex multi-enzyme defence mechanism to eliminate foreign/toxic chemicals. These drug metabolising enzymes are comprised of two types, phase I (cytochrome P450s or mixed function oxidases), and phase II enzymes (UDP-glucuronosyltransferases and sulphotransferases).

Phase I reactions are catalysed predominately by a superfamily of structurally related haem protein mono-oxygenases (Lu and West 1980). These haemproteins are located in the membrane of the endoplasmic reticulum and are known collectively as the cytochrome P450s. Phase I reactions are important in the hydrolysis, oxidation, peroxidation and reduction of numerous endogenous compounds such as bile acids, biogenic amines, fatty acids, leukotrienes, prostaglandins and steroids (Gonzalez, 1990; Guengerich, 1991). Cytochrome P450s have a major role in the detoxification or inactivation of biologically active compounds to aid excretion from the body.

Phase I enzymes are responsible for adding functional groups to xenobiotic molecules by introducing [O]; leading to the availability of polar functional groups such as, (-OH, -SH, -NH_2, or -COOH) in the molecule undergoing biotransformation. This initial stage converts the foreign molecule into a derivative that is slightly more hydrophilic and allows further metabolism to occur via phase II reactions. Phase II reactions are biosynthetic reactions involving the conjugation of an endogenous group of molecules such as glucuronic acid, amino acids, sulphates or glutathione to the phase I derived molecule. This introduction of an endogenous moiety usually confers increased water solubility and the ability to undergo yet further metabolism to

promote excretion (Gibson and Skett, 1994). The action of the cytochrome P450 enzymes upon xenobiotics sometimes produces detrimental intermediates, as is the case in the metabolism of certain carcinogens and mutagens. There are also beneficial intermediates that arise from such action, as is the case of certain prodrugs like the anticancer agent cyclophosphamide.

Section 1.1.2 Isolation of cytochrome P450

The history of the cytochrome P450s began back in the 1950s. A cellular pigment in rat and pig microsomes was discovered to give an unusual red-shifted visible absorption maximum at about 450nm when complexed with carbon monoxide (Klingenberg, 1958; Garfinkel, 1958). This absorption maximum was quite different from that exhibited by typical haemproteins such as myoglobin (435nm). Its unique 450nm-absorption spectrum was how cytochrome P450 (CYP450) obtained its name. It was further characterised and purified by Omura and Sato in 1964 (Omura and Sato, 1964 a, b) and was shown to be a b-type cytochrome containing an iron-protoporphyrin IX as the prosthetic group. In 1965 cytochrome P450 was established as being the terminal oxidase of the liver microsomal drug-metabolising enzyme system (Cooper *et al.,* 1965).

Over the last 20-30 years multiple forms of cytochrome P450 enzymes have been analysed and identified by such techniques as protein purification and monoclonal antibody analysis. There are many distinct forms of these cytochrome P450 enzymes present in all organisms and each cytochrome P450 enzyme exhibits very distinct but broad substrate specificities. Individual forms of the enzyme have a role in a variety of tightly regulated pathways, such as developmental regulation and modulation by steroid or polypeptide hormones. Others are important in the metabolism of foreign hydrophobic compounds which may include dietary components, xenobiotics, therapeutic drugs (e.g. phenobarbital, PB) or chemical carcinogens (Nebert and Gonzalez, 1987). It was the isolation and characterisation of the various components of the cytochrome P450 system which led to the current understanding of the mechanism of action of

the cytochrome P450 enzymes.

Section 1.1.3 Mechanism of action

The cytochrome P450 system is composed of two enzymes i.e. the haem containing enzyme cytochrome P450 and NADPH-cytochrome P450 reductase. These enzymes are present in all organs, but predominantly in the liver, embedded in the phospholipid matrix of the endoplasmic reticulum and other organelles such as the mitochondria. It is the phospholipid layer that facilitates the interaction between the two enzymes. Cytochrome P450 forms the main component of the microsomal mixed function mono-oxygenase system (MFO). There is an extensive array of reactions catalysed by the MFO. These include not only the hydroxylation of aliphatic, aromatic and cyclic compounds, but the oxidation, N-/S-/O-dealkylation, sulphoxidation and deamination of many other compounds. These reactions start with the introduction of a single atom of molecular oxygen derived from O_2, into the substrate, with the simultaneous reduction of the accompanying oxygen atom to water. The generic equation for the cytochrome P450 catalysed mixed function oxidase reaction is shown below:

$$RH + O_2 + H^+ + NADPH \xrightleftharpoons{\text{cytochrome P450}} ROH + H_2O + NADP^+$$

where RH represents the substrate and ROH represents the oxidised product.

This equation suggests a simple mechanism. However,this is not the case. The reaction is best represented as a cyclic reaction showing the interactions that occur between cytochrome P450 and the NADPH-cytochrome P450 reductase (Figure 1.1) (Porter and Coon, 1991; Coon *et al.*, 1992; Guengerich *et al.*, 1998; Schenkman, 1981). The reaction begins when the substrate binds to the active site of the cytochrome P450 close to the haem bound region of the molecule (Figure 1.1, step 1) and involves the reduction of molecular oxygen by the stepwise addition of two electrons. These electrons are donated to cytochrome P450 by the reducing co-factor NADPH.

NADPH is a two-electron donor but cytochrome P450 can only accept one electron at a time. This necessitates the presence of an accessory enzyme. This accessory enzyme is the flavoprotein NADPH-cytochrome P450 reductase which possesses two flavin prosthetic groups. This enables NADPH-cytochrome P450 reductase to accept two electrons from NADPH and simultaneously transfer one electron to two different cytochrome P450 molecules. The ratio of NADPH-cytochrome P450 reductase to cytochrome P450 molecules present in liver microsomes is about 20:1. It is during the first electron donation that the iron atom present in the cytochrome P450 is reduced to the ferrous state (Fe^{2+}) (Figure 1.1, Step 2) and molecular oxygen binds to the cytochrome P450 complex (Figure 1.1, Step 3). A second electron is then donated to the cytochrome P450 from NADPH-cytochrome P450 reductase (Figure 1.1, Step 4). This is followed by electron rearrangement, insertion of an oxygen atom and product release with the generation of water or hydrogen peroxide (Figure 1.1, Step 5-8).

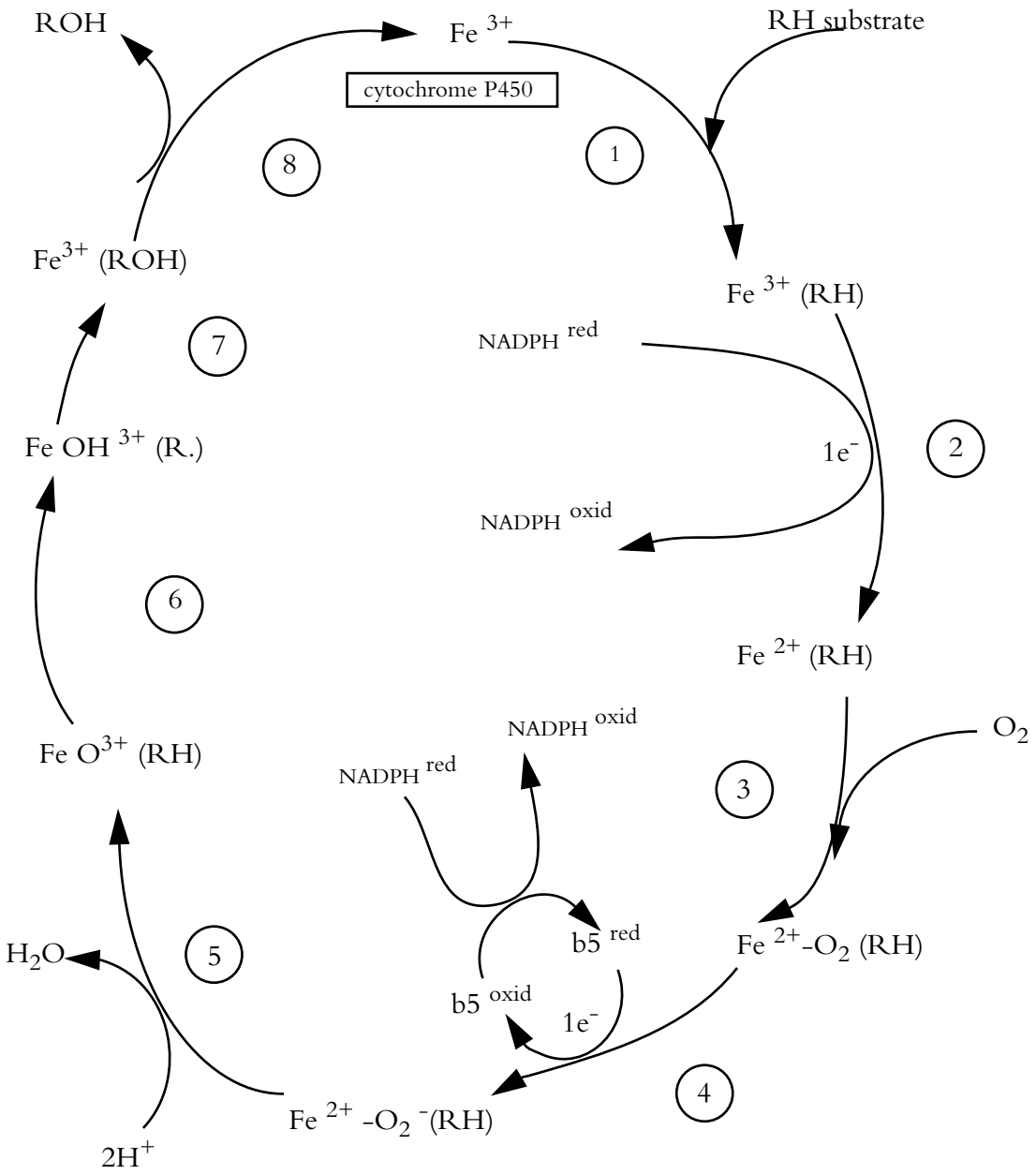

Figure 1.1 Figure 1.1 Catalytic cycle of the cytochrome P450 reactions. Step 1. Binding of substrate (RH) to Cytochrome P450 (Fe^{3+}). Step 2. Iron atom present in cytochrome P450 is reduced to the ferrous state by the addition of an electron donated by NADPH-cytochrome P450 reductase. Step 3. Molecular oxygen binds to the reduced cytochrome P450 complex. Step 4. A second electron is donated to the cytochrome P450 complex by NADPH-cytochrome P450 reductase. Step 5-8. Electron rearrangement, within the cytochrome P450 with the insertion of oxygen into the substrate and the production of the product (ROH) and water. NADPH = NADPH cytochrome-P450 reductase. b5 = cytochrome b5 reductase. Diagram adapted from Porter and Coon, 1992.

Figure 1.1 also shows the presence of another haemprotein at step 4, NADH-cytochrome b5 reductase (cytochrome b5). Cytochrome b5 may donate electrons to the cytochrome P450 catalytic cycle using NADH as the reducing co-factor. Some cytochrome P450s (such as CYP3A4, CYP2E1) have shown an apparent dependence upon cytochrome b5 for maximal catalytic activity (Guengerich and Johnson, 1997). Cytochrome P450-like activity is not just confined to the endoplasmic reticulum. These enzymes have been shown to be active in the adrenal cortex mitochondria e.g. steroid hydroxylase-P450s. These mitochondrial P450s receive their supply of electrons from the iron sulphur ferredoxin-like protein adrenodoxin which acts as an accessory enzyme between the reductase and cytochrome P450. The CYP450 component of both the mitochondrial and microsomal cytochrome P450s is present in an insoluble membrane form. By way of contrast, the bacterial cytochrome P450s in general have no apparent membrane association and are soluble.

The cytochrome P450 enzyme activity as an oxygenase rather than an electron carrier led to the proposal for a more suitable name that encompassed the enzymes catalytic activities. Standard methods for enzyme nomenclature do not apply due to their broad catalytic activities. To date, the name cytochrome P450 is still universally accepted and a systematic nomenclature system has been devised based on structural homology (Nelson *et al.*, 1996).

Section 1.1.4 1.1.4 Nomenclature of the cytochrome P450 superfamily

Omura and Sato (1964 a, b) had originally intended the name P450 to be a temporary one until more was discovered about the properties of the enzyme. Since its discovery, P450 work has increased exponentially. This has led to certain cytochrome P450s acquiring several names from different workers e.g. the PB induced rat cytochrome P450 has been given such names as CYP2B1/2 (Nelson *et al.*, 1996), P450-b/e (Ryan *et al.*, 1979), PB-B/D (Guengerich *et al.*, 1982), PB-4/5 (Waxman *et al.*, 1982). It was only with the more recent developments in the

isolation and sequencing of P450 genes, cDNAs and proteins that a unified nomenclature system could be devised based on primary amino acid sequence alignment data (Nebert *et al.*, 1991).

This system of nomenclature is constantly being reviewed and updated (Nebert *et al.*, 1989, 1991, and Nelson *et al.*, 1993, 1996). The recommendations for the naming of cytochrome P450s include the root symbol 'CYP' ('*Cyp*' which is italicised for both the mouse and *Drosophila*) to denote cytochrome P450. This is followed by an Arabic number designating the P450 family, a letter indicating the subfamily (when two or more exist), and an Arabic number representing the individual gene. For example the nomenclature denotes rat CYP2B2 as cytochrome P450 gene number 2 of subfamily 2B. All cytochrome P450 cDNAs, mRNAs and proteins are named in the same way except they are non-italicised and are in upper case lettering. The cytochrome P450 superfamily is divided into families and subfamilies, done on the basis of amino acid sequence identity. Members within the same family usually exhibit >40% amino acid sequence identity whilst members of the same subfamily are at least >55% identical.

Cytochromes P450 are ubiquitously distributed in nature and have been sequenced and isolated in prokaryotes, unicellular eukaryotes, plants, insects, invertebrates, fish, fowl and mammals (Nelson *et al* 1996 and refs therein). To date 481 CYP450 genes and 22 pseudogenes have been reported. These genes have been described in 85 eukaryote and 20 prokaryote species. The cytochrome P450 enzymes have been divided into 74 gene families of which 14 families comprise 26 mammalian subfamilies. Families CYP1-4 contribute the greatest involvement to xenobiotic metabolism. Other CYP450s typically do not contribute to xenobiotic metabolism but rather metabolise physiological endogenous substances such as the newly identified CYP5 and 8, which are important for thromboxane and prostacyclin biosynthesis respectively. CYPs 11,17,19 and 21 catalyse hydroxylation reactions required for steroid hormone biosynthesis from cholesterol (Nelson *et al.*, 1996).

Section 1.1.5 Evolution of the cytochrome P450 superfamily

It has been estimated that the first ancestral gene has its origins over 1360 million years ago, before the divergence of the bacterial, plant and animal kingdoms (Nelson and Stobel, 1987). It has been hypothesized that the first gene duplication event in eukaryotes gave rise to the presence of cytochrome P450 in two different cellular organelles, i.e. the mitochondrion and the endoplasmic reticulum. The explosion in CYP genes (particularly the CYP2 family) has been attributed to the increase in interactions between plants and animals. *C.elegans* has 100 P450s, more than that found in rat or humans. The greatest impact came initially from the plant/animal conflict. This meant that plants evolved defence mechanisms such as phytoalexins which (in turn) forced the animals to evolve biotransformation enzymes to help detoxify these compounds (Nebert and Gonzalez, 1987; Nebert, 1994). As described previously, the earliest cytochrome P450s were not concerned with detoxification processes but were more engaged in the metabolism of endogenous substrates such as cholesterol and steroids (Soucek and Gut, 1992; Nebert and Gonzarlez, 1987). The divergence separating the metabolism of endogenous versus exogenous substrates has been estimated to have occurred about 100 million years ago. It has been suggested that these early P450s evolved to maintain cell membrane integrity through the metabolism of lipids and steroids (Gonzalez and Nebert, 1990).

The divergence of particular genes and their relationship to others can be estimated by comparing primary amino acid sequence data of all the known P450s. Using computer alignment programs in conjunction with estimated species divergence (based on fossil evidence), phylogenetic trees can be constructed. The construction of phylogenetic trees has helped to determine where the major gene divergences have occurred. An indication of the divergence from the ancestral gene of the separate CYP450s is that they occur on different chromosomes and have sequence homology of < 30%, whereas members within families which diverged later have sequence homology > 55%. It is believed that the divergence within the subfamilies is a combination of

gene duplication, gene conversion and gene mutation events (Gonzalez, 1990).

Section 1.1.6 Xenobiotic induction of cytochrome P450 genes

The ability of the drug metabolising system to alter its activity in response to various xenobiotics is a key component in the regulation of cytochrome P450 gene expression. Many P450 genes are expressed constitutively in the liver (Gonzales and Lee, 1996) and can also be stage-, tissue-, strain- and sex-specific in their expression. However, many cytochrome P450 genes are involved in xenobiotic metabolism and can be induced (in the case of CYP2B1) up to 100-fold above their constitutive levels. In the liver CYP2B2 protein is present at a low constitutive level whereas CYP2B1 is 5-10 times lower by comparison. However, when induced by PB these levels increase to 20 and >100 fold above constitutive levels respectively (Christou *et al.*, 1987; Yamazoe *et al.*, 1987). It is likely that there are multiple mechanisms involved in the regulation of a particular cytochrome P450.

Inducers can be categorised into several distinct classes, which include polycyclic hydrocarbons, barbiturates, glucocorticoids, peroxisomal proliferators and ethanol (Gonzales, 1990; Whitlock *et al.*, 1986). These xenobiotics may either induce their own metabolism, or act to increase the metabolism of other xenobiotics by the induction of a particular cytochrome P450.

Regulatory control of cytochrome P450s can be exerted at a number of different levels of gene expression. These include gene transcription, mRNA processing, mRNA stabilisation, translation and protein stabilisation (Porter and Coon, 1991; Goldfarb, 1990). Induction of the cytochrome P450 families CYP1-4 occurs predominantly at the level of transcriptional activation (Okey, 1990; Denison and Whitlock, 1995; Waxman and Azaroff, 1992) with only a few exceptions such as CYP2E1. CYP2E1 is induced by ethanol and related compounds by a post-transcriptional mechanism (Roberts *et al.*, 1995; Song *et al.*, 1989). Xenobiotics induce CYP1-4 gene expression through one of four distinct receptor-mediated mechanisms (Figure 1.2).

Specific xenobiotic receptor proteins have now been identified which display a link to the four mechanistically distinct classes of cytochrome P450 inducers (Dogra *et al.*, 1998). The mechanism by which these xenobiotics interact with these receptor proteins and how the induction of the cytochrome P450 genes occurs is under investigation.

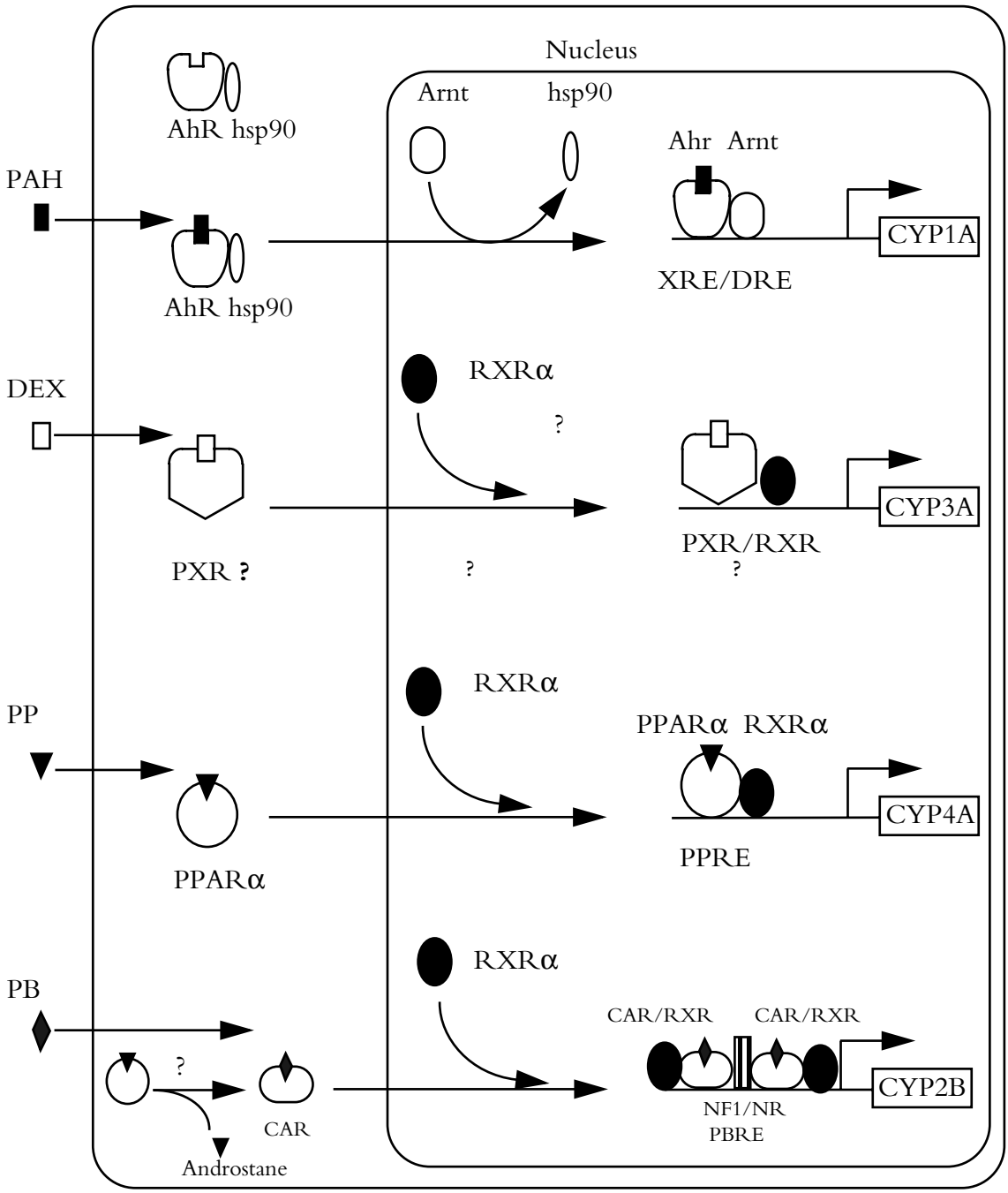

Figure 1.2 Cartoon of the four proposed models for transcriptional activation of *CYP* genes.
PAH, polycyclic aromatic hydrocarbon; DEX, dexamethasone; PP, peroxisome proliferator; PB, phenobarbital; AhR, arylhydrocarbon receptor; hsp90, heat shock protein 90; PXR, pregnane X receptor; PPARα, peroxisome proliferator-activated receptorα; CAR, constitutively activated receptor; Arnt, AhR-nuclear translocator protein; RXRα, retinoid X receptor; XRE/DRE, xenobiotic/dioxin-responsive element; PPRE, peroxisome proliferator response element; PBRE, phenobarbital response element; NR, nuclear receptor binding motifs; NF1, nuclear factor 1 binding site. (Diagram adapted from Dogra, et al., 1998)

Section 1.1.6.1 Aromatic hydrocarbon inducible cytochrome P450 genes

In the 1950s it was observed that the administration of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) resulted in the induction of a number of liver microsomal enzymes, referred to as the aryl hydrocarbon hydroxylases (AHH). AHH metabolise numerous polycyclic aromatic hydrocarbons, such as the carcinogens 3-methylcholanthrene (3-MC) and benzo(a)pyrene (Poland *et al.*, 1976). TCDD is an extremely potent inducer of CYP1A1 (30,000 times more potent than 3-MC). The receptor protein associated with the induction of CYP1A1 was shown to bind TCDD saturably, reversibly, and with high affinity and has been designated the aromatic hydrocarbon receptor (AhR) (Poland and Knutson, 1982).

The molecular mechanism regulating CYP1A1 gene expression is extremely well characterised. It begins when the ligand enters the cell cytoplasm and triggers the transformation of the AhR complex to a form which can activate the transcription of the CYP1A1/2 genes. The AhR is present in the cytoplasm bound to a dimer of hsp90 (Perdew, 1988, Perdew and Poland, 1988); the hsp90 maintains the AhR in a ligand binding conformation whilst repressing nuclear translocation and subsequent dimerisation with Ah receptor nuclear translocator (Arnt) (Pongratz *et al.*, 1992; Whitelaw *et al.*, 1995). Arnt is a nuclear protein that dimerises with liganded AhR, thereby enabling AhR to bind DNA; this leads to the subsequent shift in the distribution of AhR towards the nucleus (Pollenz *et al.*, 1991). Thus, Arnt acts as an essential heterodimeric partner for ligand-bound AhR.

The AhR ligand enters the cell cytoplasm and interacts with AhR at the ligand-binding site within the PAS region; this region is shared with the hsp90-binding site. AhR will not accept the ligand in the absence of hsp90. A conformational change occurs in AhR to a DNA-binding form once the ligand has bound. It is thought that this allows exposure of the nuclear localisation signal and subsequent nuclear translocation. Once the AhR/hsp90/ligand complex is translocated to

the nucleus an AhR/Arnt DNA binding heterodimer is formed with disassociation and recycling of hsp90. The heterodimer occurs through the bHLH and PAS domains. The AhR/Arnt heterodimer then binds to specific DNA sequences known as dioxin response elements (DRE) or xenobiotic response elements (XRE) and activates transcription. The core DRE consensus, TNGCGTG, contains one E-box half-site and one non-consensus half site. The CGTG consensus sequence is absolutely required for the inducible, AhR-dependent, Arnt-dependent protein-DNA interactions. Protein phosphorylation may be required for heterodimerisation (Berghard *et al.*, 1993), DRE-binding (Pongratz *et al.*, 1991) and transcriptional activation to occur. The chromatin structure of the enhancer and promoter regions in the CYP1A1 gene assumes a nucleosomal configuration in the uninduced state. When CYP1A1 is induced, the AhR/Arnt heterodimer binding to the DRE disrupts the organisation of the nucleosome and alters the conformation of the DNA. Once the nucleosome at the promoter region is disrupted, there follows a recruitment of general transcription factors to their cognate binding sites, followed by transcription. Unlike the xenobiotics that induce the CYP4A and CYP2B families, the xenobiotics that induce CYP1A1/2 possess very similar chemical structures, i.e. uniformly planar and aromatic.

Section 1.1.6.2 Steroid inducible cytochrome P450 genes

The steroid Pregnenolone-16α-carbonitrile (PCN) has been shown to induce cytochrome P450 enzyme activity (Okey, 1990; Gonzalez, 1988). The PCN-inducible enzymes that metabolise structurally diverse xenobiotics, including testosterone, antibiotics, dexamethasone, anti-glucocorticoids and numerous other drugs, have been designated inducers of the CYP3A family. The CYP3A genes induced by these chemicals include CYP3A4, 5, 7 (present in humans), CYP3A1, 2, 23 (present in rats) and CYP3A6 (present in rabbits). Early studies on CYP3A1 induction highlighted the paradoxical observation that both glucocorticoids (such as high levels of dexamethasone) and anti-glucocorticoids (such as PCN) could have an inductive effect (Burger *et al.,* 1992; Quattrochi *et al.,* 1995). The observation that high concentrations of dexamethasone were

required for the induction of CYP3A1 and that PCN induced CYP3A1 still further, but inhibited tyrosine aminotransferase (a classical glucocorticoid receptor-dependent gene) induction was recognised as an inconsistency in the concept that CYP3A induction was mediated via the classical glucocorticoid receptor (GR) mechanism (Schuetz and Guzelian, 1984).

Species differences in the induction of the CYP3A family have been observed (Wrighton *et al.,* 1985). In humans CYP3A5 induction has been reported to require the glucocorticoid receptor (Schuetz *et al.,* 1996), whereas rat CYP3A23 induction has been reported to occur not via the classical glucocorticoid receptor but by a novel orphan receptor, designated the pregnane X receptor (PXR) (Schuetz *et al.,* 1998). A dexamethasone responsive element was found in the CYP3A23 gene, which did not contain a GR element but contained an imperfect direct repeat DR4 motif (AGGTCA direct repeat separated by 4 bp) characteristic of the nuclear orphan receptors (Huss and Kasper, 1998). A second functional element had earlier been identified which consisted of a direct repeat of ATGAAT separated by 2 bp which was also involved in the dexamethasone response element (Huss *et al.*,1996). A PXR/retinoid X receptor (RXR) heterodimer with the dexamethasone response element present in the promoter sequence of the CYP3A23 gene was shown in transfection experiments to activate reporter gene transcription (Quattrochi *et al.*, 1998). The human homologue nuclear receptor designated hPXR (Lehmann *et al.,* 1998), or hPAR (Bertilsson *et al.,* 1998) has also been shown to heterodimerise with the RXR and apparently regulates CYP3A expression via the dexamethasone response element.

Section 1.1.6.3 Peroxisome proliferator inducible cytochrome P450 genes

Peroxisome proliferators (PP) encompass a broad spectrum of structurally dissimilar compounds, which include fibrate hypolipidaemic drugs, phthalate ester plasticizers and halogenated aromatic solvents. Exposure to PPs increases the size and number of peroxisomes present in the liver cells and leads to an increase in peroxisomal (β)-oxidation of fatty acids as well as microsomal lauric

acid (ω)-hydroxylase activity (Lock *et al.,* 1989; Johnson *et al.,* 1996; Hardwick *et al.*, 1987). It has been shown that the induction mechanism of the CYP4A enzyme precedes the β-oxidation enzymes such as acyl-CoA oxidase (Bell and Elcombe, 1991a; Milton *et al.,* 1990). The CYP4A genes that are induced by these chemicals include CYP4A1, 2, 3 (rats), and *C*yp4a10, 11, 12 (mouse) (Kimura *et al.,* 1989a, b).

A member of the nuclear receptor superfamily responsible for CYP4A induction, peroxisomal enzyme induction and hepatic PP was isolated in 1990 and designated the PPARα (peroxisome proliferator-activated receptor-α) (Issemann and Green, 1990). Experiments utilising PPARα-knockout mice showed that the PPARα was an essential factor in peroxisomal proliferation (Lee *et al.,* 1995). The possibility that PPs and fatty acids act as ligands for PPARα has recently been confirmed (Forman *et al.*, 1997; Devchand *et al.*, 1996; Keller *et al.*, 1993). Response elements present in the 5' flanking region of the rat CYP4A1 and rabbit CYP4A6 genes designated the peroxisome proliferator response elements (PPRE) have been shown to bind the heterodimer that forms between PPARα and RXR (Palmer *et al.*, 1994; Aldridge *et al.*, 1995). This ligand-induced binding of the PPARα/RXR heterodimer results in the stimulation of CYP4A transcription.

Section 1.1.6.4 Phenobarbital inducible cytochrome P450 genes

Exposure to the sedative drug PB increases the transcription of many P450 genes such as CYP2A1, 2B1/2, 2C6, and 3A1/2 as well as causing pleiotropic effects. These effects include proliferation of hepatic endoplasmic reticulum, stabilisation of microsomal proteins, cell growth, cell-cell communication, liver hypertrophy, tumour formation, glucose metabolism and haem synthesis and metabolism (Conney, 1967; Honkakoski and Negishi, 1998a). Many other genes are also induced such as epoxide hydrolase, cytochrome P450 oxidoreductase, specific forms of UDP-glucoronosyl transferase (UDPGT), glutathione S-transferase (GSTs) and aldehyde

dehydrogenase (Waxman and Azaroff, 1992; Honkakoski and Negishi, 1998a). Transcriptional activation of cytochrome P450 genes by PB occurs in a number of species including rat CYP2B1/2, CYP3A1 (Hardwick *et al.*, 1983; Gonzalez *et al.*, 1986) rabbit CYP2C1/2 (Zhao *et al.*, 1990; Leighton and Kemper, 1984), chicken CYP2H1/2 (Hahn *et al.*, 1991; Dogra *et al.*, 1999), and mouse *Cyp*2b10 (Honkakoski *et al.*, 1996; Honkakoski *et al.*, 1998b). There are also several diverse chemicals that have no obvious structural similarity to PB but which elicit parallel responses of varying intensity, which can serve as 'PB-like' inducers. These include chlordane, isosafrole, terpenes, organochlorine pesticides, 2-allyl-2-isopropylacetamide, and nonplanar polychlorinated biphenyls (Waxman and Azaroff, 1992; Honkakoski and Negishi, 1998a). This structural difference suggests that PB and PB-like inducers may bind to a common receptor.

It had been proposed that the mechanism whereby PB acted upon cytochrome P450s of different species from bacteria to birds and mammals (with the exception of fish) was highly conserved, as evidenced by the widespread occurrence of a 17 bp element (Barbie box) present in many species (He and Fulco, 1991). However, recent work has shown this not to be the case (Park and Kemper, 1996; Honkakoski *et al.*, 1996). Here, deletion of the Barbie box element did not result in an alteration in the response to PB. Although PB induces transcription of various P450s, the mechanism of induction are very different. For example, ongoing protein synthesis is required for CYP2B1/2 induction but not for CYP3A (Waxman and Azaroff, 1992). Data presented by Padmanaban's group (Bhat *et al.*, 1987) indicated that PB induction of CYP2B1/2 was blocked by cycloheximide indicating that ongoing protein synthesis is required for CYP2B induction. For the CYP3A1 and CYP2H1/2 genes, cycloheximide was shown to synergise with PB leading to a 2-19 and 7-fold superinduction respectively of these genes (Burger *et al.*, 1990; Hamilton *et al.*, 1992). Omiecinski's work (Sidhu and Omiecinski 1998) later contradicted this data in showing that *de novo* protein synthesis was not required for PB induction of CYP3A1 and CYP2B1/2 genes.

Phenobarbital response elements upstream of the CYP2B1/2 and CYP2H1 genes have been identified (Waxman and Azaroff, 1992). Relatively little is understood about the mechanism of induction of the CYP2B genes. Ligand binding studies have failed to detect a specific PB-binding protein. It is possible that PB-related compounds act indirectly by causing an accumulation of an endogenous steroid, as has been suggested by Shaw *et al.* (1993), who demonstrated that PB responsiveness was influenced by glucocorticoids.

Section 1.2 PB-inducible rat CYP2B1 and CYP2B2 genes

When rats are administered PB there is a rapid increase in CYP P450 expression, particularly CYP2B1 and 2B2 genes (Phillips *et al.*, 1983; Thomas *et al.*, 1981; Ryan *et al.*, 1982a,b). Recombinant DNA technology using genomic DNA and cloned cDNAs has enabled the determination of the coding nucleotide sequence of these two genes (Fujii-Kuriyama *et al.*, 1982; Mizukami *et al.*, 1983; Yuan *et al.*, 1983; Suwa *et al.*, 1985; Jaiswel *et al.*, 1987). CYP2B1 and CYP2B2 are closely linked on rat chromosome 1 (Rampersaud and Walz, 1987). Despite their sequence similarity, genetic cross-experiments undertaken by Rampersaud and Walz indicated that they are non-allelic (Rampersaud and Walz, 1983). CYP2B1 has at least six alleles, is 23 kb long and contains 9 exons and 8 introns. CYP2B2 has at least two alleles, is 14 kb long and has a gene structure very similar to CYP2B1. The difference in size of the two CYP2B genes lies in the length of intron 1 (12 kb in CYP2B1 and 3.2 kb in CYP2B2) (Suwa *et al.*, 1985). The nucleotide sequence in all 9 exons show very close homology between the two CYP2B genes with only 40 base-pair substitutions present in 1900 nucleotides of the exonic sequence. They are identical over the first 309 residues, differing in only 14 of the 491 amino acids present, i.e. they share 97% amino acid sequence similarity. Distribution of these differences is limited to exons 6-9, but the greatest incidence of differences occurs in exon 7 (Mizukami *et al.*, 1983).

CYP2B1 and 2B2 proteins have been shown to be immunochemically cross-reactive, although

they can be distinguished by their electrophoretic mobilities on SDS-PAGE gels (Waxman and Walsh, 1982). The corresponding mRNAs can be qualitatively monitored using RNAse protection assays (Friedberg *et al.*, 1990).

Section 1.2.1 CYP2B1 and CYP2B2 5'-flanking sequences

Sequence comparisons of the 5'-flanking regions of the CYP2B1 and CYP2B2 genes demonstrated that the 97% sequence similarity existing between the CYP2B1 and CYP2B2 coding regions extends into the promoter region as far as -2.3 kb (Shaw *et al.*, 1996; Sommer *et al.*, 1996). Beyond -2.3 kb the sequence homology between the two genes diverges significantly. The transcriptional initiation site of both genes is approximately 30 bp upstream from the ATG translation initiation site. At about 50 bp upstream from the ATG site a modified TATA box sequence, CATAAAA can be found (Mizukami *et al.*, 1983). Suwa and co-workers have suggested that a section of the 5-flanking region at about -255 bp may play a role in the regulation of gene expression, as it has the potential to form a Z-helical DNA structure (Suwa *et al.*, 1985). This section contains an alternating pyrimidine/purine sequence (CA) which is repeated several times in each gene (5 times in CYP2B1 and 19 times in CYP2B2).

Section 1.2.2 Other CYP2B genes and regulatory elements

There are at least another 6-8 genes or pseudogenes homologous to the CYP2B1/2 genes which have been identified in the rat genome (Atchison and Adesnik, 1983; Giachelli *et al.*, 1989). CYP2B3 is 77% similar to CYP2B1/2 and is expressed constitutively in the liver but it is not PB-inducible (Labbe *et al.*, 1988), whereas CYP2B8 (whose constitutive expression can only be detected by PCR) is inducible by PB by at least 6-fold (Giachelli *et al.*, 1989). The 5'-flanking sequence of CYP2B8 is only similar to CYP2B2 from the transcription start site to -77 bp (73% homology). Beyond this, i.e. from -311 to -503 bp the similarity drops to 36%. In the *Cyp*2b10 mouse gene the first exon consists of 197 bp and is 94% similar to the PB-inducible CYP2B2 gene and 82% similar to the non PB-inducible mouse *Cyp*2b9 gene. The 5'-flanking sequence

of *Cyp*2b10 showed 83% homology to CYP2B2, and in *Cyp*2b10 contained a much shorter repeat (CA) region at -303 to -300 bp (Honkakoski *et al.*, 1996).

Human forms of the CYP2B genes have also been isolated; CYP2B6 shows 73-78% amino acid sequence similarity with CYP2B1 and has been described at the cDNA level (Miles *et al.*, 1988), although it is not known whether it is PB-inducible. CYP2B6 and CYP2B7 are another two human orthologues that are expressed in the liver and lung respectively. CYP2B6 shows a 76% sequence homology with CYP2B1 and a 93% homology with CYP2B7.

The CYP2B1/2 genes have also been reported to contain many non PB-inducible regulatory elements. A putative glucocorticoid receptor element (GRE) located between -1339 and -1357 bp has been shown to confer glucocorticoid responsiveness to a reporter gene (Jaiswel *et al.*, 1990; Hoffmann *et al.*, 1992). Work carried out by Park and Kemper using transient transfections and immunological studies indicated that two regions (-82 to -67 bp and -64 to -45 bp) were found to contribute to the promoter activity of CYP2B1/2 (Park and Kemper, 1996). The element present at -45 to -64 bp in both CYP2B1 and CYP2B2 promoters binds members of the CCAAT/Enhancer Binding Protein (C/EBP) transcription factor family. Mutational studies on the -82 to -67 bp regulatory element indicated that this was essential for basal promoter activity (Park and Kemper, 1996; Luc *et al.*, 1996). An element similar to this was also found in the chicken CYP2H1 gene at -204 to -208 bp (Dogra and May, 1997; Dogra *et al.*, 1999). The mouse *Cyp*2b10 gene, has a 42 bp insert occurring at -80 bp; correcting for this insert a similar C/EBP like element present at -154 to -180 bp has been identified (Honkakoski *et al.*, 1996). Experiments carried out by this group also highlighted a response element in the *Cyp*2b10 gene present at -775 to -971 bp, which decreased its basal activity.

Section 1.2.3 Pharmacology of phenobarbital

PB was initially synthesised at the beginning of the century and was the prototype of barbiturate drugs. Its sedative/anticonvulsant properties exert their effects over prolonged time periods. It has been useful in the treatment of epilepsy due to its sedative, hypnotic effects. These effects are mediated by suppression of the polysynaptic responses, especially the mesencephalic reticular activating system. Due to their clinical importance and their various pharmacological side effects, PB and the barbiturates as a whole have been investigated quite extensively (Conney, 1967). The effects of PB, in particular, include the proliferation of smooth endoplasmic reticulum, stimulationof liver weight gain and liver tumour promotion.

Chronic administration of PB produces hepatocellular adenomas in rats and hepatocellular adenomas and carcinomas in mice. However, PB did not increase the incidence of any type of tumours in hamsters (Diwan et al., 1986). Similarly, substantial doses of PB administered to humans (even over many years) also result in no increase in liver or other cancers (Whysner *et al.*, 1996). PB is not considered to be DNA-reactive (i.e. a mutagen) although it has been found to be a liver tumour promoter when administered after various carcinogens e.g. 2-acetylamino-fluorene (Williams and Furuya, 1984).

When barbiturates are co-administered with other drugs, there may be drug-drug interactions. This can mean that the presence of a barbiturate like PB can alter the action of another drug. The drugs action may modify its pharmacodynamic interactions by altering the pharmacological action of the drug without altering its concentration in the tissue fluid. The barbiturate may affect the concentration of the other drug that reaches the site of action (pharmacokinetic interactions) or the barbiturate may have a pharmaceutical interaction where drug-drug interactions occur so that one or both drugs are inactivated.

It is the complex structural relationships and interactions of these compounds, which are the key

to elucidating their tumour-promoting activities. Mechanisms must firstly be assessed in rodents, with a view to their extrapolation to the human metabolic system.

Section 1.3 Structural organisation of the liver

The liver is the largest gland in the body and constitutes approximately 2-5% of body weight of the adult man. Hepatocytes are estimated to number 250 billion in a normal adult human liver. The liver receives blood through a dual blood supply. The portal vein carries blood that has passed through the capillary beds of the alimentary tract, spleen and pancreas, and constitutes 75% of the afferent blood volume of the liver. The hepatic artery is a branch of the celiac trunk and contains well-oxygenated blood and supplies the remaining blood to the liver.

The functional unit of the liver is the hepatic acinus. This consists of a small irregular mass of encapsulated parenchymal tissue lying between two or more terminal hepatic venules. It comprises a terminal portal vein, hepatic arteriole, bile ductule, lymph vessels and nerves. In the periphery of the hepatic acinus, the sinusoids are drained by terminal hepatic venules, which form the hepatic microcirculation units. Bile flows from the periphery towards the centre. Perfusion within the acinus is unidirectional and therefore sequential, from the acinar axis to the acinar periphery where the blood is emptied into the terminal venules. This unidirectional flow of blood within the acinus creates a microenvironment; cells within each acinus appear to be grouped into concentric zones around the axis of the acinus (arbitrarily zones 1 to 3). Zone 1 is closest to the vascular axis (periportal) and cells here are the first to receive blood and nutrients, the last to die, and the first to regenerate. Zone 3 is the furthest away (perivenous, centrilobular) and receives blood of poorer quality and appear to be less resilient to damage. A diffusion gradient appears to exist between zones 1 and 3 leading to differences in concentrations of enzymes, glucose, hormones, metabolites, oxygen and substrates.

The hepatocytes or parenchyma cells are polyhedral, and constitute approximately 60-65% of the

total number of cells present in the liver. These cells range in size from 15-30μm in diameter and occupy up to 80% of the volume of the human liver. Hepatocytes differ in size within the liver depending upon where they are situated. The smaller cells usually border perforations in the liver plate, and the larger cells are normally polyploid or located in the corners where three cell plates touch. Hepatocytes have three types of surface, those neighbouring other hepatocytes, those bordering bile canaliculi (which are essentially secretory), and those touching the perisinusoidal space (space of Disse). This last type of hepatocyte surface is covered in microvilli, which provide a large surface area to facilitate absorption from the bloodstream. The remaining 35-40% of cells are non-parenchymal and comprise endothelial, Kupffer, pit cells and lipocytes.

The three-dimensional architecture of the liver is maintained by a combination of extracellular fibroconnective skeleton (composed mostly of collagenous fibres and extracellular matrix) and cellular adhesion. Several junctional complexes are involved in cellular adhesion. These include tight junctions (zona occludens) where the cell membranes of two cells are united but not fused, thus forming an impermeable junction. Another form is the intermediate junction (zona adherens) which possess a true intercellular space. The presence of gap junctions allows communication between cells, and desmosomes (macula adherens), which are button-like points of contact, mechanically hold the cells together.

As the majority of biotransformations occur in the liver, models of drug metabolism have focused on the liver and the enzymes contained within it. The use of *in vitro* models, which mimic the liver in their response, would lead to a better understanding of the biotransformation processes in a more controlled environment.

Section 1.4 Development of a PB-responsive *in vitro* hepatocyte culture system

Isolated hepatocytes are used as either suspension cultures or as monolayer cultures when studying drug metabolism. Suspension cultures provide a more convenient and sophisticated drug metabolism model than subcellular fractions, such as microsomes. However, suspension cultures only remain viable for up to 6-8 hours before showing severe degenerative changes, visible in the rapid decline in total cytochrome P450 content. Consequently, suspension cultures have proved to be inadequate when considering long term culture studies. This problem has been partially alleviated by the use of primary hepatocyte cultures. Hepatocytes maintained under appropriate culture conditions have the advantage of remaining viable for several days. It is this ability to maintain cultures over long periods of time that allows changes in gene expression and enzyme induction to be studied.

Many groups have been unable to develop cell culture systems demonstrating responsiveness to PB. In fact, most cell lines do not express either constitutive or PB-induced levels of CYP2B1/2. Primary hepatocytes in particular demonstrate accelerated loss of microsomal cytochrome P450 content along with progressive "foetalisation" of several cellular processes (Sirca and Pitot, 1980). These alterations are also associated with the loss of PB-responsiveness. The characteristics of isolated hepatocytes alter both morphologically and biochemically relatively quickly, tending to become phenotypically unstable and eventually undergoing dedifferentiation.

Paine and co-workers have shown that there is an alteration in the expression of hepatic transcription factors and cytochrome P450 mRNAs resulting from the hepatocyte isolation procedure (Padgham *et al.*, 1993). This study showed the induction of mRNA encoding the transcription factor c-jun during hepatocyte isolation. This c-jun induction was shown to subside and a continual decline of mRNAs encoding cytochrome P450 2E1, 3A1, 3A2, 4A1 and 2C13, (as well as the major constitutively expressed 2C11) was observed. CYP1A2, 2A1/2 and 2B1/2

mRNA levels were shown to stabilise for 2-3 hours before slowly declining. The mRNAs encoding the liver-specific transcription factors C/EBPα and HNF-1 were also shown to decline after cell attachment, in a similar fashion to CYP1A1/2 and CYP2B1/2. Paine and co-workers suggest that the initial increase in the expression of c-jun followed by the decline in C/EBPα and HNF-1 are characteristic of the changes in the expression of these transcription factor mRNAs following the stimulation of hepatocyte proliferation after partial hepatectomy. Thus the hepatocyte isolation procedure appears to prompt the normally quiescent hepatocytes to enter the cell cycle and undergo dedifferentiation.

There are many publications addressing the maintenance of cellular viability and liver-specific functions in cell culture (Skett, 1994; Chenery, 1988). Some of these factors have consistently been shown to effect cytochrome P450 enzyme expression and induction *in vitro*. These include the nature and composition of the extracellular matrix present (Ben-Ze'ev *et al.*, 1988; Schuetz *et al.*, 1988), the formulation of culture medium used (Jauregui *et al.*, 1986; Lecluyse *et al.*, 1999), and the combination and concentration of various hormones and trace elements (Waxman *et al.*, 1990; Nakajima and Shimbara, 1996).

Many workers have shown that high levels of CYP2B2 induction are possible without the need for extracellular matrix components in the culture system (Bars *et al.*, 1992; Park and Kemper, 1996; Sommer *et al.*, 1996; Dogra and May, 1997). There are other workers who are unable to obtain CYP2B2 induction without the use of extracellular matrix substrata (see below).

Section 1.4.1 Extracellular matrix substrata

Matrigel™ and collagen both represent components found in the space of Disse present in the *in vivo* liver, albeit at different stages of hepatocyte differentiation (laminin is present at the feotal stage and collagen at the mature stage). It has been proposed that they are important in restoring PB-responsiveness in cultured hepatocytes (Schuetz *et al.*, 1988; Ben-Ze'ev *et al.*, 1988;

Brown *et al.*, 1995).

The attachment of primary hepatocytes onto the surface of culture flasks is one of the first hurdles that needs to be overcome when establishing long-term cultures of functional hepatocytes. Many groups have reported that the coating of tissue-culture plates with various matrix components greatly improves the efficiency of cell attachment and cell function (Ben-Ze'ev *et al.*, 1988; Dunn *et al.*, 1989; Kleinman e*t al.*, 1986). In the intact liver, cells are surrounded by a support matrix, which is composed of (amongst other factors) collagen, laminin, fibronectin, and heparin-containing and chondroitin sulfate-containing proteoglycans. The basement matrices used to coat tissue culture flasks try to mimic the conditions found *in vivo*. Many different matrix components have been assessed for there ability to enhance cell attachment (Reid and Rojkind, 1979; Bissell *et al.*, 1986). Collagen is one of the most frequently used basement matrices. Hepatocytes have been shown to survive longer on collagen-coated flasks than cells which are attached directly to plastic. In the former case, liver specific functions such as albumin synthesis and tyrosine aminotransferase activity were better maintained (Michalopoulos and Pitot, 1975). Preparations of collagen are usually obtained from calf-skin (type I) or rat-tail (type I). However, type IV collagen (which constitutes the predominant liver extracellular matrix collagen) has also been investigated (Bissell *et al.*, 1987).

Dunn *et al.* (1989) have shown that adult rat hepatocytes cultured in a collagen sandwich remained viable for at least 42 days. Here the hepatocytes exhibit a polygonal morphology more typical of *in vivo* hepatocytes, unlike the spread-out, more flattened morphology typical of hepatocytes cultured on tissue-culture plastic. This technique showed the maintenance of albumin secretion and increased hepatocyte longevity was significantly better than non-sandwiched hepatocyte cultures. Unfortunately this method could only delay dedifferentiation, not prevent it. This data tends to suggest that the matrix geometry of the cultured hepatocytes in sandwich

cultures more closely mimics that of the liver architecture when present in this conformation.

Laminin-rich matrices containing type IV collagen and heparin sulphate proteoglycan (Matrigel^{TM}) have been shown to be very effective at maintaining cytochrome P450 and liver specific activity (Ben-Ze'ev *et al.*, 1988; Schuetz *et al.*, 1988; Sidhu *et al.*, 1994). Matrigel^{TM} is a complex biomatrix derived from the Engelbreth-Holm-Swarm (EHS) sarcoma. Hepatocytes cultured using Matrigel^{TM} as the support matrix retain their polygonal morphology and are more PB-responsive, as detected by the appearance of CYP2B1/2 mRNAs and proteins (Schuetz *et al.*, 1988) and through the transcriptional activation of the CYP2B1/2 genes (Schuetz *et al.*, 1990). Brown and co-workers showed that purified laminin, a major component of Matrigel^{TM}, or two peptides (YIGSR or SIKVAV) which mimic various activities attributable to laminin, could restore PB responsiveness to hepatocytes cultured on collagen type I cultures (Brown *et al.*, 1995).

Recent reports have shown that the presence of an extracellular matrix overlay rather than a specific type is more critical for restoration of cytochrome P450 activity (Lecluyse *et al.*, 1999). These results are in agreement with findings by Sinclair *et al.* (1990) which suggested that PB induction of CYP2B1/2 cultured on either collagen or Matrigel^{TM} were a close approximation to that found *in vivo* (Sinclair *et al.*, 1990). Lecluyse *et al.* (1999) also suggested that media formulations as well as extracellular matrix conditions together influenced the ability of rat hepatocyte cultures to express CYP2B1/2 enzymes.

Section 1.4.2 Hepatocyte culture media

Various modifications to the type of culture medium used in culturing cells have been assessed for their ability to maintain differentiation in primary rat hepatocytes.

There are many commercially available media which form the basis of hepatocyte cultures such as Dulbecco's modified Eagle's medium (DMEM), Williams' E medium (WEM), modified Chee's medium (MCM) and Leibovitz medium (L15). These media all contain the essential components needed to maintain cell survival. Some media such as MCM are composed of "enriched" formulations, which contain super-physiological concentrations of certain compounds, such as amino acids that facilitate protein synthesis. Different groups have shown variations between media formulations with respect to cytochrome P450 activity. WEM was shown to be superior to DMEM and MCM in terms of the level of induction of CYP2B and in the specific content of CYP2B present in microsome preparations. (Lecluyse *et al.*, 1999). Previous to this Turner and Pitot (1989) showed that T1 medium was more effective than modified Waymouth's medium and much better than L15 in its capacity to sustain CYP2B induction. Hepatocytes cultured on collagen coated plates and maintained in serum-free, modifed Chee's medium have also been shown to exhibit long term viability and the ability to respond to PB by induction of near-physiological levels of enzymatically active CYP2B1/2 proteins and mRNA (Waxman *et al.,* 1990). It was initially thought that it was necessary to have Matrigel^{TM} present in primary hepatocyte cultures to obtain PB-inducible CYP2B expression, but reports such as that of Waxman *et al.* (1990) have shown this not to be the case. These reports only highlight the immense diversity of culture conditions which show PB-inducible CYP2B expression currently in use. However, the most successful PB-inducible hepatocyte culture systems currently employed use Matrigel^{TM} as an extracellular substrata but differ in their choice of media, Honkakoski *et al.* (1996) prefer Williams' E medium, while Trottier *et al.* (1995) prefer modified Chee's medium.

Section 1.4.3 Hepatocyte culture medium additives

The addition of certain hormones or trace elements to basic culture medium can often facilitate retention of many liver-specific functions. Of the many hormones that have been tested the two that have received the most attention are insulin and glucocorticoids. Glucocorticoids have a considerable effect on the morphology, function and survival of hepatocytes in simple media. Some workers have highlighted the possiblility of inductive effects of certain glucocorticoids such as dexamethasone upon cytochrome P450 levels (Bars *et al.*, 1989). The use of trace elements such as selenium has also been shown to be required for the induction of cytochrome P450 by PB (Nakajima and Shimbara, 1996; Newman and Guzelian, 1982; Engelmann *et al.*, 1985). It is thought that the hepatocytes in culture gradually become selenium-deficient and the introduction of selenium counter acts this phenomenon and helps to maintain the stability of the hepatocyte membranes.

Section 1.5 PB-responsive elements in PB-inducible genes

To understand the molecular basis of PB induction, it is necessary to determine the roles of both *cis* and *trans* acting factors that may interact with the CYP2B2 DNA. Given the problems entailed with cell culture, many studies have resorted to approaches based on transcription of minigene constructs of the CYP2B2 gene in whole nuclei, run-on and cell free systems. DNA-protein interactions have also been studied by the use of gel shift assays, DNA footprint assays and south-Western blot analyses (reviewed by Kemper, 1998).

Section 1.5.1 Proximal PB-responsive promoter elements

In vitro studies have targeted the 5' proximal regions of the PB-induced cytochrome P450 genes. These studies focus on the role of PB in altering the binding of nuclear proteins to DNA response elements and how these binding proteins may enhance transcriptional activation. The bacterium *Bacillus megaterium* has been shown to contain two cytochrome P450 genes, BM1 (*CYP*106) and BM3 (*CYP*102) which are inducible by PB (Fulco, 1991). The mechanism by which PB activates these genes at the molecular level has been examined extensively and several central factors have been identified (Liang *et al.*, 1998). Gel retardation studies have located a sequence known as the "Barbie box". The Barbie box is composed of a 17 bp sequence located at -318 to -302 bp in the BM1 and -243 to -227 bp in the BM3 promoter region respectively (with +1 being equivalent to the translation start site). It has been suggested that PB modulates protein bound to this sequence (He and Fulco, 1991; Liang *et al.*, 1995; Liang and Fulco, 1995) and that sequences that display a high degree of identity are present in the rat CYP2B1/2 genes. Barbie box-like sequences have been reported in multiple PB-inducible mammalian genes (rat CYP2B1/2, CYP3A2, rabbit CYP2C1). Despite the fact that Barbie box-like sequences are present in the rat CYP2B1/2 genes, it should be noted that PB treatment reduced binding of bacterial proteins to the Barbie box, whereas the reverse was observed with mammalian proteins to the same element (He and Fulco, 1991). The significance of the Barbie box is questionable as the PB inducible

response was not abolished when the Barbie box sequence was deleted or mutated *in situ* or in cultured hepatocytes (Park *et al.*, 1996; Honkakoski *et al.*, 1996). This observation suggests that BM1 PB-induction in *Bacillus megaterium* may occur via a different mechanism than that of the mammalian CYP2B genes.

Barbiturates have been shown to prevent binding of the Bm3R1 repressor protein to its operator site which consists of a 20 bp inverted repeat near the transcription site of the BM3 gene (Shaw and Fulco, 1993). Shaw and co-workers have demonstrated that BM3 was more PB-responsive than BM1 and that the PB-mediated induction in the BM1 gene was not affected by the deletion of the Barbie box (Shaw *et al.*, 1998). This data also confirmed that the Barbie box sequence was important for the negative regulation of BM1 gene expression in the absence of pentobarbital. The presence of a repressor protein in the bacterial system does have some similarity to recent work carried out on the mammalian nuclear receptor known as constitutively activated receptor (CAR). PB-like inducers activate CAR by reversing transcriptional repression of the receptor by certain endogenous steroids (see section 1.5.2).

CYP2B2 mini-genes containing a fragment of the CYP2B1/2 gene from -179 to +181 nucleotides conferred PB-responsiveness in cell-free transcription assays derived from freeze-thawed rat liver nuclei (Rangarajan and Padmanaban, 1989). Western blot analysis detected an 85 to 95 kDa protein which exhibited greater binding to nuclear extracts derived from PB treated rats. A DNase I footprint was obtained when comparing control and PB treated nuclear extracts with a Sal I-Nco I fragment of the CYP2B1/2 DNA. A 32 bp region from -56 to -88 bp was protected in this experiment (Rangarajan and Padmanaban, 1989). Work carried out by Padmanaban and colleagues showed that transcriptional activation of the -179 to +181 bp CYP2B1/2 promoter fragment targeted to the liver in a complex with asialoglycoprotein/polylysine was increased in PB-treated livers (Prabhu *et al.*, 1995). A segment of the CYP2B1/2 promoter (-69 to -98 bp)

which includes the Barbie box sequence was determined by competitive gel-shift assays to bind PB responsive proteins. UV cross-link analysis detected proteins of 42 and 39 kDa, which bound this 29 bp fragment. The evidence presented in these reports is consistent with a positive element that mediates a PB response in the -69 to -98 bp region of the CYP2B gene (Upadhya, *et al.*, 1992). A negative element (-160 to -127 bp) of the CYP2B promoter has been found by DNase I footprint and 5' deletion analysis in freeze-thaw nuclei transcription studies. Proteins of 68 and 44 kDa that bind to this region were detected using UV cross-linking (Ram *et al.*, 1995).

Studies by Prabhu *et al.*, 1995 showed by affinity chromatography that a 26 to 28 kDa protein extracted from nuclear extracts bound to the positive element present in the CYP2B promoter. Direct binding and competition studies showed that this purified protein factor also bound to the negative element present in the CYP2B promoter in the absence of PB. When this protein factor was phosphorylated, binding favoured the positive element; in the dephosphorylated form binding favoured the negative element. This indicated that regulation would involve phosphorylation of the protein factor in response to PB, with subsequent dissociation from the negative element and preferential binding to the positive element (Prabhu *et al.*, 1995).

These *in vitro* experiments may indicate the existence of common components to the mechanism of cytochrome P450 induction by barbiturates between genetically diverse organisms. However, the structure-activity relationships of barbiturates are different in the mammalian and bacterial systems, and the dose-response relationship is saturable in mammals, but not saturable to the limits of the solubility of the barbiturates in bacteria (Waxman and Azaroff, 1992).

Using DNase I footprint analysis Shephard *et al.* (1994) have shown protein binding to multiple elements in the CYP2B1/2 proximal promoter region from -150 to -368 bp. DNase I and gel-shift assays also showed that two of these elements were positive elements (-31 to -72 bp and -183 to -199 bp), binding rat liver nuclear proteins more strongly in PB-treated rats than the

corresponding controls (Shephard *et al.*, 1994). Two more elements (-178 to -368 bp and -368 to -984 bp) were highlighted as being important in regulating basal transcriptional control of the CYP2B2 gene (Shervington, 1998). However, the -178 to -368 bp region was also shown to be important in regulating the induction of the CYP2B2 gene in response to PB.

Shephard *et al.* (1994) have also described three footprints which are very similar to protein binding sites reported by other workers (Hoffmann *et al.*, 1992; Honkakoski *et al.*, 1996; Luc *et al.*, 1996; Park and Kemper, 1996; Sommer *et al.*, 1996). DNase I footprint analysis by these groups showed that the region containing the Barbie box (-73 to -89 bp) played no part in the PB-mediated regulation of the CYP2B genes. However, in some of these studies (Shephard *et al.*, 1994; Luc *et al.*, 1996; Park and Kemper, 1996) there were regions of the CYP2B promoter that possessed some hypersensitive sites which may have relevance in protein binding. Analysis of the PB-responsive mouse *Cyp*2b10 proximal promoter using *Cyp*2b10-driven chloramphenicol acetyl transferase (CAT) reporter genes constructs, revealed similar results to those outlined above (Honkakoski *et al.*, 1996). These transfection studies demonstrated the presence of a basal transcription element between -64 and -34 bp and that elimination of the Barbie box region of the *Cyp*2b10 construct did not affect the transcriptional activity of the reporter. The Barbie box sequence present in the *Cyp*2b10 and the non-responsive *Cyp*2b9 gene is disrupted by a 42 bp insertion. This insertion contains a 4 bp repeat at the beginning and end which means that the Barbie box is largely conserved at the 5' end of the insertion. DNase I protection of heparin-agarose-enriched nuclear extracts revealed footprints at -45 to -64 bp, -154 to -180 bp, and -215 to -235 bp, which are in keeping with Shephard's work. No difference in binding was observed in these assays between the control and PB-induced mice. The regions resembling the Barbie box sequence showed no binding of nuclear proteins and hence no protection in any of the samples. Mutations of the Barbie box sequence present in HepG2 cells transfected with the proximal regions of the rat CYP2B genes did not reduce transcriptional activation of the

reporters. Again no difference in binding between proteins from control and PB treated animals was evident (Park and Kemper, 1996; Park *et al.*, 1996).

Section 1.5.2 Distal PB-responsive promoter elements

The controversy surrounding studies concerning the proximal elements is not seen with the distal elements of the 5' flanking regions of the CYP2B2 gene. Here, there is a general consensus upon the elements concerned with PB induction. The first evidence for the presence of PB-responsive distal elements came from studies on the transcriptional regulation of the chicken CYP2H1 gene. These studies demonstrated the presence of three strongly PB-induced CYP mRNAs of 3.5, 2.5, and 2.2 kb. The 3.5 kb mRNA was shown to be the result of enhanced transcription of the CYP2H1 gene. Chick embryo primary hepatocytes were transfected with a CYP2H1-CAT construct containing -8.9 kb of the 5' flanking region of the CYP2H1 gene, which gave a two-fold induction when exposed to PB. Deletion analysis showed that the PB response was diminished when the 5' flanking region was reduced from -4.7 to -1.1 kb. A 4.8 kb section (-5.9 to -1.1 kb) of the 5' flanking region of the CYP2H1 gene was able to confer PB-responsiveness upon a heterologous SV40 promoter. This PB-responsiveness was found to give an 8 to 14 fold induction depending upon the position of the fragment. The orientation of the fragment relative to the promoter did not affect the level of induction obtained, indicating that the PB-responsive domain may posses the properties of an enhancer (Hahn *et al.*, 1991; Dogra *et al.*, 1999). Hahn *et al.* (1991) also showed that this 4.8 kb domain when fused to its own proximal promoter gave a weak inductive response when transfected into chick embryo hepatocytes. This was thought by May and co-workers to be due to the high basal expression of the CYP2H1 proximal promoter sequence present in the *in vitro* system, compared to the endogenous *in vivo* CYP2H1 expression (Dogra and May, 1997).

Transgenic technology provides another means of studying *in vivo* responses. Transgenic mice

incorporating a rat CYP2B2 transgene containing 800 bp of 5' flanking region (including the proximal elements discussed previously) have these transgenes expressed constitutively and at high levels, although they were not shown to be PB-inducible. Transgenes containing 19 kb the 5' flanking region of the CYP2B2 gene were shown to be PB-responsive indicating that sequences far upstream (in excess of -800 bp) are necessary for PB induction (Ramsden *et al.*, 1993). These two transgenic lines were showed to display differences in PB induction and tissue selective expression profiles. In the -800 bp construct, expression was not increased with PB treatment. Expression of the construct was found to be highest in the kidney (twice that of the liver) and liver. In the 19 kb construct, expression was shown to be PB-inducible and restricted to the liver. Later work carried out by Ramsden *et al.* (1999) in transgenic mice, showed that sequences between -2.5 and -17 kb of the 5' flanking sequence of the rat CYP2B2 gene were important in PB-induction (Ramsden *et al.*, 1999). The -2271 to -2286 bp 5' flanking region of the rat CYP2B1/2 gene was footprinted with *in vitro* DNase I methods and showed a protected pattern for both PB and control-treated rats centred around a NF-1 binding motif. Using immunoblotting and electromobility shift assays this NF-1 site was shown to interact strongly with a site at the centre of the PB response unit (PBRU discussed later) of CYP2B2. The importance of this NF-1 element was examined by introducing mutations in the NF-1 sequence, which had the effect of ablating this binding interaction with the CYP2B2 sequence.

Recent advances in the development of primary hepatocyte culture conditions as described in Section 1.4 and the application of transient transfection technology has lead to a greater understanding of the PB regulation of CYP2B genes. A similar strategy to that of Hahn *et al.* (1991) was carried out by Trottier *et al.* (1995) on primary rat hepatocytes using transfection protocols. These authors showed by Northern blotting techniques that endogenous induction of CYP2B1/2 genes by PB was detectable in primary cultures (Trottier *et al.*, 1995). Transfection of the primary rat hepatocytes with 4.5 kb of the 5'flanking region of the CYP2B2 gene fused to a CAT

reporter gave a 5-fold induction after PB treatment. Deletion analysis of this 5' flanking area showed that deletions to -2.5 kb still gave a PB response which was abolished when further deleted to -2.0 kb. Further deletion analysis showed that an internal deletion of 825 bp in the region -2.5 to -1.7 kb also eliminated the PB response. However constructs with deleted sequence from -0.2 to -1.7 kb or sections -1.6 to -2.1 kb and -2.3 to -4.5 kb showed only a slight decrease in PB induction (3-4-fold). A 163 bp Sau3AI fragment from -2318 to -2155 bp coupled to -1.7 kb of the 5'flanking sequence was still able to confer PB-inducibility (3.5-fold). This 163 bp sequence was designated the PB response element (PBRE). Tandem repeats of this 163 bp area were found to double the fold induction measured. As with the CYP2H1 PBRU study, this PBRE was able to confer PB-responsiveness on a heterologous thymidine kinase (TK) promoter with approximately 3 to 9-fold induction being measured depending upon the position of the fragment. The interaction of a NF-1 site present within the PBRE was also highlighted using electromobility shift assays (EMSA), demonstrating that sequence-specific recognition sites for proteins other than NF-1 were also present.

The recently isolated and sequenced mouse PB-inducible *Cyp*2b10 gene promoter was shown to be inducible in a liver-specific manner using Northern blot analysis (Honkakoski *et al.*, 1996). The 1405 bp *Cyp*2b10 promoter sequence was shown to be 83% identical to the rat CYP2B2 gene. A primary mouse hepatocyte culture system was developed which supported endogenous *Cyp*2b10 mRNA induction by PB. This culture system also supported a PB-inducible *Cyp*2b10-driven CAT reporter gene. The findings of this paper and a more recent publication (Honkakoski and Negishi, 1997) showed that successive 5' deletion of the *Cyp*2b10 promoter allowed the determination of a region that was PB-responsive. Constructs containing either -4.3 or -1.4 kb of the 5' flanking sequence gave a 2 to 3-fold induction. Deletion to -0.7 kb abolished the PB-induction and increased the basal CAT activity 10-fold. Another construct containing -2.4 kb of the 5' flanking sequence was shown to be 7.6-fold more inducible by PB. The

sequence between -2.397 to -1.850 kb which mediates PB-induction was found to contain sequence which was 91% similar to the 163 bp region documented by Trottier *et al.* (1995). DNase I studies using the 177 bp (-2426 to -2250 bp) fragment of the *Cyp*2b10 DNA identified three weakly protected and three strongly protected nuclear protein binding regions. None of these regions showed any difference in binding between PB or control treatments.

Deletion analysis of these six DNA regions (designated pA, pB, pB', pC, pD, pE) indicated that regions pC and pB' have a major role in determining PB-inducibility. Fragments pB and pD were shown to contribute to the *Cyp*2b10 enhancer activity by modulating the basal and PB-induced activity levels. These elements involved in mediating PB-induction covered a region of 132 bp (-2397 to -2265 bp) of the *Cyp*2b10 promoter region. As this *Cyp*2b10 DNA region was composed of a multi-component enhancer the response unit was re-designated the phenobarbital-responsive enhancer module (PBREM) in mouse (Honkakoski and Negishi, 1997) and the phenobarbital-responsive unit in rat (Stoltz *et al.*, 1998; Liu *et al.*, 1998).

The PBREM of the non-inducible mouse *Cyp*2b9 gene was analysed and found to be mutated and non-functional with a 6 bp difference between itself and the *Cyp*2b10 DNA. This 33 bp region of the *Cyp*2b10 PBREM sequence (-2332 to -2300 bp) contains a NF-1 consensus sequence ($TGGN_7CCA$) as in the CYP2B2 gene, and a putative nuclear receptor (NR1 and NR2) binding motif (AGGTCA). Mutation of the NF-1 sequence to that of the *Cyp*2b9 gene reduced the basal activity by 65% and abolished the PB-responsiveness of the element. This further indicated the importance of this region for PB-inducibility (Honkakoski and Negishi, 1997). Mutation of the putative nuclear receptor (NR) binding motif eliminated PB induction and increase basal activity by 2-fold. These mutations were also shown to alter the nuclear binding patterns in this region as assessed by DNase I footprinting and gel-shift assays. Mutation of the NF-1 site reduced binding in this region leaving only the NR motif protected. By contrast,

mutation of the NR site did not affect the NF-1 binding but reduced the extent of protection at the 5' end of the pC element. The 33 bp core element described by Honkakoski and Negishi (1997) displays only about 3-fold induction by PB. This indicated that there were sequences present outside of this core 33 bp which were required for full enhancer activity of the PBREM. Honkakoski *et al.* used transfection studies and deletion analysis of constructs containing the 132 bp of the 5' flanking sequence of the *Cyp*2b10 gene to determine the position of the PBREM (Honkakoski *et al.*, 1998b). This study delimited the PBREM to a 51 bp enhancer element to (-2339 to -2289 bp) and was shown to exhibit an 11-fold induction in response to PB. Sequence analysis of this element showed that it was composed of two (NR1 and NR2) NR binding sites (composed of imperfect direct repeats of AGGTCA-like half sites with a spacing of 4 nucleotides (DR4)) which are found in the flanking region on each side of the NF-1 site. A CAR/RXR heterodimer has been reported to bind to the NR1 site of the mouse *Cyp*2b10 (Honkakoski *et al.*, 1998a, c) as well as both NR1 and NR2 sites of the human CYP2B6 gene (Sueyoshi *et al.*, 1999). The binding of both CAR and RXR was shown to increase rapidly in PB-treated mice and to occur before the increase in *Cyp*2b10 mRNA (Honkakoski *et al.*, 1998b). The interaction of CAR/RXR and the NF-1 region of the 5' flanking sequence of the CYP2B6 and *Cyp*2b10 are discussed in more detail below.

The importance of the NF-1 site and surrounding nearby sequences in PB induction was further demonstrated by *in situ* transfection assays in rat livers (Park *et al.*, 1996; Liu *et al.*, 1998), and by transient transfection of primary rat hepatocytes (Trottier *et al.*, 1995; Stoltz *et al.*, 1998). The *in situ* transfection protocol used by Park *et al.* (1996) consisted of the direct injection of CYP2C1/CYP2B2-driven luciferase reporter genes into rat livers. It is thought that this technique has the advantage of assaying the transcription of these chimeric genes in hepatocytes, which are within the normal architecture of the liver. Luciferase reporter genes containing up to -3.5 kb of the 5'flanking sequence of CYP2C1 and -1.4 kb of CYP2B1 showed minimal

expression of luciferase activity even after PB treatment (100mg/kg) in these studies. However, the CYP2B2 sequence from -2318 to -2155 bp was shown to confer PB-responsiveness to both the CYP2C1 and CYP2B1 promoter in these reporter genes. The insertion of one to three copies of the PBRU in front of either CYP2C1 (-272 to +1 bp) or CYP2B1 (-110 to +1 bp) gave 5-15 and 2.5-5-fold induction respectively when the rat livers were treated with PB.

Kemper and co-workers further dissected this element using the *in situ* transfection technique and site-specific mutagenesis (Liu *et al.*, 1998). In this study deletion analysis of the 5' and 3' ends of single copies of the PBRU showed that an 88 bp fragment (-2258 to -2170 bp) was required for a maximal response to PB. Deletion of the 5' sequence to -2231 bp or of the 3' sequence to -2194 bp had little effect on PB-responsiveness. This data indicated that a minimal core sequence centred about the NF-1 site of 37 bp (-2231 to -2194 bp) was required for PB-responsiveness. However, a fragment containing three copies of the 37 bp core sequence alone could not confer PB-responsiveness and required additional flanking regions. These results are consistent with studies carried out by Honkakoski and Negishi (1997). The importance of the bipartite NF-1 site, which is central to the 88 bp sequence, was examined by mutation of the two bipartite sites and the intervening region. PB-responsiveness was lost with mutations of the bipartite recognition sites but was retained with the mutated intervening region. When these mutations were tested as three copies, PB-responsiveness was found to be reduced not abolished, thus indicating the importance of the NF-1 site as well as suggesting that other elements may be required for PB induction. The interaction of the NF-1 site present within the PBRU was also highlighted using the 37 bp fragment as a probe in gel-shift assays. Here multiple protein-DNA complexes were observed which were eliminated using an excess of probe; a supershift in the binding complex was observed when antiserum to NF-1 was added. When a specific NF-1 competitor was introduced, other complexes became evident, demonstrating that sequence-specific recognition sites for proteins other than NF-1 were also present.

Linker scanning mutagenesis in which a Kpn I site substituted in 6 bp blocks between -2255 to -2170 bp was employed to determine the importance of sequences flanking the NF-1 site in the PBRU. These 12 mutants were fused as triple copies to the CYP2C1-driven luciferase reporter and the PB-responsiveness of each construct assessed. The PB-response was not eliminated by any of the mutants with only two (LS4 and LS5) showing a 50% reduction in activity. Basal activity of the mutants within the region LS7 to LS9 was shown to increase in the untreated animals suggesting this region may harbour some repressor effects (Liu *et al.*, 1998). Once again this data along with the NF-1 mutation data suggests the presence of a complex of factors which function as a unit to confer PB-responsiveness in the CYP2B2 gene.

The CYP2B2 163 bp Sau3AI PBRU was further dissected by Anderson and co-workers using transfection protocols and primary rat hepatocytes (Stoltz *et al.*, 1998). In this study, 5' deletion to -2257 bp or 3' deletions to -2208 bp of the PBRU were still able to confer PB-responsiveness. These data indicated that a minimal core sequence centred about the NF-1 site of 51 bp (-2257 to -2208 bp) was required for PB-responsiveness. However a construct containing only this 51 bp element could not confer PB-responsiveness suggesting the need for sequences either 5' of -2257 bp or 3' of -2208 bp. An element between -2188 and -2172 bp when combined with the central core was found to be PB-responsive. This accessory site was designated AF1. Other potential regulatory motifs were also identified within the 163 bp Sau3AI PBRU. A perfect hexamer half-site (AGGTCA at -2223 bp to -2218 bp, lower strand) for members of the orphan nuclear receptor superfamily was found adjacent to the NF-1 site. This site in combination with the NF-1 site was designated the HX-NF1 complex. There was also an everted repeat arrangement present at -2282 to -2264 bp, (AGGTCA, designated ER-7) which possessed a 7 bp spacing. A glucocorticoid-binding site was also shown to be present at co-ordinates -2244 to -2226 bp on the upper strand and -2246 to 2228 bp on the lower strand.

DNase I footprint analysis using rat liver nuclear extracts showed protected regions very similar to those found in the *Cyp*2b10 mouse homologue (Honkakoski and Negishi, 1997) on both the upper (designated F0', F1', F2' and F3') and lower (designated F0, F1, F2, F3, F4) strands. The F0 and F0' footprints were shown to overlap with the AF1 site defined by transfection analysis. The F1 and F1' footprints extend beyond the HX-NF1 complex but the F1 footprint was eliminated by an excess of NF1 competitor, indicating that the footprint is in part composed of NF-1 protein. The F3/F4 and F3' regions correspond to the two halves of the ER-7 sites and both F3/F4 were competed by oligo HX. Hence these three footprints may be due to proteins of the nuclear receptor superfamily binding to the ER-7 site. Site-directed mutagenesis of the glucocorticoid-binding site lead to virtual abolition of the PB-response. This indicated that protein binding in the region of the putative glucocorticoid response element may be required to confer PB-responsiveness.

Mutation of the NF-1 site in either the distal portion (NF1m1) or both the distal and proximal portions (NF1dm2) of the NF-1 site was seen only to reduce PB-responsiveness. The same response was observed when mutations were introduced in the HX-NF1 complex (in either the HX site (HXm), or both the HX and NF-1 site (Hxm-NFdm2)). These data imply that the two components of the HX-NF1 complex play the role of accessory elements. In contrast, Honkakoski and Negishi (1997) reported that mutation of the equivalent element (NR site) in the mouse *Cyp*2b10 sequence abolished PB responsiveness and increased basal activity by 221%, suggesting that the NR (HX) site acts as a repressor of PB responsiveness. The possibility that the different attributes exhibited by the HX mutation used by Stoltz *et al.* (1998) was a consequence of the mutation introduced, when compared to the mutant used by Honkakoski and Negishi (1997), was examined by Stoltz and Anderson (1999). These workers introduced a new mutant (Hxdm3) into the primary hepatocyte system, which consisted of the same mutations as those used by Honkakoski and Negishi (1997). The effect of this new mutation was to reduce PB

responsiveness and decrease the basal level of expression. Hence the apparent discrepancy between the roles of the HX (NR) site in the rat CYP2B2 and the Mouse *Cyp*2b10 genes is not entirely clear, although these differences may be related to species differences, culture conditions or transfection protocols.

Work carried out by Roe *et al.* (1996) has shown that binding of rat liver nuclear proteins to an AP-1 site (TGACTCA) located at -1441 bp to -1447 bp of the 5' flanking region of the CYP2B2 gene was increased after *in vivo* PB-treatment. This increase was shown to occur within 18 hours of acute *in vivo* exposure to PB and persisted with chronic treatment for 5 days, thus indicating the existence of other possible regulatory sites. Despite data presented by both Anderson's and Kemper's groups which implicates the PBRU in the PB response, analysis of protein binding by either gel-shift assays or *in vitro* DNase I footprinting assays shows that there is no change in protein binding in this region after PB treatment. The importance of chromatin structure in the activation of the rat CYP2B genes was investigated recently by Kim and Kemper (1997). Comparisons between the effects of PB-treatment on protein binding to the PBRU (assessed by *in vitro* DNase I footprinting) and binding to the native chromatin (assessed by *in vivo* DNase I footprinting) were carried out. The results obtained with *in vitro* footprinting were similar to those of other workers namely a protected region in the vicinity of the NF-1 site which was unaffected by PB treatment. In contrast, *in vivo* footprinting to the native chromatin showed that in control samples a 25 bp region centred on the NF-1 site was protected. After PB-treatment the protected region was extended by 20 bp on each side to give a protected fragment of 60 bp overall. These data indicate that PB-treatment alters chromatin structure, facilitating additional binding of other proteins to the PBRU thus leading to transcriptional activation. The proposed model for ligand-mediated transcriptional activation consists of the chromatin structure being held in a closed state due to regulatory proteins which are bound either in an inactive conformation or suppressed by repressor factors. PB is thought to cause the binding of a factor

that alters chromatin conformation so that NF-1 and other positively acting factors (coregulators) can bind. This is followed by the recruitment of the basal transcription machinery. This proposed model is consistent with the data presented which indicate no change in binding to the DNA *in vitro* after PB treatment.

During the course of my studies, a greater understanding of the PB induction mechanism was provided when Negishi and co-workers (Honkakoski *et al.*, 1998c) discovered the involvement of the orphan nuclear receptor CAR in the PB induction mechanism of the *Cyp*2b10 gene. Their work showed that CAR binds as a heterodimer with RXRα to the nuclear receptor (NR) sites present in the PB-responsive enhancer module PBREM. This element has also been identified within the rat CYP2B2 gene and designated the PB-responsive element (PBRE) (Trottier *et al.*, 1995). RXR has been shown previously to heterodimerise with PXR (CYP3A) and PPAR (CYP4A) (section 1.1.6.2 and 1.1.6.3), and as a dimer with CAR binds certain retinoic acid response elements (RARE) (Baes *et al.*, 1994; Choi *et al.*, 1997), rather like the CYP4A PPARα complex. The heterodimer formed between CAR and RXR and the PBREM/PBRE is functional as determined by experiments utilising a CAR expression plasmid and a PBRE-containing CAT reporter gene transfected into HepG2 cells. These experiments showed that the presence of PB was not required to activate the PBRE reporter gene, although gel mobility shift assays on PB-treated liver nuclear extracts showed a 10-fold increase in CAR-RXR DNA-binding activity when compared to control treated mice (Honkakoski *et al.*, 1998c). This PB-independent CAR-stimulated PBRE-reporter gene activation may stem from the fact that CAR (unlike most nuclear receptors) has been characterised as a constitutively active receptor that does not require a ligand to bind in order for transactivation to occur (Choi *et al.*, 1997).

Work carried out by Forman *et al* 1998 (Forman *et al.*, 1998) demonstrated that androstane

metabolites act as ligands for an isoform of CAR called CARβ. These ligands were shown to inhibit CAR's transcriptional activity by dissociating the CAR-RXR-DNA complex from transcriptional co-activators like steroid receptor co-activator-1 (SRC-1). These androstane ligands were found to be examples of natural inverse agonists that have the ability to reverse the transcriptional activation by certain nuclear receptors (Klein *et al.*, 1996). SRC-1 is a protein that interacts with multiple nuclear receptors in a ligand-dependent manner and functions as a co-activator of transcription (Onate *et al.*, 1995). CAR, unlike most nuclear receptors, has been shown to adopt an active conformation in the absence of ligand. It is thought that androstanes bind directly to the CAR-RXR heterodimer in a manner which prevents the interaction between CAR and SRC-1, maintaining the receptor in an inactive conformational state (Forman *et al.*, 1998). PB and PB-like inducers have been shown to abolish the inhibitory binding of androstanes to CAR with the derepression of the receptor's activity.

The proposed model for the interaction between CAR/RXR and the PBREM received strong experimental support by Sueyoshi and coworkers in 1999. Here HepG2 cells were stably transfected with CAR and the endogenous level of CYP2B6 mRNA expression determined compared to untransfected cells (Sueyoshi *et al.*, 1999). Once HepG2 cells were transfected with CAR, the normally non-detectable endogenous levels of CYP2B6 mRNA were being expressed at a level that was detectable. Androstenol treatment (4μM) was shown to repress the CYP2B6 mRNA expression in these cells. The expression of CYP2B6 mRNA was derepressed if 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) was added after androstenol treatment. These results were mirrored in further experiments in which the cotransfection of CAR and luciferase reporter genes containing either the *Cyp*2b10 PBREM sequence, or a PBRE found 1.7 kb upstream of the human CYP2B6 gene, was carried out in HepG2 cells. Here the addition of 5mM PB to androstenol-treated CAR transfected cells overcame the suppressive effect of the androstenol, inducing reporter activity and endogenous CYP2B6

mRNA expression. CAR-dependent derepression of CYP2B6 expression and the transactivation of PBRE-linked reporter genes is also apparent with other PB-like inducers (Sueyoshi *et al.*, 1999). The evidence presented in these experiments suggests that CAR's transcriptional activity is suppressed by the presence of inhibitory steroids. However, the concentrations of androstenol used to inhibit CAR in these *in vitro* studies (0.4μM) exceeds that reported in the circulation of man (Gower and Ruparelia, 1993), raising questions about how CAR is repressed in the liver.

Section 1.6 Summary of introduction

Section 1.6.1 PB induction of cytochrome P450s

PB is a prototype of a group of structurally unrelated chemicals that share the property of inducing many members of the cytochrome P450 subfamilies in particular the CYP2B, 2C and 3A. CYP2B1 and CYP2B2 are most highly induced by PB in the liver. When rats are administered PB there is a rapid increase in the CYP P450 system, particularly CYP2B1 and 2B2 genes. When barbiturates are co-administered there is a possibility that the inductive effects of the barbiturate may be altered due to drug-drug interactions. These effects may have a major impact on drug metabolism and pharmacokinetics. In humans, barbiturates can be used for treatment of pain, insomnia, anxiety and epilepsy. Their usual high prescription dose taken over long time periods increases the risk of any resultant toxic effects within the metabolic system. Chronic effects may include tumour promotion, or drug-drug interactions.

Section 1.6.2 CYP2B expression and transcriptional regulation

CYP2B2 protein is present at a very low constitutive level in the liver whereas CYP2B1 is 5-10 times lower in comparison. However, when induced by PB these levels increase to 20 and >100 fold above constitutive levels respectively. The expression and PB-induction of both CYP2B1 and CYP2B2 are subject to gender, tissue and developmental regulation. It has been known for a long time that PB induces CYP2B1 and CYP2B2 mRNAs by increasing transcription of their genes, although the details of the molecular mechanisms by which PB-mediates transcriptional activation are not well understood. Progress towards understanding the molecular mechanisms underlying the PB induction of the CYP2B genes has been made with the development of *in vitro* culture systems which support PB induction of the endogenous CYP2B1/2 genes. Extensive studies have been carried out on the 5' flanking regions of both the rat CYP2B2 and mouse *Cyp*2b10 genes to try and identify any *cis*-acting elements and *trans*-acting factors which may be involved in PB induction. A PB-responsive enhancer element (PBRU) composed of a

163 bp fragment (-2318 to -2155 bp) was found to be present in the distal region of the CYP2B2 promoter in primary rat hepatocytes. A corresponding element (PREM) composed of a 177 bp fragment (-2426 to -2250 bp) was found in the mouse *Cyp*2b10 gene. The enhancer activity of both of these elements was subsequently delimited to a core 51 bp element. The nuclear orphan receptor heterodimer CAR/RXR has also been identified as a transactivator of the PBREM in the mouse *Cyp*2b10 gene.

Section 1.6.3 Aims of the project

An aim of this thesis was to develop an *in vitro* primary hepatocyte culture system that would support PB-mediated induction of a CYP2B2-driven reporter gene. It was hoped that this hepatocyte culture system in conjunction with reporter gene technology could be utilised to elucidate the mechanism(s) which regulate PB-induced gene transcription of CYP2B2. This system could then also be used to develop a *trans*-activation assay for high-throughput drug screening to identify potential inducers of the CYP2B2 gene.

An *in vitro* primary hepatocyte culture system was set up in connection with a sensitive and specific RNAse protection assay to demonstrate that CYP2B2 RNA was highly inducible by PB in this system. The sequence of the 5' flanking region of the CYP2B2 promoter was determined by automated sequencing; the position of the putative PBREM was then defined using this sequence. This PBREM was subcloned into a number of luciferase and CAT reporter genes. A suitable transfection protocol for introducing the CYP2B2-driven reporter constructs into the primary hepatocytes was developed in this thesis. After the transfection protocol had been optimised the effects of different CYP2B2 inducers were assessed. Finally the initial stages of the subcloning and analysis of a CAR containing construct were carried out to ascertain its interaction with the CYP2B2 PBREM.

Chapter 2 Materials and Methods

Section 2.1 Materials

All reagents were obtained at AnalaR grade purity or greater. Ammonium sulphate, ethanol, glycerol, β-mercaptoethanol and sodium potassium tartate were from BDH. Agarose, ammonium acetate, chloramphenical, BSA, DTT, DMSO, EDTA, ferrous cyanide, ferric cyanide, hydrogen peroxide, isoamylalcohol, imidazole, PMSF, Sodium phenobarbital, TCA, TEMED, tetracycline and mixed xylenes were all obtained from Sigma. Xylene cyanol FF and the Immun-Blot assay kit were obtained from Bio-Rad. Bacto-agar, Bacto-typtone and Bacto-yeast extract were obtained from Difco. All restriction enzymes were obtained from either NBL or Promega. Sequagel solutions were manufactured by National Diagnostics and obtained from Flowgen. X-ray film was manufactured by Fuji and obtained from Amersham. X-ray film developer and fixer were obtained from Ilford. Alkaline phosphatase, proteinase K, 1Kb and 1Kb+ DNA marker ladder were obtianed from Gibco-BRL. 3MM paper and GF/A glass fibre filters were obtained from Whatman. Ammonium persulphate, ampillicin, bromophenol blue, glycine, guanidine hydrochloride, SDS, Tris-base, Triton-X100, urea were all obtained from ICN Flow. IPTG was supplied by NBL. Pharmacia supplied RNAguard. Promega provided all luciferase transfection kits and commercial plasmids. Stratagene provided *E.coli*. Strain XL1-blue. Other chemicals of AnalaR grade were obtained from Sigma if not stated in the text. Oligonucleotides were synthesised by John Keyte of the Biopolymer Synthesis and Analysis Unit, Department of Biochemistry, University of Nottingham. Ultra high purity (UHP) grade water (> 13 Mohms/cm^3) was produced using a Purite Select Bio system. The following radioactive isotopes were obtained from Amersham at the stated specific activities: [D-threo-[dichloroacetyl-1-^{14}C] Chloramphenicol, 54miCi/mmol, [α-^{32}P]-CTP (3000Ci/mmol) and [α-^{32}P]-dCTP (6-800Ci/mmol).

Section 2.2 Treatment of animals

Adult male Wistar rats (180-220g, obtained from Harlan UK) were kept in a standard 12 hours dark/light cycle and were provided with food and water *ad libitum*. Groups of two Wistar rats were dosed daily by inter-peritoneal injection with phenobarbital in saline (80mg/kg body weight), for 4 consecutive days. Control animals received vehicle alone (0.5ml saline). Twenty-four hours after the last injection, animals were sacrificed by exsanguination under terminal anaesthesia with diethyl ether. The livers were removed and immediately frozen in liquid nitrogen and stored at -70^{o}C. Naive rats were sacrificed and the livers perfused as described in section 2.6.1 and 2.6.2.

Section 2.3 Methods

Section 2.3.1 Bacterial growth media and culture conditions

Luria-Bertanti (LB) broth is the main culture medium used in genetic manipulation experiments with LB agar being used to culture single colonies. LB broth (10g/L Bacto-tryptone, 5g/L Bacto-yeast extract and 10g/L NaCl) was made using ultra high purity (UHP) water and autoclaved for an hour at 15 lb./in^2. LB broth was allowed to cool to room temperature before use. LB agar plates were made by adding a further 15g Bacto-agar to a litre of LB broth and autoclaved as for LB broth. Autoclaved LB agar was allowed to cool before the appropriate antibiotics were added and then poured into Petri dishes. Antibiotics were used at the following final concentrations: tetracycline at 20μg/ml, ampicillin at 50μg/ml. When blue white selection colour screening (with vectors capable of α-complementation) was utilised, 30μl of a 20mg/ml IPTG solution and 30μl of a 20mg/ml 5-bromo-4-chloro-3-indolyl-β–D-galactoside (X-Gal) solution in DMF was added to the plate and spread evenly over the surface.

Section 2.3.2 *Eschericha coli* strains and their manipulation

E. coli strain XL-1 Blue (recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lac^q ZΔM15 Tn10 (Tet^R)] was used for subcloning most expression vectors. DH5α (ϕ80δlacZΔM15 recA1 endA1 gyrA96 thi-1 hsdR17 (rk^-,mk^+) supE44 relA1 deoR δ(lacZYA-argFv169), F^-, λ^-) cells were also used. Glycerol stocks (made by placing 10% glycerol into the bacterial culture stock) were used for the long-term storage of bacteria. Typically a 5ml culture of inoculated media was incubated overnight at 37°C, then centrifuged at 16000g for one minute and the cells resuspended in LB broth containing a 15% solution of glycerol. The glycerol stock of the bacteria could be stored at -20°C for 3 months or -80°C indefinitely. In order to culture a glycerol stock of a bacterial strain, a 1-2μl aliquot of the glycerol stock was taken up onto a sterile inoculation loop and streaked onto a LB agar plate containing the appropriate antibiotics. The streaked plate was inverted and incubated at 37°C overnight. Once colonies of a suitable size were obtained the plate was stored at 4°C for a maximum of a month. A single colony could then be 'picked' from the plate and used to inoculate a liquid culture, which was then placed in a shaking incubator at 37°C until the desired OD_{600} was obtained.

Section 2.3.3 Preparation of $CaCl_2$ competent XL-1 blue *E.coli*

A single colony of XL-1 Blue *E.Coli* from a LB/tetracycline plate was picked and inoculated into a 5ml LB/tetracycline broth culture. The culture was grown overnight in a shaking incubator at 37°C until a thick liquid culture was obtained. A 500ml culture of LB/tetracycline medium was inoculated with 1ml of the fresh overnight culture of bacteria and grown until an OD_{600} of 0.6-0.8 was reached. The cultures were placed on ice for 15 minutes and then centrifuged at 4000g for 15 minutes at 4°C. The supernatant was discarded and the pellet gently resuspended in 50ml of ice cold sterile 0.1M $CaCl_2$. Cells were again centrifuged and resuspended as before. Cells were then pelleted once more, the supernatant discarded and the final pellet resuspended in 2ml of sterile 0.1M $CaCl_2$, 10% glycerol. Cells were placed in 200μl aliquots and stored at -80°C.

Section 2.3.4 Transformation of $CaCl_2$ competent *E.coli*

A 100µl aliquot of cells was thawed on ice. To these cells 10-50ng of DNA or 5µl of a ligation reaction was added and allowed to incubate on ice for 10 minutes. The DNA/cell mixture was then heat shocked at 42°C for 90 seconds and immediately placed on ice for 2 minutes. The cells were then incubated at 37°C for an hour in 1ml of LB broth, 50-100µl of the transformed cells were then plated out onto 10cm LB agar petri dishes containing the corresponding selection antibiotics and incubated overnight at 37°C.

Section 2.3.5 Preparation of electro-competent XL-1 blue *E.coli*

Colonies were treated as in section 2.3.3, except that after the first centrifugation step the pelleted cells were resuspended in 50ml of ice cold sterile UHP water. Cells were then pelleted by centrifugation at 4000g for 15 minutes at 4°C and resuspended in ice cold sterile UHP water a further 5 times. Cells were finally resuspended in 2ml of sterile UHP water, 20% glycerol and stored as 200µl aliquots at -80°C.

Section 2.3.6 Electro-transformation of XL-1 blue *E.coli*

This method of transforming bacteria is far more efficient than that of calcium competent transformation but is very susceptible to high salt concentrations, which may affect the transformation process. An aliquot of XL-1 Blue electro-competent cells was thawed and placed on ice for 5 minutes along with the electroporation cuvettes and cuvette holder. To these cells 10-50ng of DNA or 5µl of a ligation reaction was added and allowed to incubate on ice for a further 10 minutes. The mixture of DNA and cells were placed in a Biorad Genepulser cuvette (1mm path length) and electroporated with a voltage of 1.8 kV using the Biorad electroporator. The cells were immediately resuspended in 1ml of LB broth and allowed to recover at 37°C for an hour. The transformed cells were selected by growing the electroporated bacteria on LB agar plates, containing the appropriate selection antibiotics.

Section 2.4 Manipulation of DNA

Section 2.4.1 Phenol:chloroform extraction of nucleic acids

Tris buffered phenol (pH 8.0) is prepared as in 2.7.3 and this solution was mixed in a 1:1 (v:v) ratio with chloroform. This solution was then used to remove protein contamination from nucleic acid samples by a sequential phenol:chloroform extraction. One volume of the phenol:chloroform solution was added to the nucleic acid sample and the mixture vortexed thoroughly. The organic and aqueous phases were separated by centrifugation at 15000g for 5 minutes using a benchtop centrifuge. The aqueous phase was removed and retained.

Section 2.4.2 Ethanol precipitation of nucleic acids

Ethanol precipitation was utilised to reclaim nucleic acids samples from small liquid volumes. Two volumes of absolute ethanol and 1/4 volume of 3M sodium acetate pH 5.2 (or $1/10^{th}$ volume of 7M NH_4SO_4) was added to the DNA solution. The solution was vortexed and then placed on ice or -20°C for 25 minutes to precipitate the nucleic acid. The chilled solution was then centrifuged at room temperature for 25 minutes at 14000g. The pellet formed was then washed in 200-500µl of 70% ethanol and re-spun as before for a further 5 minutes. The pellet was allowed to air dry to remove trace quantities of ethanol and the pellet resuspended in its respective liquid medum. One volume of 100% isopropanol may also be used instead of the ethanol to precipitate nucleic acids.

Section 2.4.3 Isolation of plasmid DNA by the alkaline lysis method

Clones were used to inoculate 5ml of LB broth containing the appropriate antibiotics and incubated at 37°C overnight. Plasmid DNA was extracted using a modified version of the Birnboim (1983) alkaline lysis miniprep method. A 1.5ml aliquot of this overnight culture was taken and the cells harvested into a 1.5ml eppendorf tube by centrifugation at 12000g for 1minute. The resultant pellet was then completely resuspended in 100µl of solution I (50mM glucose, 25mM Tris-HCl pH 8.0, 10mM EDTA and RNAse A 100µg/ml). To this solution, 200µl of freshly

prepared solution II (200mM NaOH, 1% SDS) was added and gently mixed by inversion of the tube. This mixture was allowed to stand for 5 minutes before 150µl of solution III (5M potassium acetate, 11.5% glacial acetic acid) was added. The sample was mixed immediately by inversion of the tube and the bacterial lysate was allowed to precipitate on ice for 10 minutes. The viscous precipitate was removed by centrifugation at 15000g for 10 minutes and the supernatant transferred to a fresh tube. Samples were phenol/chloroform extracted (section 2.4.1) and ethanol precipitated (section 2.4.2). Pellets were washed in 70% ethanol and allowed to air dry. The pelleted plasmid DNA was resuspended in 20-30µl of UHP water and stored at -20°C until required.

Section 2.4.4 Purification of plasmid DNA using the Qiagen mini-prep columns

Small scale sequencing grade plasmid DNA was prepared using a Qiagen-tip 20 plasmid mini-kit. In this protocol 3ml of an overnight culture was pelleted by centrifugation at 15000g for one minute and resuspended in 0.3ml of buffer P1 (50mM Tris-HCl pH 8.0, 10mM EDTA and 100µg/ml RNAse A). To this solution 0.3ml of buffer P2 (200mM NaOH, 1% SDS) was added, the tube mixed by inversion and then incubated at room temperature for 5 minutes. 0.3ml of chilled buffer P3 (3M-potassium acetate pH 5.5) was then added, the tube mixed and incubated on ice for 10 minutes. The sample was then centrifuged for 15 minutes at 15000g. Meanwhile a Qiagen-tip 20 was equilibrated with 1ml of buffer QBT (750mM NaCl, 50mM MOPS pH 7.0, 15% ethanol and 0.15% Triton X-100) which was allowed to drain though the column. The supernatant resulting from the centrifugation step was then immediately placed onto the Qiagen-Tip 20 column and allowed to drain through. The column was then washed 4 times with 1ml of buffer QC (1.0M NaCl, 50mM MOPS pH 7.0 and 15% ethanol). The plasmid DNA was then eluted from the column using 0.8ml of buffer QF (1.25M NaCl, 50mM Tris-HCl pH 8.5 and 15% ethanol) and collected into a fresh eppendorf. The collected fraction was then precipitated in 0.7 volumes of isopropanol and allowed to stand at room temperature for 20 minutes. The

solution was then centrifuged at 15000g for 30 minutes, washed in ice cold 70% ethanol and re-pelleted. Once air-dried, the pellet was resuspended in 25µl of autoclaved UHP water and was of a quality that could then be used for sequence analysis using an ABI 373A fluorescent sequencer.

Section 2.4.5 Plasmid purification using Qiagen-tip 500 maxi-prep column

A Qiagen-tip column is utilised to extract sequence grade plasmid DNA from bacterial cultures. Here a large 500ml bacterial culture was used and a similar protocol to that of section 2.4.4 was carried out, according to the manufacture's instructions. The final pellet was resuspended in 250µl of autoclaved UHP water and stored at -20°C for future use.

Section 2.4.6 Plasmid purification by isopycnic density gradient centrifugation

The caesium chloride-ethidium bromide (CsCl/EtBr) centrifugation method was used to prepare plasmids that were endotoxin-free as described by Weeks *et al.* (1986). All plasmids prepared by this method were first pre-treated using a Qiagen-tip 500 maxi-prep column as described in section 2.4.5. The Qiagen prepared plasmids were then split into 100µg aliquots, which were mixed into 1.5ml of a 1.65g/ml CsCl solution and centrifuged at 8000rpm (3000g) for 5 minutes at room temperature. A furry scum may be present at the top of the CsCl solution after centrifugation, consisting of complexes formed between the EtBr and bacterial proteins. The DNA band present in the lower section of the solution obtained from the first spin was extracted and mixed with 1.5ml of a 1.63g/ml CsCl solution containing 450µg of ethidium bromide. The samples were mixed thoroughly and loaded into 2.1ml polycarbonate tubes which were balanced to within a mg of each other. The tubes were then loaded into the Optima TLA 120.2 ultracentrifuge rotor and centrifuged at 80,000rpm (22000g) for 6 hours at 20°C. The speed at which this solution is centrifuged and density of the CsCl solution is critical and precipitation curves for CsCl must be consulted before any centrifugation is carried out. After the first spin, the DNA bands were located using an UV light source and removed using an 18

gauge needle connected to a 1.0 ml syringe. The DNA was removed in the smallest volume possible and subjected to a further centrifugation step at 80,000rpm (22000g) for 6 hours at 20°C. The DNA was further purified as described in section 2.4.6.1.

Section 2.4.6.1 Removal of EtBr and CsCl from DNA

The DNA solution from the CsCl/EtBr centrifugation step was treated with an equal volume of water-saturated 1-butanol. Water-saturated 1-butanol is prepared by mixing 1-butanol with water in a 1:1 ratio, giving rise to a biphasic medium of which the top phase is comprised of water-saturated 1-butanol. The two layers formed by the CsCl/EtBr DNA preparation and the water-saturated 1-butanol solution are mixed by vortexing for 30 seconds and then centrifuged at 1500rpm for 3 minutes at room temperature in a benchtop centrifuge. After centrifugation the solution consists of a red upper organic phase and a lower 'pink tinge' coloured aqueous phase. The lower phase was removed and extraction of EtBr from the solution was carried out a further 6-10 times using both water-saturated 1-butanol and centrifugation. The CsCl was removed from the DNA solution by dialysis for 24-48 hours against several changes of water. It may also be removed by diluting with 3 volumes of water and precipitating the DNA with 2 volumes of ethanol (final volume is equal to 6 times the original volume of the undiluted aqueous phase) for 15 minutes at 4°C. This was then followed by centrifugation at 10000g for 15 minutes at 4°C. The precipitated DNA was then dissolved in an appropriate amount of water.

Section 2.4.7 Restriction enzyme digest of plasmid DNA

Typically a restriction digest would take place in a reaction volume of 30µl in an eppendorf at 37°C for 1-2 hours. The digest would contain 5-10µl of plasmid DNA mixed with 3µl of the appropriate 10X restriction enzyme buffer and diluted to the final volume with water. Normally about 4U/µg DNA of restriction enzyme would completely digest the plasmid DNA. If non-compatible double digests were to be carried out; the enzyme with the lowest salt concentration requirement was carried out first; or alternatively the DNA could be ethanol precipitated

between digests. The reaction was normally terminated by either ethanol precipitation of the sample or by simply adding 10X loading buffer. The following restriction digest buffers were used with the appropriate manufacture's restriction enzyme. Buffer 5 (NBL) contained, 10mM Tris-HCl (pH 7.8), 50mM NaCl, 10mM $MgCl_2$ and 1mM DTT. Buffer 6 (NBL) contained, 50mM Tris-HCl (pH 7.8), 100mM NaCl, 10mM $MgCl_2$ and 1mM DTT. Buffer A (Boehringer Mannheim) contained, 33mM Tris-OAc (pH 7.9), 10mM $Mg(OAc)_2$, 66mM KOAc and 0.5mM DTT. Buffer B (Boehringer Mannheim) contained, 10mM Tris-HCl (pH 8.0), 5mM $MgCl_2$, 100mM NaCl and 1mM 2-Mercaptoethanol. Multicore-Buffer (1x) contained, 25mM Tris-OAc (pH 7.8), 100mM KOAc, 10mM $Mg(OAc)_2$ and 1mM DTT.

Section 2.4.8 Agarose gel electrophoresis

DNA and RNA was separated by agarose gel electrophoresis. DNA/RNA agarose electrophoresis was performed using a submarine Pharmacia GNA-100 gel electrophoresis kit or a Stratagene gel kit. Agarose was dissolved in the appropriate amount of 1X TBE (45mM Tris-HCl, 44mM Boric acid and 2mM EDTA (pH 8.0)) to give a 0.7-2.0% (w/v) gel. A 1X TAE (40mM Tris-HCl, 1mM EDTA and 5.71% glacial acetic acid) agarose gel was used for the gel excision of DNA fragments. Agarose was dissolved into the 1X TBE/TAE by microwave heating of the solution. The dissolved agarose solution is allowed to cool to about 55°C before the addition of ethidium bromide (10mg/ml stock) to give a final concentration of 0.5μg/ml. If the gel is being poured for RNA samples, then the 1X TBE or 1X TAE was made using DEPC-treated water and 0.1% SDS (w/v) was included in the gel mixture. The agarose mix was then poured into the appropriate gel casting mould containing the well forming comb and allowed to set. After the gel had solidified it was placed in the electrophoresis tank and immersed in the appropriate running buffer (1X TAE, 1X TBE). DNA samples were prepared in a 10% solution of 10X loading buffers (30% glycerol, 0.25% Bromophenol Blue and 0.25% Xylene Cyanol FF) and loaded into the wells formed by the comb. The samples were run at a constant voltage of

6V/cm until the desired resolution of DNA was formed. DNA bands were visualised by illumination with an UV trans-illuminator and photographed.

Section 2.4.9 Acrylamide gel electrophoresis

Acrylamide electrophoresis was carried out so that DNA sequences of between 6-2000 bp could be separated. These gels were made so that the final percentage of acrylamide was between 5 and 20% depending upon the size resolution required. Acrylamide electrophoresis was performed using a mini Biorad Protean II vertical electrophoresis kit. A 15ml, 12% acrylamide gel was made using 3ml of 5X TBE, 6ml 30% acrylamide, 5.9ml UHP water. The acrylamide was polymerised by the addition of 6µl of TEMED and 105µl of 10% ammonium persulphate and mixed. The acrylamide solution was then pipetted into the gel casting kit according to the manufacture's instructions using 10-well combs and allowed to polymerise for at least an hour.

The wells of the gel were flushed out using 1X TBE before loading the DNA samples. The samples were run at a constant voltage of 15V/cm until the desired resolution of DNA banding patterns were formed. After electrophoresis the gels were stained with ethidium bromide (10mg/ml stock) in 100ml of UHP water to give a concentration of 0.5µg/ml for 10 minutes. The gel was rinsed in 50ml of UHP water for 5 minutes and DNA bands were visualised by illumination with an UV trans-illuminator and photographed.

Section 2.4.10 Purification of DNA from an agarose gel

Plasmid DNA that has been restriction digested can be purified using the GENECLEAN II kit (BIO 101 inc.). This system works only on 1X TAE gels; if a 1X TBE gel has been used then the fragment may only be extracted by using a TBE modified protocol (see manufactures instructions). The kit contains a specially formulated silica matrix (glassmilk) to which DNA binds without contaminants. Restriction digested DNA was resolved on a 1X TAE agarose gel until the band of interest could be visualised using an UV trans-illuminator. This band was then excised

using a clean scalpel and placed in an eppendorf and weighed. This excised gel fragment was then treated with 3 volumes of NaI solution (6.0M). The tube was then incubated at 55°C with periodic mixing for 5 minutes or until the gel fragment was dissolved. To this solution 5µl of glassmilk solution was added and mixed. The solution was then incubated at room temperature with periodic mixing for 10 minutes. This was to allow the DNA to bind to the silica matrix. The glassmilk-DNA complex was then pelleted by centrifugation at 15000 rpm for 10-15 seconds. The supernatant was then removed and the glassmilk pellet resuspended in 500µl of ice cold New Wash Buffer (50% 20mM Tris-HCl pH 7.2, 0.2M NaCl, 2mM EDTA and 50% ethanol) and centrifuged again. This step was repeated a further 2 times before the final pellet was resuspended in 15µl of UHP water. Incubation of the glassmilk solution at 55°C for 2-3 minutes will elute the DNA from the glassmilk complex. The DNA is extracted from the glassmilk solution by centrifugation at 15000rpm for 15 seconds. The purified DNA solution is then transferred to a clean eppendorf where further DNA manipulations were carried out.

Section 2.4.11 Phosphatase treatment of DNA

Shrimp alkaline phosphatase (SAP) was used to remove the 5'-terminal phosphate from nucleic acids. Linearised cloning vectors can be prevented from recircularising by dephosphorylation with alkaline phosphatase. To the 15µl of GENECLEAN sample, 1µl of 10X shrimp alkaline phosphatase buffer [United States Biochemical-USB] (200mM Tris-HCl (pH 8.0), 100mM $MgCl_2$) and 1U of SAP (USB) were added and the reaction diluted with UHP water to give a final volume of 20µl. The reaction was incubated at 37°C for 30 minutes and heat inactivated by incubation at 65°C for 15 minutes. The final solution can now be used for further manipulations. Alternatively, the solution can be cleaned up still further by a phenol:chloroform extraction (section 2.4.1), followed by an ethanol precipitation (section 2.4.2) with the pellet being taken up in a final volume of 15ul of UHP water.

Section 2.4.12 Ligation of DNA

During ligation the formation of a phosphodiester bond between the 5'-phosphoryl group and the 3'-hydroxyl group of two double stranded DNA fragments occurs. This reaction is catalysed by the presence of T4 ligase, which requires ATP to be present. A balance between the ratio of vector DNA to insert DNA must be found when performing a ligation reaction. The simplest ligation is formed when a 1:1 ratio of vector DNA to insert DNA is formed. Here the reaction volume takes place in 10µl and consists of 4µl of vector DNA, 4µl of insert DNA, 1µl of T4 ligase (1U/ul) and 1µl 5x T4 ligase buffer (250mM Tris-HCl (pH 7.6), 50mM $MgCl_2$, 5mM ATP, 5mM DTT and 25% (w/v) polyethylene glycol-8000) made up with UHP water. The tube was vortexed and then centrifuged for 10 seconds. The solution was incubated overnight at 4^{o}C. This solution can then either be used directly for transforming calcium competent cells, or ethanol precipitated and resuspended in 10µl UHP water if using electro-competent cells.

Section 2.4.13 Automated fluorescent sequencing

DNA was purified using a Qiagen mini-prep kit (see section 2.4.4) before sequencing could be carried out. J. Keyte, Biochemistry Dept. Nottingham University performed automated sequencing, using the Perkin Elmer ABI 373A Fluorescent Sequencer and an ABI Cycle sequencing Kit. DNA was sequenced on both strands, unless otherwise stated. Sequencing was performed using the forward and reverse primers, T7 and SP6 as well as custom made primers for oligonucleotide directed sequencing.

Section 2.4.14 Sequence analysis

Raw DNA sequences were inspected and edited using the GCG (University of Wisconsin) sequence analysis software and Straden software suites (Bonfield *et al.*, 1995). The software programs used were TED, SEQED, BESTFIT, GELASSEMBLE and MAP (Wisconsin package Version 9.0). Putative protein factor binding sites were searched for using WWW based software packages (at their respective default values) such as Transcription Element Search Software

(TESS) and the TRANSFAC database (Heinemeyer *et al.*, 1990).

Section 2.5 Polymerase chain reaction (PCR)

Short segments of DNA were amplified using PCR primers. PCR primers were designed so that a high sequence identity to the target DNA was achieved. Base-pair mismatches were incorporated into the primers to generate particular restriction enzyme sites. These sites could be used subsequently for subcloning purposes. PCR was performed using 5µl of 10X KlenTaq PCR reaction buffer (400mM Tricine-KOH (pH 9.2 at 25°C), 150mM KOAc, 35mM $Mg(OAc)_2$, 750µg/ml bovine serum albumin), 1µl 5'-Primer (~70pmol), 1µl 3'-Prime (~70pmol), 4µl dNTP mix (1.25mM), 1.5µl 163bp-Pluc DNA (1ng), 0.5µl KlenTaq polymerase and diluted to 50µl with UHP water. The PCR reaction was overlaid with 50µl of mineral oil to prevent the contents from evaporating. PCR was performed on a Perkin Elmer DNA Thermal Cycler 480 for 30 cycles at 1.0 minute denaturing at 95°C, 1.0 minute annealing at 55-70°C (depending upon the primers used) and 1.0-minute polymerisation at 72°C. The final cycle consisted of an extension cycle of 5 minutes at the appropiate annealing temperature and 10 minutes at 72°C. 5µl of the PCR products were analysed on a 1.2% agarose/TBE gel.

Primer names and DNA sequences (5'-3'):-

Hind5 5' GAG ATG AAG CTT GCA GAT CGT GGA CAC AAC CTT 3'

Hind3 5' CTA GCC AAG CTT CGA GAT CAC CCA GCC TGG CAG 3'

Conditions:-

95°C for 1 minute (1)

55°C for 1 minute (2)

72°C for 1 minute (3)

Steps 1-3 are repeated for 30 cycles

55°C for an additional 5 minutes

72°C for an additional 10 minutes.

Section 2.5.1 Purification of PCR products

PCR products were purified using the Qiagen Qiaquick PCR spin columns. 250µl of buffer PB was added to the PCR reaction. This was then placed into a Qiagen Qiaquick PCR spin column which was placed inside an eppendorf tube and centrifuged at 14000rpm for one minute in a Heraeus benchtop centrifuge. The flow through was discarded and 750µl of buffer PE was added to the spin column and centrifuged at 14000rpm for one minute in a benchtop centrifuge. The flow through was discarded and the centrifugation step repeated to remove traces of residual buffer PE. The column was transferred to a fresh eppendorf tube and 50µl of UHP water was added to the column. The centrifugation step was repeated once more to elute the bound DNA from the column. The PCR reaction products could now be used for other DNA manipulation protocols.

Section 2.5.2 First strand cDNA synthesis

Mouse liver total RNA (a kind gift from M. Choudhury) was diluted to give a stock solution of 1µg/ml. To a microcentrifuge tube 5, 2.5 and 1µg of mouse total RNA were added along with 500ng of Oligo-dT (12-18) primer and each tube made up to 12µl with DEPC-treated water. Each tube was heated to 70°C for 10 minutes and then snap cooled on ice. The contents of the tubes were collected by pulse centrifugation and 4µl of 5X First strand buffer (250mM Tris-HCl (pH 8.3), 375mM KCl, 15mM $MgCl_2$ and 100mM dithiothreitol (DTT)), 2µl of 0.1M DTT, and 1µl of 10mM dNTP mix (10mM each dATP, dCTP, dGTP, dTTP at neutral pH) added.

To the 2.5μg total RNA tube 1μl of [^{3}H] dCTP (50μM stock) was added to trace the efficiency of the reaction. To the other two samples 1μl of DEPC water was added. All three tubes were gently mixed and incubated at 42°C for 2 minutes, 1μl (200units) of Superscript II RNAse H-reverse transcriptase (Gibco BRL, Life Technologies) was added to each tube and the reaction mixed by gentle pipetting. The solutions were incubated at 42°C for a further 50 minutes. Heating at 70°C for 15 minutes and cooling on ice inactivated the reaction. RNA complementary to the cDNA was removed by the addition of 1μl (2 units) of *E. coli*. RNAse H with incubation at 37°C for 20 minutes. A DE81 test (see section 2.7.9) was preformed on the reaction tube containing the radiolabelled dCTP to verify that first strand cDNA had been made.

Section 2.5.3 PCR amplification of cDNA

PCR was performed using the Advantage cDNA polymerase mix from Clontech laboratories, USA (Cat N° 8417-1). The PCR reaction contained 5μl of 10X cDNA PCR reaction buffer (400mM Tricine-KOH (pH 9.2 at 25°C), 150mM KOAc, 35mM $Mg(OAc)_2$, 750μg/ml bovine serum albumin), 2μl 5'-Primer (~70pmol), 2μl 3'-Prime (~70pmol), 1μl 50X dNTP mix (10mM each dATP, dCTP, dGTP, dTTP at neutral pH; 1X concentration: 0.2mM each), 1μl 50X Advantage cDNA polymerase mix which includes KlenTaq-1 DNA polymerase and TaqStart antibody (1.1μg/μl) in the following storage buffer (50% glycerol, 40mM Tris-HCl (pH 7.5), 50mM KCl, 25mM $(NH_4)_2SO_4$, 0.1mM EDTA, 5.0mM β-mercaptoethanol and 0.25% Thesit), 2-3μl of DNA template generated in section 2.5.2 made up to a reaction volume of 50μl with UHP water. The PCR reaction was overlaid with 50μl of mineral oil to prevent the contents from evaporating. PCR was performed using a Perkin Elmer DNA Thermal Cycler 480 with a 1-minute at 94°C step then for 25 cycles at 3.0 minute denaturing at 94°C, 3.0 minute annealing at 68°C and 3.0-minute polymerisation at 68°C. 5μl of the PCR products were analysed on a 1.2% agarose/TBE gel.

Primer names and DNA sequences (5'-3'):-

BAMPR1 5' GCC TGC TGC CTA AGG GA**T** **C**CA GGA GAC CAT GAC AGC 3'

BAMPR2 5' GGT CTG GGG AAA GGA T**C**C AAG CCT GGG CCT CAA GTG 3'

Conditions:-

94°C for 1 minute (1)

94°C for 3 minute (2)

68°C for 3 minute (3)

68°C for 3 minutes (4)

Steps 2-4 are repeated for 25 cycles

Section 2.6 Primary tissue culture

Section 2.6.1 Isolation of primary rat hepatocytes

Hepatocytes were isolated from the livers of adult male Wistar rats (180-220g) by the two step *in situ* perfusion technique described by Seglan (1976) and modified according to Mitchell *et al.* (1984).

Strict aseptic techniques were maintained throughout the preparation. The buffers used were Ca^{2+}-free Krebs Ringer Phosphate Buffer, pH 7.4, (KRPB) containing 150mM NaCl, 4.97mM KCl, 1.24mM KH_2PO_4, 0.62mM $MgSO_4$, 0.62mM $MgCl_2$, 3.73mM $NaHCO_3$ and 4.84mM $Na_2H_3PO_4$; and Ca^{2+}-free Krebs Ringer hydrogen carbonate Buffer pH 7.4 (KRHB), containing 142mM NaCl, 4.37mM KCl, 1.24mM KH_2PO_4, 0.62mM $MgSO_4$, 0.62mM $MgCl_2$ and 24.0mM $NaHCO_3$. These buffers were maintained at 37°C and gassed continuously with 95% O_2/ 5% CO_2. The rats were killed by diethyl ether anaesthesia and prepared for perfusion immediately after expiration. Firstly the abdominal cavity was opened and the liver exposed. The hepatic portal vein was located and cannulated followed by ligation of the inferior vena cava above the branch of the renal vein. The right atrium of the heart was then exposed and a catheter inserted into the superior vena cava via the right atrium. The liver was perfused with Ca^{2+} free KRPB at a rate of 40ml/min for 10 minutes. The flow rate was adjusted to ensure that the liver did not incur any excessive pressure fluctuations, which may have resulted in decreased viability due to cell damage. Following this, perfusion was continued with Ca^{2+} free KRHB for 10 minutes. 96mg collagenase H (0.15U/mg lyophilizate) and 117mg $CaCl_2.H_2O$ were dissolved in 20ml of Ca^{2+} free KRHB, filtered and added to the remaining 200ml of KRHB. This was then re-circulated for up to 20 minutes. Re-circulation was continued until the liver exhibited signs that it was sufficiently digested to remove it from the rat. This is generally indicated by the liver's inability to resist pressure when applied with the blunt edge of a pair of forceps, resulting in a depression occurring for a number of seconds. The softened liver was dissected from the rat and

transferred to a sterile 200ml beaker containing 20ml of KRHB. The tissue was gently teased apart and passed though a 120-μm bolting cloth (Lockertex, Warrington UK), which was secured over a sterile beaker. Approximately 100ml of KRHB was passed through the blotting cloth with gentle agitation of the tissue using a glass rod. This allowed free single hepatocytes from the digested liver to pass through the bolting cloth. The filtrate was divided into four 30ml tubes and centrifuged at 50g for 3 minutes. The resultant pellet was washed in ice cold KRHB and pelleted as above twice more. The final pellet was resuspended in ice cold CL15 medium (see section 2.6.4) and the cell viability was determined by Trypan Blue exclusion (see section 2.6.3). Cells of viability > 85% were plated out on 25cm^2 flasks (see section 2.6.4).

Section 2.6.2 Modified isolation of primary rat hepatocytes

A second modified hepatocyte protocol was carried out using individual rat liver lobes and based on techniques described by Strom *et al.* (1982) and Oldham *et al.* (1985). Strict aseptic techniques were maintained throughout the preparation. The buffers used were perfusion buffer, pH 7.4, containing Earle's balanced salt solution (EBSS, 10X concentrate, Ca^{2+} and Mg^{2+} free) with $NaHCO_3$ (3% v/v of a 7.5% w/v stock solution). Chelating buffer pH 7.4 comprised of perfusion buffer containing 0.5mM EGTA as the chelating agent. Digestion buffer pH 7.4 comprised of perfusion buffer containing collagenase A (Boehringer Mannheim, 0.19-0.23 units/ml final concentration (about 40-60mg)), 2mM $CaCl_2$ and 10mg trypsin inhibitor (per 150ml). A Dispersal buffer (pH 7.4) was used after the digestion step and consisted of 10mM HEPES, 140mM NaCl, 9.6mM KCl and 1% w/v BSA. With the exception of the dispersal buffer which was kept at 4°C, these buffers were maintained at 37°C and gassed continuously with 95% O_2/ 5% CO_2. Two rats were killed by diethyl ether anaesthesia and the complete liver excised and placed in a beaker containing 50-100ml of ice cold perfusion buffer. Care was taken not to damage the capsule. Perfusion was carried out within 10 minutes of the livers being excised from the animals. The largest two lobes of the liver were separated by dissection and the major exposed vessels can-

nulated with 20 gauge catheters, this was repeated for the other excised liver. The lobes were perfused simultaneously with chelating buffer at a flow rate of 6ml/min/cannula for 10 minutes to remove calcium. The flow rate was adjusted to ensure that the liver did not incur any excessive pressure fluctuations, which may have resulted in decreased viability due to cell damage. The lobes were then switched to perfusion buffer for 7 minutes to remove the chelating buffer and allow for system dead volume clearance. Following this, perfusion was continued with digestion buffer for 3 minutes (to remove the system dead volume) and then re-circulated for up to 25 minutes to remove the extracellular matrix of the liver lobes. Re-circulation was continued until the lobes exhibited signs that they were sufficiently digested for perfusion to be stopped. Once collagenase digestion was complete, the lobes were carefully transferred to a sterile Petri dish containing 50ml of dispersal buffer containing deoxyribonuclease I (DNAse I, 4mg/100ml). Each individual lobe capsule was broken and gently teased apart with forceps thus releasing isolated cells into the dispersal buffer. The remaining vascular mass was then gently 'combed' using forceps to further release more hepatocytes from the tissue. The cell suspension was then filtered through pre-wetted 64μm nylon bolting cloth (Brunner Scientific, Yorkshire). Approximately 50ml of dispersal buffer were passed through the bolting cloth to wash through the remaining isolated hepatocytes. The filtrate was divided into four 30ml tubes and centrifuged at 50g for 5 minutes at 4^{o}C. The supernatant was poured off and the resultant pellet was resuspended and washed in ice cold dispersal buffer containing DNAse I. The suspension was passed though a fresh nylon blotting cloth and centrifuged once more. The supernatant was poured off and the pellet resuspended in dispersal buffer (without DNAse I), filtered though bolting cloth and re-centrifuged. The cell pellets were resuspended in the appropriate culture medium (CL15 or Chees) (see section 2.6.4) and the cell viability was determined by Trypan Blue exclusion (see section 2.6.3). Cell populations of > 85% viability were plated out on 25cm^2 flasks (See section 2.6.4).

Section 2.6.3 Enumeration of isolated hepatocytes

The viability of isolated primary hepatocytes must be determined before cells can be seeded; this is to ensure that the correct numbers of viable cells are present in the culture system. Viability is tested for via the use of a vital stain such as Trypan blue. Trypan blue stain is only taken up by non-viable cells which have damaged plasma membranes and as a consequence do not exclude the polar stain. Percentage viability can be ascertained with this exclusion dye using a haemocytometer. Relative numbers of blue to white stained hepatocytes following Trypan blue treatment enables both the viability and concentration of the cell suspension to be determined. To test the viability of the hepatocyte suspension, 250µl of hepatocyte suspension is mixed with 100µl of Trypan blue dye (0.4% (w/v) in Phosphate buffered saline). This solution is placed into an 'Improved Neubauer haemocytometer' and counted. The 'Improved Neubauer haemocytometer' uses a five by five (50µm x 50µm) grid. Five of these squares were counted for both total numbers of cells and numbers of non-viable cells. Counts from the five squares were totalled and the viability and concentration of the cell suspension calculated:

$$\text{Viability (\%)} = \left[1 - \left(\frac{\text{number of blue cells}}{\text{total number of cells}} \right) \right] \times 100$$

$$\text{Concentration of viable cells (cells/ml)} = \frac{\left[\left(\text{total number of cells} \times \frac{7}{5} \times 5 \times 10^4 \right) \right] \times \text{viability}}{100}$$

Section 2.6.4 Primary Rat Hepatocyte Culture

Primary monolayer cultures of hepatocytes were prepared in the following manner: Cells were diluted to a concentration of 2×10^6 viable cells/ml (as calculated in section 2.6.3) in CL15 medium*. CL15 medium is composed of Liebowitz L15 media containing foetal bovine serum (8.3%), tryptose phosphate broth (8.3%), penicillin G (41.3 IU/ml), streptomycin sulphate (8.2µg/ml), L-glutamine (241 µg/ml), insulin (10^{-6}M), and hydrocortisone-21-hemisuccinate

(10^{-5}M). 3ml of CL15 media was added to the required number of flasks to which a further 1ml of diluted cells was added, giving an initial plating density of $2x10^6$ viable hepatocytes per $25cm^2$ Falcon flask. Flask lids were loosely tightened to allow gaseous exchange to occur between the incubator and the cell culture system. Flasks were gently agitated to ensure even distribution of the hepatocytes across the flask surface. The flasks were then incubated at 37°C in a humidified 100% air incubator. If a 95% O_2/5% CO_2: air humidified incubator is used in the culture system, then sodium hydrogen carbonate (20mM) must also be present in the CL15 medium to act as a buffer. Four hours after seeding the spent media and unattached cells were carefully removed and fresh media added. Cells were left overnight to allow complete attachment to occur. Further media changes were made at 24, 48 and 72 hours after seeding.

⋆ Chees medium is purchased as a complete medium and only required the addition of foetal bovine serum (8.3%), penicillin G (41.3 IU/ml), streptomycin sulphate (8.2μg/ml) and L-glutamine (241 μg/ml)

Section 2.6.5 Matrix components for hepatocyte culture

Section 2.6.5.1 No substratum

Here no pre-treatment of the flasks is required to culture primary rat hepatocytes. The flasks are composed of polystyrene and cells are seeded as described in section 2.6.4

Section 2.6.5.2 Type-I collagen coated substratum

A collagen type I (or type IV) solution can be prepared in the laboratory or purchased commercially, in this case collagen type-I from rat-tail (Sigma cat n° C7761) was used. Collagen type I coated flasks were prepared as described by Sidhu *et al.* (1993) and as detailed by the Sigma protocol sheet. Collagen was dissolved in 0.2% glacial acetic acid (for 6-8 hours or overnight) to give a 2mg/ml stock solution, which was stored at 4°C. A 75μl aliquot of the stock solution was diluted into 600μl with sterile phosphate-buffered saline (PBS) to give a working solution of

150μg/25 cm^2 per flask. The working solution was applied evenly to the surface of the flask with a 1ml sterile pipette. The collagen solution was allowed to bind for several hours at room temperature, 37°C in an incubator or overnight at 2-8°C. The excess collagen was aspirated off and the flask left drying overnight. The dried collagen coated surface was sterilised by overnight exposure to UV light in a sterile tissue culture hood. The flasks were then rinsed 4 times with 3-4ml of sterile distilled water to remove residual acidity. The water was aspirated and the flasks were covered with 3-4ml of sterile culture medium containing foetal bovine serum (8.3%). This final step was carried out 3-4 hours before primary hepatocyte culture suspensions are seeded on to the base membrane of the flasks. Flasks can be stored at 4°C until needed. It is important that the prepared collagen-coated flasks are not allowed to dry out as this results in reduced hepatocyte viability.

Section 2.6.5.3 Matrigel overlay

Matrigel overlay was carried out as described by Sidhu *et al.* (1994). An 11mg/ml stock solution was supplied by Sigma (cat n° E1270). This solution was kept at -20°C and was thawed to 0°C on ice. Care must be taken with the stock solution as above 4°C the solution solidifies, hence all manipulations with matrigel were carried out over ice and all pipette tips were pre-cooled at -80°C. Matrigel was diluted to give a working solution of 5mg/ml. Matrigel was added to the hepatocyte monolayers 4 hours after plating out. A 67μl aliquot of the stock solution was diluted into 1ml with CL15 culture medium (pre-chilled to 4°C) to give a working solution of 333μg/ml. The culture medium was removed from the hepatocyte monolayers and the 1ml working solution applied evenly over the surface of the flask with a 1ml pre-cooled sterile pipette. The matrigel solution was allowed to come to room temperature and a further 3ml of CL15 added to the monolayer and the flasks returned to the 37°C incubator. Daily additions of the matrigel solution were made after each medium change.

Section 2.6.6 Dosing primary hepatocytes

Chemical substances were added after 24 hours in culture and included in all subsequent media changes. Chemical stocks were kept at 4°C for up to a week, while diluted chemicals were made fresh daily. Compounds were either added directly to the culture media in a homogeneous manner or dissolved in a suitable organic solvent such as dimethyl sulphoxide (DMSO) or dimethyl formamide (DMF). Additions of DMF or DMSO never exceeded 10µl per flask, giving a final working concentration of solvent at 0.25% (v/v). This concentration produced no cytotoxicity in the culture system (Nick Plant, Thesis results The University of Nottingham, 1994).

Section 2.7 RNA manipulation

Section 2.7.1 Extraction of RNA

Solutions used in the extraction of RNA must be RNAse free. Glassware and RNA solutions were pre-treated with diethylpyrocarbonate (DEPC) to ethoxyformylate them, and thus inactivate any RNAse present. Plasticware can be treated in a 2-3% hydrogen peroxide solution to remove any RNAse contamination.

Section 2.7.2 Diethylpyrocarbonate treatment of solutions for RNA manipulation

DEPC is a potent inhibitor of RNAse activity. DEPC treated water and solutions were used extensively throughout all RNA studies. A stock solution of DEPC was prepared as a 1:10 solution in 100% ethanol, this solution was then added to solutions to give a final concentration of 0.1% (v/v), and left at room temperature for a minimum of 12 hours or at 37°C for 2-3 hours. DEPC was then deactivated by autoclaving the treated solutions for 20 minutes at 120°C. Solutions such as Tris cannot be DEPC treated as the amine group reacts with the DEPC. In such cases the solutions are prepared from fresh stocks using DEPC-treated water.

Section 2.7.3 Preparation of phenol

Phenol must be equilibrated prior to use to remove contaminant substances and to adjust the pH. Liquefied phenol's acidic nature prevents efficient separation of proteins from nucleotides and so

must be equilibrated to a pH greater than 7.8, to prevent DNA from partitioning into the organic phase. 500g of crystalline phenol was melted at 68°C and to this solution 0.1% (w/v) hydroxyquinoline was added. To the melted phenol an equal volume of 0.5M Tris-HCl (pH 8.0) was added and stirred for 15 minutes. The mixture was then allowed to separate and the aqueous phase was discarded. To the phenolic phase an equal volume of 0.1M Tris-HCl (pH 8.0) was added and stirred for 15 minutes. The mixture was again left to separate and the pH of the phenolic phase was tested, this final extraction step was repeated until the phenolic phase was greater than 7.8. After the phenol is equilibrated and the final aqueous phase has been removed, 0.1 volumes of 0.1M Tris-HCl (pH 8.0) was added. This solution was stored in this form under 0.1M Tris-HCl (pH 8.0) in the dark at 4°C for up to one month.

Note: For the Xie and Rothblum (1991) method of extracting RNA, phenol at pH 7.0 is required. This was produced using the above protocol, except that Tris-HCl (pH 7.0) is used in place of Tris-HCl (pH 8.0).

Section 2.7.4 RNA extraction from tissue sample

RNA was extracted from tissue using the method described by Green *et al.* (1976) and modified by Aufray and Rougeon (1980). Tissue samples were excised from treated animals and immediately flash frozen in liquid nitrogen. The samples were then either processed or stored at -80°C to prevent degradation of the RNA. For the extraction of RNA, 0.5-1.0g of tissue was placed in a sterile RNAse-free glass vial containing 15ml of lysis buffer (6M urea, 3M lithium chloride, 3M sodium acetate and 10% SDS) and homogenised for 30 seconds using a Silverston homogeniser. The solution was homogenised repeatedly in blocks of 30 seconds to prevent overheating, until the sample became a thick homogeneous solution. The homogenate was then mixed by inversion, transferred to an RNAse-free 50ml centrifuge tube and stored at 4°C overnight. The homogenate was then spun at 11000g for 20 minutes at 4°C. The supernatant was discarded to

reveal a clear white pellet, which was then resuspended in 5ml of aqueous buffer (10mM Tris-HCl pH 7.4, 0.2% SDS). This solution was then transferred to a clean 50ml centrifuge tube and mixed with 2.5ml Tris-buffered phenol, pH 8.0 and 2.5ml chloroform: IAA, (24:1). This solution was then shaken vigorously (on a rotary shaker) for 15 minutes, and then spun at 11000g for 20 minutes at 4°C. The aqueous phase was then removed to a fresh tube and phenol: chloroform: IAA (24:24:1) added. This was then mixed and spun as before. The resultant aqueous phase was then added to 5ml of chloroform: IAA (24:1) in a fresh tube, mixed and spun for 10 minutes at 11000g at 4°C. The aqueous phase was then removed and ethanol precipitated (using two volumes of ethanol and 1/4 volume of 3M sodium acetate pH 5.2) overnight at -20°C. This was then spun at 11000g for 30 minutes at 4°C and the pellet washed in 70% ethanol (v/v), and re-pelleted. The final pellet was air-dried and then resuspended in 250µl of DEPC-treated water. The sample was then placed in a 65°C water-bath for 15 minutes to ensure the RNA was dissolved and then stored at -70°C until required.

Section 2.7.5 RNA extraction from primary cell cultures

An alternative protocol was used to extract RNA from cultured hepatocytes, as the yield of RNA from other methods was insufficient (unpublished observations, NJH). This method incorporated the use of guanidinium thiocyanate, which is a strong inhibitor of ribonucleases and is based on the method described by Chomczynski and Sacchi (1987), and modified by Xie and Rothblum (1991). The cell monolayer is harvested using a denaturing solution which must be prepared before the monolayers are rinsed. Solution B consists of 4M-guanidinium thiocyanate and 25mM sodium citrate (pH 7.0). Solution A was prepared by mixing Tris-saturated phenol (section 2.7.3), solution B and 2M sodium acetate (pH 4.0) in a ratio of 1:1:0.1. This mixture was supplemented with 720µl of β-mercaptoethanol per 100ml of solution B and could be stored at 4°C for up to several months. The cell monolayer was harvested by first draining the monolayers of culture medium, followed by washing twice with 5ml Ca^{2+} and Mg^{2+} free PBS. The cells

were lysed using 0.9ml of solution A, a disposable cell scraper was used to dislodge the cells from the flask's surface and the lysate was then transferred to a microfuge tube and placed on ice. To the lysate 100µl of chloroform: IAA, 24:1 was added and the phases mixed by vortexing for 20 seconds. The mixture was kept on ice for 25-30 minutes and the phases separated by centrifuging at 12000g for 20 minutes at 4°C. The aqueous phase was removed to an eppendorf containing 800µl of 100% ethanol. The sample was then vortexed and placed at -70°C for 1-2 hours or at -20°C overnight. The thawed solution was then centrifuged at 12000g for 20 minutes at 4°C to pellet the RNA. The pelleted RNA was rinsed in 70% ethanol, centrifuged for 5 minutes as before and excess moisture blotted. The final pellet was then resuspended in 25µl of DEPC treated water and the sample placed in a 65°C water-bath for 15 minutes to ensure the RNA was dissolved and then stored at -70°C until required.

Section 2.7.6 RNA quantification and integrity

Quantification of the extracted RNA was carried out using the absorbance at 260nm of a diluted sample of RNA. 1µl of RNA was added to 999µl of DEPC water and the mixture placed in a quartz cuvette, DEPC water was used as the blank sample. The A_{260}nm was then determined for the sample and the concentration of the RNA sample was determined as shown below,

RNA concentration (µg/µl) = (A260nm X 40[1]) X dilution factor

[1] For the calculation of DNA concentration the value of 50µg/µl per 1 unit O.D. reading instead of 40.

The integrity of the RNA was determined by visualisation of the 18S and 28S rRNA bands. RNA was analysed by running 1µl of the sample RNA on a 1.0 % agarose TBE gel (DEPC treated, 1 X TBE, 0.1% SDS, 0.1µg/ml ethidium bromide) which was ran at 65 volts for 2-3 hours.

Section 2.7.7 Preparation of riboprobe template DNA

Template DNA which was used to synthesis the RNA probe, was prepared as a large stock solution from which aliquots could be taken. This template DNA stock, must firstly be restriction digested. A master preparation of the restriction digest was prepared thus. 10-15μg of template DNA was digested using 3μl of restriction digest buffer (10X), 4U of restriction digest enzyme, and the solution was made up to 30μl with DEPC water. This mixture was incubated for 1-2 hours at 37°C followed by analysis of 1μl of the restriction digest mixture on an appropiate percentrage TBE-agarose gel.

The linearised template DNA was then purified by inactivating the restriction enzyme by the addition of 100μg/ml of Proteinase K and 0.1% SDS (w/v) to the reaction mixture and incubation at 37°C for an hour. Protein contamination was then removed by phenol:chloroform (1:1, v/v) extraction, followed by centrifugation at 14000g at 4°C for one minute. The aqueous phase containing the purified linear template was then added to 2 volumes of absolute ethanol and 1/4 volume 3M sodium acetate and precipitated for 20 minutes at -20°C. The template was then pelleted by centrifugation at 14000g for 30 minutes at 4°C. The pellet formed was then washed in 200-500μl of 70% ethanol and re-spun as before for a further 5 minutes. The resulting pellet was resuspended in 15μl of DEPC water. This stock of linearised probe template would then be enough for fifteen RNAse protection assays.

Section 2.7.8 Synthesis of RNA probe

In vitro transcription was performed at room temperature by the addition of 1μl of 5X-transcription buffer (200mM Tris-HCl, pH 7.9, 30mM $MgCl_2$, 10mM spermidine, 50mM NaCl), 2μl 100mM DTT, 1μl RNAguard (Pharmacia), 2μl NTP mix (5mM ATP, GTP, UTP), 1μl 100μM CTP, 6μl DEPC water, 5μl [α-^{32}P] CTP (12.5 μM [α-^{32}P] CTP, specific activity 600Ci/mmol), 1.5μl (1-2μg) template DNA and 1μl SP6 or T7 RNA polymerase. The solution was mixed and incubated for an hour according to which polymerase is being used, 42°C for SP6 or

37°C for T7 RNA polymerase. After this time 1µl of DNase I was added and incubated for a further 30 minutes at 37°C, to remove the template DNA. Probe synthesis was tested for efficiency of incorporation of labelled nucleotides by carrying out the DE81 test (section 2.7.9). If an incorporation of 10% or more is achieved then the probe is suitable for use in a protection assay. The probe is purified using 30µl of a phenol:chloroform (1:1 v/v) solution, which is vortexed and centrifuged for one minute at 14000g. The resultant aqueous phase was transfered to a clean eppendorf and ethanol precipitated by the addition of 2 volumes (120µl) of absolute ethanol and 1/4 volume (15µl) of 3M sodium acetate, and left at -20°C for 25 minutes. The probe was pelleted by centrifugation at 14000g for 30 minutes and finally washed in 70% ethanol and spun as before, the resultant pellet was resuspended in 15µl of deionised formamide and stored at -20°C.

Section 2.7.9 Radionucleotide incorporation test

Incorporation of radioactive CTP into the probe was estimated using Whatman DE81 filter paper. DE81 ion-exchange filters have a high affinity for nucleotide chains and a low affinity for single nucleotides. 1µl of the synthesised probe was applied to each of the two halves of the DE81 filter paper and allowed to dry. One half of the filter was then washed in 20ml of 0.5M Na_2HPO_4 for one minute, the wash solution was changed and the procedure repeated a further 4 times. The residual Na_2HPO_4 was then removed by washing the filter twice with 20ml of DEPC water for one minute. The filter was then rinsed twice in 100% ethanol for one minute and then allowed to air-dry. Both filters (washed and unwashed) were then placed into scintillation vials containing 3ml of scintillation fluid and the radioactivity determined for 1 minute on a ^{32}P program using a 1900 TR liquid scintillation analyzer. The amount of incorporation was calculated as detailed below.

$$\frac{\text{counts per second of washed filter}}{\text{counts per second of unwashed filter}} \times 100\% = \text{Incorporation rate (\%)}$$

An incorporation efficiency of greater than 10% was acceptable.

Section 2.7.10 RNAse protection assay

RNAse protection assays were based on a method described by Melton *et al.* (1984) and modified by Bell *et al.* (1991a). 30µg samples of the RNA of interest were ethanol precipitated (two volumes of ethanol and 1/4 volume of 3M sodium acetate pH 5.2) and the pellets stored at -20°C. The pGEM7-2B2-X-lig riboprobe was diluted 100 fold in hybridisation buffer (80% formamide (v/v), 40mM Pipes (pH=6.7), 0.4 mM NaCl, 1mM EDTA (pH 7.5)). 30µl of this solution was then used to resuspend each of the RNA pellets. Samples were heated to 85°C for 3 minutes and then incubated overnight at 45°C. 30µg of yeast tRNA was also ethanol precipitated and resuspended in 30µl of hybridisation buffer, these samples were then heated to 85°C for 3 minutes and incubated overnight at 45°C as with the other RNA samples. These samples were included as the tRNA positive and negative controls. Following hybridisation, 300µl of RNAse buffer (0.35 NaCl, 10mM Tris-HCl (pH =7.5), and 5mM EDTA) was then added to the negative control tRNA sample. The positive control tRNA and the RNA samples of interest were then treated with an RNAse enzyme such as RNAse A or RNAse T1. RNAse A (10mg/ml) was added to 300µl of RNAse buffer at a 1/10000 dilution to give a 1µg/ml working concentration and incubated at 37°C for an hour per RNA sample. RNAse T1 was used at 15 units per 300µl of RNAse buffer and incubated at 37°C for 45 minutes. RNAse activity was terminated by the addition of 20µl of 10% SDS (w/v) and 5µl (50µg) of a 10mg/ml Proteinase K solution to each tube and incubation at 50°C for an hour. Each protected sample was then treated with 800µl of phenol:chloroform (1:1 v/v), vortexed and spun. The aqueous layer was removed and precipitated using two volumes (800µl) of absolute ethanol and one quarter volume (100µl) of 3M sodium acetate. Following precipitation at -20°C for 25 minutes, the samples were spun for 30 minutes at 14000g, the supernatant was discarded and the pellet was washed in 70% ethanol. The pellet was allowed to air dry and then resuspended in 10µl of load buffer (90% formamide (v/v),

0.25% Xylene Cyanol FF (w/v), 0.25% Bromophenol Blue (w/v), 1X TBE). A serial dilution of the probe was also produced and 10µl of this was mixed with 5µl of load buffer, a labelled ladder (see 2.7.13) was also mixed with 5µl of load buffer. All samples were heated to 85°C for 3 minutes and then cooled on ice immediately and pulse centrifuged. Denaturing acrylamide gel electrophoresis (section 2.7.11) was then used to resolve the RNA samples.

Section 2.7.11 Denaturing acrylamide electrophoresis

A denaturing acrylamide electrophoresis gel is used to ensure that separation of RNA is based on absolute size and not secondary structure. Acrylamide electrophoresis was performed using a Biorad Protean II vertical electrophoresis kit. Glass plates used for casting gels were cleaned with acetone to remove all traces of grease and dirt that may interfere with the casting of the gels. Sequagel concentrate (237.5g acrylamide, 12.5g methylene bisacrylamide, and 450g urea (7.5M) per litre) and Sequagel diluent (450g urea (7.5M) per litre) were used to make the 6% gels. The following equations were used to determine the appropriate amounts of concentrate and diluent that was needed to obtain a certain percentage gel.

$$\text{Volume of concentrate needed} = \frac{[(\%\text{ acrylamide gel needed}) \times (\text{volume of gel used})]}{25}$$

$$\text{Volume of 10 X TBE} = 0.1\ (\text{Total gel volume})$$

$$\text{Volume of diluent} = \text{Volume of gel} - (1/10\text{th volume of gel} + \text{volume of concentrate})$$

A 60ml, 6% denaturing acrylamide gel was made using 6ml of 10 X TBE, 14.4ml of sequagel concentrate, 39.6ml of sequagel diluent. The acrylamide was polymerised by the addition of 40µl of TEMED and 500µl of 10% ammonium persulphate and mixed. The acrylamide solution was then pipetted into the gel casting kit according to the manufacture's instructions using 25-well

combs and allowed to polymerise for at least an hour. The gel casting kit was dismantled and the gels connected to the Biorad model E4860 recirculator according to the manufacture's instructions. The lower reservoir was stirred continually and the gel was heated to 55°C when in use. The gels were pre-run at 15V/cm using a Biorad model 3000Xi power pack for at least 30 minutes before use. Prior to loading, wells were flushed with 1 X TBE to remove salts from the sample wells. Samples were heat treated and loaded into the wells. Separation was carried out at 15V/cm (300 V) at 55°C until the Xylene Cyanol FF maker dye had travelled at least 60% through the gel. The gels were placed in fixing solution (10% (v/v) methanol, 10% (v/v) acetic acid) for 30 minutes. Gels were transferred onto 3MM Whatman paper and dried for 4 hours at 85°C, under vacuum, using the Biorad model 583 flatbed gel dryer. Smaller gels were run under similar conditions at 20V/cm in the mini Protean II gel kit. Autoradiographs were treated as detailed in section 2.7.12.

Section 2.7.12 Autoradiograph development

The dried gels were exposed to pre-flashed hyperfilm at -70°C with intensifying screens for between 1-5 days. After exposure, film cassettes were allowed to warm to room temperature and developed in the darkroom. Hyperfilm was immersed in 1X Kodak LX24 X-ray developer for approximately 3 minutes or until bands were clearly visible. The hyperfilm was then rinsed in tap water for 2 minutes, before being immersed in 1X Rapid fixer (Ilford Hypam Fixer) for 3 minutes. The film was finally rinsed thoroughly in tap water and hung up to dry.

Section 2.7.13 [^{32}P] labeling of marker

Klenow labelling was performed on ice by adding 3µl of 5X labelling buffer (250mM NaCl, 150mM Tris-HCl (pH=7.5), 50mM $MgCl_2$), 3.5µl Nuclease-free water, 1.5µl 10mM dATP,dGTP,dTTP, 5µl [α-^{32}P] dCTP (3.3µM [α-^{32}P] dCTP, specific activity 600Ci/mmol), 5µg 100bp ladder (Gibco BRL, Life Technologies) and 1µl of Klenow DNA polymerase. The reaction was then mixed and incubated at room temperature for 2 hours. It is at this point that

the ladder is tested for efficiency of incorporation of labelled nucleotides by carrying out the DE81 test (see section 2.7.9). Typically incorporations of between 40-80% were achieved. 2µl of labelled ladder is mixed with 10µl of 1X TBE and 5µl of loading buffer, heated at 85°C for 3 minutes and then cooled on ice before being loaded onto a denaturing acrylamide electrophoresis gel.

Section 2.8 Transfection protocols

Section 2.8.1 Preparation of cationic liposome formulations

There are various commercially prepared lipofection reagents available such as lipofectin^{TM} and LipofectAMINE^{TM} (Gibco-BRL). Dioleoylphosphatidylethanolamine (DOPE) was obtained from Sigma (Poole, UK) as a liposome (10mg/ml) solution dissolved in chloroform. 1,2-dioley-loxypropyl-3-trimethyl ammonium bromide (DOTMA) was supplied as a lipid solid and was a kind gift from Dr Cliff Elcombe, Zeneca, CTL. The cationic liposomes were prepared as detailed in Felgner *et al.* (1987, 1994) and Hofland *et al.* (1996) by mixing 10mg of solid DOTMA with 10mg of DOPE in 1ml of chloroform. This solution was evaporated to dryness under a stream of nitrogen, in the dark, to produce a greasy yellow lipid residue. Liposomes were prepared by re-suspending the lipid residue in 1-2ml of filter sterilised de-ionised water, which was then vortexed for one minute at room temperature. The suspension was then split into two eppendorfs and sonicated in a 50-60Hz, 80watts Polaron Sonibath for 5 minutes or until the suspension became clear in appearance. Sterile preparations of the cationic liposomes can be stored at 4^{o}C for at least 6 months. This liposome preparation was referred to as DOTMA/DOPE. Each batch of DOTMA/DOPE was verified for its effectiveness by determining transfection efficiency as detailed in section 2.9.1.

Section 2.8.2 Transfection optimisation

The most important aspect for obtaining efficient transfection is the optimisation of transfection conditions for each cell culture system. The nature and stability of the lipid-DNA complex formed is sensitive to the DNA concentration, liposome concentration, time of incubation and cell density. Each of these parameters must be altered so that the greatest transfection efficiency may be obtained. This is normally determined by optimising the liposome concentration whilst maintaining a constant DNA concentration initially, then altering the various parameters based on these findings.

Section 2.8.3 Transient transfection of primary rat hepatocytes

Isolated supercoiled Qiagen prepared plasmid DNA's (pRSV-β-galactosidase, β-gal; chloramphenicol acetyltransferase, pCAT-cont; luciferase, pGL3-LUC) were transfected into primary rat hepatocytes by cationic-liposomes (DOTMA/DOPE; Felgner *et al.,* 1987). Primary rat hepatocytes were prepared as detailed in section 2.6.1 and 2.6.2 and incubated overnight before transfection was carried out. This was to allow the freshly prepared hepatocytes to become stabilised in their new environment. For each transfection the lipid-DNA complexes were prepared as follows. Solution A was prepared by diluting 5-8μg of DNA into 300μl -ve L15 medium. -ve L15 is comprised of CL15 medium without foetal calf serum, tryptose phosphate buffer, penicillin G or streptomycin sulphate. Solution B was prepared by diluting 25-50μg of DOTMA/DOPE into 300μl -ve L15 medium. These two solutions were allowed to stand for 30-40 minutes at room temperature. Solution A and solution B were then gently mixed together and incubated at room temperature for a further 10-15 minutes to give solution C. Solution C may appear cloudy; however, this will not impede the efficiency of transfection. Immediately prior to lipid-DNA addition, the monolayers were washed with 4-6ml of -ve L15 media. 2.4ml of -ve L15 was then added to Solution C, mixed gently, and overlaid onto the drained monolayer. The monolayers were then incubated at 37^{o}C for 5-24 hours (typically 5-6 hours is adequate to obtain good transfection efficiency). Medium was replaced with CL15 post-transfection and dosed if necessary (section 2.6.6). The monolayers were incubated for a further 24-72 hours for transient expression to occur.

Section 2.9 Reporter gene assays

Section 2.9.1 β-Galactosidase histochemistry

β-Galactosidase (β-gal) is a useful internal control for normalising variability in reporter gene activity due to transfection efficiency or cell extract preparation. β-galactosidase can be monitored using a simple photometric assay, by measuring enzymatic hydrolysis of the substrate ONPG (O-nitrophenyl-β-D-galactopyranoside) by β-galactosidase in cell-free extracts. β-galactosidase activity can also be monitored histochemically by the cleavage of the substrate X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactoside). This substrate is hydrolysed to an indolyl, which oxidises to an indoxyl, which then self-couples to form an indigo blue derivative. Histochemical analysis of β-galactosidase activity was carried out as described by Sanes *et al.* (1986). Cultured cells were first rinsed in phosphate buffed saline (PBS) containing 100mM NaCl, 81mM Na_2HPO_4 and 20mM $NaH_2PO_4.2H_2O$, (pH= 7.3) and then fixed for 5 minutes at 4°C in 2% formaldehyde plus 0.2% gluteraldehyde in PBS. The cells were then rinsed with PBS to remove the fixing solution. The monolayer was then overlaid with 3ml of a histochemical reaction mixture containing; 1mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal), 5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6.3H_2O$ and 2mM $MgCl_2$. X-Gal was kept as a stock solution dissolved in dimethoxysulfoxide (DMSO) at 40mg/ml, and then diluted into the reaction mixture. The histochemical reaction mixture was then incubated at 37°C for 12-24 hours or until β-galactosidase activity was evident by the blue colouration of the cell cytoplasm in the fixed monolayer. The β-galactosidase mixture was then replaced with PBS and the numbers of transfected cells were identified at 40X magnification under a dissecting microscope. Percentage transfection was determined by the number of blue transfected cells expressed as a percentage of the total number of cells present.

Section 2.9.2 Harvesting of cell monolayers

The method chosen for harvesting cells was dependent upon the reporter constructs that had been transfected. The first method utilised was based upon the Firefly luciferase assay. This system

used a Reporter Lysis Buffer (RLB), which was specifically developed to lyse cultured mammalian cells and to be compatible with the luciferase, CAT, and β-galactosidase enzyme assays. The second system used a Passive Lysis Buffer (PLB), so that the Dual Luciferase Reporter Assay system could be utilised. Here mammalian cells could be efficiently harvested without the need for freeze-thaw cycles allowing both transfected reporter vectors to be assayed at the same time. For monolayers that were transfected with CAT, LUC or β-gal the monolayer were harvested by first washing the monolayer with 5ml Ca^{2+} and Mg^{2+} free PBS. This was then removed and 900µl of 1X RLB were added to each flask. The flasks where then rocked gently several times to ensure complete coverage of the monolayer for 5 minutes. The cell monolayer was then disrupted using a clean tissue culture cell scraper and the cell extract transferred to a microfuge tube. The cell extract was then subjected to a single freeze-thaw in liquid nitrogen and thawed at room temperature. The thawed extract was vortexed for 15 seconds then centrifuged at 15000rpm for a further 15 seconds. The resultant supernatant was then transferred to a fresh tube and placed on ice for immediate use or stored at -70°C. For cell extracts that were used in the Dual luciferase reporter assay (DLRA) system containing firefly luciferase and *Renilla* luciferase a similar harvest protocol is followed. After the Ca^{2}+ and Mg^{2+} free PBS was removed from each flask, 900µl of 1X PLB were added. The cell monolayer was rocked for 5 minutes, then disrupted using a clean tissue culture cell scraper. The cell extract was then transferred to a fresh microfuge tube and placed on ice for immediate use or stored at -70°C.

Section 2.9.3 Assay for chloramphenicol acetyl transferase

Chloramphenicol acetyl transferase is used as an internal control to determine the transfection efficiency. It is used widely, as eukaryotic cells have no equivalent enzyme to complicate the interpretation of assays for CAT activity. The assay is based on the enzymatic butyrylation of radiolabelled chloramphenicol as detailed by Seed and Sheen (1988). The protein concentration of each sample was determined by the modified Bradford method (Bradford, 1976) as described in

section 2.10.2. A 40μg protein sample of cell extract was mixed with ultra pure water to give a final volume of 50μl and incubated at 65°C for 15 minutes. This was then added to a solution of substrates constituted to give a final volume of 100μl, and comprised of 10μl of 1M Tris-HCl pH 8.0, 5μl [D-threo-[dichloroacetyl-1-^{14}C] chloramphenicol, 54miCi/mmol (Amersham), 25μl of 0.33mM chloramphenicol and 10μl of a 2.5mM n-butyryl-Coenzyme A. Cell extract was then incubated at 37°C for between 5-24 hours. The reaction was terminated by the addition of 2 vols. (200μl) of a 2:1 mixture of 2,6,10,14-tetramethyl-pentadecane: mixed xylenes, then mixed vigorously. After centrifugation for 3 minutes at 15000rpm in a bench-top centrifuge, 90% of the organic phase was removed to a scintillation vial containing 3ml of scintillation fluid and radioactivity determined for 1 minute on a ^{14}C program using a 1900 TR liquid scintillation analyzer. Each sample was carried out in triplicate and a mean taken of the results. A purer organic phase may be obtained by terminating the reaction with two volumes (200μl) of mixed xylenes followed by centrifugation for 3 minutes at 15000rpm in a bench-top centrifuge. The organic phase was removed and extracted twice with one volume (100μl) of 10mM Tris-HCl pH 7.5, 1mM EDTA solution, with complete removal of the aqueous phase after each extraction. The final organic phase was removed to a scintillation vial containing 3ml of scintillation fluid and radioactivity determined for 1 minute on a ^{14}C program using a 1900 TR liquid scintillation analyzer

Section 2.9.4 Luciferase assay system with reporter lysis buffer

Luciferase activity was assayed according to the DeWet *et al.* (1987) protocol and modified according to Promega technical bulletin 161. All reactions were carried out at room temperature, as enzyme activity will decrease by 5-10% if carried out at lower temperatures. Cell extracts were prepared using 1X RLB and protein concentration determined according to section 2.10.2 or section 2.10.3. 100ml of reconstituted luciferase assay reagent (Promega) containing 20mM tricine, 1.07mM $(MgCO_3)_4Mg(OH)_2.5H_2O$, 2.67mM $MgSO_4$, 0.1mM EDTA, 33.3mM DTT,

270mM coenzyme A, 470mM Luciferin and 530mM ATP at pH 7.8 was mixed with 20µl of cell extract in a polystyrene cuvette. This cuvette was then placed immediately in a Packard Picolite luminometer analyzer and the light produced measured. The peak light emission and time course of the reaction was recorded over 10 second intervals for at least five readings or until the luminescence began to decline.

Section 2.9.5 Dual-luciferase reporter assay system (DLRA)

The dual-luciferase assay is based on the work of DeWet *et al.* (1985) and modified according to the Promega technical DLRA manual. All reactions were carried out at room temperature. Cell extracts were prepared using 1X PLB and protein concentration determined according to section 2.10.2 or section 2.10.3. 20µl of cell extract was added to 100µl of luciferase assay reagent II (Promega) and the luminescence generated by the firefly luciferase reaction was measured over four 10-second intervals. The firefly luciferase activity was stopped by adding 100µl of Stop & Glo reagent and the luminescence generated by the *Renilla* luciferase reaction was measured over four 10-second intervals in a Packard Picolite luminometer. Readings were also taken using the Top Count HTS Packard microplate scintillation and luminescence counter using 96 well plate (Corning Costar Corporation, Cambridge). The average luminescence for each luciferase assay was used as the final measurement.

Section 2.9.6 Normalisation of luciferase activity

The effectiveness of a transfection can be monitored, by the use of two reporter genes. The first reporter is used to monitor gene function and related cellular events. The second reporter is used so that a normalisation of the first reporter activity can be made. This is carried out by first subtracting background counts from each reporter assay and then taking the first reporter gene assay value and dividing this value by that of the second reporter gene assay value.

Section 2.10 Protein methodologies

Section 2.10.1 Purification of hepatocyte microsomes

Hepatocytes were cultured for 3 days as detailed in section 2.6.4 before they were harvested. Microsomes were prepared as outlined by Waxman *et al.* (1990). The culture medium was removed and the monolayers rinsed with 3-5ml of ice cold phosphate buffered saline (PBS) containing 100mM NaCl, 81mM Na_2HPO_4 and 20mM $NaH_2PO_4.2H_2O$ (pH 7.3), this was then removed and replaced with a further 3ml of PBS. The monolayers were then harvested by scraping the cells off (all solutions were kept over ice) and pooling 3 flasks to give approximately 8 x 10^6 cells per microsomal sample. A 0.1M sodium phosphate buffer solution was made by mixing 77.4ml Na_2HPO_4 (1M) and 22.6ml $NaH_2PO_4.H_2O$ (1M) together. The harvested cells were made up to 15ml with homogenisation buffer which contained 0.1M sodium phosphate buffer pH 7.4, 10mM EDTA and 1.15% v/v KCl. This solution was placed into a 20ml-Dounce homogenisation tube and the monolayer homogenised with 5-7 stokes of the homogeniser. The total homogenate was placed into polycarbonate centrifuge tubes and spun for 20 minutes at 4°C at 9000g using a pre-chilled centrifuge. The supernatant (S9 fraction) was removed to another tube and balanced using homogenisation buffer. The S9 fraction was then spun at 100,000g for 1 hour at 4°C. The supernatant was discarded and the pellet formed inverted to remove the remnants of the homogenisation buffer. The microsomal pellet was then transferred to a 2ml Dounce homogenisation tube and carefully resuspended in 50µl of Storage buffer which contained 0.1M sodium phosphate buffer pH 7.4, 0.1mM EDTA and 20% v/v glycerol by 5-7 stokes of the homogeniser. The micosomal fraction was stored at -70°C.

Section 2.10.2 Bradford (Coomassie blue) protein assay

Protein concentration was determined using the method described by Bradford, 1967. Bradford reagent is the main component of the Bradford assay, comprised of 100mg Serva blue G dissolved in 100ml of 85% phosphoric acid and 50ml of 95% ethanol, made up to 1 litre. 30µl sample of

cell extract was added to 50µl of 1M NaOH. To this 950µl of Bradford reagent was added and the assay solution vortexed immediately. Absorbance was read at 590nm in an UV cuvette. The protein concentration was determined from a standard curve of bovine serum albumin (BSA) generated between the range of 0-40µg/ml. All assay samples and standards were carried out in triplicate and the resultant means determined. The standard curve of BSA concentration against the absorbance at 590nm produces a linear plot upon which linear regression was carried out. Linear regression was used on the sample readings so that unknown protein sample concentrations could be calculated. Only standard curves that gave rise to $R^2 > 0.95$ were used.

Section 2.10.3 Bicinchoninic acid (BCA) kit for protein determination

This is a commercial kit and is an alternative to the Folin-Ciocalteu reagent for protein determination. The assay is designed for 96 well format with the absorbance of the final solution being read at 562nm on an UV plate reader. The protein concentration was determined from a standard curve of bovine serum albumin (BSA 1mg/ml supplied with the kit) generated between the range of 0-1000µg/ml. The standards were prepared directly into the 96 well plate in the designated wells. Microsomal samples were serially diluted with water to give a maximum volume of 10µl. These 10µl samples were placed into the appropriately designated wells and assayed. BCA reagent must be prepared immediately prior to use by mixing 50 parts BCA with 1 part copper sulphate solution (present in kit). 200µl of this solution were immediately added to the standards and diluted samples and the plate incubated at 37°C for 30 minutes. The plate was removed from the incubator and allowed 5 minutes to return to room temperature. The absorbance of each standard and sample were read at 562nm using an UV plate reader. All assay samples and standards were carried out in duplicate and the resultant means determined. The standard curve of BSA concentration against the mean OD value at 562nm produces a linear plot upon which linear regression was carried out. Linear regression was used on the sample readings so that unknown protein sample concentrations could be calculated. Only standard curves that gave rise to

R^2> 0.95 were used. All samples were diluted down to give stock solutions of 2mg/ml.

Section 2.10.4 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Based on a modified version of that described by Laemmli (1970), SDS-PAGE was performed using a SE 250 Mighty small II electrophoresis kit. Glass plates used for casting gels were cleaned with 100% methanol to remove all traces of grease and dirt that may interfere with the casting of the gels. Using a multi-caster up to 12 separating gels may be cast at the same time. Once these gels were prepared they were stored for up to 2 months at 4°C wrapped in cling-film and placed in a moist airtight container. Separating gels consisted of a final concentration of 7.5% acrylamide/bis-acrylamide (30%), 375mM Tris-HCl (pH 8.8), 0.1% SDS, 0.001% TEMED and 0.005% Ammonium persulphate (APS), and were poured into the casting units to within 0.5-1.0cm from the bottom edge of the well comb then overlaid with 100% ethanol. The gels were left to polymerise for about an hour and the ethanol overlay removed once this has occurred. The stacking gel component of the gel was made immediately prior to electrophoresis. The stacking gel consists of 4% acrylamide/bis-acrylamide (30%), 125mM Tris-HCl (pH 6.8), 0.1% SDS, 0.001% TEMED and 0.005% APS and was poured over the separating gel 0.25cm from the top of the gel kit, the well comb was then inserted before the gel sets. Polymerisation of the gel takes about an hour. The completed gel running apparatus was then placed into its gel tank and the upper and lower reservoirs were filled with the appropriate amounts of electrophoresis buffer which consisted of 25mM Tris-HCl (pH 8.6), 192mM glycine and 0.1 % SDS (w/v). The lower reservoir was stirred continually. The gels were pre-run at 8mA per gel and pre-cooled to 4°C before use. Prior to loading, wells were flushed with electrophoresis buffer to clear the sample wells. Samples for electrophoresis that were at concentrations of 1-2mg/ml were diluted 1:1 using 1X Laemmli sample buffer consisting of 2% SDS, 10% gylcerol, 5% 2-mercaptoethanol, 0.002% bromophenol blue and 0.125M Tris-HCl, (pH 6.8). These diluted samples and standards were then boiled for 3 minutes to denature the proteins and then cooled on ice. 10µl of the

boiled samples, PB standard (rat microsomes prepared from rats dosed with PB at 80mg/kg) and 10µl of high range (M.W 6,500-205,000) coloured markers were loaded into each lane. Electrophoresis was performed using constant current. For stacking the proteins, the gels were run at 8mA per gel until the loading dye had concentrated at the top of the separating gel. The current was then increased to 16mA per gel until the blue running dye had reached the bottom of the gel or the yellow dye marker had reached a suitable resolving distance.

Section 2.10.5 Western blotting and immunodetection

10-20µg of micromal protein was separated using denaturing SDS-PAGE on a 7.5% gel as described in section 2.10.4. The stacking gel was scraped away from the separating gel and discarded. The separating gel was then transferred onto two pieces of 3MM Whatman blotting paper pre-soaked with transfer buffer. Transfer buffer consisted of 25mM Tris-HCl, 192mM glycine, 20% methanol (v/v) and 0.1% SDS (w/v). A piece of nitrocellulose (cut to the size of the gel) was soaked in transfer buffer for 2-3 minutes, then layered over the separating gel, ensuring that no air bubbles were trapped between the gel and the nitro-cellulose sheet. Two more pieces of pre-soaked 3MM Whatman blotting paper were placed over the top of the nitro-cellulose sheet and the resulting 'sandwich' was encased in an Electro-transfer cassette. The cassette was placed into the transfer tank with the gel side of the sandwich closest to the cathode electrode and the nitro-cellulose side closest to the anode electrode. The tank was filled with transfer buffer and the proteins transferred to the nitro-cellulose membrane by Electro-transfer in transfer buffer at 4^{o}C and at a constant current of 200mA for 2-3 hours. Tris buffered saline (TBS) consisted of 20mM Tris-HCl (pH 7.5), 500mM NaCL. Blocking solution consisted of 3% gelatin in Tris buffered saline (TBS) were prepared immediately before transfer was complete. 3% gelatin solution was prepared by dissolving 3g of gelatin in 100ml of TBS; the solution was heated to 50^{o}C with stirring. Once the gelatin had dissolved the solution was cooled sufficiently enough to use for blocking. The Electro-transfer cassette was disassembled once transfer was complete and the

nitro-cellulose sheet was placed into a plastic tray containing 50ml of blocking solution and incubated for 2 hours at room temperature. This step ensured the remaining protein binding sites on the nitro-cellulose surface were blocked. After 2 hours the blocking solution was discarded and the blot washed twice in 50ml of Tween-Tris buffered saline (TTBS) which consisted of 0.5% Tween 20 (polyoxyethylene-sorbitan monolaurate) in TBS. The blot was then incubated in 25ml of antibody solution containing rabbit anti-rat CYP2B1/2 (a kind gift from Professor C.R. Wolf, Dundee) at a 1:1000 dilution in 1.0% gelatin TTBS for 2 hours with rocking. The primary antibody solution could be used several times, depending on the depletion of the anti-body so this was retained and stored at -20^{o}C, the blot was then washed twice in 50ml of TTBS. The blot was then incubated in 30ml of secondary antibody solution containing goat anti-rabbit-alkaline phosphatase (BioRad Immun-Blot kit Cat n^{o} 170-6460) at a 1:3000 dilution in 1% gelatin TTBS for 2 hours at room temperature with rocking. The secondary antibody solution was discarded and the blot washed twice in 50ml of TTBS and twice in 50ml of TBS, this last step was to remove the final traces of Tween 20. The blot was developed using the BioRad Immun-Blot assay kit. Alkaline phosphatase (AP) reagent A contains nitroblue tetrazolium (NBT) in aqueous dimethylformamide (DMF), containing magnesium chloride. AP reagent B contains 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in DMF. Colour development reagents A and B (0.5ml each) were added to 50ml of colour development solution (CDS) and the blot incubated at room temperature until purple bands begin to appear. This colour development generally took about 30 minutes, although blots could be incubated overnight at 4^{o}C if coloured bands were very faint. Once incubation in the CDS did not intensify the coloured bands further the CDS was discarded and the blot rinsed thoroughly in distilled water. The blot was dried on paper towels and stored between 2 sheets of blotting paper in a plastic bag.

Section 2.11 Lactate dehydrogenase (LDH) assay

This method of LDH assay is a modification of the lactate to pyruvate procedure first reported by Wacker *et al.* (1956) and modified later by Gay *et al.* (1968). The assay is based on the ability of LDH (EC 1.1.1.27) to catalyse the reduction of pyruvate to L-lactate with simultaneous oxidaion of NADH to NAD.

$$\text{Pyruvate} + \text{NADH} + H^+ = \text{L-lactate} + NAD^+$$

The change in absorbance at 340nm due to the disappearance of reduced NAD (NADH) over time is directly proportional to the LDH activity and is measured photometrically. The results of the LDH assay are expressed in international units per litre (U/L). LDH can be measured in tissues of various origins, such as liver or kidney. This assay was carried out upon hepatocyte monolayers and extracted culture medium. Aliquots of the cell culture media (500µl) were removed at suitable time points during the experiment and stored at 4°C until all samples were ready to be assayed for LDH activity. At the end of the experiment (48 hours), a final aliquot of cell culture media was removed and the cell monolayer lysed using 0.6% Triton X-100. This was carried out to determine the percentage of total intracellular LDH content left in the cell culture system at 48 hours. The 200µl culture medium samples and the lysed cells were centrifuged at 7000g for 5 minutes. After centrifugation 200µl of the supernatant was removed and placed into clean eppendorfs ready for LDH assay. LDH assay was carried out using the Unimate 3 LDH SFBC kit (Roche Products Limited, UK). Reagent R1 consisted of 0.2mM NADH and reagent R2 consisted of 80mM Tris-HCl buffer (pH 7.2), 1.6mM pyruvate, 200mM NaCl and 0.1% NaN_3. The contents of vial R1 were reconstituted with 6ml of vial R2 (these regents were stable for 3 weeks at 4°C, or 3 days at 25°C). The assay was carried out using 1ml of combined reagent R1 and R2, 100µl distilled water and 20µl of sample. The absorbance was read using a Cobas Fara II centrifugal analyser according to the manufacture's instructions.

Section 2.12 Statistical analysis

Data was collected and analysed using Microsoft Excel 5 and mathematical functions undertaken within the program to determine percentages and standard deviation within groups. Differences between individual groups were tested by the Student t-Test, with a confidence limit of 95% using the computer package Instat.

Chapter 3 Results

Section 3.1 Isolation of primary rat hepatocytes

Primary rat hepatocyte cultures have been used in an attempt to elucidate the molecular processes involved in regulating the induction of CYP2B1/2 genes by chemicals such as phenobarbital. Under conventional culture conditions hepatocytes lose their ability to express CYP2B proteins and are unable to respond to phenobarbital (PB) (Waxman and Azaroff, 1992). The induction of the CYP2B genes by PB, in our primary rat hepatocyte culture system was investigated using modifications of standard culture techniques.

In this thesis two modifications of the primary hepatocyte isolation procedure were utilised : -

(i) Primary rat hepatocytes were isolated as described in section 2.6.1 which consisted of the modified two step *in situ* perfusion technique as described by Mitchell *et al.* (1984). Viability was routinely greater than 85%; preparations of lower viability were discarded. Typically $3.0x10^8$ viable hepatocytes were isolated from 200g-250g male Wistar rats.

(ii) A second hepatocyte isolation protocol was carried out using individual rat liver lobes and consisted of a modification of the Strom *et al.* (1982) and the Oldham *et al.* (1985) isolation procedures as described in section 2.6.2. This method produced slightly lower yields of typically $8.5x10^7$ viable hepatocytes from the excised livers of two rats; typically isolations of greater than 80% viability were obtained.

Section 3.2 Expression of CYP2B2 mRNA

Section 3.2.1 Generation of a CYP2B2 probe

The CYP2B1 and CYP2B2 genes have 97% DNA sequence identity. To examine the expression of the CYP2B2 gene a protocol was required with sufficient sequence specificity to distinguish between transcripts of high sequence similarity. This discrimination was achieved using the RNAse protection assay. This assay has been shown to detect >50% of single base-pair

mismatches (Myers *et al.*, 1985). For RNAse protection studies, an RNA probe (riboprobe) was constructed which contained highly divergent regions of the CYP2B transcripts (Kumar *et al.*, 1983).

The construct for RNAse protection assays was generated from a rat CYP2B2 cDNA in the pBR322 vector at the BamH I -EcoR I site (a kind gift from Dr. M. Adesnik, pR17) (Adesnik *et al.*, 1981; Kumar *et a*l., 1983). This was transformed into DH5α cells and plasmid DNA was isolated by alkaline lysis mini-preparation. A fragment of the CYP2B family coding region from 1063 bp to 1440 bp was chosen as it contains 17 mismatches between 2B1 and 2B2 out of 379 bases, or 4.6% divergence (Kumar *et al.,* 1983; Fujii-kuriyama *et al.,* 1982). The Bgl II restriction enzyme sites flanking this region enabled a 379 bp fragment to be excised.

This 379 bp fragment was gel excised as described in section 2.4.10 and sub-cloned into the cloning vector pGEM-7Zf(+), at the BamH I site, and designated pGEM7-2B2-Xlig (Xlig). The positive clone generated was then EcoR I/Sac I double digested as a diagnostic test for the inserted fragment (Figure 3.1).

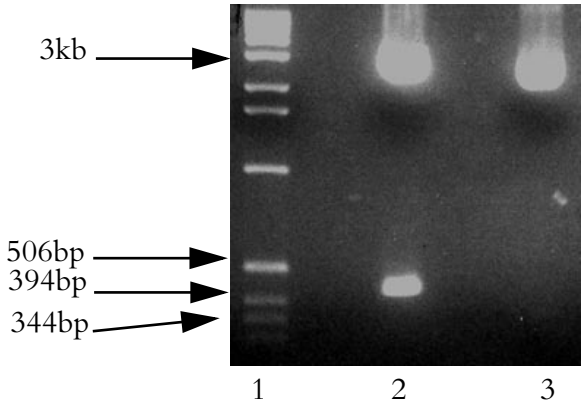

Figure 3.1 A restriction digest of the pGEM7-2B2-Xlig plasmid . DNA was restriction digested with EcoR I and Sac I enzymes and run on a 1.5 % pre-stained 0.1µg/ml ethidium bromide (EtBr) 1xTBE agarose gel at constant voltage of 90V for 2 hours to separate the fragments as described in section 2.4.7. Lane 1 contains 1kb ladder. Lane 2 contains EcoRI /Sac I digested pGEM7-2B2-Xlig giving rise to a 379 bp fragment. Lane 3 contains the uncut plasmid.

Sequence analysis of Xlig plasmid using the SP6 primer confirmed that the 379 bp fragment of the CYP2B2 cDNA was in the correct anti-sense orientation necessary for the generation of an effective riboprobe. This sequence was compared to the published (Kumar *et al.,* 1983) cDNA sequence as shown in Figure 3.2 for verification.

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 28 CAGATCTGGTATGTTGGAGGTATTTTTGCAATGTCACTCTCCTTGGGCGT 77
    ||||||||||||||||||||||||||||||||||||||||||||||||||
620 CAGATCTGGTATGTTGGAGGTATTTTTGCAATGTCACTCTCCTTGGGCGT 571
           .         .         .         .         .
 78 GAGGTCAATGTCCTTGGGAGCCAAATGGCTTGACACAGAGAAGTTCTGGA 127
    ||||||||||||||||||||||||||||||||||||||||||||||||||
570 GAGGTCAATGTCCTTGGGAGCCAAATGGCTTGACACAGAGAAGTTCTGGA 521
           .         .         .         .         .
128 GGGTGGTGGTGAAGAAGAGGAACAATTCATTTCGGGCAATGCCTTCGTCA 177
    ||||||||||||||||||||||||||||||||||||||||||||||||||
520 GGATGGTGGTGAAGAAGAGGAACAATTCATTTCGGGCAATGCCTTCGTCA 471
           .         .         .         .         .
178 AGACAAATGCGCTTTCCTGTGGAGAAGGGCATAAAAGCTTCACTCTTTTT 227
    ||||||||||||||||||||||||||||||||||||||||||||||||||
470 AGACAAATGCGCTTTCCTGTGGAGAAGGGCATAAAAGCTTCACTCTTTTT 421
           .         .         .         .         .
228 CAGTGTCCCATCGGCATCCAGGAAGTGCTCAGGATTGAAGGTGTCTGGA 277
    ||||||||||||||||||||||||||||||||||||||||||||||||||
420 CAGTGTCCCATCGGCATCCAGGAAGTGCTCAGGATTGAAGGTGTCTGGA 372
           .         .         .         .         .
278 TGGTCAAAGTACTGTGGGTCATGGAGAGCTGAACTCAGGATGGGATACA 327
    ||||||||||||||||||||||||||||||||||||||||||||||||||
371 TGGTCAAAGTACTGTGGGTCATGGAGAGCTGAACTCAGGATGGGATACA 323
           .         .         .         .         .
328 CCTCAGTGTTCTTGGGGAAGCAGGTACCCTCCGGAACATGGTGTTCTTTG 377
    ||||||||||||||||||||||||||||||||||||||||||||||||||
322 CCTCAGTGTTCTTGGGGAAGCAGGTACCCTCCGGAACATGGTGTTCTTTG 276
           .         .         .
378 GTGACTCTGTGTGGTAAACCAATTGGGGCAAGATC 412
    |||||||||||||||||||||||||||||||||||
275 GTGACTCTGTGTGGTAAACCAATTGGGGCAAGATC 241
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Figure 3.2 Alignment of CYP2B2 cDNA sequence with Xlig sequence. Purified Xlig CYP2B2 DNA was sequenced using an SP6 primer on an ABI cycle sequencing kit and the ABI 373A fluorescent Sequencer. CYP2B2 cDNA and Xlig cDNA insert were aligned and compared using the BESTFIT tool with the GCG sequence analysis program (Daresbury). The lower sequence is the published CYP2B2 cDNA sequence (Kumar *et al.,* 1983) and the upper sequence is the sub-cloned Xlig sequence. Numbering is based on the values quoted in (Kumar *et al.,* 1983) for the CYP2B2 cDNA.

Xlig anti-sense templates were produced by linearising the plasmid by a Cla I digest, which was visualised on a 1.0 % TBE agarose gel (not shown). The linearised template was prepared and [α-^{32}P] labelled probes (riboprobes) were generated by *in vitro* transcription as described in section 2.7.8.

Section 3.2.2 Extraction of RNA

Total RNA was extracted from both PB treated (80mg/kg i.p. for 3 days) and control rat livers, using the method described in section 2.7.4. Primary cultures of rat hepatocytes were treated for 3 days with 0-1.0mM PB and samples collected as described in section 2.7.5. RNA quantification was determined by spectroscopy at 260nm and the integrity of the RNA was visualised by agarose electrophoresis (Figure 3.3). Typically 0.6-0.8mg of RNA was isolated per 1.5g of liver processed; or 30μg of RNA was produced per tissue culture flask ($2x10^6$ cells) used. RNA produced by these methods gave rise to high purity intact RNA samples, as can be seen in Figure 3.3 where several species of RNA are visible (e.g. 28S, 18S and 5S).

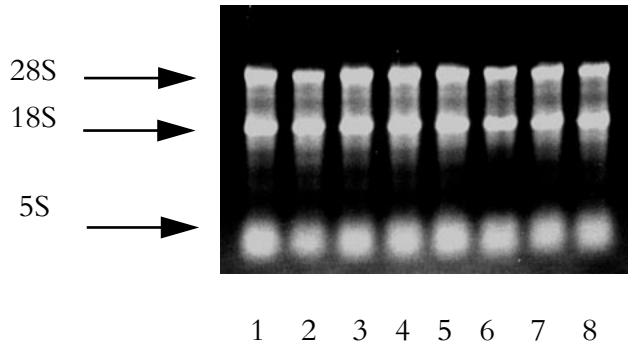

Figure 3.3 Analysis of total primary rat hepatocyte RNA. RNA was prepared from $2x10^6$ rat hepatocytes and resuspended in 30μl of DEPC water. 2μl of the RNA samples were analysed on a 1.0 % denaturing agarose gel (1x TBE, 0.1 % SDS, 0.1μg/ml EtBr) and run at 70V for 2 hours. Lanes 1-8 contain 2μg of RNA from each flask respectively. Arrows indicate 28S, 18S and 5S from top to bottom.

Section 3.2.3 Induction of CYP2B2 RNA by PB

RNAse protection assay was used to examine the expression of CYP2B RNA in both *in vitro* and *in vivo* systems. This technique is able to distinguish between transcripts that exhibit a high degree of sequence similarity (Myers *et al.*, 1985; Lee and Costow, 1987). Data presented by D. Bell (Bars *et al.*, 1993) has shown that the RNAse protection assay could be used to discriminate between CYP2B1 and CYP2B2 RNA species. No data has been presented in this thesis to demonstrate the specificty of the riboprobe for CYP2B2 mRNA. However, based on the data by

D. Bell, it is likely that the riboprobe used currently in this assay reflects a specificity for the CYP2B2 mRNA. An anti-sense riboprobe transcribed from the SP6 polymerase promoter by *in vitro* transcription (section 2.7.8) resulted in a transcript of 426 bp which gave rise to a 379 bp protected fragment. Hybridisation of the riboprobe to RNA, followed by RNAse treatment, results in a protected fragment corresponding to the RNA duplex complex which is resistant to RNAse A. The resulting protected fragments were resolved on a denaturing polyacrylamide gel and visualised by autoradiography. Multiple autoradiograph exposures were taken to ensure the signal was occurring within the linear range of the film.

RNA was obtained from both *in vitro* and *in vivo* samples and treated as detailed in section 2.7.8 and 2.7.10. All samples were treated with 10μg/ml of RNAse A. Yeast tRNA was also included to demonstrate the specificity of the Xlig probe. The negative control lane (T-ve) indicates that the probe is full length and intact. However, upon addition of RNAse A the probe is destroyed, as can be seen in the positive control lane (T+ve). The two bands present in the T+ lane (A1 +A4) are by definition non-specific. The probe produced was intact and full-length, indicating the absence of any exogenous nuclease contamination (Figure 3.4). Figure 3.4 demonstrates that CYP2B2 mRNA is highly induced in the liver of PB treated rats but is only present in lower quantities in the control liver samples. The *in vitro* samples indicate that our primary rat hepatocyte culture system supports CYP2B2 induction albeit at a lower level than *in vivo*. The band indicated by A3 occurs in both *in vitro* and *in vivo* samples. This band was thought to be primarily due to over-digestion of the 2B2:2B2 hybrids during the RNase A step of the protocol. The gel is a composite of the protection assay as a number of lanes have been removed between the T+ve lane and the ladder lanes in the interest of clarity.

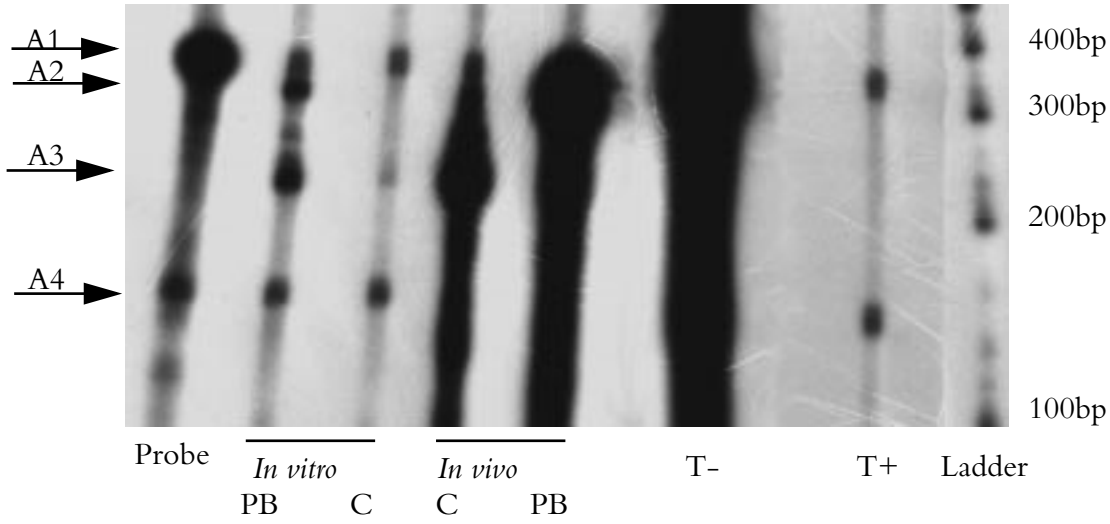

Figure 3.4 Comparison of *in vitro* and *in vivo* PB induced CYP2B2 mRNA samples. RNAse protection assays were carried out on both *in vitro* and *in vivo* rat total RNA samples using an [α-^{32}P] labelled anti-sense riboprobe to CYP2B2 cDNA. Hepatocyte monolayers were treated with control or 1.0mM PB for 3 days and RNA harvested as described in section 2.7.5. Rats were dosed with vehicle, PB 80mg/kg i.p. for 3 days and RNA extracted as described in section 2.7.4. Anti-sense CYP2B2 probe was transcribed using a Cla I cut Xlig plasmid with SP6 polymerase. Full-length Xlig probe was 426 bp in length giving rise to a 379 bp protected fragment. 30µg of total RNA was used for each lane. T+ and T- represent probe incubated with 30µg of yeast tRNA either with or without the addition of RNAse A (10µg/ml). RNAse protection assays were run on a 6% denaturing polyacrylamide gel in 1x TBE at 300V for 3 hours. The gel was fixed, dried and exposed to hyperfilm for 3 days at -70^{o}C. Arrow A1 indicates full-length probe. Arrow A2 indicates protected probe-mRNA complex with the accompanying vector DNA digested away by RNAse A. Arrow A3 + A4 indicates a non-specific protected fragment. The picture is a composite as a number of lanes have been removed between the T+ve lane and the ladder lanes in the interest of clarity.

Section 3.2.4 Optimising the RNAse protection assay

The result presented in Figure 3.4 indicated that there was a difference in the intensity of the response between *in vitro* and *in vivo* samples. This was further examined in subsequent protection assays to determine whether the expression of CYP2B2 mRNA could be optimised. Figure 3.5 demonstrates that the induction of CYP2B2 mRNA was significantly increased above basal levels when comparing 1.0mM PB and 1.0mM clofibric acid (CFA) induced primary rat hepatocytes cultures over 3 days. CFA was used in this experiment as it has previously been shown using Western blotting (Bars *et al.*, 1989) and RNAse protection assays (Bars *et al.*, 1993) to be a strong CYP2B inducer. Here a distinct protected fragment was present for both chemicals used (A2), which was absent in the T+ yeast RNA and control hepatocyte lanes. However, once again multiple protected fragments (A3 +A4) were present at smaller molecular weights. It was thought

that these bands of variable intensity were the result of hybridisation to other CYP2B species which would indicate that the probe was not as specific as first believed, or that the probe was hybridising to other RNA transcripts. Another possibility was that the bands represent over digestion of the 2B2:2B2 hybrids, during the ribonucease digestion step resulting in cleavage of the RNA:RNA hybrids (Kaga *et al.,* 1992; Ambion technical documents on http://www.ambion.com). This overdigestion may be the result of contaminant DNAses present in the stock solution of RNAse A.

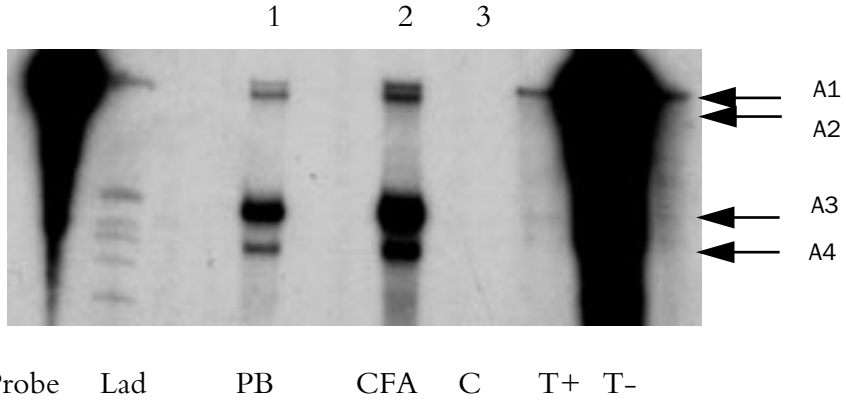

Figure 3.5 Expression of CYP2B2 in CFA and PB treated hepatocyte cultures . RNAse protection assays were carried out on *in vitro* hepatocyte total RNA samples using an [α–^{32}P] labelled anti-sense riboprobe to CYP2B2 cDNA. Hepatocyte monolayers were treated with control, 1.0mM PB or 1.0mM CFA for 3 days and RNA harvested as described in section 2.7.5. Probe was transcribed as in Figure 3.4. 30µg of total RNA was used for each lane. T+ and T- represent probe incubated with 30µg of yeast tRNA either with or without the addition of RNAse A (10µg/ml). Lane 1- 1.0mM PB, Lane 2- 1.0mM CFA. Lane 3- Control. Arrow A1 indicates full-length probe. Arrow A2 indicates protected probe-mRNA complex with the accompanying vector DNA digested away by RNAse A. Arrow A3 + A4 are thought to be non-specific protected fragments.

It was decided that further optimisation of the protection assays was necessary in order to determine the reason for these artefactual bands that were present at the lower molecular weights. Various concentrations of RNAse A were investigated for their ability to generate the lower molecular weight protected fragments. Hepatocytes were dosed with 1.0mM PB for 3 days and RNA collected for analysis. RNA protection assays were carried out as described in section 2.7.8 and 2.7.10 with RNAse A being used at concentrations ranging from 5µg-1µg/ml. Figure 3.6a shows a gradual shift of intensity from the low molecular weight bands (Arrow A3 and A4) to an intensification of the CYP2B2 protected fragment (Arrow A2).

The lower bands were still quite intense, so a second form of ribonuclease digestion was tested involving RNAse T1. Again hepatocytes were treated as before and the protection was carried out as before with various RNAse T1 concentrations from 1unit/μl-100units/μl per 30μg RNA. Figure 3.6b shows that the protected fragments obtained in this case lacked any of the previous artefactual lower weight bands. The gradual increase in RNAse T1 concentration also gave rise to an increase in the protected fragments intensification. Excessive background is present in lane 3 of Figure 3.6b, this is probably due to degradation of the target RNA during the protection protocol. Based on the results of these two experiments it was decided that only RNAse T1 would be used in all subsequent experiments.

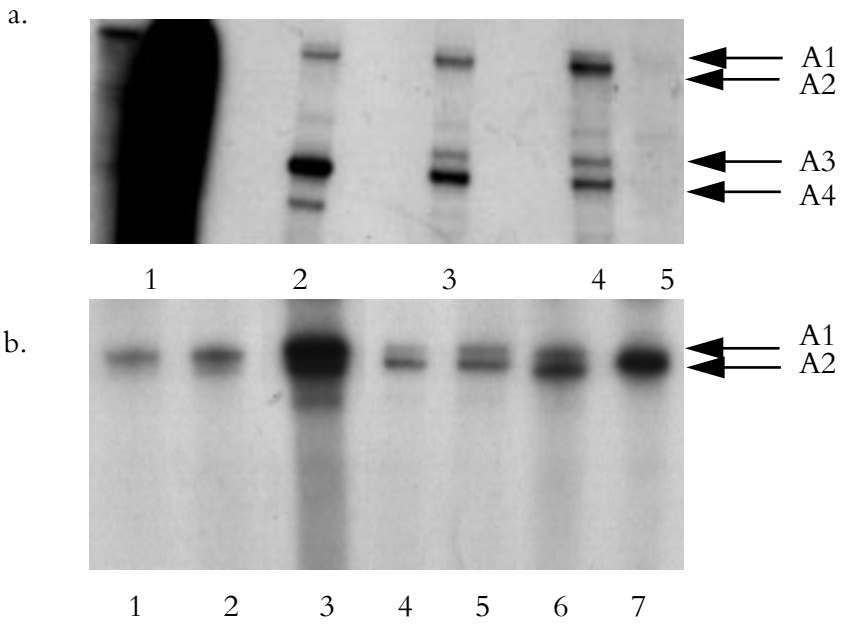

Figure 3.6 Effect of RNAse concentration on RNAse protection for CYP2B2 RNA. Protection assay of CYP2B2 probe against a range of ribonuclease concentrations (a) RNAse A and (b) RNAse T1. Protections were performed as in Figure 3.4. Hepatocytes were treated as in Figure 3.5. (a) RNAse A treated samples, Lane 1 contains full-length probe. Lane 2 contains 5μg/ml RNAse A. Lane 3 contains 2μg/ml RNAse A. Lane 4 contains 1μg/ml RNAse A. Lane 5 control. The gel shows a slow shift from low molecular weight fragments to the full length protected fragment. (b) RNAse T1 treatment, Lane 1 contains 1 unit RNAse T1. Lane 2 contains 2 units RNAse T1. Lane 3 contains 10 units RNAse T1. Lane 4 contains 25 units RNAse T1. Lane 5 contains 50 units RNAse T1. Lane 6 contains 100 units RNAse T1. Lane 7 contains full-length probe.

Section 3.2.5 Xenobiotic induction of CYP2B2 mRNA

The induction of CYP2B2 mRNA by various phenobarbital-like inducers was investigated using the RNAse protection assay. Structurally different xenobiotics such as those shown in Figure 3.7 were applied to primary rat hepatocyte cultures. Analysis of CYP2B mRNA induction by RNAse protection assay allowed determination of whether a range of dissimilar compounds induced CYP2B mRNA.

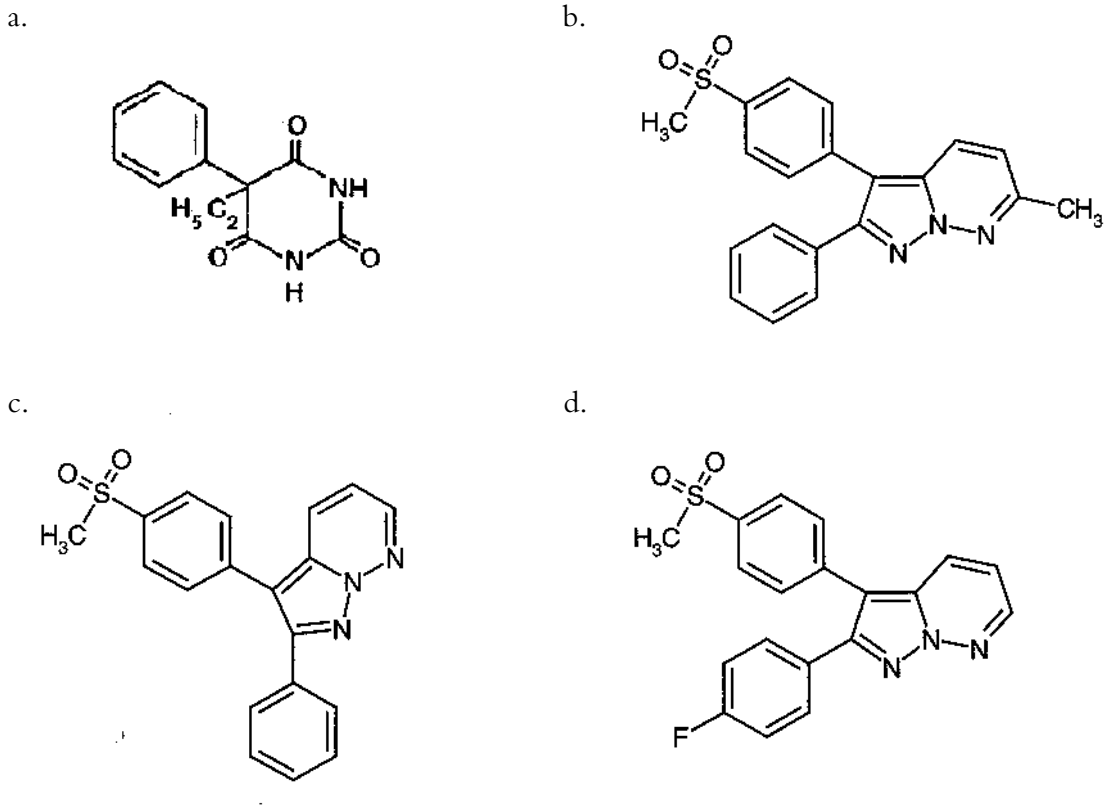

Figure 3.7 Structure of phenobarbital and possible phenobarbital-like inducers.

a. Phenobarbital. b. GWX. c. GWY. d. GWZ.

Rat hepatocytes were dosed for 3 days with three compounds at 25μM and the induction of CYP2B2 mRNA was directly compared with the known CY2B inducer PB. Figure 3.8 shows that of the three compounds tested, only two were shown to be CYP2B2 inducers. The chemical structures of the three Glaxo-Wellcome compounds are very different from that of PB. The result presented in Figure 3.8 shows that the induction of CYP2B mRNA was not dependent upon one specific chemical structure. The comparison of the induction of CYP2B2 mRNA by PB,

GWX, GWY and GWZ treated hepatocytes shows that the Glaxo-Wellcome compounds GWY and GWZ do elicit CYP2B2 induction at the mRNA level. This induction occurs in the μM dose range for the Glaxo-Wellcome compounds and in the mM range for PB. The data also suggests that the two drugs that induce CYP2B2 do so at a level comparable to PB. The absence of intense bands at smaller molecular weights indicates that the artefactual bands present in earlier experiments were due to RNAse A contamination.

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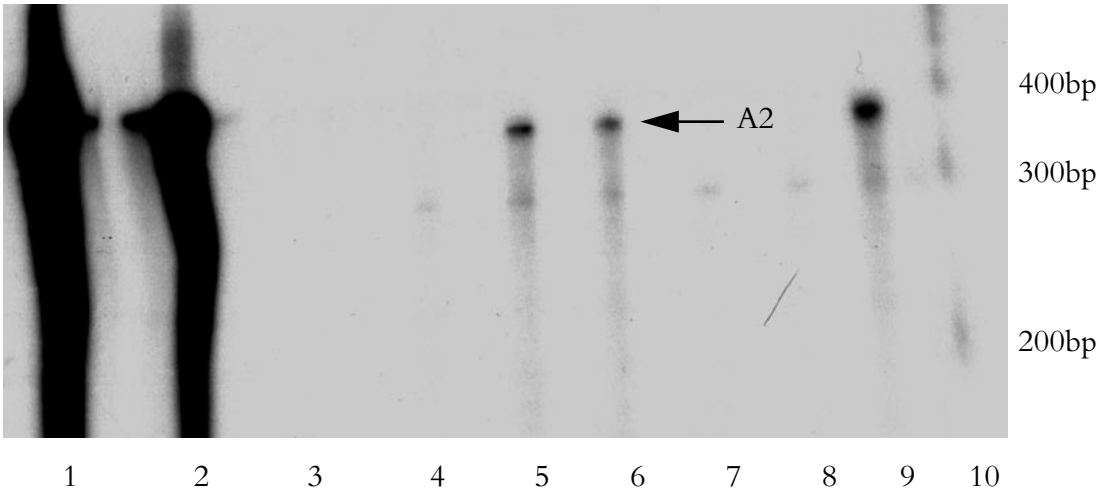


Figure 3.8 Comparison of CYP2B2 induction. Hepatocytes were cultured for 3 days in the presence of 25μM of each drug or 1.0mM PB. 30μg of RNA was isolated and a protection assay carried out as in Figure 3.4, except in this case 15 units of RNAse T1 was used. RNAse protection assays were run on a 6% denaturing polyacrylamide gel in 1x TBE at 280V for 3 hours. The gel was fixed, dried and exposed to hyperfilm for 3 days at -70^{o}C. Lane 1 full-length probe. Lane 2 yeast tRNA without RNAse T1. Lane 3 yeast tRNA with RNAse T1. Lane 4 25μM drug GWX. Lane 5 25μM drug GWY. Lane 6 25μM drug GWZ. Lane 7 control. Lane 8 the drug vehicle DMSO. Lane 9 1.0mM PB. Lane 10 100bp ladder. Arrow A2 indicates protected fragment.

Section 3.2.6 Characterisation of the CYP2B2 response by various xenobiotics

Section 3.2.6.1 Dose response curves of four xenobiotics

The data presented thus far demonstrates that the current *in vitro* system supports CYP2B2 induction at the mRNA level and that differential induction can be monitored using the RNAse protection system. To further characterise the effectiveness of this system, dose response curves were constructed for four structurally dissimilar compounds. The results of these response curves are shown in Figures 3.9-3.12. The first compound to be tested was that of the known CYP2B2

inducer PB (see Figure 3.9). This dose response shows that the basal level of CYP2B2 is too faint to be identified. The same is not true for the PB induced cultures, even at 0.1mM there is a highly induced level of CYP2B2 present.

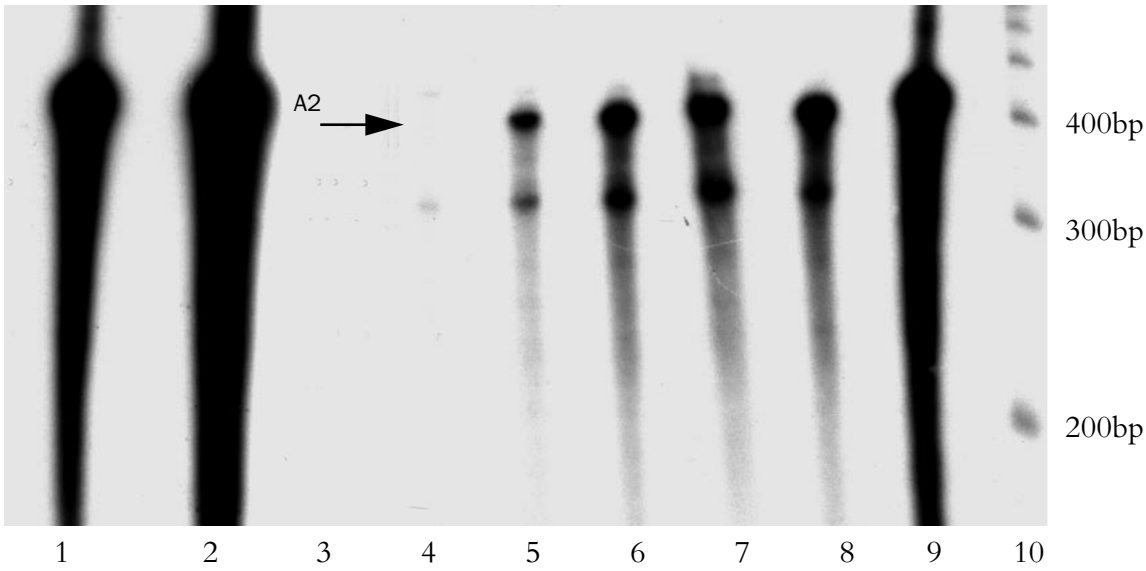


Figure 3.9 Dose response for PB. Hepatocytes were cultured for 3 days in the presence of various concentrations of PB. CYP2B2 mRNA induction was measured as previously by RNAse protection assays using 15 units of RNAse T1. RNAse protection assays were run on a 6% denaturing polyacrylamide gel in 1x TBE at 280V for 3 hours. The gel was fixed, dried and exposed to hyperfilm for 3 days at -70 ^{o}C. Lane 1 Probe. Lane 2 T-ve. Lane 3 T+ve. Lane 4 control. Lane 5 0.1mM. Lane 6 0.25mM. Lane 7 0.5mM. Lane 8 1.0mM. Lane 9 Probe. Lane 10 100bp ladder. Arrow A2 indicates protected fragment.

Figure 3.10 shows the dose response for CFA. This ranges from 0-1.0mM with a visible induction occurring at 0.05mM and steadily increasing to a maximal at 1.0mM. CFA does not dissolve directly into CL15 media (like PB) and so must be dissolved in DMSO; this protection assay shows that DMSO has no effect on CYP2B2 induction.

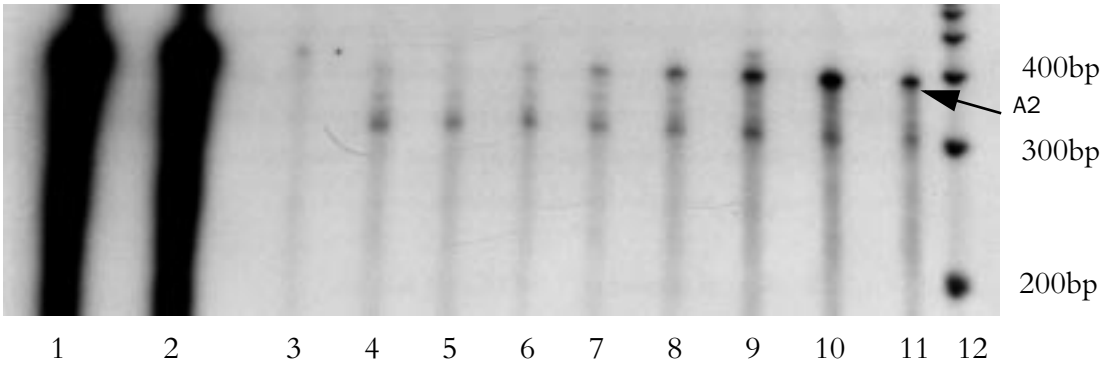


Figure 3.10 Dose response for CFA. Hepatocytes were cultured for 3 days in the presence of various concentrations of CFA. CYP2B2 mRNA induction was measured as previously by RNAse protection assays using 15 units of RNAse T1. RNAse protection assays were run on a 6% denaturing polyacrylamide gel in 1x TBE at 280V for 3 hours. The gel was fixed, dried and exposed to hyperfilm for 3 days at -70 ^{o}C. Lane 1 Probe. Lane 2 T-ve. Lane 3 T+ve. Lane 4 control. Lane 5 DMSO. Lane 6 0.05mM. Lane 7 0.1mM. Lane 8 0.25mM. Lane 9 0.5mM. Lane 10 1.0mM. Lane 11 1.0mM PB. Lane 12 100bp ladder. Arrow A2 indicates protected fragment.

Figure 3.11 shows the dose response for the Glaxo-Wellcome candidate drug GWY. The potency of this drug is far greater than that of the classical CYP2B2 inducer, PB, with a response occurring at 1.0μM. This response increases in intensity up to a maximal at 25μM, diminishing at 50μM possible due to cytotoxicity.

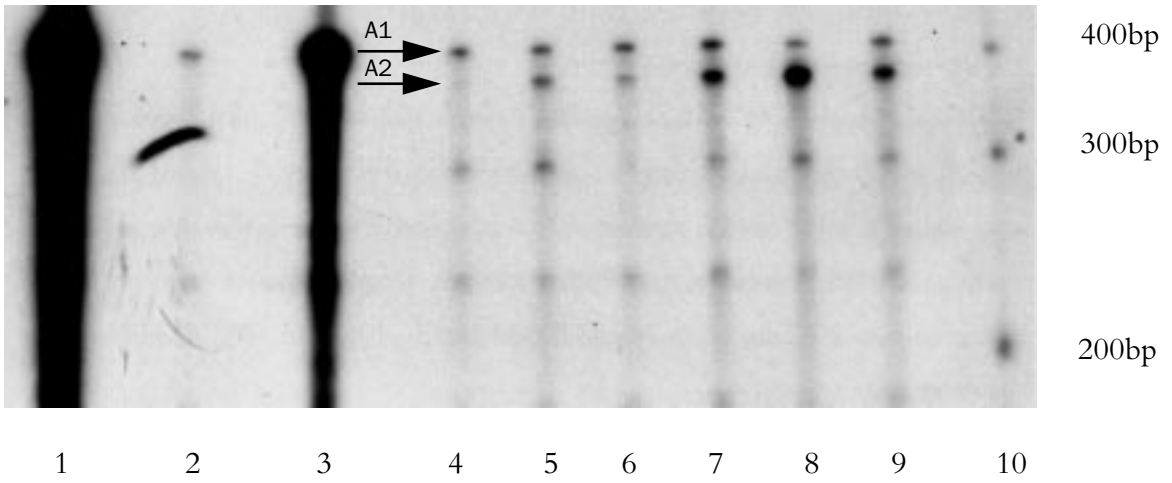


Figure 3.11 Dose response for GWY. Hepatocytes were cultured for 3 days in the presence of various concentrations of GWY. CYP2B2 mRNA induction was measured as previously by RNAse protection assays using 15 units of RNAse T1. RNAse protection assays were run on a 6% denaturing polyacrylamide gel in 1x TBE at 280V for 3 hours. The gel was fixed, dried and exposed to hyperfilm for 3 days at -70 ^{o}C. Lane 1 Probe. Lane 2 T+ve. Lane 3 T-ve. Lane 4 DMSO. Lane 5 1μM. Lane 6 5μM. Lane 7 10μM. Lane 8 25μM. Lane 9 50μM. Lane 10 100bp ladder. Arrow A1 indicates full lenght probe. Arrow A2 indicates protected fragment.

Figure 3.12 shows the final dose response for the Glaxo-Wellcome candidate drug GWZ. This final compound appears to have the greatest potency of all with a response visible at 1.0μM increasing to a maximal at 5-10μM, rapidly tailing off thereafter. With all of the candidate drugs minimal signal with hepatocytes treated with DMSO was apparent.

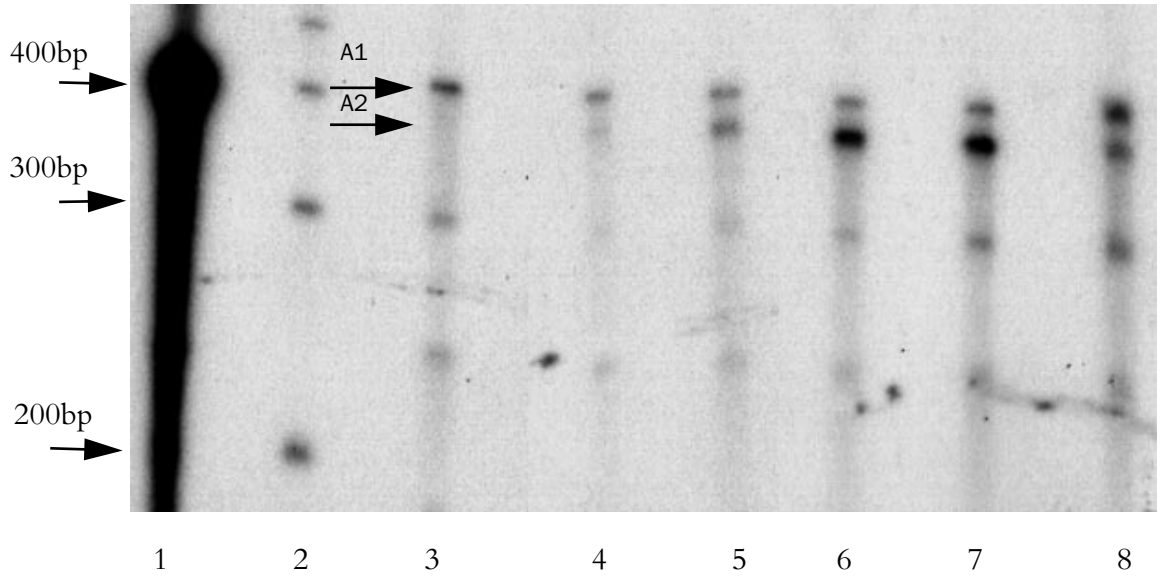


Figure 3.12 Dose response for GWZ. Hepatocytes were cultured for 3 days in the presence of various concentrations of GWZ. CYP2B2 mRNA induction was measured as previously by RNAse protection assays using 15 units of RNAse T1. RNAse protection assays were run on a 6% denaturing polyacrylamide gel in 1x TBE at 280V for 3 hours. The gel was fixed, dried and exposed to hyperfilm for 3 days at -70 ^{o}C. Lane 1 Probe. Lane 2 100bp ladder Lane 3 DMSO. Lane 4 50μM. Lane 5 25μM. Lane 6 10μM. Lane 7 5.0μM. Lane 8 1.0μM. Arrow A1 indicates full lenght probe. Arrow A2 indicates protected fragment.

Section 3.2.6.2 Time course of CYP2B2 mRNA induced by PB

Results obtained from the dose response of PB indicated that a concentration of 1.0mM PB would elicit a strong response in the cultured hepatocytes. This concentration was subsequently used to determine the time course of the CYP2B2 induction by PB. Hepatocytes were dosed with 1.0mM PB from 0-72 hours and the CYP2B2 induction determined by RNAse protection assays as shown in Figure 3.13. The dose response showed that CYP2B2 induction does not increase above basal levels until 24 hours and increases to a maximum at 48 hours before the response begins to decrease. Bands present in the T+ve lane are non-specific.

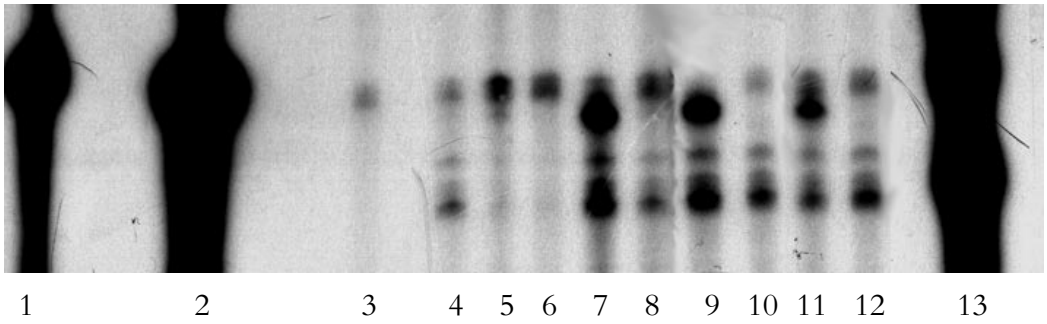


Figure 3.13 Time course of induction of CYP2B2 mRNA by 1.0mM PB. Hepatocytes were cultured in the presence of 1.0mM PB over 72 hours. CYP2B2 mRNA was obtained at the intervals shown in the protection, and induction was measured as previously by RNAse protection assays using 15 units of RNAse T1. Lane 1 probe. Lane 2 T-ve. Lane 3 T+ve. Lane 4 Control (Con) 0 hrs. Lane 5 PB 6 hrs. Lane 6 Con 6 hrs. Lane 7 PB 24 hrs. Lane 8 Con 24 hrs. Lane 9 PB 48 hrs. Lane 10 Con 48 hrs. Lane 11 PB 72 hrs. Lane 12 Con 72 hrs. Lane 13 100bp ladder. Arrow A2 indicates position of protected fragment. The picture is a composite, as a number of lanes have been removed in the interest of clarity.

Section 3.3 Expression and induction of CYP2B proteins

Many groups have reported that specific culture conditions are required for CYP2B induction to occur (Waxman *et al.,* 1990; Sinclair *et al.*, 1990; Sidhu *et al.,* 1993). Western blotting was employed to investigate whether the observed increase in CYP2B mRNA was reflected in a corresponding increase in CYP2B protein in our hepatocyte culture system. Here a comparison between the media used by our group (CL15) and Chee's media was made. Chee's media has been shown to support PB-mediated induction of CYP2B genes by many groups (Waxman *et al.*, 1990; Trottier *et al.*, 1995). Hepatocytes were cultured for 3 days in the presence of 0.5-1.0mM PB in either CL15 or Chee's medium. Rat hepatocyte microsomes were prepared as detailed in section 2.10.1 and CYP2B proteins were separated by electrophoresis through a 7.5% SDS-PAGE prior to electro-blotting onto a nitrocellulose filter. CYP2B protein was detected using a rabbit anti-rat CYP2B1/2 and a goat anti-rabbit alkaline phosphatase kit.

Figure 3.14 demonstrates that a protein of approximately 50 kDa is detected by the antiserum against CYP2B. This result shows that the induction of CYP2B protein in PB induced hepatocytes in our culture system is comparable to the levels obtained with CHEE's media. The level of CYP2B protein expressed in the cultured samples appears to be at a lower level than the

80mg/kg PB *in vivo* rat liver microsome standard. Thus, the CYP2B protein induction presented here mirrors that observed for the CYP2B mRNA.

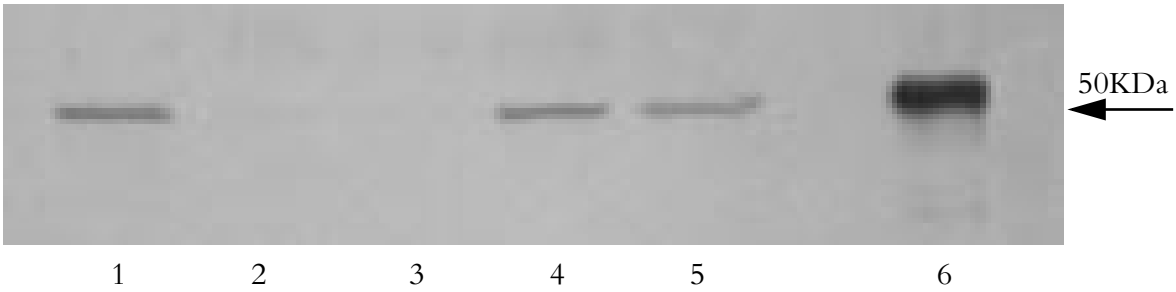


Figure 3.14 Western blot analysis of CYP2B induction. Rat hepatocytes were cultured as described in section 2.6.4 with either CL15 or CHEE's medium and exposed to PB for 72 hours. Microsomal protein was prepared from these cultures ($8x10^6$ cells each sample) and 10µg run on a 7.5% SDS-polyacrylamide gel and blotted onto a nitrocellulose membrane. This membrane was then probed by western blotting using a rabbit anti-rat CYP2B1/2 antibody (1:1000) and goat anti-rabbit alkaline phosphatase antibody (1:3000) as described in section 2.10.5. Lane 1-2 Microsome (10µg) prepared from 1.0mM PB and control hepatocytes cultured in CL15 media. Lane 3-5 Microsome (10µg) prepared from control, 0.5mM and 1.0mM PB treated hepatocytes cultured in Chees media. Lane 6 *in vivo* (80mg/kg, i.p. dosed) PB induced rat liver microsome standard (10µg). Molecular weight of detected protein is indicated by an arrow and is at about 50 kDa as determined by Rainbow molecular weight markers (Sigma), not shown in figure as bands are too faint to detect when scanned.

Section 3.4 Analysis of the 5' flanking region of the rat CYP2B2 gene

Hardwick et al. (1983) has shown that the transcription of the gene encoding CYP2B2 in the rat liver is induced upon treatment with PB. In order to understand the mechanism of CYP2B2 gene regulation that is occurring at the level of transcription, it is necessary to determine the roles of both *cis* and *trans* acting factors that may interact with the CYP2B2 DNA. The sequencing and cloning of the 5'flanking region of the CYP2B2 gene would enable an examination of the elements present and an understanding of the roles that they may play.

CYP2B1 and CYP2B2 comprise the two major PB-inducible genes in the CYP2B subfamily. However, 6 to 8 additional rat genes showing sequence similarity to CYP2B1/2 cDNAs have been identified (Atchison and Adesnik, 1983; Adesnik and Atcinson, 1986; Mizukami *et al.*, 1983; Labbe *et al.*, 1988). Hence, it was necessary to confirm the 5' flanking sequence of our CYP2B2-like gene. This sequence could then be compared to that of other published CYP2B2 gene sequences, so that a comprehensive analysis of the promoter region of the CYP2B2 gene

could be made.

A clone designated λH5b1 was isolated from the library and shown to contain an 11.7 kb EcoR I DNA insert containing the 5' flanking region of a CYP2B2-like gene. A pUC19 vector was linearised with the restriction endonuclease EcoR I and the 11.7 kb λ fragment was ligated into the pUC19 vector at the EcoR I polylinker site (Dr D.R. Bell, Ph.D Thesis 1988, University of London). This was designated the CYP2B2 construct. This CYP2B2 construct (a kind gift from Dr Lesley Forrest) was transformed into XL-1 Blue *E.coli* cells and plasmid DNA isolated so sequencing of the promoter region could be carried out. Figure 3.15 confirms the presence of the 11.7 kb insert with an EcoR I endonuclease digest giving rise to the insert plus the 3 kb vector.

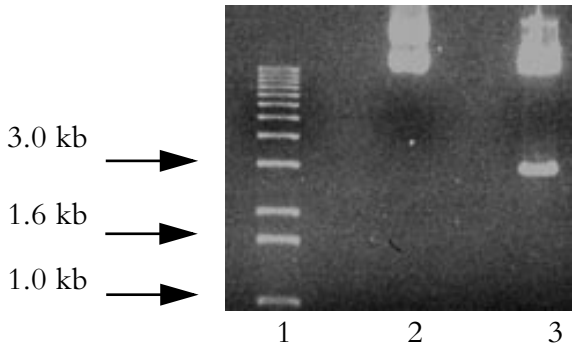


Figure 3.15 A confirmation EcoR I digest of the CYP2B2 construct. CYP2B2 DNA was restriction digested with EcoR I enzyme to confirm that the 11.7 kb insert was present and run on a 0.6 % pre-stained 0.1µg/ml EtBr, 1xTBE agarose gel at constant voltage of 90V for 2 hours to separate the fragments as described in section 2.4.7. Lane 1 1kb ladder. Lane 2 CYP2B2 construct uncut. Lane 3 EcoR1 digested CYP2B2 construct giving rise to 11.7 kb insert.

Once the CYP2B2 construct had been Qiagen miniprepped it was sequenced as described in section 2.4.13 + 2.4.14. The 5' flanking sequence was determined using primers designed initially from published data (Jaiswal *et al.,* 1987). Automated fluorescent sequencing using oligonucleotide walking was used to obtain subsequent sequence data. Sequencing errors were minimised by sequencing on both the forward and reverse strands of the gene, followed by manual evaluation of the fluorescent sequence. Table 3.1 shows the primers used to achieve the 1.3 kb of CYP2B2 sequence.

Name	Primer design 5'-3'	Derivation	Sequence obtained
F1	caccagcccaccactacagtg	Known sequence	531
F2	ggtggcaggacacttagagga	Known sequence	374
F3	ccatttacagctcagactggcc	F2 sequence	415
F4	ggtcttagacaaggtctccc	F3 sequence	330
F5	cctgagctggactccagtgcc	F4 sequence	361
F6	cctctctgtacttctgtc	Consensus sequence	400
F7	gccatctgggaccctggtgcatg	F5 sequence	233
R1	cattgcaggggaggtatgac	F2 sequence	475
R2	ccagcagtggacatgccagag	F3 sequence	407
R3	ggcactggagtccagctcagg	F4 sequence	307
R4	caatgcctttctggatcgtgg	F5 sequence	342
R5	ctttcaactgttcctctgggc	F7 sequence	260
R6	gagcccccatgcaccagggtc	R4 sequence	270

Table 3.1 Primers used to determine the CYP2B2 5'flanking sequence. Primers were designed for use in automated fluorescent sequencing and designated either F or R with regards to the forward or reverse direction of sequencing. The fourth column denoted the length of sequence obtained with each sequence run. Sequencing was carried out as detailed in section 2.4.13 + 2.4.14.

The sequence data obtained from oligonucleotide walking was compiled to give 1.3 kb of consensus CYP2B2 DNA. Figure 3.16a shows a cartoon summarising the position of each primer in Table 3.1 and the approximate length of sequence obtained. Figure 3.16b shows a physical map of the CYP2B2 5'flanking region outlining some of the restriction sites used in subsequent sub-cloning strategies.

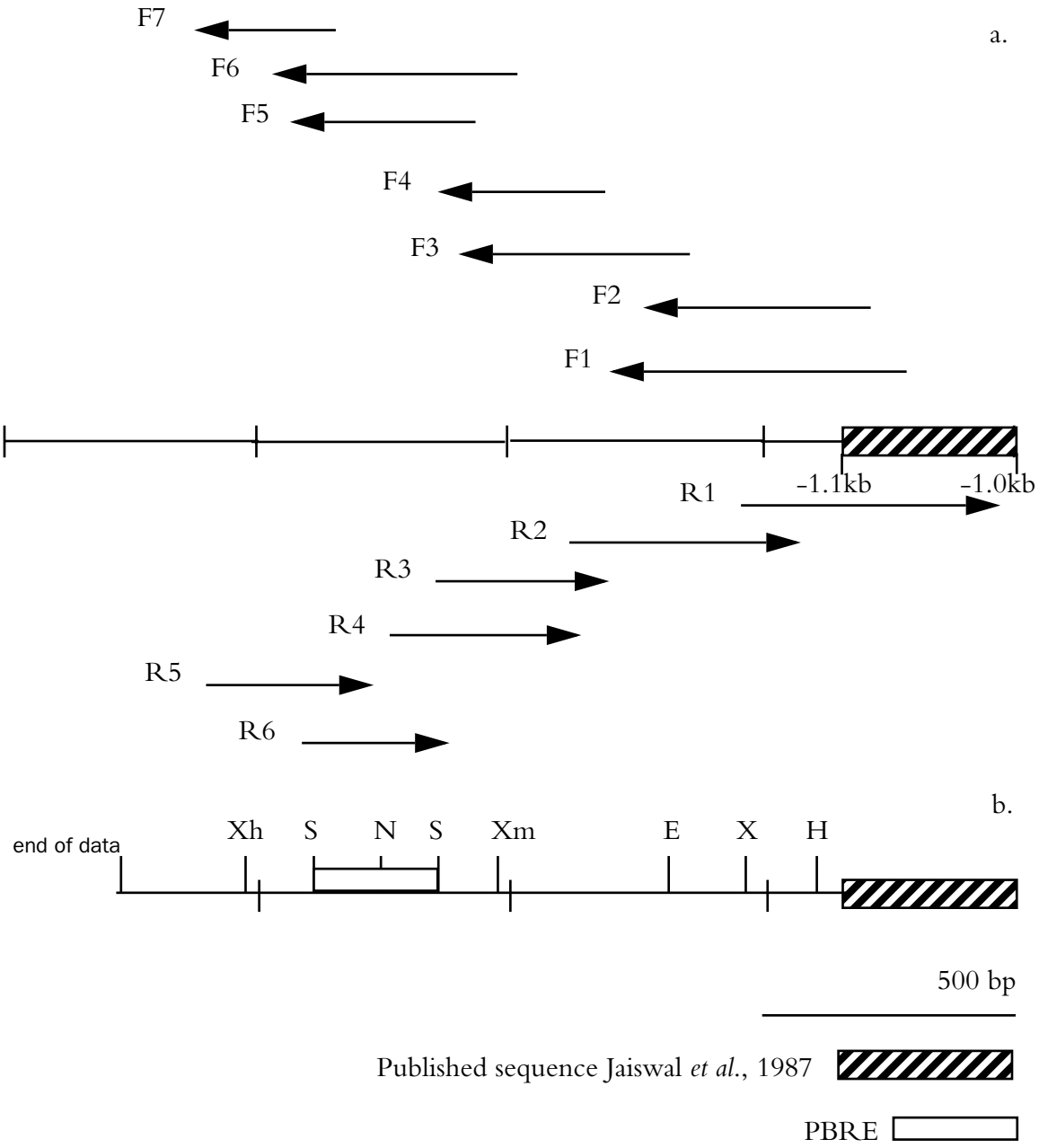
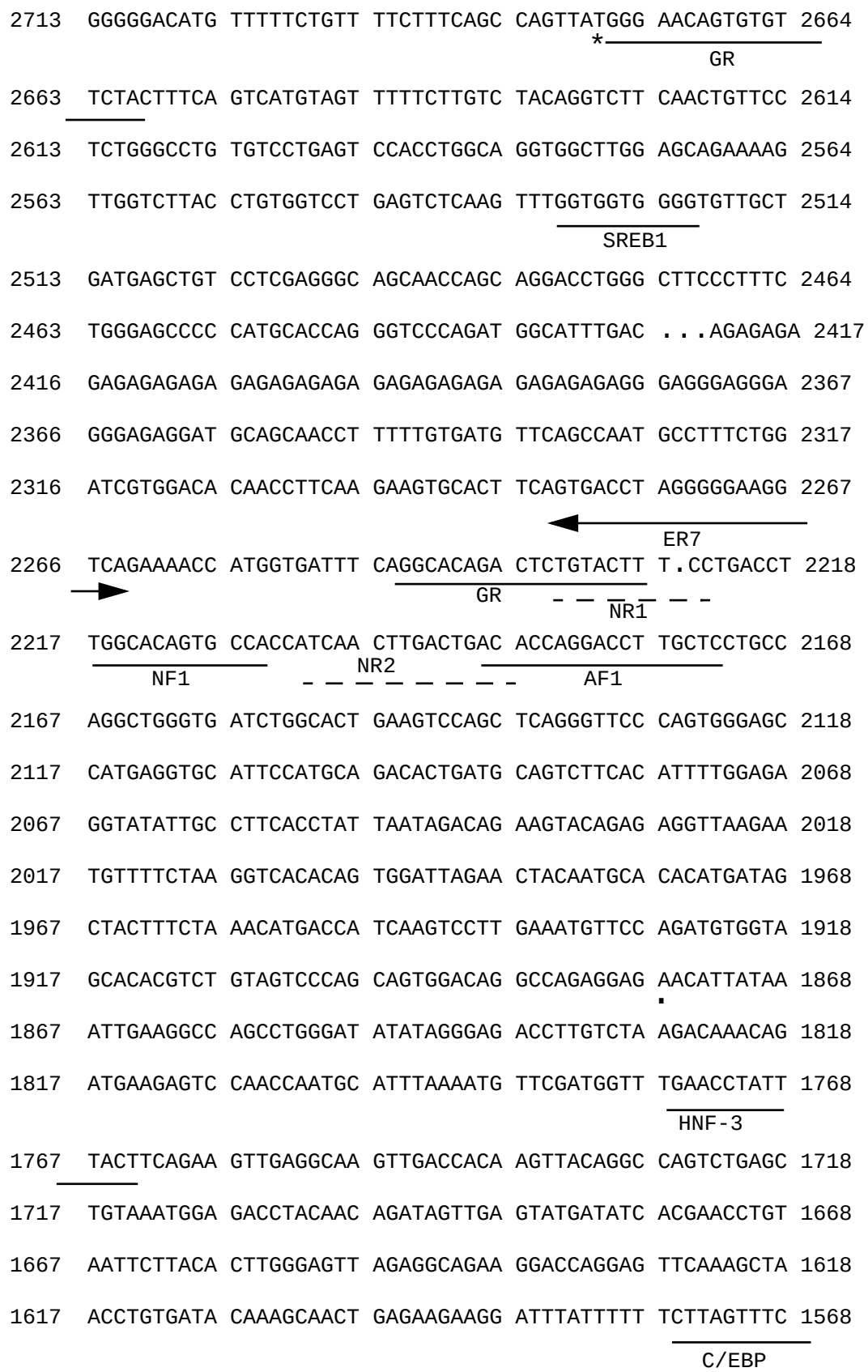


Figure 3.16 Sequencing strategy for the CYP2B2 5' flanking sequence. (a). A cartoon of the CYP2B2 gene is shown indicating the primers used to determine its sequence in both the forward and reverse direction by oligonucleotide-directed automated sequencing. Arrows indicate direction and approximate length of sequencing with primer names shown beside each arrow. (b). The physical map of the 5'flanking region of the CYP2B2 gene is shown. This diagram shows the gene from 1047 bp to 2714 bp upstream including some of the restriction sites used in subsequent sub-cloning strategies. Blank box indicates approximate position of the putative PB response element (PBRE). Restriction sites are labelled thus H: HindIII, X: XbaI, E: EcoRV, Xm: XmnI, S: Sau3AI, N: NcoI, Xh: XhoI. Diagram not to scale.

The promoter region of the CYP2B2 gene whose sequence covers nucleotides -1047 to -2714 bp was determined. This 5' flanking sequence was elucidated by sequence data derived from the primer directed sequencing strategy. Using GELMERGE and GELASSEMBLE tools within the

GCG analysis program, the 1663 nucleotide sequence data was assembled into a single contiguous sequence. The sequence of the CYP2B2 genomic DNA determined by automated sequencing is shown in Figure 3.17. Position 1047 is 1047 bp downstream from the CYP2B2 transcription start site. The sequence of bases 1047-1395 bp is published data (Jaiswal *et al.,* 1987). However, repeat sequencing within this area served to verify that our isolated fragment was in agreement with the published data.

The CYP2B2 sequence was compared to the nucleotide sequence of the published 5' flanking regions of the CYP2B2 gene (Jaiswal *et al.,* 1987; Hoffman *et al.,* 1992) in Figure 3.17. The data presented here highlights the need to sequence the 5' flanking region of the CYP2B2 gene, so that the discrepancies in data published thus far by different groups can be resolved. In the data presented in Figure 3.17 the presence of a * below a nucleotide indicates a base-pair mismatch, here present at -2676 bp where this sequence shows a G nucleotide instead of the published C nucleotide (Hoffman *et al.*, 1992). The presence of a dot (.) in the place of a nucleotide or below a nucleotide indicates an area of sequence where a deletion or an insertion has occurred respectively in comparison to Hoffman's data. Here, insertions are present at -1876 bp (A) and -1527 bp (G). A row of three extra bases at -2423 bp (GAG) is present in Hoffman's data (Hoffman *et al.*, 1992). The deletion of a T residue at -2226 bp in our data is in agreement with that of Stoltz *et al.* (1998). A (+) indicates an unresolved nucleotide in Hoffman's data, these are present at -1447, -1353, and -1264 bp and consist of G nucleotides in our sequence. These G nucleotides are in agreement with data published by Jaiswal *et al.* (1987).


2713 GGGGGACATG TTTTTCTGTT TTCTTTCAGC CAGTTATGGG AACAGTGTGT 2664
GR
2663 TCTACTTTCA GTCATGTAGT TTTTCTTGTC TACAGGTCTT CAACTGTTCC 2614
2613 TCTGGGCCTG TGTCCTGAGT CCACCTGGCA GGTGGCTTGG AGCAGAAAAG 2564
2563 TTGGTCTTAC CTGTGGTCCT GAGTCTCAAG TTTGGTGGTG GGGTGTTGCT 2514
SREB1
2513 GATGAGCTGT CCTCGAGGGC AGCAACCAGC AGGACCTGGG CTTCCCTTTC 2464
2463 TGGGAGCCCC CATGCACCAG GGTCCCAGAT GGCATTTGAC ...AGAGAGA 2417
2416 GAGAGAGAGA GAGAGAGAGA GAGAGAGAGA GAGAGAGAGG GAGGGAGGGA 2367
2366 GGGAGAGGAT GCAGCAACCT TTTTGTGATG TTCAGCCAAT GCCTTTCTGG 2317
2316 ATCGTGGACA CAACCTTCAA GAAGTGCACT TCAGTGACCT AGGGGGAAGG 2267
ER7
2266 TCAGAAAACC ATGGTGATTT CAGGCACAGA CTCTGTACTT T.CCTGACCT 2218
GR
NR1
2217 TGGCACAGTG CCACCATCAA CTTGACTGAC ACCAGGACCT TGCTCCTGCC 2168
NF1
NR2
AF1
2167 AGGCTGGGTG ATCTGGCACT GAAGTCCAGC TCAGGGTTCC CAGTGGGAGC 2118
2117 CATGAGGTGC ATTCCATGCA GACACTGATG CAGTCTTCAC ATTTTGGAGA 2068
2067 GGTATATTGC CTTCACCTAT TAATAGACAG AAGTACAGAG AGGTTAAGAA 2018
2017 TGTTTTCTAA GGTCACACAG TGGATTAGAA CTACAATGCA CACATGATAG 1968
1967 CTACTTTCTA AACATGACCA TCAAGTCCTT GAAATGTTCC AGATGTGGTA 1918
1917 GCACACGTCT GTAGTCCCAG CAGTGGACAG GCCAGAGGAG AACATTATAA 1868
1867 ATTGAAGGCC AGCCTGGGAT ATATAGGGAG ACCTTGTCTA AGACAAACAG 1818
1817 ATGAAGAGTC CAACCAATGC ATTTAAAATG TTCGATGGTT TGAACCTATT 1768
HNF-3
1767 TACTTCAGAA GTTGAGGCAA GTTGACCACA AGTTACAGGC CAGTCTGAGC 1718
1717 TGTAAATGGA GACCTACAAC AGATAGTTGA GTATGATATC ACGAACCTGT 1668
1667 AATTCTTACA CTTGGGAGTT AGAGGCAGAA GGACCAGGAG TTCAAAGCTA 1618
1617 ACCTGTGATA CAAAGCAACT GAGAAGAAGG ATTTATTTTT TCTTAGTTTC 1568
C/EBP

```
1567  ATTGCAGGGG AGGTATGACA TTTGGGGCTT TGGTCTAGAT GTGGGAGCTT 1518

1517  GTGGTTGTTT CTCTATATGG CAGATGGAAC AAGGAGGAGG AAGAGGATAT 1468

1467  ATGTTTATAT GTAGTGGATT TGTGACTCAT TAGGTTGACT CCAAGAATCT 1418
                           +  AP1

1417  GCTTCTTTCT TGCAAGCTTT TCCTCTAAGT GTCCTGCCAC CCCCCACCCC 1368
                                                  Sp1

1367  AATAATATCA GTTAGGGTAC AAAGTGTTCA AACATGAACT TCTGAGACAG 1318
                      +          GR

1317  ATTTCACATT CAAATAGAAC ACATTATGAA TAGATTAATG TTATCACTGT 1268


1267  AGTGGTGGGC TGGTGAAGAA TGTTCAATTC CTTTTAGCAA GATGGAAGGT 1218
        +
      Sp1                          C/EBP          HNF-4
1217  CAAAGAACTT CCTGTGCTAT GAACAAATCA GAAGGATGAA GGGAACCATT 1168

1167  TGTCATTAGA CACAGTGTTC AGAGACTATC TTTGTTAGGT TCACTATTTC 1118
                        GR

1117  TGTGATAAAA CCTCACAGCA GAAAGCAACT TGGGGAAAAG GGTTTATTTT 1068

1067  GCTTGCACTT TACAGTTTGT C 1047
```

Figure 3.17 Nucleotide sequence of the 5' flanking region of the PB-inducible CYP2B2 gene . The sequence shown is 1047 bp downstream of the transcription start site, of which 1.3 kb is beyond that published by Jaiswal *et al.,* 1987. Purified DNA was sequenced using an ABI cycle sequencing kit and an ABI 373A fluorescent sequencer. Areas of mismatch have been resolved by sequencing in both directions. The sequence shown is that derived from the primer directed sequence strategy and has been compared to that of the data published by Hoffman *et al*., 1992. Where base-pair mismatches in sequence occur a * appears. A dot (.) indicates an area of sequence where either a deletion or insertion has occurred. A (+) indicates a matching of a nucleotide with an area in the published data where the nucleotide has not been resolved. Position 1047 is 1047 bp downstream from the CYP2B2 transcription start site. Putative regulatory motifs are underlined and include: GR = glucocorticoid response element. SREBP = sterol regulatory element binding protein. ER = everted repeat. NR = nuclear receptors. NF1 =nuclear factor binding site. AF1 = accessory factor binding site. HNF = hepatocyte nuclear factor. C/EBP = CCAAT enhancer binding protein. Sp1 = transcription factor binding sites.

To further understand the regulation of the CYP2B2 gene, the promoter region of the gene was analysed for any transcription factor binding sites and other putative regulatory motifs (Figure 3.17). By utilising the Transcription Element Search Software (TESS) and the Transfac database (threshold, 0.85), the search revealed the presence of several putative protein factor binding sites. These sites may serve as *cis* acting elements for transcription, with many sequences resembling the binding sites for transcription factors including Sp1, AP1, C/EBP, HNF-3, HNF-4, and GR present.

Upon closer inspection of the 5' flanking sequence, within the section which has been reported to confer PB-responsiveness (Honkakoski and Negishi, 1997; Troittier *et al.,* 1995; Sueyoshi *et al.,* 1999), the PB-responsive enhancer module (PBREM) was found to be present. The PBREM present at -2318 to -2155 bp was shown to contain multiple potential enhancer elements, the central core consisting of a Nuclear Factor 1 binding site (NF1) ($TGGN_7CCA$ on the sense strand) at - 2218 to -2205 bp. This NF1 element was flanked on both sides by accessory elements such as the downstream (AF1) accessory factor 1 element and the upstream glucocorticoid response element (GR). Two Nuclear receptor binding motifs (AGGTCA) designated NR1 and NR2 (dashed lines) are found flanking either side of the NF1 core element (Honkakoski *et al.,* 1998b.). A section of the 5' flanking region was shown to contain two sets of repeat sequences which comprised of an alternating purines $(GA)_{21}$ section followed by a $(GGGA)_4$ repeat sequence. The AP1 site present at -1441 bp is also in agreement with data presented by Roe *et al.* (1996). It is not known if the GR at -2664 to -2682 bp and the sterol regulatory element binding protein at -2523 to -2534 bp have any significance in the PB response.

Once the 5' flanking sequence of the CYP2B gene had been determined it was then possible to construct reporter genes containing sections of the PBREM region. These reporter genes would enable us to determine and dissect the significance of each of the response elements upon PB mediated CYP2B induction.

Section 3.5 Isolation of the CYP2B2 putative PB response element (PBRE)

Trottier *et al.* (1995) localised a phenobarbital-responsive element (PBRE) in the 5' flanking region of the rat CYP2B2 gene. This work was also mirrored by the localisation of the phenobarbital responsive enhancer module (PBREM) in the mouse *Cyp*2b10 gene by Honkakoski and Negishi (1997). Based on the data presented by these groups and coupled to the elucidation of the full restriction map of the 5' flanking region, it was possible to devise a sub-cloning strategy to further characterise the PBRE/PBREM. The PBRE has been localised to a 163 bp Sau3AI flanked region situated 2155-2318 bp upstream from the rat CYP2B2 transcription start site. Using the map shown in Figure 3.16b it was possible to isolate a 969 bp fragment using Xba I/Xho I digest of the pUC19-CYP2B2 construct (Figure 3.18) which included the putative PBRE.

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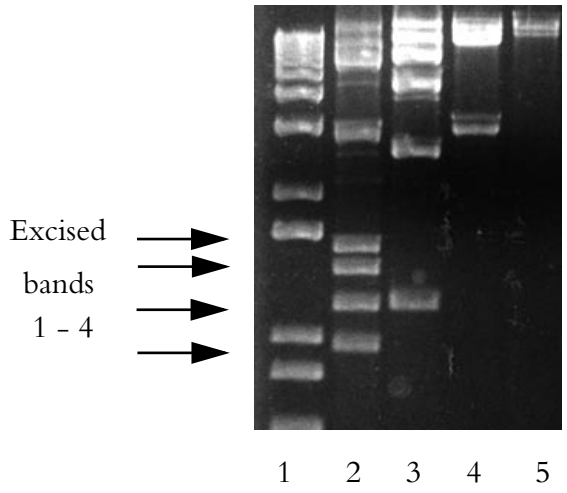

Figure 3.18 Restriction digest of the 969 bp PBRE containing region. DNA was restriction digested with Xba I and Xho I enzymes and run on a 1.5 % pre-stained 0.1µg/ml EtBr, 1xTBE agarose gel at constant voltage of 70V for 2 hours to separate the fragments of the pUC19-CYP2B2 DNA. Lane 1 1 kb+ ladder. Lane 2 Xba I/Xho I digest. Lane 3 Xba I digest. Lane 4 Xho I digest. Lane 5 uncut plasmid. Arrow 1-4 indicated fragments that were Gene-cleaned and used for sub-cloning.

Multiple bands were obtained from the Xba I/ Xho I digest, so each band was Gene-cleaned and ligated into the alkaline phosphatased Xba I/Xho I site of the pGEM-7 vector. Only with the lowest extracted band was the XXpGem7 clone produced. The 969 bp insert was cut out of

XXpGEM7 using a Kpn I/Xba I digest and cloned into the Kpn I/ Nhe I cut pGL3-promoter vector, producing the clone 1Kb-Pluc. Diagnostic restriction digests as shown by Figure 3.19 verified this 1Kb-Pluc clone contained the 969 bp. A cartoon of this sub-cloning strategy is given in Figure 3.20a This clone was then sequenced using the primer RVP3 5' CTT TAT GTT TTT GGC GTC TTC 3' as confirmation of the clone's integrity.

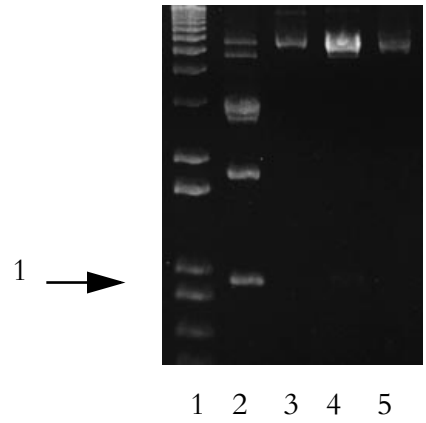

Figure 3.19 Restriction digest of the 1Kb-Pluc clone. 1Kb-Pluc DNA was restriction digested with Xba I and Xho I enzymes and run on a 1.5 % pre-stained 0.1µg/ml EtBr, 1xTBE agarose gel at constant voltage of 70V for 2 hours to show the presence of the Xba I/Xho I fragment from 1Kb-Pluc. Lane 1 1 kb+ ladder. Lane 2 Xba I/ Xho I digest. Lane 3 Xba I digest. Lane 4 Xho I digest. Lane 5 uncut plasmid. Arrow 1 indicates the PBRE containing fragment.

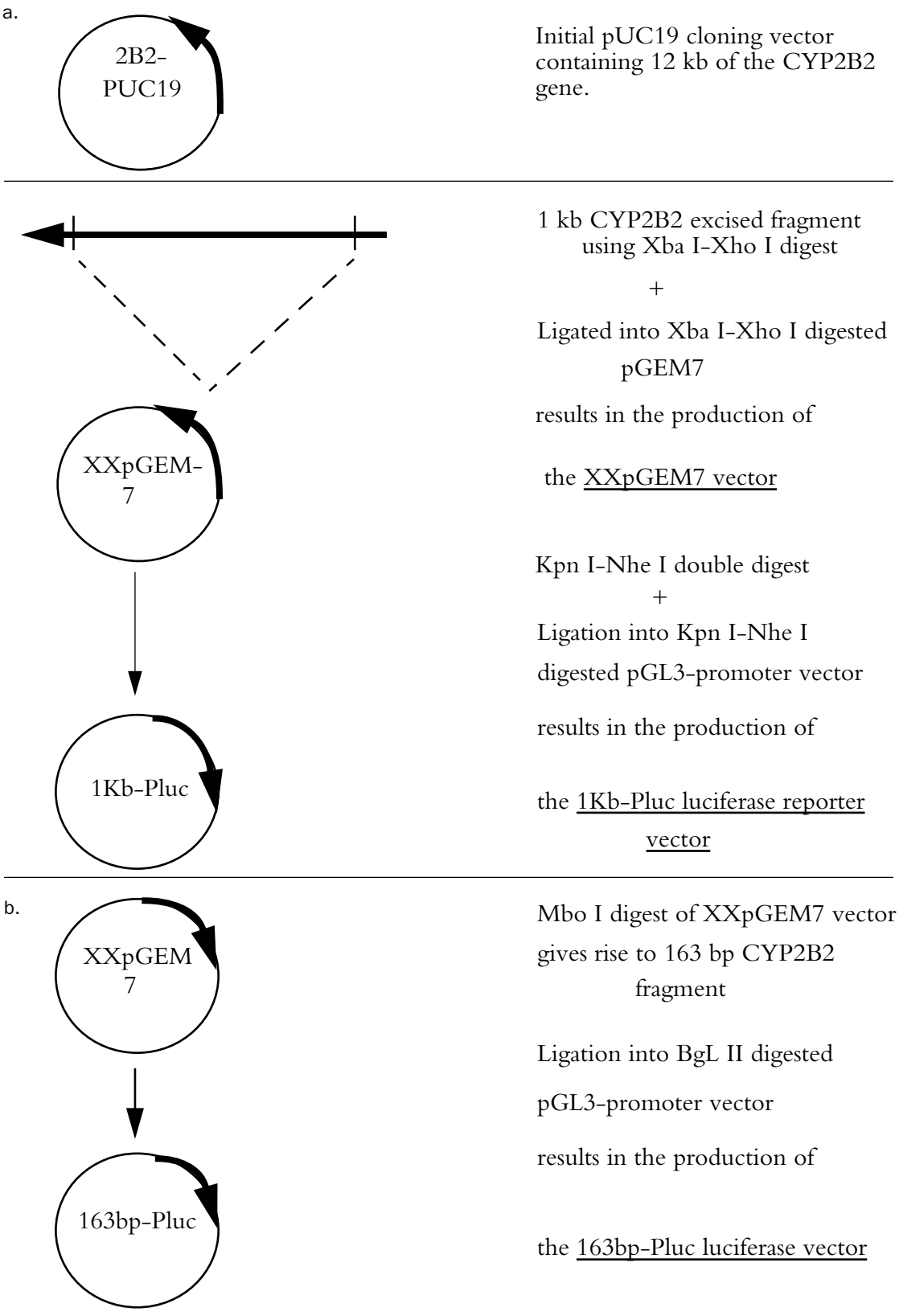

Figure 3.20 Cartoon depicting the sub-cloning of the PBRE containing CYP2B2 plasmids.

(a) Generation of the 1Kb-Pluc plasmid. (b) Generation of the 163bp-Pluc plasmids. Thin lines represent cloning vectors. Dark thick arrow represents the CYP2B2 gene fragment.

Section 3.5.1 Truncation of the 969 bp CYP2B2 fragment

The 1Kb Pluc clone was further truncated so that the 163 bp putative PBRE element could be examined using transfection protocols. The XXpGEM7 clone was restriction digested with a Xba I/Xho I digest, which gave rise to a 969 bp fragment. This fragment was further restriction digested using Mbo I, This restriction digest gave rise to a 163 bp Sau3AI flanked putative PBRE fragment (163SAI). This 163SAI fragment was ligated into the Bgl II site of the pGL3-promoter vector which gave rise to the 163 bp-Pluc clone (Figure 3.20b). Candidate clones were verified using an Nco I digest (Figure 3.21). Depending upon the orientation of the fragment, two different size fragments were produced of either 300 or 347 bp. Clones were sequenced using the primer RVP3 5' CTT TAT GTT TTT GGC GTC TTC 3', three clones 163bp-D-Pluc, 163bp-G-Pluc and 163bp-M-Pluc were confirmed as containing the 163 bp PBRE. The clones 163bp-M-Pluc and 163bp-G-Pluc were shown to contain CYP2B2 sequence present in the reverse orientation. Transfection procedures could now be carried out upon the 1Kb-Pluc and 163bp-Pluc clones.

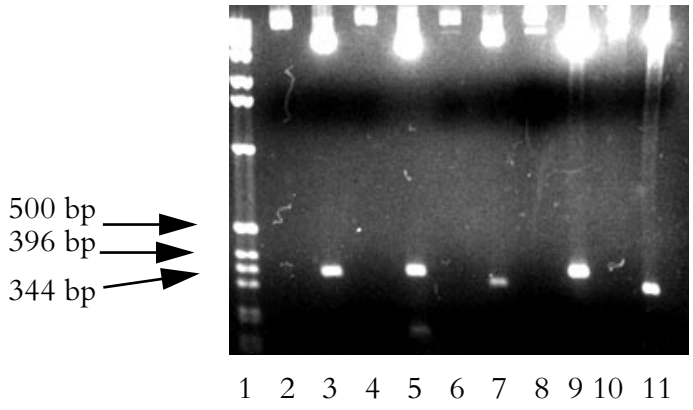

Figure 3.21 Restriction digest of the 1Kb-Pluc clone. 163 bp-Pluc DNA were digested with Nco I restriction enzyme and run on a 2.0 % pre-stained 0.1µg/ml EtBr, 1xTBE agarose gel at constant voltage of 70V for 2 hours to show the presence of the 300/347 bp Nco I fragment from 163 bp-Pluc. Lane 1 1 kb ladder. Lane 2,4,6,8,10 uncut plasmids. Lane 3,5,7,9,11 Nco I cut plasmids. Each clone has a letter attached to it corresponding to each of the 24 colonies picked. Plasmids 163 bp-M-Pluc, 163 bp-D-Pluc, 163 bp-G-Pluc were shown to contain the correct fragments as confirmed by sequence analysis.

Section 3.6 Optimisation of transfection of primary hepatocytes

A protocol for the transfection of primary rat hepatocytes was established so that characterisation of the PBRE could be undertaken. Although several transfection methods have been developed e.g. calcium phosphate, electroporation and liposome fusion (Watannabe *et al.,* 1994), it is generally considered difficult to transfect DNA into primary rat hepatocytes. Optimisation of transfection efficiency was necessary to determine the important variables present in the system. By minimising variation in the number of parameters, it should be possible to derive a reproducible system for the induction of the CYP2B2 gene.

Preliminary data showed that transfection efficiency was greatly retarded by the presence of high salt concentrations in the DNA or by poor quality DNA. The presence of fetal calf serum in the transfection medium was also shown to reduce transfection efficiency, possibly by preventing DNA: liposome complex formation. The effects of temperature and duration of DNA: liposome complex formation was shown to have negligible effects on transfection efficiency (data not shown).

Section 3.6.1 Effect of concentration of DOTMA/DOPE on transfection efficiency

In this system, we have chosen a non-commercial form of lipofectin reagent designated DOTMA/DOPE. This reagent was prepared as detailed in section 2.8.1. In preliminary experiments, the reporter vector pRSV-β-GAL was used as the source of plasmid DNA, as its uptake into hepatocytes could be monitored by the expression of β-galactosidase using a simple histochemical assay. Primary rat hepatocytes were isolated and transfected as described in section 2.8.3 with various modifications made as detailed in each experiment. pRSV-β-GAL plasmid concentration was kept constant at 6μg/flask whilst DOTMA/DOPE concentrations (0-80μg/flask) were altered to determine the effect on transfection efficiency. Transfection efficiency was measured 48 hours post transfection by the percentage of hepatocytes which stained positive after X-Gal

histochemical assay (Sanes *et al.,* 1986). The results of these experiments are summarised in Figure 3.22. The data indicates that a broad range of concentrations (10-40μg/flask) of DOTA/DOPE could be used to obtain high transfection efficiencies. This data also suggested that some form of cytotoxicity is occurring at the higher concentrations beyond 40μg/flask. Tandem experiments have been carried out in this study to compare non-commercial to commercial forms of lipofectin (data not shown). It has been found that the non-commercial form was as efficient at DNA transfer (data not shown) and a less expensive alternative to the commercial products available.

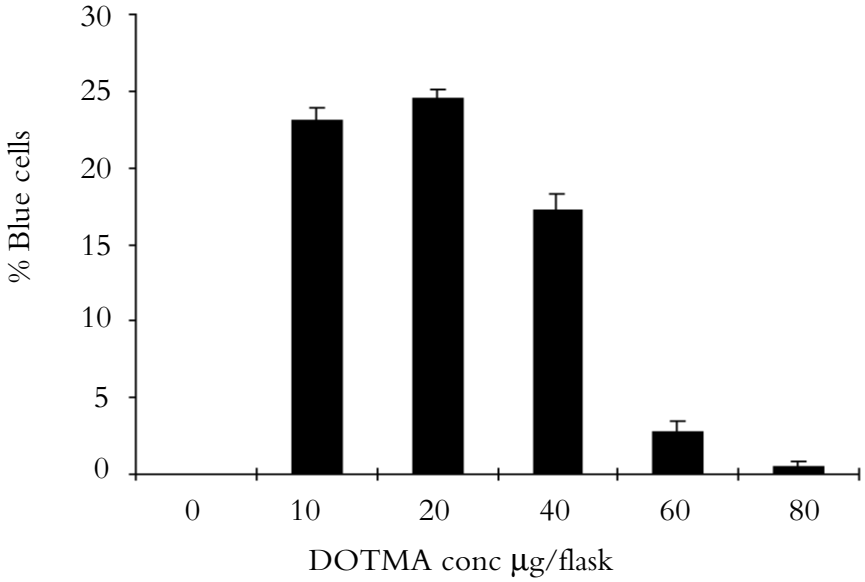

Figure 3.22 The influence of DOTMA/DOPE concentration on transfection efficiency. Primary hepatocytes were isolated and transfected as detailed in section 2.8.3. Hepatocytes were transfected in -ve L15 medium over 24 hours with a complex of 6μg of pRSV-βGAL and 0-80μg DOTMA/DOPE reagent at 37°C. Cells were cultured in CL15 medium for 48 hours post-transfection before β-galactosidase activity was measured by histochemical reaction with X-Gal. The % transfection efficiency was determined by recording the number of blue stained cells in at least eight fields of vision and representing this as a percentage of the total number of cells per field. Optimum transfection appears to occur at 10-40μg DOTMA/DOPE per flask. The mean of 3 flasks was plotted. Data points were analysed using Student's t-test. Error bars represent the standard error about the mean.

Section 3.6.2 Effect of reporter DNA concentration upon transfection efficiency

The next parameter to be assessed was the concentration of plasmid DNA. The previous data suggests some form of cytotoxicity is occurring at higher concentrations of DOTMA/DOPE so only 20μg/flask was used for all subsequent experiments. Primary rat hepatocytes were isolated and transfected as described in section 2.8.3. The concentration of DOTMA/DOPE was kept constant at 20μg/flask whilst pRSV-β-GAL plasmid concentrations (0-8μg/flask) were altered

to determine the effect on transfection efficiency. Transfection efficiency was measured 48 hours post transfection by the percentage of hepatocytes which stained positive after X-Gal histochemical assay. The results of these experiments are summarised in Figure 3.23. Here, an increase in the pRSV-β-GAL concentration from 0-6μg/flask indicated an increase in transfection efficiency without any apparent deleterious effects on cell viability. This data also indicates the variability in the transfection protocol from each hepatocyte preparation and transfection procedure, with a maximum of only 17% being obtained in this experiment compared to 28% in the previous experiment.

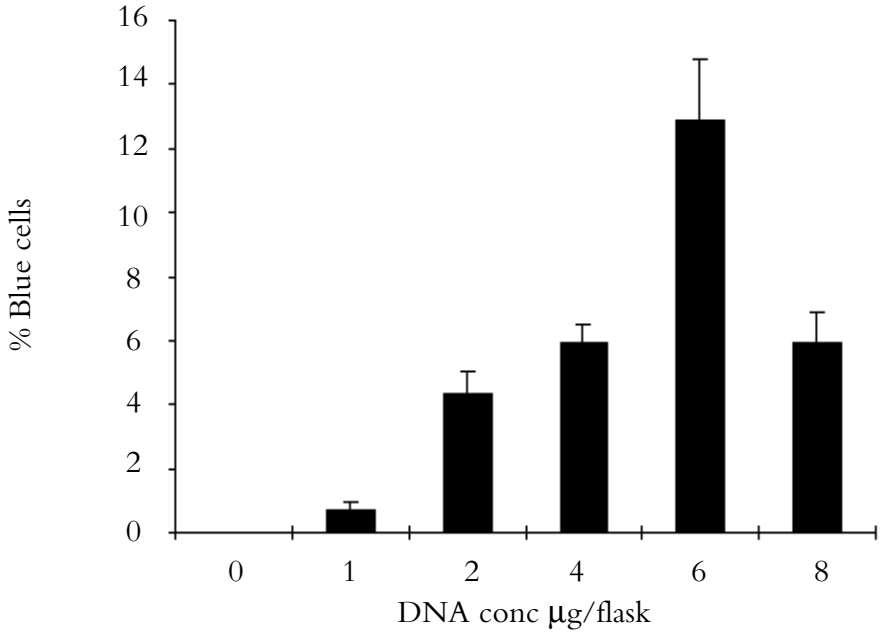

Figure 3.23 The influence of DNA concentration on transfection efficiency . Primary hepatocytes were isolated and transfected as detailed in section 2.8.3. Hepatocytes were transfected in -ve L15 medium over 24 hours with a complex of 20μg of DOTMA/DOPE reagent and 0-8μg of pRSV-β-GAL DNA at 37°C. Cells were cultured in CL15 medium for 48 hours post-transfection before β-galactosidase activity was measured by histochemical reaction with X-Gal. The % transfection efficiency was determined by recording the number of blue stained cells in at least eight fields of vision and representing this as a percentage of the total number of cells per field. Optimum transfection appears to occur at 6μg of pRSV-β-GAL per flask. The mean of 3 flasks was plotted. Data points were analysed using Student's t-test. Error bars represent the standard error about the mean.

Section 3.6.3 Effect of incubation time upon transfection efficiency

The final variable tested to obtain optimal transfection efficiency was the time of incubation with the DNA-liposome complex. Maximal transfection levels obtained for the previous experiments were measured at a specific arbitrary time-point (24 hours). The results presented in Figure 3.22

suggest that a minimum transfection incubation time would be advantageous so as to minimise deleterious effects on cell viability. Primary rat hepatocytes were isolated and transfected as described in section 2.8.3. The concentration of DOTMA/DOPE and pRSV-β-GAL plasmid were kept constant at 20μg and 6μg/flask respectively whilst the transfection incubation time was varied between 0-24 hours to test the effects on transfection efficiency. Transfection efficiency was measured 48 hours post transfection by the percentage of hepatocytes which stained positive after X-Gal histochemical assay. The results of these experiments are summarised in Figure 3.24. Hence, the shortest incubation time allowing for sufficient transfection was determined and found to be 4-5 hours. All subsequent experiments used the optimal values obtained for these three parameters.

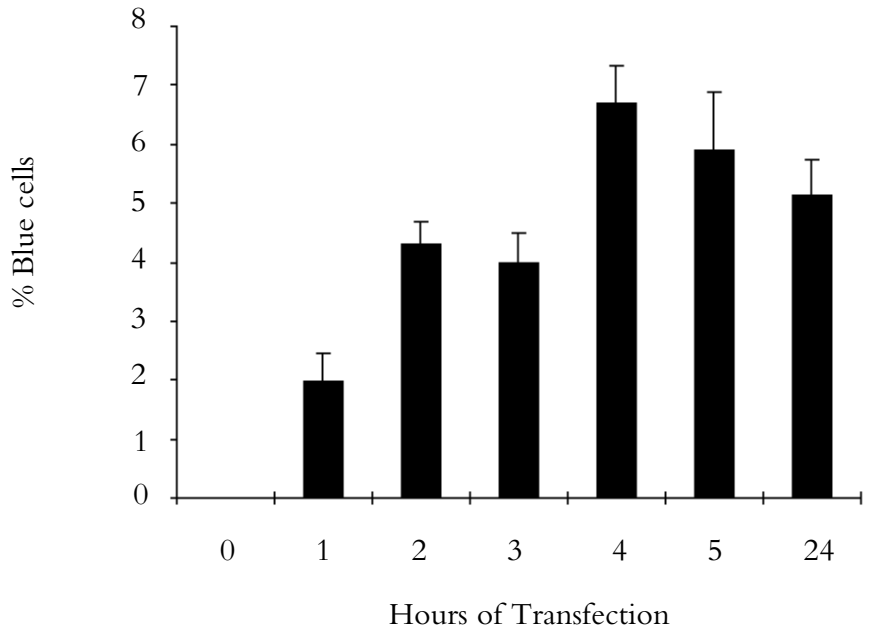

Figure 3.24 The influence of incubation time on transfection efficiency. Primary hepatocytes were isolated and transfected as detailed in section 2.8.3. Hepatocytes were transfected in -ve L15 medium with varying incubation times of 0-24 hours with a complex of 20μg of DOTMA/DOPE reagent and 6μg of pRSV-β-GAL DNA at 37^{o}C. Cells were cultured in CL15 medium for 48 hours post-transfection before β-galactosidase activity was measured by histochemical reaction with X-Gal. The % transfection efficiency was determined by recording the number of blue stained cells in at least eight fields of vision and representing this as a percentage of the total number of cells per field. Optimum transfection incubation time appears to occur at around 5 hours. The mean of 3 flasks was plotted. Data points were analysed using Student's t-test. Error bars represent the standard error about the mean.

Section 3.6.4 Morphology of hepatocytes post-transfection

Primary rat hepatocytes may exhibit some cytotoxic effects due to the transfection protocol. Maximal transfection incubation times, DOTMA/DOPE and DNA concentrations were all assessed for their degenerative effects on the morphology of the monolayer. A visual examination of the monolayers both before (Figure 3.25a) and after (Figure 3.25b) transfection was carried out; no phenotypic alterations were apparent at the optimised parameter values, despite the high transfection efficiency indicated by the histochemically developed blue colouration of the cell cytoplasm. Routine transfection efficiencies varied between 10-40 % in all subsequent experiments.

A. B.

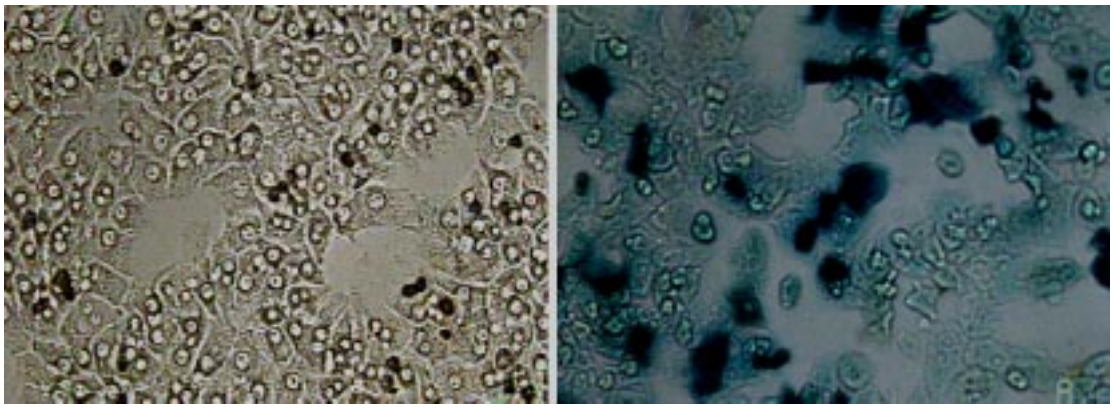

Figure 3.25 Intact morphology both before (A) and after (B) transfection . Primary hepatocytes were isolated and transfected as detailed in section 2.8.3. Hepatocytes were transfected in -ve L15 medium for 5 hours with a complex of 20μg of DOTMA/DOPE reagent and 6μg of pRSV-β-GAL DNA at 37^{o}C. Cells were cultured in CL15 medium for 48 hours post-transfection before they were fixed with glutaraldehyde and stained for β-galactosidase activity using standard techniques (section 2.9.1). Cells expressing β-galactosidase activity are stained blue as shown in (b), which is an indication of positive transfection of pRSV-β–GAL DNA. The % transfection efficiency was determined by recording the number of blue stained cells in at least eight fields of vision and representing this as a percentage of the total number of cells per field. (Magnification x 40)

Section 3.6.5 Optimisation of reporter system assays

Initial experiments were also carried out to optimise the method of harvesting the primary rat hepatocyte monolayers for analysis of reporter genes. The chloramphenicol acetyltransferase (CAT) control and luciferase reporter vectors pCAT3-control and pGL3-control respectively were used in these preliminary experiments. Several cell disruption techniques were assessed for their efficiency of enzyme harvest. Cell lysates were extracted by one of three methods: scraped

cells were disrupted by either sonication; or by repeated (x3) freeze/thaw cycles; or detergent (Triton-X-100) were used to lyse cells in the flask. Freeze /thaw was found to be the most efficient method initially (data not shown).

Luciferase and CAT assays were carried as described by DeWet *et al.* (1987) and Seed and Sheen (1988) respectively. The results of these assay optimisation experiments were often inconsistent and unrepeatable, particularly with the luciferase assay. Many different parameters were changed to alleviate this problem but it was not possible to obtain consistent results. A commercial luciferase assay kit (Promega) was utilised which contained a lysis system that enabled CAT assays to be carried out in tandem and this yielded consistent data.

Section 3.7 Functional characterisation of the PBRE in primary rat hepatocytes

The transfection system that has been developed and refined in this thesis could now be utilised to determine the responsiveness of the putative PBRE present with our culture system. The isolated clones described in section 3.5 could now be transfected into the primary rat hepatocytes and the effect of PB treatment analysed. Although the transfection protocol had been optimised using the pRSV-β-GAL vector for efficient transfections, the interaction of the reporter construct containing the response element and the normalising reporter vector in the system must also be investigated. Normalisation of the transfection procedure was carried out using CAT reporter vectors.

Section 3.7.1 Determination of the PB responsiveness of CYP2B2 PBRE containing constructs

The 1Kb-Pluc clone and the 163bp-Pluc PBRE containing constructs were now tested for PB responsiveness using the optimised transfection system. Here, 3μg of either 1Kb-Pluc or 163bp-D-Pluc and 3μg of the pCAT3-control normalising vector were combined with 20μg of DOTMA/DOPE allowing liposome: DNA complexes to form. After 5 hours incubation in

-ve L15 media, the cells were transferred to CL15 media and immediately dosed with 1.0mM PB. They were incubated for 30 hours before the cell monolayer was harvested. Cell extracts were harvested and firefly luciferase activity measured according to the instructions given in the luciferase firefly assay kit and as detailed in section 2.9.4.

The results of experiments in which CYP2B2 PBRE containing constructs were transfected into primary rat hepatocytes are summarised in Figure 3.26. Two PBRE-containing constructs were assayed and the normalised responses to PB measured. The results obtained from these experiments were rather unexpected. When treated with 1.0mM PB both the 1Kb-Pluc construct and the 163 bp PBRE containing constructs showed very little net induction when compared to control values. Notably, with the 163bp-D-Pluc construct the expression obtained was over 2.5 fold greater than that obtained for the 1Kb-Pluc construct for both control and dosed groups.

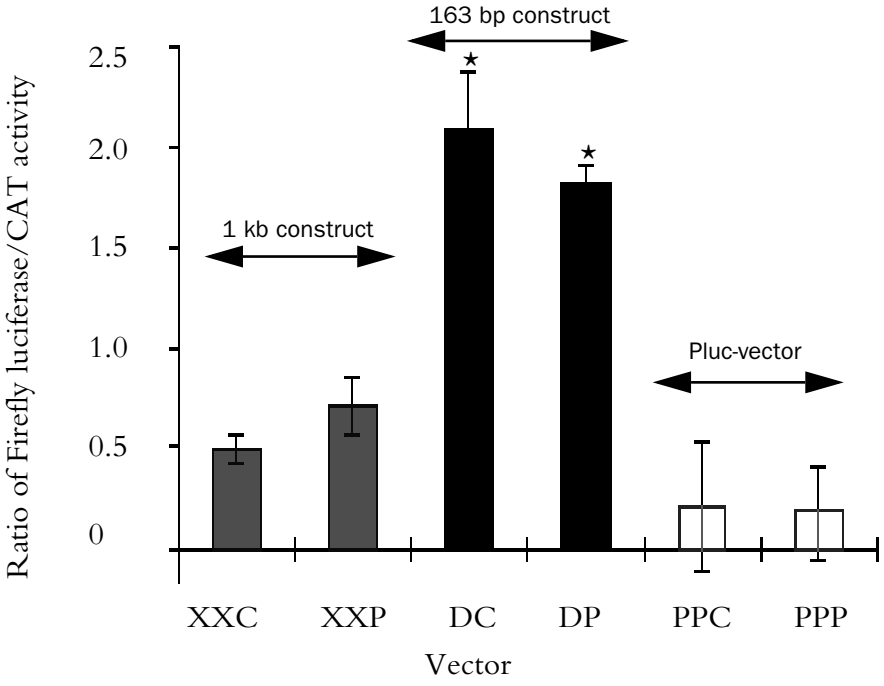

Figure 3.26 Determination of PB responsiveness of two PBRE containing constructs. Primary rat hepatocytes were transfected with 3µg of either 1Kb-Pluc or 163bp-D-Pluc and 3µg of pCAT3 control vector using 20µg of DOTMA/DOPE reagent for 5 hours. Flasks were dosed with 1.0mM PB and incubated for 30 hours. Cell extracts were harvested and assayed for firefly luciferase activity as described in section 2.9.4. The results of the luciferase assay were normalised using the results of the CAT assay; the mean of 3 experiments was plotted. Data points were analysed using Student's t-test. C = control, P = 1.0mM phenobarbital, XX = 1Kb-Pluc construct, D= 163bp-D-Pluc construct, PP = PGL-3-Pluc control vector. Error bars represent S.E. Reporter activity of 163bp-D-Pluc is significantly higher than that of 1Kb-Pluc (p=0.01, df=5, Two-tailed t-Test).

The presence of insulin within the hepatocyte culture system has been shown to strongly suppress the induction of the CYP2B1/2 gene in primary rat hepatocytes (Yoshida *et al.,* 1996). Experiments were carried out to investigate whether or not insulin, present in our hepatocyte culture system, was suppressing CYP2B2 gene expression. The results of these experiments showed that the level of expression of the 163bp-D-Pluc construct in hepatocytes dosed with 1.0mM PB was unaffected by the presence ($1x10^{-6}$ M) or absence of insulin in the culture media (data not shown).

The result presented in Figure 3.26 showed that hepatocytes transfected with PBRE-containing constructs were giving minimal or no induction responses when cells were treated with PB. It was thought that there may have been some interaction between the two expression vectors within the reporter system. This interaction may have been the result of a titration of general transcription factors by one of the reporter genes or a *trans* effect brought about by the presence of the other reporter gene (Natesan *et al*., 1997).

Section 3.7.2 Generation of a HSV-thymidine kinase promoter-containing PBRE construct

Farr and Roman (1991) highlighted that *trans* effects occurring between promoters on co-transfected plasmids could affect reporter gene expression of one or both of the reporter genes involved. The *trans* effects brought about by the interaction of the two reporter genes may result in suppression in the activity of either of the reporter genes involved. This is of particular concern when working with reporter genes containing strong promoter/enhancer elements such as Simian virus 40 (SV40) or cytomegalovirus (CMV) promoter elements which may reside on one or other, or both, of the control and experimental reporter vectors. The HSV-thymidine kinase (TK) promoter is useful for providing neutral constitutive expression which may allow for a reduction in the amount of *trans* effects occurring between the different promoter elements. The possibility of reporter gene promoter 'cross-talk' was addressed by designing a sub-cloning

strategy that would enable this to be investigated. Reporter genes were designed so that a combination of HSV-TK, SV40 or CMV-driven promoters were present on both the control and experimental reporter genes. It was thought these reporters would help determine whether the unexpected effects that were being observed in section 3.7.1 had arisen due to *trans* effects occurring between reporters containing strong promoter elements. A sub-cloning strategy was undertaken to generate reporter constructs of varying promoter strengths that contained the 163 bp PBRE fragment of the CYP2B2 gene.

The reporter constructs sub-cloned in section 3.5.1 were used to generate a PBRE HSV-TK promoter reporter. A PCR sub-cloning strategy incorporating the PBRE fragment of the 163bp-G-Pluc construct and a HSV-thymidine kinase promoter driven firefly luciferase reporter (TK-Luc, a kind gift from Ronald M. Evans of the Howard Hughes Medical Institute) was devised.

Oligonucleotide primers were designed based around the 163bp-G-Pluc ligated PBRE fragment. This meant that the primer contained partial sequence homologous to the PGL3-promoter vector and partial sequence homologous to the PBRE insert. The primers were also designed so that a Hind III restriction site would be engineered into the PCR-generated insert.

PCR was carried out as described in section 2.5 on the 163bp-G-Pluc construct using primers, Hind 5 and Hind 3. PCR products gave rise to a 207 bp fragment (Figure 3.27). This PCR product was restriction digested using Hind III and ligated into the Hind III site of the TK-Luc vector. Clones produced from this ligation were restriction digested with Hind III and shown to contain a fragment of 193 bp (Figure 3.28). Positive clones were designated TK2B2-Luc. This sub-cloning strategy was non-directional, so two clones were sequenced using primer Hind 5 as a confirmation of the clone's integrity. These two clones were shown to be in the same orientation as the CYP2B2 gene and were designated TK2B2-F-Luc and TK2B2-D-Luc.

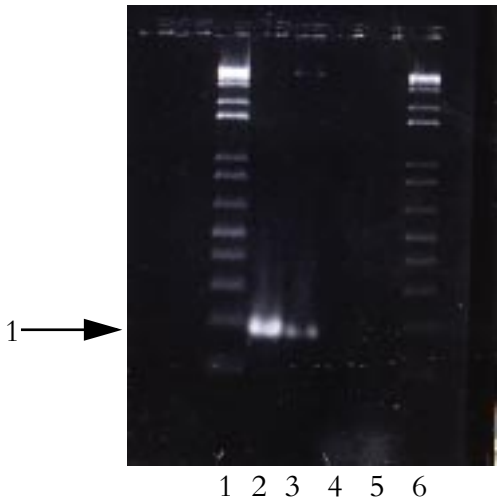

Figure 3.27 PCR amplification of CYP2B2 PBRE DNA. PCR of the PBRE containing fragment amplified from 163bp-G-Pluc used primers Hind 5 (5' GAG ATG **A**AG **C**T**T** GCA GAT CGT GGA CAC AAC CCT C 3') containing 3 base-pair mis-matches (shown in bold) and primer Hind 3 (5' CTA GCC **AA**G **CT**T CGA GAT CAC CCA GCC TGG CAG 3') containing 4 base-pair mis-matches (shown in bold). PCR was carried out as described in section 2.5 and consisted of 30 cycles of 94^{o}C, 1 min; 55^{o}C, 1 min; and 72^{o}C, 1 min. 5µl of the 50µl PCR reaction was run on a 1.0 % pre-stained 0.1µg/ml EtBr, 1X TBE agarose gel at constant voltage of 90V for 2 hours. Lane 1 contains 1 kb+ ladder. Lane 2 contains 0.5ng of 163bp-G-Pluc template DNA. Lane 3 contains 3.0 ng of 163bp-G-Pluc template DNA. Lane 4 contains product from the PCR reaction with no template DNA. Lane 5 contains product from the PCR reaction with 163bp-G-Pluc template DNA but no Taq. Lane 6 contains 1 kb+ ladder. Arrow 1 indicates position of 207 bp PCR fragment amplified from the 163bp-G-Pluc PBRE containing region.

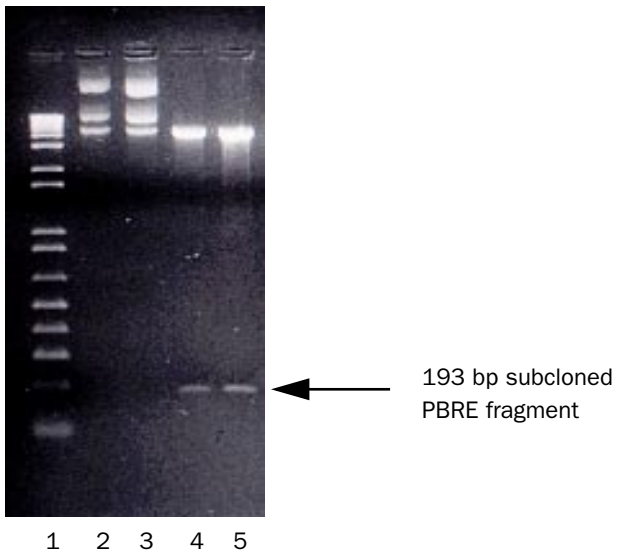

Figure 3.28 Cloning of the PBRE containing TK163-Luc plasmid. PCR clones were restriction digested with Hind III and ran on a 1.2%- agarose gel. The 193 bp band was gel excised and cloned into the Hind III site of the TK-Luc vector and designated TK163-Luc. TK163-Luc clones were digested with the Hind III restriction enzyme. The products of each restriction digest were analysed on a 1.5%-prestained 0.1 µg/ml EtBr, 1X TBE agarose gel at constant voltage of 70V for 2 hours to show the presence of a 193 bp fragment from the TK163-Luc clone. Each TK163-Luc clone had a letter attached to it corresponding to each of the 14 clones picked. Plasmid TK2B2-D-Luc and TK2B2-F-Luc are shown. Lane 1 contains 1 kb+ ladder. Lane 2-3 contain TK2B2-D-Luc and TK2B2-F-Luc uncut DNA. Lane 4-5 contain TK2B2-D-Luc and TK2B2-F-Luc plasmid DNA cut with Hind III. Arrow indicates position of 193 bp subcloned PBRE fragment.

Section 3.7.3 Optimisation of the Dual luciferase reporter assay (DLRA)

The transfection system used thus far incorporated the use of multiple reporter constructs with multiple reporter assays, this meant that cross-talk between the two reporter gene promoters was a distinct possibility. The DLRA was a commercial kit which had been optimised for the inter-actions that may occur between various reporter constructs. The kit also had the added advantage that the two assays were performed on the same sample in sequence. The system consisted of an experimental reporter containing a segment of PBRE attached to the firefly luciferase reporter and the control reporter consisting of the *Renilla* (sea pansy) luciferase gene which acted as the normalising vector (pRL-CMV).

An experiment was designed to find out if an alteration in the ratios of the two reporter plasmids could have any affect on the overall response of the PBRE. The first parameter to be adjusted was the amount of the *Renilla* luciferase reporter plasmid present in the system. The amount of 163bp-D-Pluc firefly luciferase reporter was kept constant at 6μg /flask whilst the amount of pRL-CMV *Renilla* luciferase reporter was altered from 100ng-0.001ng /flask. The hepatocytes were transfected and dosed for 30 hours before they were harvested and analysed as described in section 2.9.5. Table 3.2 summarises the values obtained by altering the amount of pRL-CMV plasmid DNA whilst keeping the amount of 163bp-D-Pluc plasmid DNA constant. This data shows that an extremely high signal to noise ratio is obtained with these reporters. It also shows that only very small quantities of pRL-CMV needed to be present for a high signal to be produced. The *Renilla* luciferase gene had a strong constitutive expression in the hepatocyte system with values ranging from 3000 relative light units (RLU) above background to outside the range of the luminometer (approx. $1.7x10^8$) over the range of *Renilla* reporter transfected. The values obtained for the firefly luciferase reporter ranged from 9000-160000 RLU but this value did not alter greatly along with an alteration in vector ratio.

Amount of vector of 163bp-D-Pluc: pRL-CMV (μg/flask)	Treatment	Firefly luciferase activity (RLUx100)	*Renilla* luciferase (RLUx1000)
6: 0.1	PB	95-125	off scale
6: 0.1	C	50-145	off scale
6: 0.01	PB	50-65	off scale
6: 0.01	C	70-100	off scale
6: 0.001	PB	30-70	50-150
6: 0.001	C	40-150	45-285
6: 0.0001	PB	40-65	3-4
6: 0.0001	C	10-160	3-9
6: 0.000001	PB	40-130	background
6: 0.000001	C	80-100	background

Table 3.2 Relative activities of firefly and *Renilla* luciferase reporter gene activity. Hepatocytes were transfected with 6μg /flask of 163bp-D-Pluc firefly luciferase reporter and between 100-0.01ng/flask of pRL-CMV *Renilla* luciferase reporter then dosed for 30 hours and harvested. The results of a DLRA were measured in relative light units (RLU), PB = 1.0mM PB, C = control.

Once an optimum amount of pRL-CMV had been found, this was then used to test the effects of reducing the amount of 163bp-D-Pluc firefly luciferase reporter transfected into the system. The hepatocytes were transfected and dosed for 30 hours before they were harvested and analysed as described in section 2.9.5. The amount of 163bp-D-Pluc was varied between 6-0.1μg of DNA /flask whilst the amount of pRL-CMV was kept at a constant 0.1ng /flask to see if this would effect the response of the PBRE present in the 163bp-D-Pluc construct. Table 3.3 summarises the values obtained by altering the 163bp-D-Pluc plasmid DNA whilst keeping the amount of pRL-CMV plasmid DNA constant. The data indicates an increase in *Renilla* luciferase values obtained from 1.5-40000 RLU when the amount of 163bp-D-Pluc decreases from 6-3μg of DNA /flask. A further decrease in 163bp-D-Pluc concentration does not seem to effect the signal produced by the *Renilla* luciferase reporter present in the system. The results presented in Table 3.2 and 3.3, only indicate the trend in firefly luciferase and *Renilla* luciferase reporter gene

activity that was observed when different reporter gene constructs were used. As the values varied considerably no statistical analysis was carried out upon this data. The data does show that there is negligible difference between 163bp-D-Pluc expression in hepatocytes cultured under 1.0mM PB or control conditions. To prevent DLRA problems due to promoter cross-talk, the amount of firefly luciferase and *Renilla* luciferase reporter constructs were reduced to an absolute minimum in all subsequent experiments.

Amount of vector of 163bp-D-Pluc: pRL-CMV (μg/flask)	Treatment	Firefly luciferase activity (RLUx100)	*Renilla* luciferase (RLUx1000)
6.0: 0.0001	PB	80-100	1.5-2.0
6.0: 0.0001	C	65-105	1.5-2.0
3.0: 0.0001	PB	110-140	3.5-4.5
3.0: 0.0001	C	80-200	2.5-4.0
1.5: 0.0001	PB	110-165	3.2-5.2
1.5: 0.0001	C	210-225	3.2-4.3
0.1: 0.0001	PB	5-7	1.6-3.0
0.1: 0.0001	C	10-13	2.2-2.8

Table 3.3 Relative activities of firefly and *Renilla* luciferase reporter gene activity. Hepatocytes were transfected with 0.1ng /flask of pRL-CMV *Renilla* luciferase reporter and between 6-0.1μg /flask of 163bp-D-Pluc luciferase reporter then dosed for 30 hours and harvested. The results of a DLRA were measured in relative light units (RLU), PB =1.0mM PB, C= control.

Section 3.7.4 Effect of different promoter driven PBRE reporter constructs

The effects of reporter promoter 'cross-talk' upon the transfection systems ability to exhibit PB responsiveness was investigated using reporter constructs containing different promoters. Transfection experiments were carried out using different combinations of reporters containing either HSV-TK (TK), SV40 or CMV containing promoters to ascertain their effects on PB responsiveness. Using the optimised conditions from Section 3.7.3 primary rat hepatocytes were transfected with 1.5μg /flask of the firefly luciferase construct and 0.3ng /flask of the *Renilla* luciferase reporter. The 163bp-D-Pluc firefly luciferase reporter contains an SV40-driven promoter. The

TK2B2-F-Luc construct contains a TK-driven promoter. The *Renilla* luciferase reporter contains either a CMV-driven promoter or a TK-driven promoter. Combinations of TK, SV40 firefly lucferase and TK, CMV *Renilla* luciferase reporters were examined to determine their effects on overall induction ratios of control over 1.0mM-PB treated hepatocytes. Hepatocytes were transfected with the appropriate combination of reporters and DOTMA/DOPE for 5 hours and dosed with PB for a further 30 hours. The monolayers were then harvested and assayed as detailed in section 2.9.5. The effect of altering the reporter promoter on the responsiveness of the hepatocyte system is shown in Figure 3.29. Here, the effect of altering the type of promoter driving each reporter upon the overall signal obtained is quite complex.

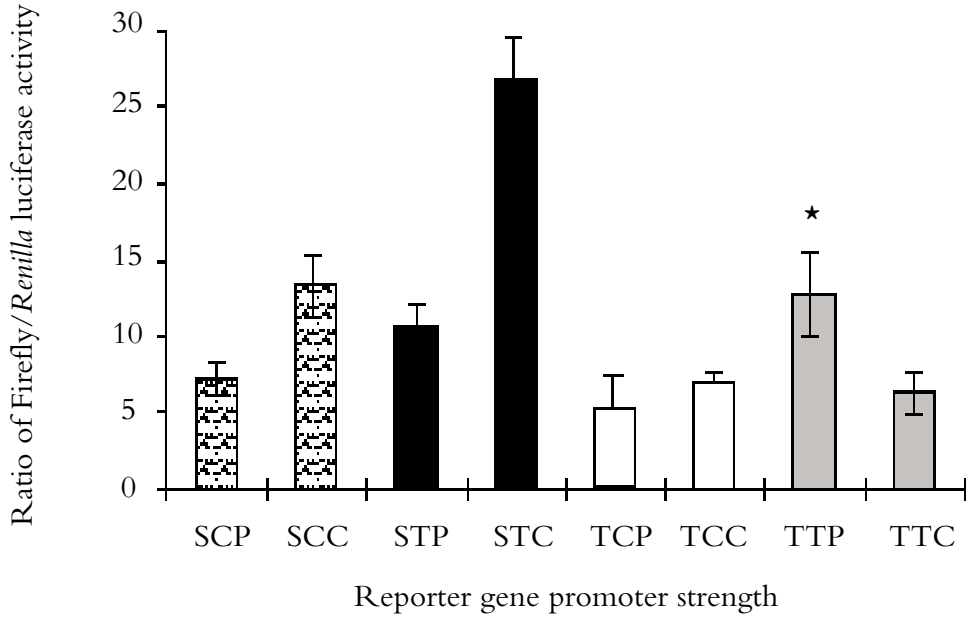

Figure 3.29 Effect of different reporter promoter activity on PBRE responsiveness to PB . Primary rat hepatocytes were transfected with a combination of 1.5µg /flask of either 163bp-D-Pluc or TK2B2-F-Luc luciferase constructs and 0.3ng /flask of pRL-CMV or pRL-TK *Renilla* luciferase constructs with 20µg of DOTMA/DOPE reagent for 5 hours. Flasks were dosed with control or 1.0mM PB and incubated for 30 hours. Cell extracts were harvested and assayed for firefly/*Renilla* luciferase activity and normalised as described in section 2.9.5. The normalised results of these assays (luminescence) are expressed as a ratio of firefly/*Renilla* luciferase activity. Data points were analysed using Student's t-test. C = control, P=1.0mM phenobarbital. The SC denotes the firefly/*Renilla* luciferase reporters driven by SV40 or CMV-driven promoters respectively, ST denotes the firefly/*Renilla* luciferase reporter driven by SV40 or TK-driven promoters respectively, TC denotes the firefly/*Renilla* luciferase reporter driven by TK or CMV-driven promoters respectively, and TT denotes the firefly/*Renilla* luciferase reporters driven by TK or TK-driven promoters respectively. *Reporter activity in the presence of 1.0mM PB is significantly higher than in its absence. ($P < 0.05$, df=2, two-tailed t-test).

The data shows the intensity of the response measured is dependent upon which combination of

promoters is used. These data suggest that the PBRE showed PB responsiveness only when both the firefly luciferase and *Renilla* luciferase reporter genes contained a TK-driven promoter. Here, treatment of hepatocytes with 1.0mM PB gave a 2-fold induction above control values. The other promoter permutations gave rise to higher values for the control cells than they did for the PB-treated hepatocyte cultures.

Section 3.7.5 Cytotoxicity of the transfection protocol

The results obtained from the previous section indicated that transcriptional activation of the PBRE containing reporter constructs could be demonstrated if the two reporters were present in the correct ratios within the transfection system.

The decreased reporter response from the PB-induced cultures might have been the result of promoter 'cross-talk' as mentioned before, although cytotoxicity induced by the transfection procedure and subsequent dosing with PB was another possibility that had to be examined. Results presented in section 3.6.4 showed that no deleterious effects brought about by the transfection protocol were apparent, but in this case the monolayers were not dosed with PB. The cytotoxic effects of the transfection procedure were assessed by indirect measurement of the release of the cytosolic enzyme lactate dehydrogenase (LDH). LDH catalyses the reduction of pyruvate to L-lactate with simultaneous oxidaion of NADH to NAD. The change in absorbance at 340nm due to the disappearance of reduced NAD (NADH) over time is directly proportional to the LDH activity.

Hepatocytes were transfected with 2μg of TK-2B2-Luc, 0.3ng of pRL-TK *Renilla* and 20μg of DOTMA/DOPE for 5 hours then dosed with PB. Aliquots of the cell culture media (200μl) were removed at various time points during transfection and post-transfection and assayed for LDH activity as described in section 2.11. At the end of the experiment (48 hours), a final aliquot of cell culture medium was removed and the cell monolayer lysed using 0.6% Triton X-100. This

was carried out to determine the percentage of total intracellular LDH content left in the cell culture system at 48 hours.

Figure 3.30 shows the results of the LDH assay expressed in international units per litre. The data shows very clearly the difference in LDH leakage that was occurring between the two phases of the transfection and dosing protocol. During the transfection stage there was a 2.7 fold increase in the amount of LDH leakage occurring in the transfected cells when compared to the non-transfected cultures. The initial increase in LDH leakage was mainly due to the effects of the hepatocyte isolation procedure. The presence of the transfection reagent and the effects it exhibits on the plasma membrane of the hepatocytes during the transfection protocol further compounds the LDH leakage. Once the transfection reagent was removed and the cells dosed with PB, the level of LDH leakage was shown to decrease. At this time point (0 hrs) there is a 1.4 fold difference in the amount of LDH leakage occurring between the transfected (T) and the non-transfected (NT) hepatocytes. The amount of LDH leakage was shown to further decrease at the 3 hour time point as the hepatocyte culture becomes acclimatised, although at this time point there is still a significant difference between T and NT hepatocytes, but there was no difference in LDH leakage between control and PB-dosed transfected hepatocytes. After 3 hours LDH leakage increases gradually with time as did the difference between the T and NT cells. The difference in LDH leakage between transfected cells that were control or PB-treated was found to be negligible over the 48 hour time period, thus, indicating that no cytotoxicity was occurring due to PB treatment post-transfection.

At 48 hours both the amount of LDH leakage occurring and the total available intra-cellular LDH activity of the monolayers was assessed by total cell lysis. This was carried out to determine if the amount of LDH leakage occurring at 48 hours was significant compared to the total available intra-cellular LDH activity present in the monolayers. Although the transfected cells showed

a 2-fold increase in LDH leakage above non-transfected cells, the amount of LDH leakage was less than 30% of the total available intra-cellular LDH activity. This final value of LDH leakage does not suggest that a significant amount of cytotoxicity was occurring in the transfected cells (Delraso, 1989).

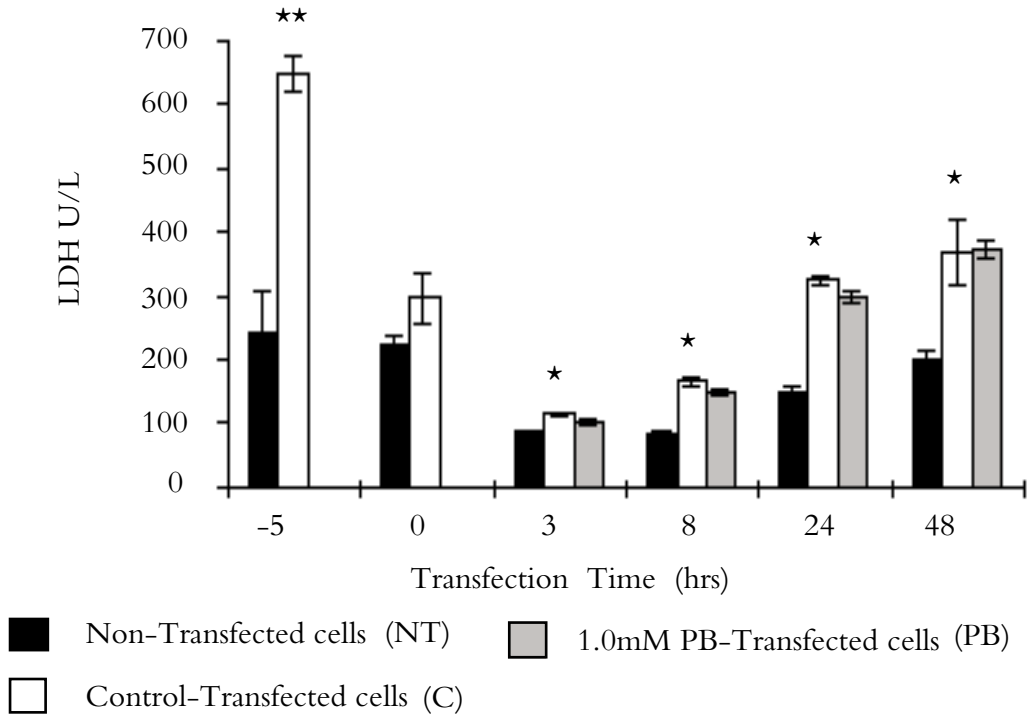

Figure 3.30 Cytotoxic effects of transfection protocol upon primary rat hepatocytes. Primary rat hepatocytes were transfected with 2µg TK2B2-F-Luc, 0.3ng of pRL-TK *Renilla* and 20µg of DOTMA/DOPE for 5 hours then dosed with 1.0mM PB. Aliquots of the cell culture media (200µl) were removed at various time points during transfection and post transfection and assayed for LDH activity in international units/litre (U/L) as described in section 2.11. Data points were analysed using Student's t-test. -5 denotes time during which transfection is occurring. 0-48 denotes time post-transfection during which the monolayers were exposed to 1.0mM PB. NT = non-transfected cells, C = control, PB = 1.0mM phenobarbital treated transfected cells. *LDH leakage of transfected cells is significantly higher than non-transfected cells ($p = 0.06$, df =2, two-tailed t-test). **LDH leakage of transfected cells is significantly higher than non-transfected cells ($p = 0.02$, df =2, two-tailed t-test).

Section 3.7.6 Determination of TK2B2-F-Luc expression over time

The results presented in Figure 3.29 indicate that the PBRE mediated induction of the firefly luciferase reporter gene may be increased if the correct reporter gene conditions were present. A time course of the induction of the reporter containing the firefly TK-driven promoter luciferase gene TK2B2-F-Luc by PB in transfected hepatocytes was carried out to determine the kinetics of the PB-mediated induction. Hepatocytes were transfected as before with 2µg of TK2B2-F-Luc, 0.3ng of pRL-TK *Renilla* and 20µg of DOTMA/DOPE for 5 hours then dosed as control

or 1.0mM PB. The monolayers were harvested and assayed, to determine if the length of time that hepatocytes were exposed to 1.0mM PB post-transfection would affect PBRE mediated induction of the firefly luciferase reporter gene. The results shown in Figure 3.31 indicate that there is minimal induction exhibited by PB-induced hepatocytes transfected with the TK2B2-F-Luc firefly luciferase construct up to 8 hours. A significant 1.6 fold induction above control hepatocytes is demonstrated at 24 hours, which decrease to 1.5 fold at 48 hours.

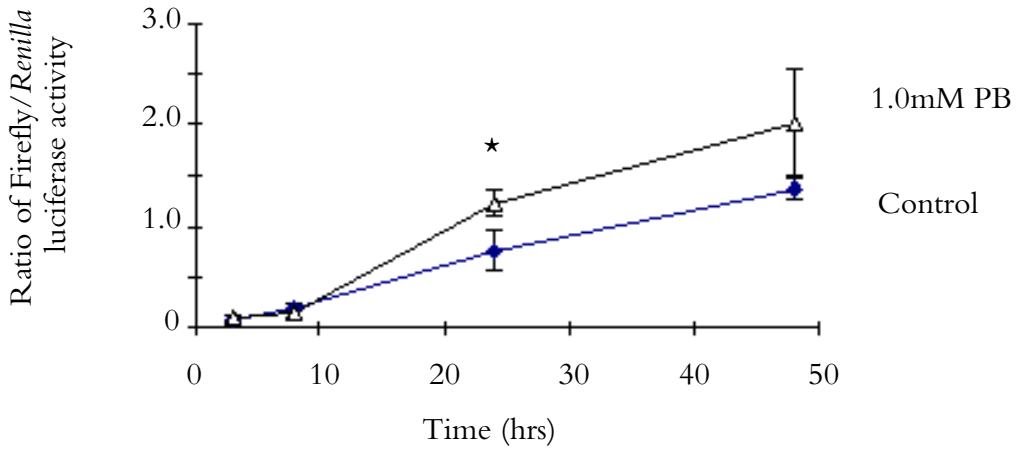

Figure 3.31 Effect of time upon expression of PB induced TK2B2-F-Luc reporter activity. Primary rat hepatocytes were transfected with 2µg of TK2B2-F-Luc, 0.3ng of pRL-TK *Renilla* and 20µg of DOTMA/DOPE for 5 hours then dosed with 1.0mM PB. Hepatocyte monolayers were harvested at the times indicated and analysed for firefly luciferase and *Renilla* luciferase reporter activity. The normalised results of these assays (luminescence) are expressed as a ratio of firefly/*Renilla* luciferase activity as described in section 2.9.6. Data points were analysed using Student's T-test. C = control, P = 1.0mM phenobarbital treated transfected cells. *Hepatocytes treated with 1.0mM PB post-transfection are 1.6 fold significantly higher than control transfected cells ($p = 0.02$, $df = 2$, two-tailed t-test).

Section 3.7.7 PB-mediated induction of two PBRE containing reporter constructs

The results presented in the previous sections demonstrate that PB-mediated induction of the PBRE is possible under specific experiment conditions in this thesis. The 1.3-1.6 fold induction obtained using various PBRE containing reporter constructs, recorded within this culture system does not compare with the results obtained in other laboratories such as that of Anderson's (Trottier *et al.,* 1995). In order to understand why we could only obtain a comparatively low induction, it was decided that a comparison between PBRE-containing constructs which have been characterised (Trottier *et al.,* 1995) and our TK2B2-F-Luc construct was necessary.

The pBSCAT plasmid carrying a portion of the CYP2B2 5'-flanking region inserted upstream from the CAT gene (a kind gift from Alan Anderson of le Centre de Recherche en Cancerologie de l' Universite Laval, L'Hotel-Dieu de Quebec, Quebec) was provided. This construct consisted of a 163 bp Sau3AI fragment from nucleotides -2318 to -2155 added to -1681 bp of the 5' flanking sequence of the CYP2B2 portion of the non-responsive EcoRV construct. This construct was designated Sa-Sa/EV in the Anderson paper but will be referred to here as the 163bpCAT construct.

The 163bpCAT construct was restriction digested so that the integrity of the positive clones could be assessed. Figure 3.32 confirms the presence of the 163bpCAT construct with a multiple restriction digest using Hind III, Nco I and EcoR I giving rise to distinct banding patterns.

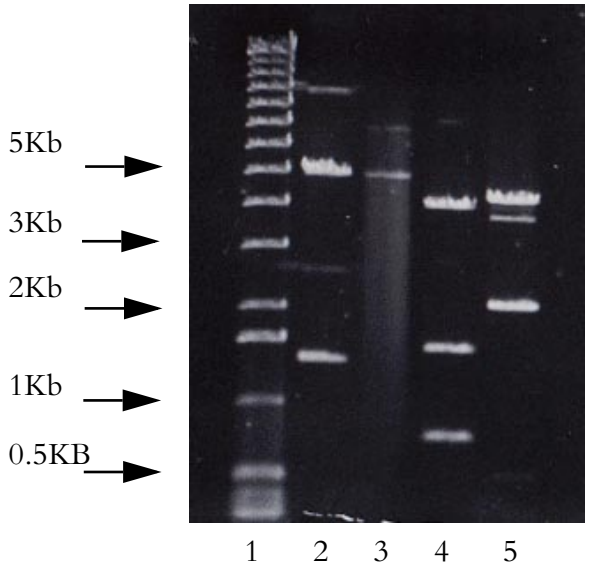

Figure 3.32 Restriction digest of the 163bpCAT clone. 163bpCAT plasmid DNA was restriction digested as described in section 2.4.7, with Hind III, Nco I and EcoR I restriction enzyme and run on a 0.6 % pre-stained ethidium bromide 1xTBE agarose gel at constant voltage of 90V for 2 hours to determine the presence of the 163 Sa-Sa/EV insert. Lane 1 1 kb ladder. Lane 2 Hind III digest giving rise to 2 bands at 1440 bp and 5010 bp. Lane 3 undigested plasmid. Lane 4 Nco I digest giving rise to 3 bands at 4082, 1572 and 798 bp. Lane 5 EcoR I digest giving rise to 2 bands at 4315 bp and 2137 bp. A positive clone was picked and prepared for transfection by plasmid Qiagen maxi-preparation.

The 163bpCAT-reporter gene activity was monitored using the CAT assay as described in section 2.9.3. Initial CAT assay results indicated that the values obtained from hepatocytes

transfected with the 163bpCAT reporter construct were at a level similar to that of mock transfected cells (hepatocytes which did not contain a CAT reporter construct) (data not shown).

These results suggested that some attribute of the 163bpCAT reporter was interfering with the CAT assay, or that the assay was not working correctly. The possibility that the 163bpCAT prepared plasmid may be exhibiting some form of endotoxin contamination was investigated.

Section 3.7.7.1 Endotoxin contamination of the 163bpCAT reporter

Endotoxins (lipopolysaccharides) are cell membrane components of Gram-negative bacteria e.g., *E.coli.*, which are released during the lysis step of plasmid preparations. It has been noted that these endotoxins strongly influence the efficiency of transfection of DNA into sensitive cultured cells (Weber *et al.*, 1995). The presence of endotoxins in the transfected DNA could specifically inhibit PB-induction of hepatocytes containing the 163bpCAT reporter. This inhibition of PB induction and decreased ability of the transfected 163bpCAT containing hepatocytes to express CAT enzyme would affect the overall assay signal.

All plasmid DNA that was involved in the transfection procedure was Qiagen maxi-prepared and further purified to remove any remaining contaminant endotoxins by caesium chloride-ethidium bromide (CsCl-EtBr) centrifugation. For each plasmid, 100μg of plasmid was mixed into 1.5ml of a 1.63g/ml solution of caesium chloride containing 450μg of ethidium bromide. This solution was then centrifuged in an Optima TLA-120.2 ultracentrifuge at 80,000 rpm for 6 hours at 20°C, DNA extracted, centrifuged again, and the DNA was then cleaned up as stated in section 2.4.6.1. The CsCl-EtBr centrifuged plasmids were visualised on a 1.0 % agarose gel to compare with plasmids before CsCl-EtBr treatment (Figure 3.33). This gel showed no apparent difference between plasmids from before and after CsCl-EtBr treatment. The CsCl-EtBr plasmids were given the prefix (Cs-) to differentiate them from the untreated plasmids during transfection experiments.

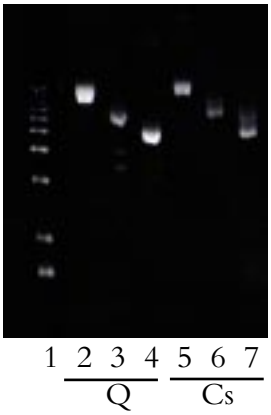

Figure 3.33 Electrophoresis of CsCl-EtBr treated reporter constructs. Plasmid DNA was prepared from isolated colonies of three separate plasmids pGL3-control, pCAT3 control, 163bpCAT. Plasmid DNA was prepared by Qiagen maxipreparation. Caesium chloride -ethidium bromide (CsCl-EtBr)) treated plasmids were Qiagen prepared plasmids that had been further treated by CsCl-EtBr centrifugation. CsCl-EtBr plasmid DNA was centrifuged in an Optima TLA-120.2 ultracentrifuge at 80000 rpm for 6 hours at 20^{o}C, twice. 2µl of each plasmid DNA was visualised on a 1.0 % pre-stained 0.1µg/ml EtBr, 1X TBE agarose gel at constant voltage of 100V for 2 hours. Lane 1 1 kb ladder. Lane 2 pGL3-control plasmid DNA. Lane 3 pCAT3 control plasmid DNA. Lane 4 163bpCAT plasmid DNA. Lane 5 pGL3-control plasmid DNA. Lane 6 pCAT3 control plasmid DNA. Lane 7 163bpCAT plasmid DNA. Cs- denotes CsCl- EtBr treated Qiagen plasmids. Q- denotes Qiagen prepared plasmids.

These CsCl-EtBr treated plasmids were then used in transfection experiments to determine if the treatment had any effect on the signal measured in the CAT assay. Primary rat hepatocytes were transfected with 5µg of Cs-163bpCAT or 163bpCAT, 0.1µg of pGL3-control luciferase and 20µg of DOTMA/DOPE for 5 hours then dosed with 1.0mM PB. The monolayers were harvested at 36 hours and CAT and luciferase assays carried out to determine if the CsCl-EtBr treatment of the reporter constructs would affect PBRE-mediated induction of the 163bpCAT-reporter gene. The results shown in Figure 3.34 indicate that 1.0mM PB treated hepatocytes gave a significant 1.5 fold induction above controls for both plasmid treatments.

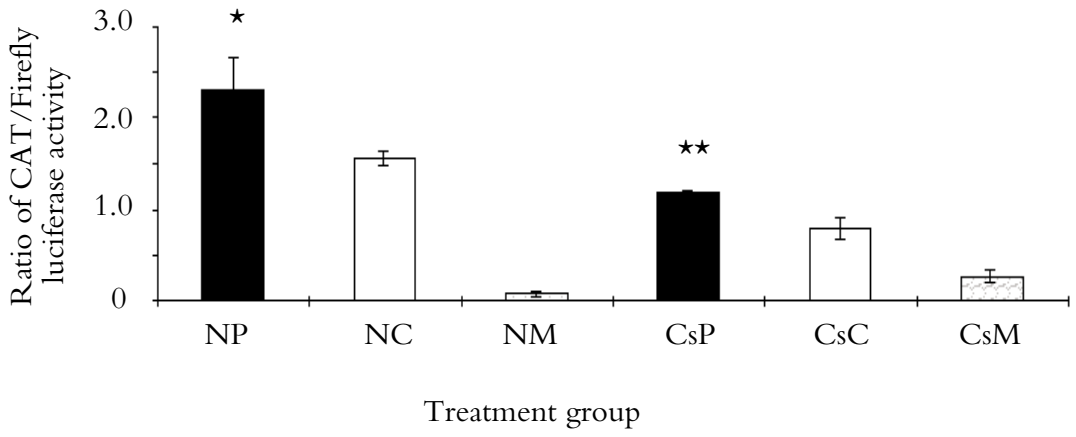

Figure 3.34 Effect of CsCl-EtBr treatment on 163bpCAT transfected hepatocytes . Primary rat hepatocytes were transfected with 5µg of Cs-163bpCAT or 163bpCAT, 0.1µg of pGL3-control luciferase and 20µg of DOTMA/DOPE for 5 hours then dosed with 1.0mM PB. Hepatocyte monolayers were harvested at 36 hours and assayed for CAT/luciferase activity and normalised as described in section 2.9.6. The normalised results of these assays (luminescence) are expressed as a ratio of CAT/firefly luciferase activity. Data points were analysed using Student's t-test. C = control, P = 1.0mM phenobarbital, M = mock transfected, N = untreated plasmid and Cs = CsCl treated reporter constructs. *Reporter activity in the presence of 1.0mM PB is significantly higher than in its absence. (P= 0.05, df=2, two-tailed t-test). **Reporter activity in the presence of 1.0mM PB is significantly higher than in its absence. (P= 0.02, df=2, two-tailed t-test).

Section 3.7.7.2 Optimisation of CAT assay

Experiments were carried out to determine if altering the cell lysate volume and the amount of time the samples were heat-treated for would have any effect on the CAT assay signals obtained. Cell lysate taken from transfection experiments describe in section 3.7.7.1 were heated at 65°C for 20 minutes and the effects of this treatment upon the CAT activity exhibited by 163bpCAT reporter constructs determined. The results shown in Figure 3.35 indicate that heat treatment of the cell extract is necessary to inactivate endogenous deacetylase activity before the CAT assay can be performed. Here different volumes of cell extract were heat-treated and compared with untreated samples and the results determined in cpm.

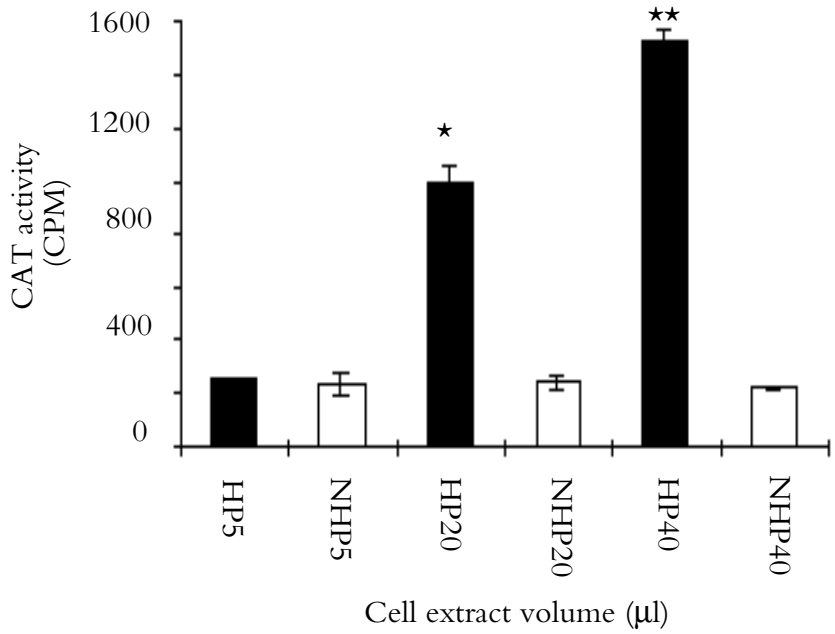

Figure 3.35 Effect of heat treatment upon CAT assay of 163bpCAT cell extracts . Primary rat hepatocytes were transfected with 5µg of 163bpCAT, 0.1µg of pGL3-control luciferase and 20µg of DOTMA/DOPE for 5 hours then dosed with 1.0mM PB. Hepatocyte monolayers were harvested at 36 hours. Various volumes of PB induced cell extracts (5µl-40µl) were heat treated at 65^{o}C for 20 minutes and assayed for CAT activity as described in section 2.9.3. The result of this assay was expressed as cpm. Data points were analysed using Student's t-test. H = heat-treated, NH = not heat-treated, Numbering denotes volume of cell extract used (µl). *Reporter activity in the presence of 1.0mM PB and heat-treated is significantly higher than in the absence of heat treatment. (P= 0.04, df=2, two-tailed t-test). **Reporter activity in the presence of 1.0mM PB and heat-treated is significantly higher than in the absence of heat treatment. (P= 0.008, df=2, two-tailed t-test).

Figure 3.35 showed that the amount of cell extract used in the CAT assay was also a crucial factor in obtaining significant CAT values. This was further investigated by altering the amount of cell extract present in the CAT assay to determine if increasing cell lysate volume would increase the signal. Cell lysate from both mock-transfected and 1.0mM PB-induced 163bpCAT containing cells were analysed. Figure 3.36 indicates that by increasing the total cell lysate volume present in the CAT assay that an increase in CAT enzyme activity was obtained, it also demonstrates that below 5µl of lysate that the signal-to-noise ratio is such that no conclusions could be drawn from the data. The values obtained beyond 60µl of lysate are not reliable, as the assay is non-linear beyond a certain concentration of CAT enzyme.

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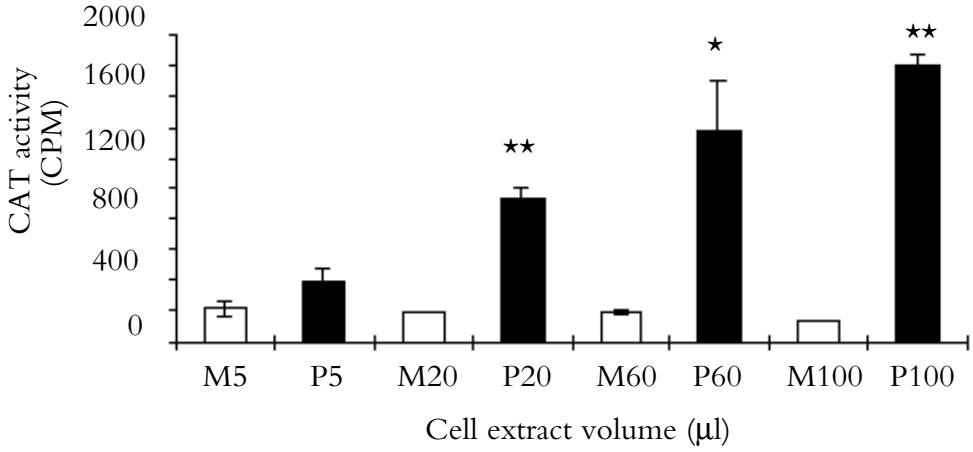

Figure 3.36 Variation of cell lysate volume effects the amount of CAT activity present . Primary rat hepatocytes were transfected with 5µg of 163bpCAT, 0.1µg of pGL3-control luciferase and 20µg of DOTMA/DOPE for 5 hours then dosed with 1.0mM PB. Hepatocyte monolayers were harvested at 36 hours. Various volumes of 1.0mM PB cell extracts (5µl-100µl) were heat treated at 65^{o}C for 20 minutes and assayed for CAT activity as described in section 2.9.3. The result of this assay was expressed as cpm. Data points were analysed using Student's t-test. M = Mock-transfected cell lysate, P = 163bpCAT-transfected cell lysate, Numbering denotes volume of cell extract used. *Reporter activity of 60µl of control 163bpCAT transfected lysate is significantly higher than mock transfected lysate. (P= 0.03, df=2, two-tailed t-test). ** Reporter activity of control 163bpCAT transfected lysate is significantly higher than mock transfected lysate. (P< 0.005, df=2, two-tailed t-test).

Section 3.7.7.3 Comparison between TK2B2-F-Luc and 163bpCAT reporter activity

The PBRE-containing reporters used in this thesis have shown 1.6-fold induction when hepatocytes are treated with 1.0mM PB. Anderson's 163bpCAT reporter, has been shown to give in excess of 6-fold inductions when treated with 1.0mM PB in primary rat hepatocytes. Experiments were carried out to compare the activation of CYP2B2 response elements in the TK2B2-F-Luc construct (generated in this thesis), to that of the 163bpCAT construct designed by Anderson's group (Trottier *et al.*, 1995).

Transfections were carried out using the two reporter constructs TK2B2-F-Luc and 163bpCAT. The 163bpCAT plasmid used in these transfection experiments was treated using the conditions outlined in the Anderson paper (Trottier *et al.,* 1995). In the case of the 163bpCAT plasmid, hepatocytes were transfected with 8µg of 163bpCAT, 5µg of pGL3-control luciferase and 20µg of DOTMA/DOPE for 5 hours then dosed with PB. Hepatocytes were transfected with 8µg of

TK2B2-F-Luc, 0.3ng of pRL-TK *Renilla* and 20μg of DOTMA/DOPE for 5 hours then dosed with 1.0mM PB. The monolayers were harvested at 48 hours and assayed according to the reporter vectors used in each case. The results of these comparative assays are shown in Figure 3.37. This data shows that there was no significant difference between the two plasmids when comparing the level of induction mediated by 1.0mM PB treated cells over control cells. As the two-plasmid systems used incorporated two different reporter vectors, only the percentage fold induction mediated by 1.0mM PB above controls was represented.

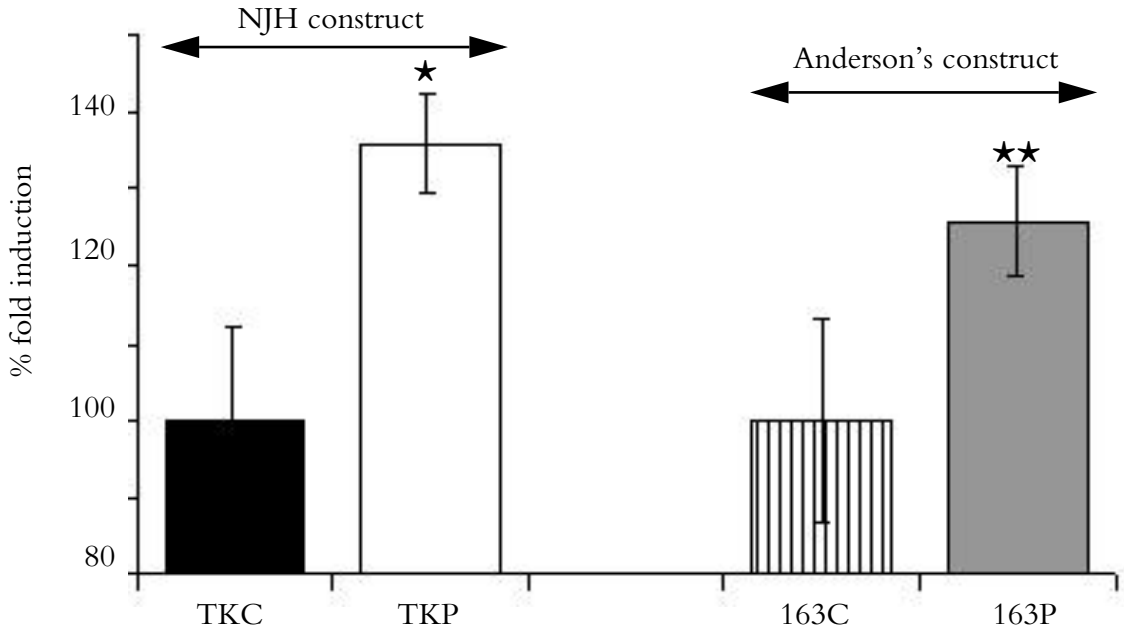

Figure 3.37 Comparison of PB mediated induction of the TK2B2-F-Luc and 163bpCAT constructs. Primary rat hepatocytes were transfected with 8μg of TK2B2-F-Luc, 0.3ng of pRL-TK *Renilla or* 8μg of 163CAT, 5μg of pGL3-control and mixed with 20μg of DOTMA/DOPE for 5 hours, then dosed with 1.0mM PB. Hepatocyte monolayers were harvested at 48 hours and analysed for firefly luciferase/*Renilla* luciferase reporter and CAT/firefly luciferase reporter activity respectively. Percentage fold inductions are expressed relative to the control hepatocytes in each assay. A value of 100% was arbitrarily assigned to the level of activity in the control hepatocytes in each assay and PB induction expressed as a percentage of this value. Data points were analysed using Student's t-test and showed no significant difference between the TK2B2-F-Luc and 163bpCAT reporters (n=3 per treatment group). C denotes control hepatocytes in each assay. P denotes hepatocytes treated with 1.0 mM phenobarbital. TK denotes those hepatocytes transfected with the TK2B2-F-Luc reporter generated in this thesis, 163 denotes those hepatocytes transfected with the Anderson contruct 163bpCAT. *Reporter activity in the presence 1.0mM PB is significantly higher than in the absence. (P = 0.04, df = 2, two-tailed t-test). ** Reporter activity in the presence 1.0mM PB is significantly higher than in the absence. (P = 0.001, df = 2, two-tailed t-test).

Section 3.7.8 Effect of culture matrix components upon PBRE-mediated induction

Adult primary rat hepatocytes lose their ability to respond to PB in primary cultures. This phenomenon has been investigated by many groups with parameters such as the composition of the culture media being altered (Waxman *et al.,* 1990; Sinclair *et al.,* 1990). The substratum to which the hepatocytes were attached to, or overlaid with (Klienman *et al.,* 1985; Schuetz *et al.,* 1988; Bissell *et al.,* 1987; Sidhu *et al.,* 1993) was also altered in an attempt to resolve this problem.

The original culture conditions used for the 163bpCAT-reporter construct utilised a matrigel substratum (Trottier *et al.,* 1995). The 163bpCAT construct had only been assessed in culture conditions in which the hepatocytes were attached directly to the plastic Falcon flasks in our system. The effect of three different substratum conditions were investigated for their effect on the PB-mediated induction of the PBRE.

Hepatocytes were cultured either upon plastic Falcon flasks or in collagen type-I coated (150μg /25cm^2 flask) Falcon flasks, or as a hepatocyte monolayer with a Matrigel overlay (83.3μg/ml solution). These solutions were prepared as detailed in section 2.6.5.2 and 2.6.5.3. Hepatocytes were either seeded directly onto the plastic flask as in the case of the conventional and Matrigel coated Falcon flasks, or seeded directly over the collagen-coated surface of the Falcon flasks. Primary rat hepatocytes were transfected with 5μg of 163bpCAT, 0.1μg of pGL3-control luciferase and 20μg of DOTMA/DOPE for 5 hours then dosed with 1.0mM PB. Hepatocyte monolayers were harvested at 48 hours and assayed for CAT/Firefly luciferase activity and normalised as described in section 2.9.6. Transfection of the Matrigel treated cells was carried out prior to Matrigel treatment as the overlay inhibits the efficiency of the transfection procedure. The Matrigel solution was added directly to the cells post-transfection.

A post-transfection microscopic examination of hepatocytes cultured on either conventional

plastic, collagen type-1, or Matrigel coated flasks (Figure 3.38) was carried out. Both 1.0mM PB-induced (Figure 3.38 a, c, e) and control monolayers (Figure 3.38 b, d, f) were analysed. The monolayers were examined at 36 hours and showed marked differences in the morphology of the hepatocyte monolayers depending upon which form of substratum was used. The appearance of each monolayer was compared with hepatocytes grown under the conventional Falcon flask environment. All flasks showed a slight difference between control and treated monolayers, with the 1.0mM PB-induced monolayers appearing more confluent and uniform. Conventionally cultured hepatocytes were rather flat in appearance and not so uniform in their distribution (Figure 3.38 a, b). The monolayers overlaid onto collagen type I flasks appeared more confluent and slightly more rounded and spread out with less cell debris (Figure 3.38 c, d). The Matrigel treated cells looked very similar to that of the conventionally cultured hepatocytes with regards to confluence, the cells also did not spread very well over the surface of the flask. Upon closer inspection the cells treated with Matrigel (Figure 3.38 e, f) were not flat in appearance but more spherical and protruding from the flasks.

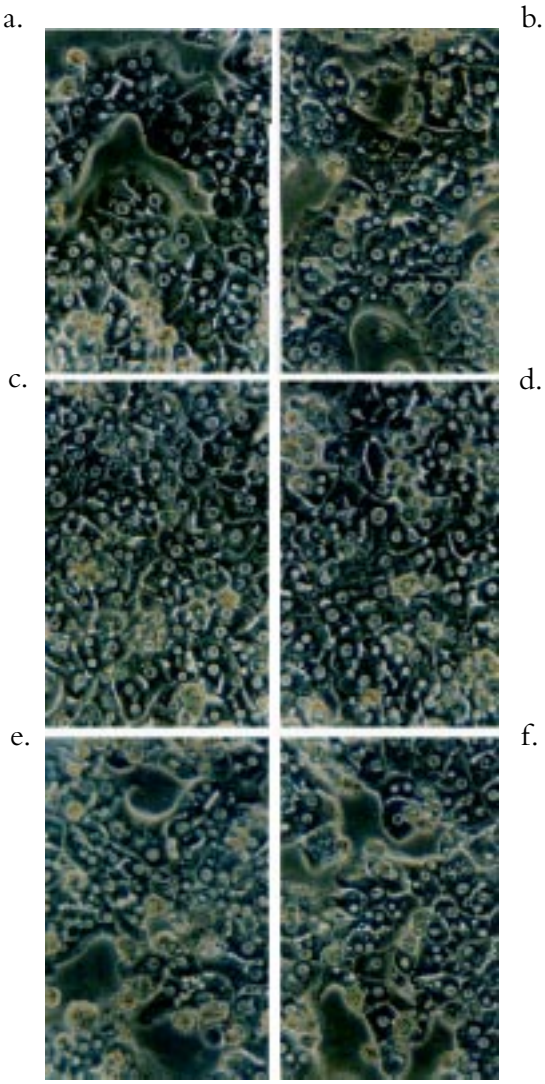

Figure 3.38 Morphological appearance of primary rat hepatocytes cultured with various matrix components. Primary rat hepatocytes were cultured either on conventional plastic Falcon flasks (a, b), collagen type I coated (150µg per 25cm^2 flask) Falcon flasks (c, d), or overlaid (post-transfection) with Matrigel in an 83.3µg/ml solution (e, f). Primary rat hepatocytes were transfected with 5µg of 163bpCAT, 0.1µg of pGL3-control luciferase and 20µg of DOTMA/DOPE for 5 hours as described in section 2.8.3 and treated with control (a, c, e) or 1.0mM PB (b, d, f). Monolayers were photographed at 36 hours at a magnification of x 32.

The appearance of each monolayer at 48 hours seemed to be consistent with that noted at 36 hours. The monolayers were harvested at 48 hours and their lysate analysed for CAT/Firefly luciferase activity. The results shown in Figure 3.39 indicate that under conventional conditions PBRE mediated induction of the 163bpCAT reporter was 1.5 fold greater than control samples. This was also mirrored in monolayers cultured in the presence of Matrigel but to a lesser extent with a 1.4 fold induction being recorded. Hepatocytes cultured in collagen type I coated flasks

did not show any significant difference between control and PB-treated monolayers. The data suggests that PBRE mediated induction of the CAT reporter gene is affected by the type of substratum incorporated in the culture conditions. There appears to be no significant difference between hepatocytes cultured in the conventional manner with those cultured in the presence of Matrigel.

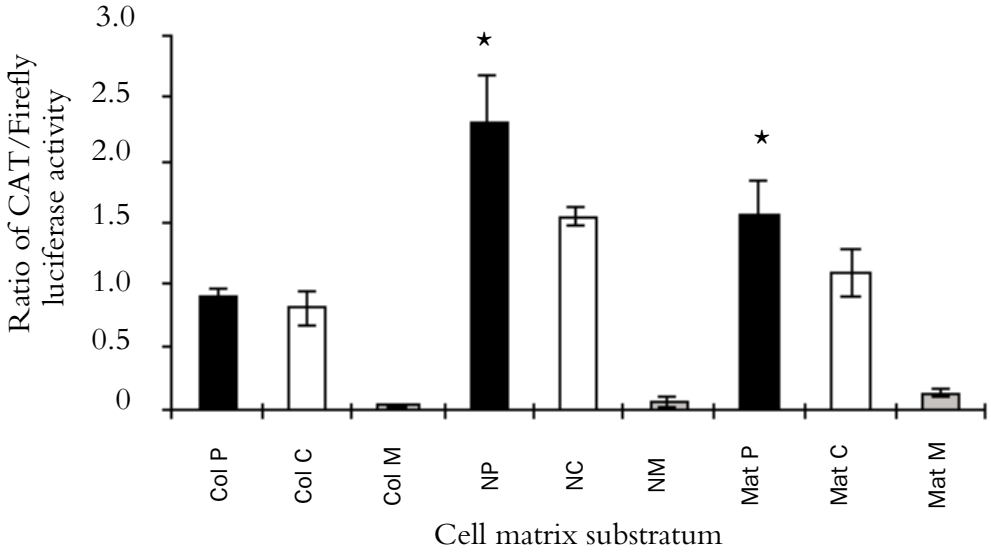

Figure 3.39 Comparison of the effects of matrix components upon PBRE-mediated induction . Primary rat hepatocytes were cultured either on conventional plastic Falcon flasks, collagen type I coated (150µg per 25cm^2 flask) Falcon flasks, or overlaid (post-transfection) with Matrigel in an 83.3µg/ml solution. Primary rat hepatocytes were transfected with 5µg of 163bpCAT, 0.1µg of pGL3-control luciferase and 20µg of DOTMA/DOPE for 5 hours and dosed with 1.0mM PB as described in section 2.8.3. The normalised results of these assays were expressed as the ratio of CAT/Firefly luciferase activity. Data points were analysed using Student's t-test. C = control, P = 1.0mM phenobarbital, M denotes mock transfected cells. Col = Collagen treated flasks, N = conventional Falcon flasks, and Mat = Matrigel overlays. *Reporter activity in the presence of 1.0mM PB is significantly higher in conventional and Matrigel treated cultures than in collagen type I treated cultures. (P= 0.02, df=2, two-tailed t-test)

The induction of CYP2B2 mRNA by phenobarbital was investigated using an RNAse protection assay using the conditions outlined in Figure 3.8, to ascertain if the transfection of the 163bpCAT-reporter construct would affect transcription of the CYP2B2 mRNA species. The results obtained from this experiment suggest the transfection of 163bpCAT reporter constructs into the primary rat hepatocyte system did not affect the induction of CYP2B mRNA by PB as measured by RNAse protection (Figure 3.40).

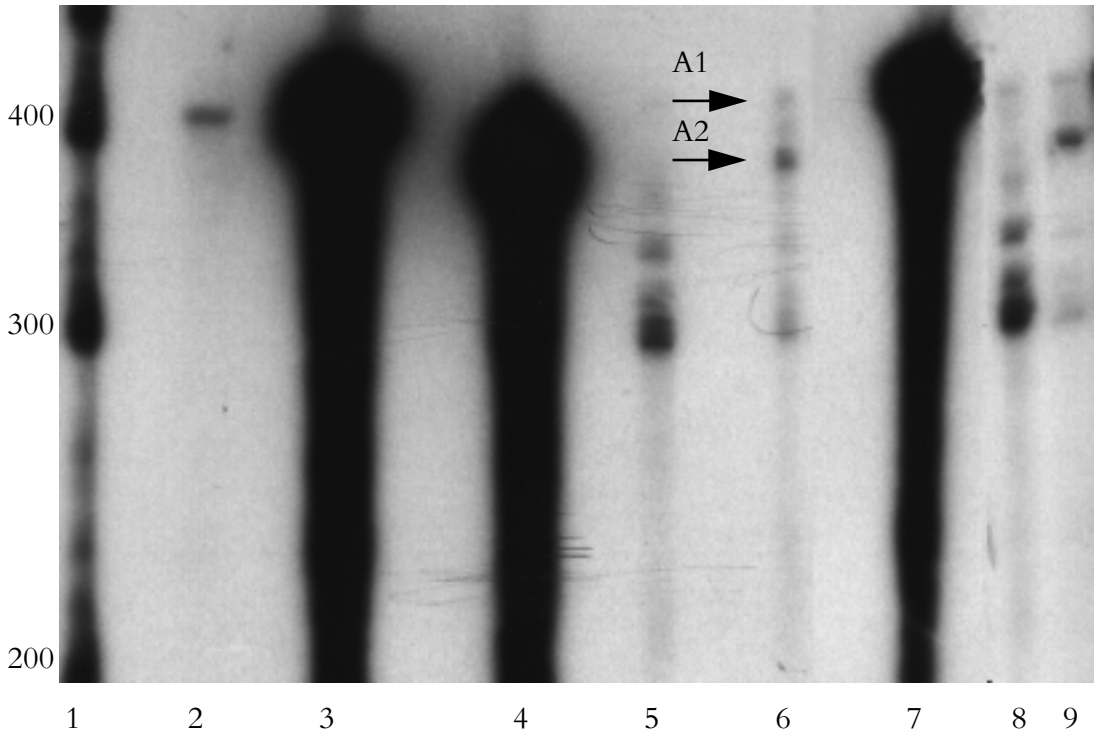

Figure 3.40 Effect of transfection protocol upon PB induction of CYP2B2 mRNA. Primary rat hepatocytes were transfected with 5μg of 163bpCAT, 0.1μg of pGL3-control luciferase and 20μg of DOTMA/DOPE for 5 hours then dosed with 1.0mM PB. These hepatocytes were then harvested as described in section 2.7.5. RNAse protection assays were carried out on both *in vitro* and *in vivo* rat total RNA samples using an [α-^{32}P] labelled anti-sense riboprobe to CYP2B2 cDNA. Hepatocyte monolayers were treated with control or 1.0mM PB for 3 days and RNA harvested as described in section 2.7.5. Rats were dosed with vehicle, PB 80mg/kg i.p. for 3 days and RNA extracted as described in section 2.7.4. Anti-sense CYP2B2 probe was transcribed using a Cla I cut Xlig plasmid with SP6 polymerase. Full-length Xlig probe was 426 bp in length giving rise to a 379 bp protected fragment. 30μg of total RNA was used for each lane. Lane 1 contains 100bp ladder. Lane 2 contains yeast tRNA with RNAse T1. Lane 3 contains yeast tRNA without RNAse T1. Lane 4 contains *in vivo* PB dosed liver RNA sample. Lane 5 contains control hepatocyte RNA. Lane 6 contains PB-dosed hepatocyte RNA. Lane 7 contains full length probe. Lane 8 contains control transfected hepatocyte RNA. Lane 9 contains PB-dosed transfected hepatocyte RNA. RNAse protection assays were run on a 6% denaturing polyacrylamide gel in 1x TBE at 300V for 3 hours. The gel was fixed, dried and exposed to hyperfilm for 3 days at -70^{o}C. Arrow A1 indicates full-length probe. Arrow A2 indicates protected probe-mRNA complex with the accompanying vector DNA digested away by RNAse T. The picture is a composite as a number of lanes have been removed between the T+ve lane and the ladder lanes in the interest of clarity.

Section 3.7.9 Effect of reporter ratios upon 163bpCAT activity

The effect of altering the amount of luciferase reporter construct and altering the time of transfection was investigated to determine if this would have an impact upon the level of induction exhibited by the 163bpCAT construct.

Primary rat hepatocytes were transfected with a constant 5.0μg of 163bpCAT reporter /flask whilst the amount of pGL3-control luciferase reporter was altered from 1.0μg-0.01μg /flask.

This combination of reporter constructs was then mixed with 20μg of DOTMA/DOPE for 5 hours at 4 hours post-isolation or 24 hours post isolation and dosed with 1.0mM PB. The hepatocytes were harvested at 48 hours and analysed for CAT and firefly luciferase activity as described in section 2.9.3 and 2.9.4.

Figure 3.41 shows the dynamic effect of altering the amount of pGL3-control firefly luciferase reporter upon the normalised activity of 163bpCAT-reporter enzyme produced in the hepatocyte cell lysate. These data clearly show the direct correlation between the amount of reporter that was present and the overall signal that was obtained. Here, as the amount of pGL3-control luciferase reporter was decreased there was an increase in normalised reporter activity. This trend was seen with both transfection time-frames, the difference between the two transfection times upon reporter activity was very subtle and can only be assessed when the induction ratios were analysed (Figure 3.42).

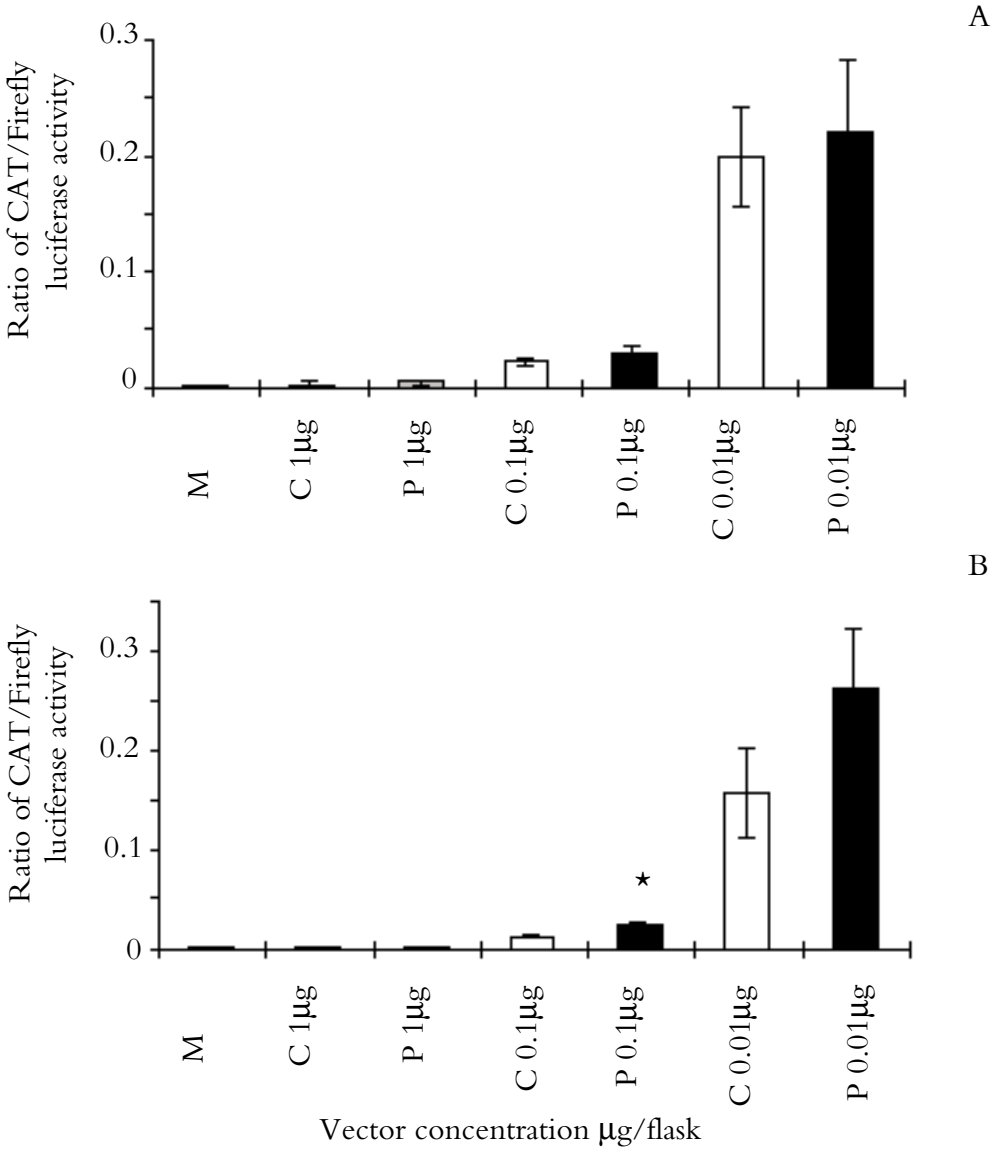

Figure 3.41 Effect of luciferase reporter concentration and time of transfection upon 163bpCAT reporter activity. Primary rat hepatocytes were transfected with a constant 5µg of 163bpCAT reporter /flask whilst the amount of pGL3-control luciferase reporter was altered from 1µg, 0.1, 0.01µg /flask. This combination of reporter constructs was then mixed with 20µg of DOTMA/DOPE for 5 hours and dosed with 1.0mM PB. Transfection was carried out at 4 hours (A) or at 24 hours (B) post isolation. The hepatocytes were harvested at 48 hours and analysed for CAT and firefly luciferase activity. The normalised results of these assays are expressed as the ratio of CAT/Firefly luciferase activity. Data points were analysed using Student's t-test. C = control, P = 1.0mM phenobarbital, M denotes mock transfected cells. *Reporter activity in the presence of 1.0mM PB is significantly higher in hepatocytes transfected at 24 hours post isolation (P = 0.03, df =2, two-tailed t-test).

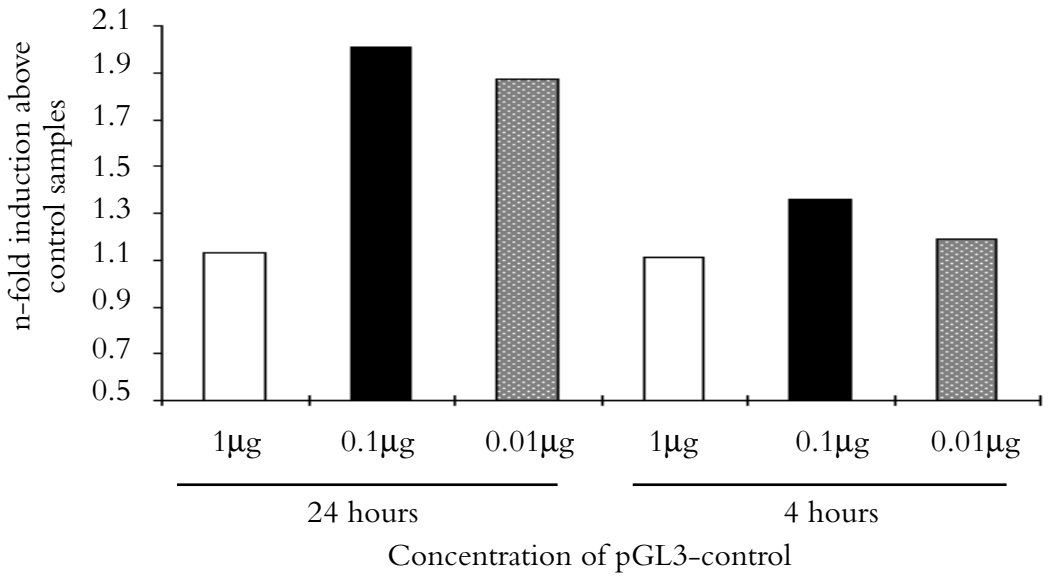

Figure 3.42 Effect on fold induction by PB of altering pGL3-control concentration and transfection time upon 163bpCAT reporter activity. Primary rat hepatocytes were transfected with 5µg of 163bpCAT reporter /flask whilst the amount of pGL3-control luciferase reporter was altered from 1µg, 0.1, 0.01µg /flask. This combination of reporter constructs were then mixed with 20µg of DOTMA/DOPE for 5 hours and dosed with 1.0mM PB. Transfection was carried out at 4 hours or at 24 hours post isolation. Hepatocyte monolayers were harvested at 48 hours and analysed for CAT\luciferase reporter activity. The normalised induction of 1.0mM PB treated hepatocytes over control hepatocytes was determined and expressed as a fold induction. The mean of three flasks has been plotted.

Section 3.7.10 Effect of 163bpCAT reporter concentration upon PB induction

The results presented in section 3.7.9 indicate that variations in the concentration of reporter construct present in the hepatocyte system during transfection can have an effect upon PBRE activation. This augmentation in the response exhibited by the PBRE to PB was also investigated by altering the concentration of the 163bpCAT-reporter construct used in each transfection.

Primary rat hepatocytes were transfected 24 hours post isolation with a varying amount of 163bpCAT reporter (10µg, 5µg, 1µg, 0.1µg) DNA /flask whilst the amount of pGL3-control luciferase reporter was kept constant at 0.01µg /flask (based on figure 3.41). This combination of reporter constructs was then mixed with 20µg of DOTMA/DOPE for 5 hours and subsequently dosed with 1.0mM PB. The hepatocytes were harvested at 48 hours and analysed for CAT and firefly luciferase activity as described in section 2.9.3 and 2.9.4. Figure 3.43 A and

Figure 3.43 B show the results of the normalised activation of the PBRE-containing reporter and the ratio of PB induction respectively. The lower limit of the assay occurs at between 0.1μg and 1μg of 163bpCAT reporter (Figure 3.43A) where the activity of the reporter is not significantly different from that of the mock-transfected cells. The normalised PBRE reporter activity increases significantly as the amount of 163bpCAT present in the transfection increases above this value.

The results presented in Figure 3.43B indicate that although there is a general increase in 163bpCAT activity linked to an increase in 163bpCAT-reporter concentration, this is not reflected in the overall induction exhibited by cells when dosed with 1.0mM PB. Here, the induction of PB-dosed cells above control samples is fairly consistent across the three orders of magnitude of 163bpCAT concentrations used.

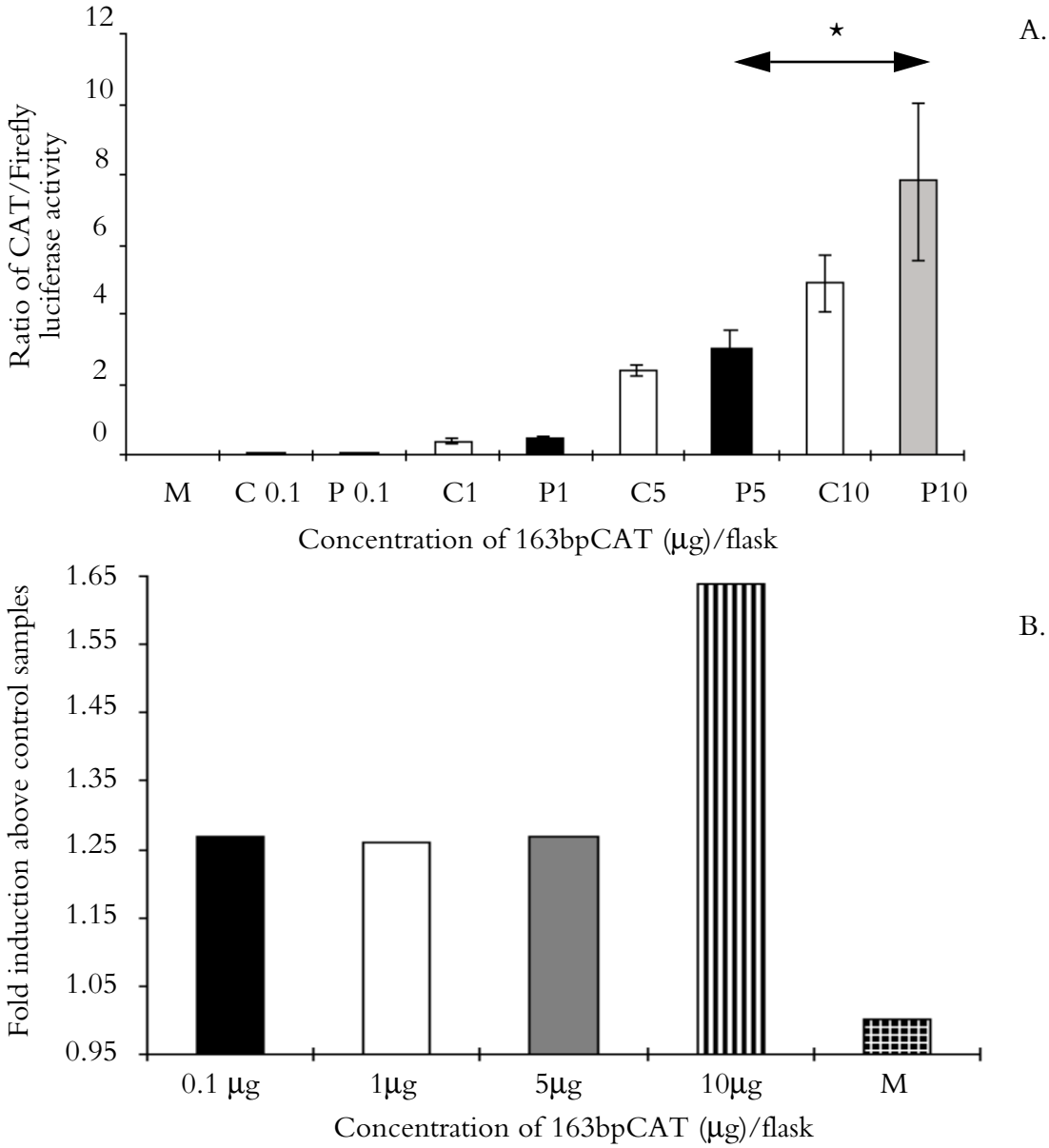

Figure 3.43 (A) Normalised activation of the PBRE containing reporter under various 163bpCAT concentrations, (B) Ratio of PB induction exhibited by these cultures. Primary rat hepatocytes were transfected with a varying amount of 163bpCAT reporter (10µg, 5µg, 1µg, 0.1µg) DNA /flask whilst the amount of pGL3-control luciferase reporter was kept constant at 0.01µg /flask (based on figure 3.41). This combination of reporter constructs was then mixed with 20µg of DOTMA/DOPE for 5 hours, 24 hours post isolation and dosed with 1.0mM PB. The hepatocytes were harvested at 48 hours and analysed for CAT and luciferase activity. (A) The normalised results of these assays are expressed as the ratio of CAT/Firefly luciferase activity. Data points were analysed using Student's t-test. C = control, P = 1.0mM phenobarbital, M denotes mock transfected cells. *Reporter activity present in cells containing 163bpCAT is significantly higher in hepatocytes than mock cells ($P < 0.009$, df =2, two-tailed t-test). (B) The normalised fold induction of 1.0mM PB treated hepatocytes over control hepatocytes was determined and expressed as fold induction above control samples.

Section 3.7.11 Xenobiotic induction of PBRE containing 163bpCAT reporter construct

The induction of the 163bpCAT-reporter gene by various phenobarbital-like inducers was investigated by integrating all of the parameters controlled for thus far in the transfection reporter assay system. Three compounds at various concentrations were tested for their activation of the PBRE reporter construct. This was carried out to see if the system could differentiate between different chemicals at different potencies. Primary rat hepatocytes were transfected 24 hours post-isolation with 10μg of 163bpCAT, 0.01μg of pGL3-control luciferase and 20μg of DOTMA/DOPE for 5 hours then dosed with one of three chemicals (0.5 mM PB, 1.0mM PB or 0.5mM CFA, 1.0mM CFA or 10μM GWY, 25μM GWY). Hepatocyte monolayers were harvested at 48 hours and assayed for CAT/firefly luciferase activity and normalised as described in section 2.9.3 and 2.9.4.

The results presented in Figure 3.44 showed that some modulation of the PBRE containing reporter construct was detectable with the current system. Hepatocytes transfected with the 163bpCAT reporter showed a significant 3.3 fold induction with 1.0mM PB and a 1.8 fold induction with 0.5mM PB. The GWY compound was found to be more potent than PB by RNAse protection assay. At 25μM and 10μM with this transfection system, GWY gave 2B2 inductions of 1.93 and 1.6 fold over control respectively. CFA at 1.0mM gave only a 1.24 fold induction above the control DMSO flasks.

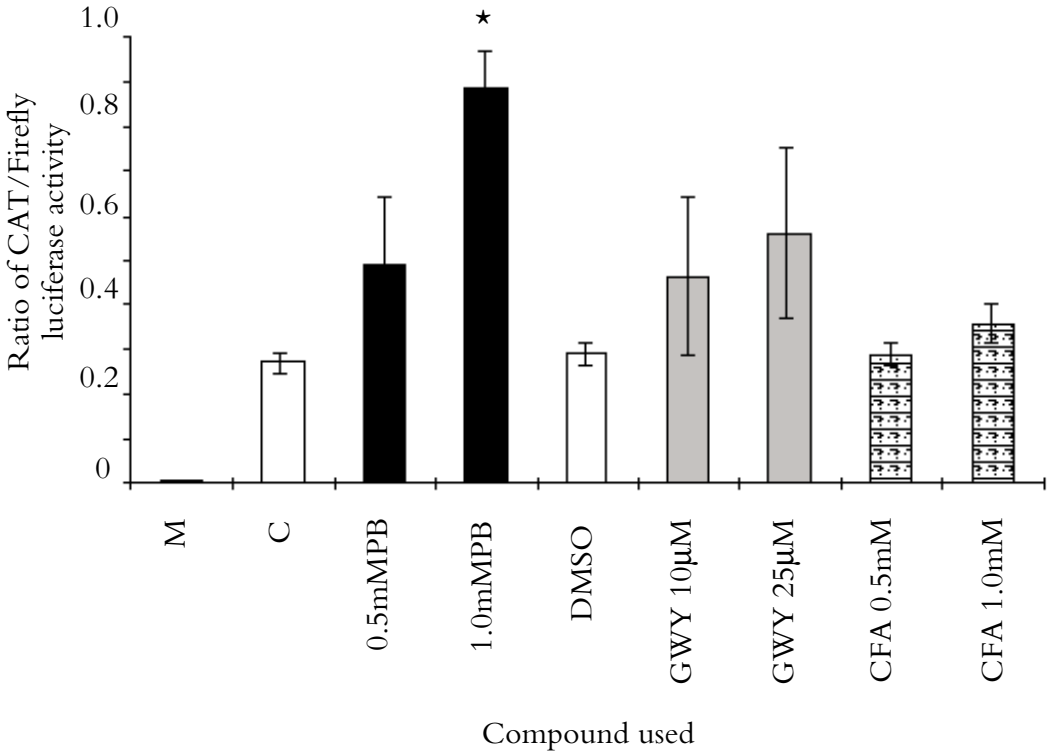

Figure 3.44 Modulation of the PBRE present in 163bpCAT by various xenobiotics. Primary rat hepatocytes were transfected 24 hours post-isolation with 10µg of 163bpCAT, 0.1µg of pGL3-control luciferase and 20µg of DOTMA/DOPE for 5 hours, then dosed with one of three chemicals (0.5 mM PB, 1.0mM PB or 0.5mM CFA, 1.0mM CFA or 10µM GWY, 25µM GWY). The hepatocytes were harvested at 48 hours and analysed for CAT and firefly luciferase activity. The normalised results of these assays are expressed as the ratio of CAT/Firefly luciferase activity. Data points were analysed using Student's t-test. C = control, PB = phenobarbital, M = mock transfected cells, DMSO = dimethyl sulfoxide, GWY = Glaxo-Wellcome compound, CFA = clofibric acid. *Reporter activity in the presence of 1.0mM PB is significantly higher than in its absence. (P = 0.008, df =2, two-tailed t-test).

Section 3.7.12 Transcriptional regulation of the PBRE by the nuclear orphan receptor mCAR

Resent publications by Negishi's group have suggested a link between the transcription regulation of the PB-inducible *Cyp*2b10 genes and the nuclear orphan receptors CAR and RXR (Honkakoski et al., 1998c). It is thought that CAR regulates the enhancer activity of the PBREM's nuclear receptor (NR1, NR2) sites as a heterodimer with the retinoid X receptor (RXR). This serves as a common heterodimerisation partner for the putative orphan nuclear receptor. Based on the findings of this group and coupled to the identification of two isoforms of the orphan nuclear hormone receptor CAR (Choi *et al.,* 1997), it was possible to devise a sub-cloning strategy to further characterise the PBRE/PBREM.

First strand cDNA was generated using 500ng of oligo-dT (12-18) primers and 1-5μg of mouse total RNA (a kind gift from M. Choudhury) as described in section 2.5.2 and this cDNA was then used as a template for PCR amplification as described by section 2.5.3. An 1160 bp fragment of the mCAR nuclear receptor (Accession number AF009327) starting at 120 bp and finishing at 1280 bp was amplified using this PCR strategy. This region was chosen as it contained the correct frame translational start and stop codons at 150 bp and 1230 bp respectively. The upper 5' GCC TGC TGC CTA AGG GA**T C**CA GGA GAC CAT GAC AGC 3'(5'-Prime) primer contained two base-pair mismatches (shown in bold) designated primer BAMPR1. The lower 5' GGT CTG GGG AAA GGA T**C**C AAG CCT GGG CCT CAA GTG 3' (3'-Prime) primer incorporated one base-pair mismatch (shown in bold) designed primer BAMPR2. This change in nucleotide sequence enabled a BamH I site to be introduced into either side of the 1160 bp fragment generated. This alteration would then make subsequent sub-cloning simpler.

PCR was carried out as described in section 2.5.3 and gave rise to a PCR product of 1160 bp (Figure 3.45). PCR products were purified using a Qiagen Qiaquick PCR spin columns as described in section 2.5.1 and ligated with pGEM-T vector (Promega), overnight at 4^{o}C. This ligation mixture was transformed into $CaCl_2$ XL-1 Blue *E. coli* cells. Several colonies were isolated (section 2.4.3) and double restriction digested (section 2.4.7) with BgL II/ Sal I to verify the presence of the mCAR PCR insert, these clones were designated mCAR-pGEM-T.

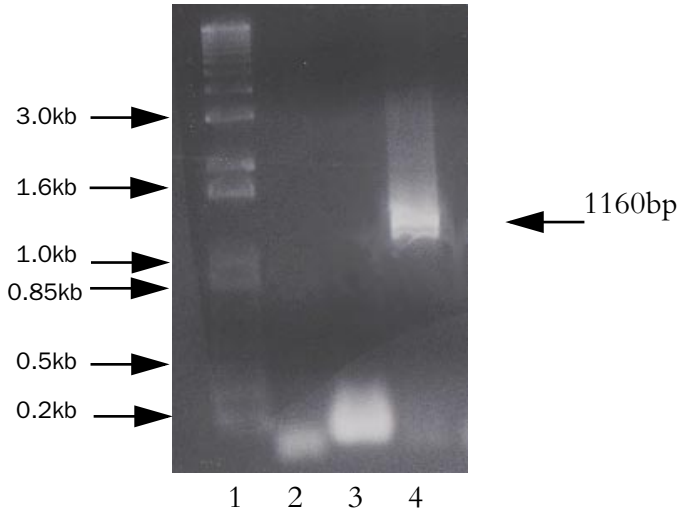

Figure 3.45 PCR amplification of 1160 bp mCAR cDNA. PCR amplification of an 1160 bp fragment of the mCAR cDNA was carried out using primer BAMPR1 5' GCC TGC TGC CTA AGG GA**T C**CA GGA GAC CAT GAC AGC 3'(5'-Prime) containing 2 base-pair mismatches (shown in bold) and primer BAMPR2 5' GGT CTG GGG AAA GGA T**C**C AAG CCT GGG CCT CAA GTG 3' (3'-Prime) containing one base-pair mismatch (shown in bold). PCR was carried out as described in section 2.5.3 and consisted of 1-minute at 94°C then 25 cycles at 3.0 minute denaturing at 94°C, 3.0 minute annealing at 68°C and 3.0-minute polymerisation at 68°C and cooling the reaction to room temperature. 5µl of the 50µl PCR reaction were run on a 1.2% pre-stained 0.1µg/ml EtBr, 1X TBE agarose gel at constant voltage of 70V for 2 hours. Lane 1 1 kb+ ladder. Lane 2 5µl PCR product without any Klen-Taq-1 DNA polymerase. 3 PCR product without any cDNA template. Lane 4 5µl of mCAR PCR product.

The pcDNA3.1 (-) (Invitrogen) expression vector and mCAR-pGEM-T vector were restriction digested using BamH I and gel purified. The gel-excised pcDNA3.1 (-) vector was ligated into the BamH I-digested mCAR-pGEM-T vector and transformed into $CaCl_2$ XL-1 Blue *E. coli* cells. Mini-preparations were made from positive cultures (section 2.4.3) and a restriction digest (section 2.4.7) with Bgl II/Xho I performed, which gave rise to fragments of 680, 909, 1589 and 4978 bp in size. This digest was carried out to verify that the plasmids contained the mCAR insert as shown in Figure 3.46. These clones were designated mCAR-pcDNA3.1 (-). This sub-cloning strategy was non-directional therefore three clones were chosen and shown to contain the correct size fragments before being sequenced using the primer 5' GAG ATG AAG CTT GCA GAT CGT GGA CAC AAC CCT C 3' as confirmation of the clone's integrity.

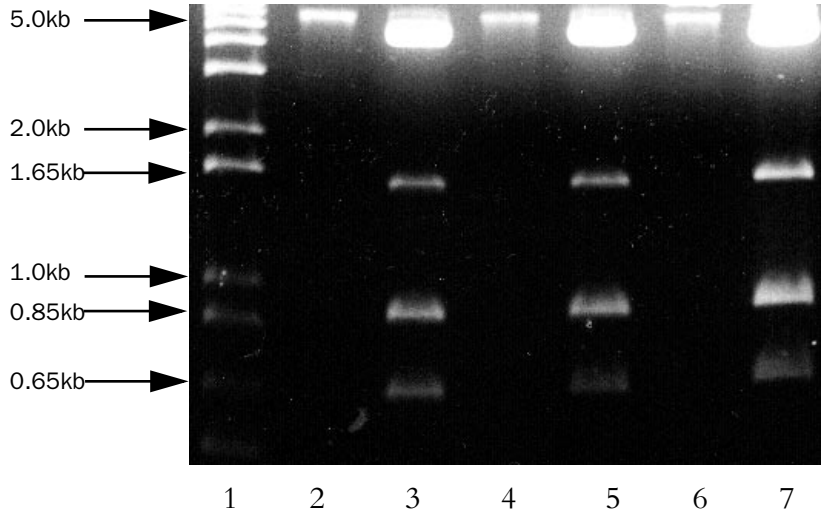

Figure 3.46 Restriction digest of the mCAR-pcDNA 3.1 (-) clones. The mCAR-pcDNA 3.1 (-) clones were double restriction digested with BgL II/Xho I and run on a 1.0 % pre-stained 0.1µg/ml EtBr, 1xTBE agarose gel at constant voltage of 70v for 2 hours to separate fragments of the mCAR-pcDNA 3.1(-) vector. This gives rise to fragments of 680, 909, 1589 and 4978 bp. Lane 1 1 kb ladder. Lanes 2, 4 and 6 uncut plasmids. Lanes 3, 5 and7 BglII/Xho I cut plasmids.

These mCAR expression vectors were now ready to be used to verify PBREM-dependent reporter gene activity in the 163bpCAT vectors used previously. The next sequence of experiments to be carried out, would have been to determine whether nuclear receptor CAR-dependent transcriptional activation does occur with these 163bpCAT vectors in the absence of PB, and to determine the role, if any, of androstenol in the culture medium and the effects on PB-mediated induction.

Chapter 4 Discussion

Section 4.1 PB induction of the rat CYP2B2 gene at the mRNA level

An aim of this thesis was to establish an *in vitro* primary rat hepatocyte culture system which was highly responsive to PB at both RNA and protein levels. This culture system would be used in the characterisation of the putative CYP2B2 phenobarbital response element (PBRE). It was anticipated that this hepatocyte system could also be used to develop a trans-activation assay for high-throughput drug screening to identify inducers of the rat CYP2B2 gene and to identify the mechanisms of induction by PB.

Section 4.1.1 Optimisation of the RNAse protection assay

Progress in understanding the molecular mechanisms involved in the regulation of the CYP2B1 and CYP2B2 genes by chemicals such as PB, has largely been hampered by the lack of a suitable cell culture system. Many groups have been unable to develop cell lines (derived from hepatoma cells), that express either constitutive or PB-induced levels of CYP2B1/2 (Schuetz *et al.*, 1988). This inability to express sufficient levels of CYP2B1/2 has been ascribed to "dedifferentiation" of the hepatoma cell lines. The implementation of a reproducible *in vitro* model to study CYP2B2 induction would provide a means by which the mechanism of CYP2B2 induction could be studied. This system must accurately model both the resting liver and changes that may occur *in vivo* following administration of PB. Such an *in vitro* model would mean that the problems encountered *in vivo* systems such as bioavailability, liver, and blood levels of the drug would be avoided.

During the course of the present studies a primary rat hepatocyte culture system was established in this laboratory to demonstrate PB-mediated induction of the CYP2B2 gene. As CYP2B1 and CYP2B2 gene DNA sequences share 97% identity a suitable technique was required, which could differentiate between transcripts of high sequence similarity. Several groups have studied the expression of CYP2B1/2 mRNA by using oligonucleotide probes, which were directed

against a sequence located in the region of 997 to 1015 base pairs from the initiation codon of the cDNA (Omiecinski *et al.*, 1985; Omeicinski, 1986; Giachelli and Omiecinski, 1987). However Atchison and Adesnik (1986) have identified four additional members of the CYP2B family, three of which hybridised to these oligonucleotide probes. There are therefore multiple CYP2B genes which are detected by these oligonucleotide probes. The problem of specificity arises as the oligonucleotide probes are short (25 bp) and so have the disadvantage that the identification of a particular mRNA is only based on a short section of sequence. By contrast, RNAse protection assays can use probes which are 300-400 bp in length, which provides specificity and allows the detection of rare transcripts as they can be labelled to a higher specific activity than oligonucleotide probes. The expression of the CYP2B2 gene was examined using the RNAse protection assay, as this sensitive technique would allow the degree of specificity required for differentiating between CYP2B1 and CYP2B2, and between other CYP2B species.

Section 4.1.1.1 Probe Specificity

The riboprobe constructed for these RNAse protection studies was designed to contain highly divergent regions between CYP2B1 and CYP2B2 (Kumar *et al.*, 1983; Fujii-Kuriyama *et al.*, 1982) and also between CYP2B3 (Labbe *et al.*, 1988) and CYP2B12 (Keeney *et al.*, 1998). Sequence analysis of the sub-cloned fragment of the CYP2B2 cDNA (Figure 3.2) showed that the sequence was in complete agreement with that of the published data for CYP2B2 (Kumar *et al.*, 1983). The RNAse protection assay has been shown to detect > 50% of single base-pair mismatches in a sequence (Myers *et al.*, 1985; Lee and Costow, 1987). The section of the CYP2B2 cDNA that was chosen for sub-cloning contains 17 bp mismatches with CYP2B1 over 379 bp; on this basis the CYP2B2 probe has a 7.6×10^{-6} chance of failing to discriminating between the CYP2B1 and CYP2B2 transcripts. RNAse protection assays carried out in this laboratory using a probe which spanned the region 967 bp to 1138 bp of the CYP2B2 cDNA showed that discrimination between CYP2B1 and CYP2B2 mRNA species was possible (Bars *et al.*, 1992).

The probe used in this thesis has been designed to contain highly divergent regions of the CYP2B cDNA. The probe used by Bars *et al.* (1992) contained 13 mismatches between the CYP2B1 and CY2B2 cDNAs whereas the probe used in this thesis contains 17 bp mismatches between CYP2B1 and CYP2B2. It is on this evidence that the assumption is made that the probe used in this thesis is specific for CYP2B2 mRNA, but this has not been demonstrated experimentally.

RNAse protection assays were performed on control and PB-induced *in vitro* and *in vivo* samples (Figure 3.4). A number of low molecular weight bands are present in this RNAse protection assay that were not anticipated (Figure 3.4, Arrow A3 and A4). There are a number of possible reasons for the presence of these low molecular weight bands in these RNAse protection assays. Firstly, the bands may have been produced as a result of hybridisation of the probe to other CYP2B species, or that the probe was hybridising to other RNA transcripts, which were not being digested by the RNAse digest step. This would indicate that the probe was not as specific as it was originally intended. It is believed this is unlikely given the specificity of the CYP2B2 probe as outlined above.

An alternative cause for the low molecular weight bands present in the RNAse protection assays is the over-digestion or non-specific digestion of 2B2: 2B2 hybrids during the RNAse digestion step of the protocol. This would lead to cleavage of the RNA: RNA hybrids and the generation of smaller protected fragments (Ambion technical documents). Examination of the Xlig cDNA insert (Figure 3.2) reveals a region containing an A/T rich region (15/20) at nucleotides 207-227 bp. Regions of the RNA: RNA hybrid that contain A/T rich sections are prone to 'breathing' or a relaxing of the double strand formation fragments (Ambion technical documents). This 're-laxing' of the A/T rich region may result in cleavage by RNAse A of the RNA: RNA hybrid, resulting in the generation of smaller protected fragments (Ambion technical documents); a sim-

ilar phenomenon was observed by Bell and Elcombe (1991b). It is known that even with a perfectly homologous probe and template that RNAse digestion can generate spurious low molecular weight bands (Bell and Elcombe, 1991b).

The low molecular weight bands visible in the protections carried out in this thesis (Figure 3.4-8) were thought to be occurring as a consequence of some contaminant component of RNAse A stock solution that was not being removed during its preparation. Experiments were carried out to control for this aspect by altering the commercial supply of the RNAse A stock (data not shown). It was found that different commercial supplies of RNAse A could affect whether the presence of low molecular weight bands were observed. Different ribonucleases attack RNA in a sequence specific manner. RNAse T1 specifically attacks the 3'-phosphate groups of G residues and cleaves the adjacent 5'-phosphate linkage, whereas RNAse A cleaves the 3' at pyrimidine residues. A comparison between two types of endoribonucleases was undertaken to determine if the incidence of spurious low molecular weight bands could be reduced. Figure 3.6 shows a comparison of the effects of using RNAse A and T1. The data present in Figure 3.6a indicate that as the concentration of RNAse A used on each sample is reduced, the intensity of the spurious bands is also reduced with a corresponding increase in the protected CYP2B2 fragment. It follows that as less RNAse A is being used that there is less of an opportunity for the samples to be exposed to unwanted RNAse A contaminants. Figure 3.6b shows that by using an alternative endoribonuclease the incidence of spurious bands is reduced. These experiments suggest that the lower molecular bands present in many of the protections assays were a consequence of RNA:RNA hybrid degradation due to some contaminant component of RNAse A stock solutions.

The data present above suggest that the RNAse protection assay used in this thesis is specific for CYP2B2 mRNA. It also demonstrates that the incidence of low molecular weight bands is a consequence of the type and source of endoribonuclease used during the RNAse digestion step.

Section 4.1.2 Comparison of CYP2B2 expression levels by PB *in vitro* and *in vivo*

The optimised RNAse protection assay was used to investigate whether PB induced CYP2B2 mRNA *in vitro* and *in vivo*. The results obtained in this assay should show a size difference between the full-length probe and the protected CYP2B2 mRNA species present in each sample. The *in vivo* samples produced by dosing rats with PB (80mg/kg (i.p.) for 3 days) show a protected fragment which appears to be of a similar size to that of the full-length probe (Figure 3.4). This result suggests that the signal obtained in the *in vivo* PB-treated sample is not protected CYP2B2 mRNA but is the signal obtained from full-length probe. Results presented in Figure 3.40 demonstrate that this is not the case. Here, the true size difference between full-length probe and protected CYP2B2 mRNA bands can be seen by comparing lane 3 and lane 4 respectively. Full-length probe and protected CYP2B2 mRNA appear to be similar in size for the *in vivo* PB-treated samples in Figure 3.4. This was due to overexposure of the autoradiograph in order to obtain a clear PB *in vitro* signal. This has the effect of making the distinction between full-length probe and protected fragments less defined. The yeast tRNA lanes present in Figure 3.4 demonstrates that the CYP2B2 probe used in this RNAse protection assay is full length and intact.

Figure 3.4 also demonstrates that CYP2B2 mRNAs is highly induced in our primary rat hepatocyte culture system when dosed for three days with 1.0mM PB compared to control cells. Control hepatocytes showed no detectable constitutive level of CYP2B2 mRNA expression in this culture system. However, PB-treated hepatocyte samples showed extremely high levels of CYP2B2 mRNA expression. The *in vivo* samples produced by dosing rats with PB (80mg/kg (i.p.) for 3 days) also showed marked PB induction of the CYP2B2 mRNA. Control *in vivo* samples also showed a very low level of constitutively expressed CYP2B2 mRNA. Comparison between *in vitro* and *in vivo* PB induced samples showed that the level of CYP2B2 mRNA expression *in vivo* was higher than that of the *in vitro* treated samples. One possible cause for this difference may have been that the *in vitro* samples were taken from cultures exposed to PB for 72

hours, which, as demonstrated in Figure 3.13 was around the time-point when the CYP2B2 mRNA signal begins to decline. Alternatively the lower *in vitro* signal may have been attributable to the culture conditions under which the hepatocytes were maintained. Nevertheless the level of PB-induced CYP2B2 mRNA is still extremely high in our *in vitro* samples.

The results obtained with this protection assay (Figure 3.4) show that the primary rat hepatocyte culture system used in this thesis is able to support PB-induced increases in CYP2B2 mRNA. The hepatocyte culture conditions used in Figure 3.4 consisted of cells grown on plastic flasks in CL15 media containing 8.3% foetal calf serum (FCS). The culture conditions used here are very different from those documented as being necessary for PB-mediated CYP2B2 induction (Newman and Guzelian, 1982; Waxman *et al.*, 1990). Both Waxman's and Newman's group include a cellular support matrix in their cultures. Waxman has also stated that CYP2B2 induction is reduced in the presence of FCS. Using protection assays I have been able to show that culture conditions such as the presence of foetal calf serum, or the absence of extracellular substratum or complex media additives have no effect on PB-mediated induction of CYP2B2 (data not shown, Figure 3.38-39 and 3.14).

It is believed that these experiments show that we have a CYP2B2 specific RNAse protection assay. They also show that our primary rat hepatocyte culture system supports PB-mediated induction of CYP2B2 mRNA. The induction seen in hepatocytes in this system suggests that the PBRE regulating CYP2B2 gene expression remains active for a minimum of 3 days. This demonstrates the validity of our system as a means for studying the mechanism of PB induction.

With the RNAse protection assay it is possible to detect several mRNA species in a single hybridisation assay provided the probes are designed to give transcripts of different lengths. Using this approach certain "house-keeping" genes could also have been included in this assay. This would have enabled the quantification of mRNA expression and interpretation of RNA

modulation, providing proof of equal RNA loading and allowing normalisation of signal. Such "house-keeping" genes as albumin, β-actin, cyclophilin, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and ribosomal proteins could be used as normalisation probes in this assay. However, these gene products have also been shown to be hormonally controlled or to vary in abundance from tissue to tissue (Savonet *et al.*, 1997; Thellin *et al.*, 1999). The use of 18s or 28s rRNA would provide a better option as a normalising probe as the abundance of the target mRNA would be paralleled by the amount of total RNA present. The use of a normalising probe may have helped determine if the different levels in signal obtained between *in vivo* and *in vitro* PB-treated samples was due to fluctuations in the amounts of mRNA used. However, the multiple bands produced by the CYP2B2 mRNA gene products may have hindered the interpretation of the bands produced by "house-keeping" gene products. The presence of more than one probe in the assay would also diminish the ability to accurately quantify mRNA, as the signal-to-noise ratio would be reduced. This is more prevalent when an abundant isoform produces a higher signal that masks the signal obtained from a low abundance isoform. The use of a normalising probe as an internal standard would have been advantageous for monitoring the fluctuations in signal between samples in this thesis. However, the caveats of using this method of normalising signal would have to be investigated further before it should be implemented.

Section 4.1.3 Induction of CYP2B2 by various chemical compounds

It has been demonstrated that our primary rat hepatocyte system supports PB-mediated induction of the CYP2B2 mRNA (Figure 3.4). It was uncertain whether the induction of CYP2B2 mRNA measured by the RNAse protection assay was a compound-specific response. Further protection assays were carried out upon primary hepatocyte cultures to determine whether this CYP2B2 response could be shown with other structurally dissimilar compounds (Figure 3.5 and Figure 3.8). Experiments carried out by Bars *et al.* (1993) had shown that CYP2B proteins and CYP2B mRNAs were induced with 1.0 mM clofibric acid (CFA). Results presented in Figure

3.5 are in agreement with these findings showing an increased induction of CYP2B2 mRNA above basal levels with both PB and CFA treated samples. Not surprisingly, CFA gave the greater induction of the two compounds. This higher induction with CFA had previously been shown by Bars *et al.* (1989).

Three structurally dissimilar compounds of varying potencies were tested for their ability to induce CYP2B2 mRNA levels. These compounds are structurally dissimilar to PB (Figure 3.7) and possess different functional groups. These compounds have been tested for their ability to induce CYP2B2 by Glaxo-Wellcome (personnal communication, Dr. Maurice Dickins) and the results of this protection assay are in complete agreement with this data. Figure 3.8 shows that two of the compounds (GWY and GWZ) were able to induce CYP2B2 mRNA above basal levels *in vitro*. However, GWX was unable to induce CYP2B2 mRNA levels. The GWX compound was shown not to be a CYP2B2 inducer and acts as a negative control for these protections, this finding is in agreement with Glaxo-Wellcome's findings. GWY is the stronger CYP2B2 inducer of the two Glaxo-Wellcome compounds. These Glaxo-Wellcome compounds were shown to induce in the μM range whereas PB was shown to induce in the mM range.

The results of these experiments demonstrates that our hepatocyte culture system supports CYP2B2 induction at the mRNA level. The assay also demonstrates that the response by the hepatocyte cultures is not a compound-specific response. The system can also differentiate between compounds of different potencies, and has specificity, insofar as the system does not induce with GWX. This protection assay has demonstrated that the hepatocyte culture system used in this thesis is able to support CYP2B2-induction by PB. However it was believed further characterisation of the hepatocyte system was required to obtain a better understanding of the mechanism of induction of the CYP2B2 gene.

Section 4.1.3.1 PB-like compounds show dose-dependent induction of CYP2B2

The induction of CYP2B2 mRNA in this primary rat hepatocyte system has been shown to occur with various compounds at a single concentration. Dose response curves were performed on the four compounds that were shown to be CYP2B2 inducers by previous RNAse protection assays (Figure 3.5 and 3.8). Induction of CYP2B2 mRNA has previously been demonstrated to occur in response to PB in a dose-dependent manner *in vitro* (Kocarek *et al.*, 1990). Dose response curves were constructed in our system for four structurally dissimilar compounds, demonstrating that our culture system exhibits CYP2B2 mRNA induction *in vitro* and that it occurs in a dose dependent manner.

Quantification and interpretation of the results generated by dose response curves would have been facilitated by phosphorimage analysis. Unfortunately this equipment was not operational throughout the duration of my studies and access to a densitometer was also unavailable. Quantification of these results would have enabled a comparison to have been made between the various concentrations of drugs used in each dose response experiment, and a clearer interpretation of the differences in expression obtained between *in vitro* and *in vivo* samples. Of the two quantification methods discussed above, phosphorimage analysis is the most accurate and convenient. The linear range of most phosphorimage scans extends over four orders of magnitude, provided care is taken to prevent overexposure of the scans. A standard curve is generated by comparing the signals obtained from known amounts of undigested, full-length radioactive probe which have been treated in exactly the same manner as the samples of interest. A comparison can then be made between the signal produced by the samples and the amount of radioactivity present in the samples, as determined by the standard curve. The use of densitomtry is less accurate, as the film used has a very narrow range in which a linear signal can be obtained. Hence, many different exposures must be taken so as to ensure that the signal obtained is within the linear range of the film.

The dose response for PB (Figure 3.9) demonstrated that basal levels of CYP2B2 were undetectable with autoradiography. However, even at 0.1mM PB there were highly induced levels of CYP2B2 present (Figure 3.9). This induction steadily increased to a maximum between 0.5-1.0mM PB with 2.0mM PB being cytotoxic (unpublished observations). This result differs from that of Kocarek *et al.* (1990) where the maximal response to PB occurred at 0.1mM. This difference in maximal PB concentrations between each system may be due to the differences in cell culture conditions. The culture conditions used to obtain the dose response for PB (Figure 3.9) during this thesis consisted of CL15 media containing 8.3% foetal calf serum, whereas Kocarek's hepatocytes were cultured on Matrigel substratum and maintained in Waymouth MB-752 media without foetal calf serum. The difference seen in maximal dose between these two culture systems may be a result of the variations in the amount of foetal calf serum present in the culture media. The absence of foetal calf serum in Kocarek's system may mean that more PB is available to the hepatocyte monolayer thus giving a greater response at a lower concentration.

A dose response of CFA was carried out (Figure 3.10). The results of this experiment showed that CYP2B2 mRNA was induced in a dose-dependent manner. The dose response of CFA demonstrates a visible induction of CYP2B2 occurring at 0.05mM steadily increasing to 1.0mM. Here also a direct comparison can be made between the induction seen with PB and that of the CFA (Figure 3.10, lanes 10 and 11). The induction of CYP2B2 mRNA in hepatocytes dosed with CFA *in vitro* is consistent with findings by Bars *et al.* (1993) and in experiments using oligonucleotide probes that were used to show CFA-induced levels of CYP2B2 in rat livers (Sritvastava *et al.*, 1990). DMSO (the vehicle used for CFA) does not induce CYP2B2 induction under these conditions.

The two Glaxo-Wellcome compounds that had already been shown to be CYP2B2 inducers by protection assays were examined to determine if the induction of CYP2B2 mRNA occurred in

a dose-dependent manner (Figure 3.11 and Figure 3.12). The dose response obtained for GWY (Figure 3.11) shows that this compound is more potent than PB with a response occurring at 1.0μM increasing to a maximal at 25μM. At 50μM the monolayers do not appear to be as confluent as they are at 25μM (data not shown) and this observation is mirrored by a decrease in CYP2B2 activity possibly due to this concentration being cytotoxic. The dose-response curve obtained with compound GWZ demonstrates that it has the greater potency of the two Glaxo-Wellcome compounds investigated. GWZ shows a marked increase in CYP2B2 mRNA levels even at 1.0μM, with a maximal response occurring between 5-10μM. These dose response curves confirm the data obtained in Figure 3.8. Here, there is less of a response with GWZ than there is with GWY. This is because in Figure 3.8 hepatocytes were dosed with GWZ at the sub-optimal concentration of 25μM, whilst GWY is at its maximal concentration. The results of these two experiments (Figure 3.11 and Figure 3.12) indicate that these compounds are more potent that PB. However, this potency is still quite low when compared to the extremely potent CYP2B inducer TCBOPOP. This compound has been shown to induce the level of *Cyp*2b10 mRNA in primary mouse hepatocytes at 0.5nM with a maximal induction occurring at 50nM (Honkakoski and Negishi, 1997).

The data present above demonstrates that the RNAse protection assay used in this thesis is able to differentiate between structurally dissimilar compounds and determine whether they produce a CYP2B2 response in primary rat hepatocytes. The assay demonstrates that the response occurs in a dose-dependent manner in these three compounds, which is consistent with the *in vivo* response. In this thesis, the data presented in Figures 3.9-3.12 demonstrate dose response curves for four compounds. The interpretation of this data would have benefited from quantitative analysis. Although unavailable, the general trend observed in dose response experiments can still be observed. This ability to differentiate between compounds containing different functional groups will provide a useful tool in identifying the putative PB receptor.

Section 4.1.3.2 Time course of PB-induced CYP2B2 induction

The primary hepatocyte culture system used in this thesis has been shown to support PB-mediated induction of CYP2B2 with several compounds in a dose-dependent manner. The duration of the PB-mediated induction of CYP2B2 in this hepatocyte culture system was unknown. An analysis of the time course of PB induction at 1.0mM was carried out to determine how long the response lasted. Hepatocytes were dosed for 3 days with 1.0mM of PB as this had been shown to be the optimum concentration for CYP2B2 induction (Figure 3.9). The protection assay of this time course shows that no induction of CYP2B2 mRNA is detectable at 6 hours but at 24 hours a significant fold induction is evident (Figure 3.13). This induction is maintained up to 72 hours where the signal appears to be diminishing. Previous work by other groups has shown slightly different time-course data, with 12 hours being shown optimal for chick embryo primary hepatocytes (Hahn *et al.*, 1991). Trottier *et al.* (1995) showed PB (1.0mM) induction of CYP2B2 to occur in a similar time-frame with CYP2B1/2 mRNA levels reaching a maximal at 48-72 hours after administration of PB.

This data demonstrates that it is possible using this primary rat hepatocyte culture system to maintain PB-mediated induction of CYP2B2 for up to 3 days, thus demonstrating the validity of this hepatocyte culture system in mimicking the *in vivo* response.

The interpretation of this RNAse protection assay data would have been enhanced by the use of phosphorimage analysis. This would have enabled direct comparisons to be made between each sample thus enabling greater quantification of the signals produced. Before any subsequent work is carried out using this RNAse protection assay, I would recommend that a thorough evaluation of the use of normalisation probes be made and that a means of quantification of the results obtained in each assay be addressed.

Section 4.1.4 PB mediated induction of CYP2B protein

Many groups have documented a requirement for specific culture conditions in order to obtain CYP2B2 induction (Waxman *et al.*, 1990; Trottier *et al.*, 1995; Kocarek *et al.*, 1990). A comparison between the levels of CYP2B protein expression was assessed in hepatocytes cultured in two different culture media. Several groups have reported that superior PB-mediated induction of CYP2B protein expression is achieved when using Chee's medium (Trottier *et al.*, 1995; Waxman *et al.*, 1990; LeCluyse *et al.*, 1999). Here we have compared Chee's medium (Gibco) to the culture medium used in this thesis CL15 (Sigma). The data presented in figure 3.14 shows a protein of approximately 50kDa is detected by the antiserum against CYP2B and shows that similar levels of CYP2B protein expression were obtained under both medium conditions. The level of CYP2B protein expressed was also seen to be at a lower level than the *in vivo* rat liver microsome standards. This difference between *in vitro* and *in viv*o levels of protein expression mirrors the trend observed in mRNA levels measured by RNAse protection assays.

The results presented in Figure 3.14 show that a 50kDa CYP2B protein is detectable with the antiserum against CYP2B. However, this data does not demonstrate whether this 50kDa CYP2B protein is functionally active. The presence of the CYP2B haemprotein could have been assayed by the use of the CO-reduced difference spectrum at 450nm as described by Omura and Sato, 1964 a, b. However, this assay would be more a measure of total cytochrome P450 content and P450 functionality than of CYP2B activity. The spectral assay, which uses the metyrapone-reduced (P446), verses reduced cytochrome P450 (P450) difference spectrum (Waxman *et al.*, 1982) would have been more specific for measuring the PB-induced CYP2B haemproteins. A determination of whether the CYP2B proteins were functional could have also been carried out by the use of enzyme activity assays. The functionality of the hepatocyte microsomes could have been tested by pentoxyresorufin O-dealklyase activity or by androstenedione 16 β-hydroxylase activity. Both of these enzyme activities have been shown to be CYP2B specific (Waxman *et al.*,

1982). Unfortunately none of the above assays were available in our laboratory during this study.

The protection assay and Western blotting data demonstrates that the primary rat hepatocyte culture system supports CYP2B2 induction at both the mRNA and protein level. This system is able to differentiate between compounds with different chemical structures and potencies and has been shown to mediate PB-induction for up to 3 days, without the need for complex medium additives and extracellular substratum supports. The system provides further evidence that the proposed hepatocyte culture system is a valid *in vitro* model, which can be used to characterise the induction of the CYP2B2 gene.

Section 4.2 Characterisation of the 5' flanking sequence of the CYP2B2 gene

PB induces transcription of the genes encoding CYP2B1/2 in the rat liver (Hardwick *et al.*, 1983). Although these two genes share 97% sequence identity, they differ both in their basal expression and in their response to PB. CYP2B2 protein is present at very low constitutive levels in the liver, whereas CYP2B1 is 5-10 times lower by comparison. The levels of these two cytochrome P450 genes are very different in the PB-induced liver. Here their levels increase to 20-fold for CYP2B2 and >100-fold for CYP2B1 (Christou *et al.*, 1987; Yamazoe *et al.*, 1987; Omiecinski *et al.*, 1985). Hence it follows that the differences between their DNA sequences and regulatory proteins affect how these genes are expressed. A better understanding of the mechanism of induction of the CYP2B2 gene would be obtained if the roles played by *cis* and *trans* acting factors that interact with the CYP2B2 DNA were elucidated.

Section 4.2.1 Cloning of the 5' flanking region of a CYP2B2-like gene

Determination of the sequence of the CYP2B2 gene would enable the functional elements constituting the promoter region of this gene to be dissected. Although CYP2B1 and CYP2B2 constitute the major PB-inducible cytochrome P450s of this subfamily, it has been estimated that 6-8 additional rat genes showing sequence identity to the CYP2B1/2 genes have been identified

(Atchison and Adesnik, 1983; Adesnik and Atchison, 1986; Mizukami *et al.*, 1983). It is not certain whether these CYP2B1/2-like genes are equally expressed or are inducible by PB.

When initial sequencing of the 5' flanking region of the CYP2B2 gene was undertaken only the region as far as -1.4 kb documented by Jaiswal *et al.* (1987) was known. Hence it was necessary to establish the sequence of the CYP2B2 clone used in this thesis to ascertain its validity and to determine the sequence of this gene beyond -1.4 kb. Once the 5' flanking sequence of this CYP2B2 gene was determined, a comparison with known data (Jaiswal *et al.*, 1987; Suwa *et al.*, 1985; Hoffmann *et al.*, 1992) could be made and a comprehensive DNA sequence for the CYP2B2 promoter region compiled. This compiled sequence could then be used to sub-clone truncated sections of the 5' flanking regions of the CYP2B2 gene into reporter constructs (see section 4.2.3.1).

A plasmid (D.R Bell, PhD thesis 1988) containing 11.7 kb of DNA was used to determine the 5' flanking sequence of the CYP2B2 gene. Primers were initially designed from previously published data (Jaiswal *et al.*, 1987) beginning at -1240 bp of this data and extended the sequence further 5' by automated fluorescent sequencing and oligonucleotide walking. Using the published data by Jaiswal *et al.* (1987) it was possible to start sequencing over -1.2 kb downstream from the transcription start site and extend this data to beyond -2.7 kb (Figure 3.17). The sequence provided by Jaiswal *et al.* (1987) data provides -1.4 kb of CYP2B2 DNA sequence. Primers were designed 160 bp from the end of this region so that sequence produced by automated fluorescent sequencing and oligonucleotide walking in this thesis could be "anchored" to this data; this also served to verify that I was sequencing the CYP2B2 gene. Sequencing was carried out using primers designed on both the forward and reverse strand so that a comprehensive CYP2B2 sequence could be compiled. Table 3.1 shows the primers used in this sequencing strategy. Figure 3.16 shows a cartoon of the oligonucleotide walking strategy and indicates some

of the key restriction enzyme sites that were used in subsequent sub-cloning strategies.

The sequence data generated by automated fluorescent sequencing and oligonucleotide walking was compiled into a single contiguous sequence and consisted of 1667 nucleotides from -1047 to -2714 bp. Once my sequence data had been assembled and manually checked it was decided to compare it to the sequence data that was now available (Jaiswal *et al.*, 1987; Hoffmann *et al.*, 1992; Shaw *et al.*, 1996; Sommer *et al.*, 1996). An initial sequence comparison was carried out between my data and the data of Jaiswal *et al.* (1987) and Hoffmann *et al.* (1992). A comparison of the overlap between my data and that of Jaiswal's data showed that the two sequences were identical. However, the comparison between my data and that of Hoffmann's showed a number of sequence discrepancies (Figure 3.17). At -2676 bp the compiled data shows a guanine residue whilst Hoffmann's data shows a cytosine residue. Analysis of my data shows two inserted residues occurring at -1876 bp (A) and -1527 bp (G). However, these insertions are in agreement with other more recent publications (Sommer *et al.*, 1996; Shaw *et al.*, 1996). The comparison also shows other sequence disparities such as the deletion of a T residue at -2226 bp. The T residue is present in the NR1 site within the phenobarbital response element (PBRE) and gives cause for concern as this deletion might affect whether or not the PBRE is functional (Figure 3.17). However, Stoltz *et al.* (1998) have recently published sequence data that is in agreement with my findings with respect to this deleted T residue, moreover their reporter constructs show PB-mediated induction of the phenobarbital response element, thus indicating that the extra T residue present in Hoffmann's data is due to a sequencing error. Three more guanine residues present at -1447, -1353, and -1264 bp could not be matched to residues in Hoffmann's data, as the nucleotides had not been assigned to a nucleotide base. These guanine residues were shown to be in agreement with data presented by Sommer *et al.* (1996).

The sequence generated in this thesis showed only one other area of mismatch (Figure 3.17).

This occurred at -2423 bp where in Hoffmann's data 3 extra bases (GAG) are present. These 3 extra bases could be present for a number of reasons. Firstly, the discrepancy occurs after a run of 21 alternating (GA) residues and so it is thought that the extra residues may simply be the consequence of sequencing errors. Hoffmann's sequence has already been shown to contain areas where sequence differences have occurred, and based on data from other groups and myself (Sommer *et al.*, 1996; Shaw *et al.*, 1996; Stoltz *et al.*, 1998) it is believed that these differences are due to sequencing errors. The extra 3 bases may also indicate that Hoffmann has identified the presence of a possible CYP2B2 allelic variant. It is possible that the GAG repeat is the occurrence of a polynucleotide polymorphism indicated by the occurrence of another 21 GA residues 3' to the extra GAG (Jeffreys and Brookfield, 1985).

I have sequenced the 5' flanking sequence of the CYP2B2 gene from -1047 to -2714 bp using primer-based automated fluorescent sequencing and oligonucleotide walking. The data presented here demonstrates the sequence of the CYP2B2 gene. This sequence has been compared to the data of many groups and has highlighted the sequence discrepancies of published data in a number of regions. It is believed that this comprehensive data is free from sequencing errors and represents the sequence of the CYP2B2 gene. The sequence data presented here also shows some of the restriction digest sites that are used to isolate the phenobarbital response element (PBRE) of the CYP2B2 gene for sub-cloning purposes.

Section 4.2.2 Possible regulatory motifs

The DNA sequence of the 5' flanking region of the CYP2B2 gene has been presented in Figure 3.17. This sequence was analysed for the presence of transcription factor binding sites or putative regulatory elements. Possible sites of regulation were highlighted using the transcription element search software (TESS) and the Transfac database (Bonfield *et al.*, 1995; Heinemeyer *et al.*, 1999). This suite of software has highlighted a tremendous number of different putative regulatory

elements, most of which were ruled out as unlikely candidates due to poor sequence matches (Figure 3.17). The regulatory elements highlighted closely resemble liver specific transcription factor binding sites HNF-4/3, C/EBP and Sp1.

A consensus GRE present between -1336 bp and -1350 bp present here has been shown to confer glucocorticoid responsiveness to reporter genes (Jaiswal *et al.*, 1990). This site was found to be in close proximity to a putative AP-1 site, which was found to have increased protein binding in rats dosed for 18 hours with PB (Roe *et al.*, 1996). Another putative GRE found at -2227 bp to -2244 bp when mutated was found to virtually abolish the PB response (Stoltz *et al.*, 1998) indicating that protein binding to this element may confer PB-responsiveness. Although TESS and the Transfac database have highlighted some regulatory elements it is unknown whether these identified elements have any functional significance. The relevance of these regions could be ascertained by sub-cloning these sections. Studies could then be undertaken to see if there is any correlation between the elements that have been identified and the proteins that interact with them.

Section 4.2.3 Determination of the location of the PBRE

The 5' flanking sequence of the CYP2B2 gene is depicted in Figure 3.16. This cartoon indicates some of the restriction enzyme sites that were used to sub-clone the phenobarbital response element (PBRE). Much attention has focused on an area designated the phenobarbital responsive enhancer module (PBREM) or the phenobarbital response unit (PBRU) (Honkakoski and Negishi, 1997; Trottier *et al.*, 1995). This sequence is present at -2155 bp to -2318 bp in the rat CYP2B2 gene and has been shown to contain many potential enhancer elements, and to confer PB inducibility to a heterologous reporter (Trottier *et al.*, 1995). This element contains a Nuclear Factor-1 binding site (NF-1) at -2218 to -2205 bp (Liu *et al.*, 1998; Stoltz *et al.*, 1998; Trottier *et al.*, 1995). This NF-1 element is found to contain sequence with a $TGGN_7CCA$ motif.

Ramsden *et al.* (1999) and Stoltz *et al.* (1999) also showed that mutation of this site affected binding of nuclear proteins indicating its involvement in the PBREM. Two nuclear receptor binding motifs (AGGTCA) designated NR 1or 2 have been shown to flank the NF-1 site (Honkakoski *et al.*, 1998b; Sueyoshi *et al.*, 1998). These NR sites have been shown to interact with the liver enriched, constitutively active receptor (CAR), that binds as a heterodimer with RXR, and have been implicated in the PBREM response mechanism (Kawamoto *et al.*, 1999; Sueyoshi *et al.*, 1998). It is thought that the ER-7 site is not essential for PB-responsiveness but may act as an accessory site (Stoltz *et al.*, 1998).

Section 4.2.3.1 Isolation of the PBREM

At the start of this study the exact position of the PBREM had not been established. The PBREM of the CYP2B2 gene was first localised to between -2318 bp and -2155 bp by Anderson and co-workers in primary rat hepatocytes (Trottier *et al.*, 1995). A similar region was later identified in the mouse *Cyp*2b10 gene (Honkakoski and Negishi, 1997). Reporter gene technology was used in this thesis to test for functional enhancer elements that may be present in the 5' flanking region of the CYP2B2 gene. Sections of the 5' flanking sequence of this gene were sub-cloned into appropriate reporter genes. These sections were then truncated so that only the PBRE was present in the reporter. It was hoped that by limiting the region of investigation of the 5' flanking sequence to the PBRE that a greater understanding of the PB-response mechanism could be gained. Two forms of reporter gene were used during the course of this work. These were the firefly luciferase vector and the CAT vector. A 1 kb Xba I-Xho I fragment of the 5' flanking region was ligated into a pGL-3-luciferase promoter vector (Promega). This construct did not contain any of the proximal regions of the CYP2B2 promoter as it had been shown that a PB-response was possible without these regions (Trottier *et al.*, 1995). A 163 bp PBRE pGL-3-luciferase promoter was also constructed to determine if a PB-response was possible with only a minimal 163 bp PBRE. Figure 3.20 outlines the basis of the sub-cloning

strategy that was used to obtain these constructs. These PBRE-containing reporters were driven by a strong SV40 promoter, a second reporter construct was designed which incorporated the same 163 bp PBRE region of the CYP2B2 gene, but with a TK promoter. This was carried out to examine the possibility that *trans* effects occurring between promoters on different co-transfected reporters were affecting reporter gene activity (Farr and Roman, 1991).

Section 4.3 Transient transfection of primary rat hepatocytes

The ability to introduce potential regulatory elements into mammalian cells in culture provides a powerful means for studying the function and control of mammalian transcription. A wide variety of techniques have been used to transfer foreign DNA into mammalian cells (Watannabe *et al.*, 1994). Although the transfer of DNA into established cell-lines is well developed, it is generally considered difficult to transfect primary cultured cells such as hepatocytes. Transfection of primary rat hepatocytes was used in this thesis as the culture system had already been shown to support PB-mediated induction of CYP2B2. The implementation of an efficient transfection procedure in primary rat hepatocytes in conjunction with reporter constructs would enable the mechanism of PB-induction of the CYP2B2 gene to be investigated. During this thesis a non-commercial 'in-house' lipofectin reagent designated DOTMA/DOPE was utilised as an alternative to the more expensive commercial lipofectin reagent (Gibco).

Section 4.3.1 Optimisation of transfection parameters

The mechanism of action by which DOTMA/DOPE enables DNA to enter a cell is not fully understood. It is thought that the reagent forms liposomes that interact spontaneously with the foreign DNA to form a positive lipid-DNA complex, which fuses with the cell membrane and is non-specifically adsorbed on to the negatively charged surface of the cells. At the start of this thesis there were no transfection protocols in use in our laboratories, it was therefore necessary to optimise this new protocol. Optimisation of the transfection protocol would indicate which variables were important in increasing transfection efficiency and enable a reproducible

transfection system to be implemented.

Section 4.3.1.1 Miscellaneous transfection variables

The presence of foetal calf serum (FCS) has been observed to increase cell attachment and prolong cell viability in our culture system (unpublished observations). However many liposome based transfection techniques must be performed in serum-free conditions, since transfection efficiency is thought to be inhibited by a polyvalent, negatively charged serum component (Felgner and Ringold, 1989). Preliminary experiments carried out in this laboratory demonstrated that the presence of foetal calf serum could affect transfection efficiency adversely (data not shown). This data was in agreement with the findings of Hofland *et al.* (1996), who demonstrated that the presence of as little as 2% of FCS could dramatically reduce the transfection of transiently transfected murine 3T3 fibroblasts. In this thesis 4 times as much FCS was used than in the Hofland study. Hofland and co-workers also showed that 2-15% of FCS in transfection medium could reduce the transfection efficiency of stable transfected fibroblasts by up to 30% (Hofland *et al.*, 1996). It was thought that the presence of the FCS might inactivate the formation of the DNA/liposome complex in this hepatocyte culture system thus reducing the transfection efficiency. All subsequent transfection experiments were carried out in the absence of FCS during the liposome/DNA step.

Transfection efficiencies were also affected by the procedure used to prepare the plasmid DNA. Comparisons were made between different commercial plasmid purification techniques (data not shown). It was noted that if DNA was prepared either with a Wizard ^{Tm} or Qiagen^{Tm} plasmid purification kits that there was a distinct difference in the transfection efficiency obtained. The Qiagen kit produced high quality DNA that could be easily transfected into primary hepatocyte cultures. Wizard kits on the other hand repeatedly produced DNA that contained an excess of ethanol and salt at the end of the protocol, which in turn would affect the efficiency of the

transfection protocol. It was suspected that an excess of salt or ethanol may have affected the formation of the DNA/liposome complex.

The pH of the transfection media was also observed to be a contributory factor to whether good transfection efficiency was obtained, with a pH of 7.4 giving the best results (data not shown). Preliminary experiments were also carried out to determine if the temperature at which the complex formation was carried out at affected the final transfection efficiency. The complex formation step was carried out at 4°C, 25°C and 37°C with no difference in final transfection efficiency being observed (data not shown). It was assumed that temperatures would be a discriminating factor as to whether complexes were formed or not, so the results of these experiments were somewhat surprising. These preliminary results suggest that the DNA/liposome complex formation is affected by the presence of FCS, excess salt and ethanol, but is temperature independent.

Section 4.3.1.2 Effect of DOTMA/DOPE concentration

During the optimisation step of the transfection protocol, the pRSV-β-GAL reporter (β-Gal) was used. This is a useful reporter gene as the transfection of the β-gal DNA can be checked visually by histochemical staining of the cell monolayer (Figure 3.25) (Sanes *et al.*, 1986). The percentage transfection efficiency is determined by counting the number of hepatocytes containing blue staining compared to those without. Of the many variables that were assessed during the optimisation of the transfection protocol, those of altering DOTMA/DOPE and DNA concentration and length of time to which the hepatocytes were exposed to the DNA/lipid complex formation were examined further still to determine their effect on transfection efficiency.

The concentration of the DOTMA/DOPE was the first parameter to be examined. Hepatocytes were transfected with 6μg of pRSV-β-GAL and between 0-80μg of DOTMA/DOPE reagent per flask for 5 hours and the expression of β-gal determined after 48 hours. The data presented

in Figure 3.22 demonstrates the effect of altering the DOTMA/DOPE concentration whilst keeping the DNA concentration constant. Here, even at 10μg per flask, extremely high (for hepatocytes) transfection efficiencies were obtained, with 23% of hepatocytes expressing β-gal. This experiment also demonstrates that there is an optimal concentration of DOTMA/DOPE reagent that can be used in each transfection. The DOTMA/DOPE has a maximal concentration of between 10-40μg per flask, with 24% of hepatocytes expressing β-gal. Higher concentrations of DOTMA/DOPE (60-80μg per flask) resulted in decreased transfection efficiency, with only 2-3% of hepatocytes expressing β-gal. This was attributed to the cytotoxic effects of increased concentrations of DOTMA/DOPE on the hepatocytes. When the monolayers were examined visually for signs of cytotoxicity it was apparent that deterioration of the monolayers was evident at the higher DOTMA/DOPE concentrations. The increase in transfection efficiency as DOTMA/DOPE concentration is increased and a corresponding decrease in cell viability at higher concentrations has been reported by many groups (Felgner *et al.*, 1987; Holmen *et al.*, 1995; Hofland *et al.*, 1996). Felgner *et al.* (1987) reported that an increase in lipofectin concentration would result in an increase in specific activity of cell extracts but this could also result in an increase in cell toxicity. This toxicity has also been reported to vary with cell type, cell density and the duration of exposure to lipofectin (Felgner *et al.*, 1987).

All transfections experiments carried out in this thesis were performed using a DOTMA/DOPE reagent made according to a modified method of Felgner *et al.* (1987). This DOTMA/DOPE reagent was compared to a commercial liposome reagent "lipofectin" (Gibco) to determine which reagent gave the higher transfection efficiency (data not shown). Here, it was found that our DOTMA/DOPE reagent was as effective as the commercial reagents and gave a similar non-toxic range of concentrations.

The data presented above demonstrates that it is possible to obtain high transfection efficiencies

with the primary hepatocyte culture system. A concentration of 10-40μg per flask of DOTMA/DOPE gives the highest transfection efficiency without any deleterious effects to the hepatocyte cultures.

The results presented in Figure 3.25 demonstrate that no phenotypical alterations were apparent at this optimised concentration of DOTMA/DOPE. However, a visual inspection such as this does not really confer whether the transfection protocol is having a cytotoxic effect upon the cells at these lower DOTMA/DOPE concentrations. At higher concentrations of DOTMA/DOPE, this clearly is the case as the monolayer shows a rapid deterioration in cell integrity. It was anticipated that if the monolayers treated with DOTMA/DOPE concentrations above 40μg per flask were to be analysed using the lactate dehydrogenase (LDH) assay, that they would all show signs of LDH leakage, indicative of cytotoxic cell culture conditions. This assay determines whether the plasma membrane of the hepatocytes has become compromised thus leading to the eventual death of the hepatocyte.

Another internal control that could be used to determine whether the transfection protocol was having cytotoxic effects upon the hepatocytes would be to perform the 3-(4, 5-dimethyl-thiaz-oyl-2-yl) -2, 5-diphenyltetrazolium bromide (MTT) assay (Fujii *et al.*, 1995). MTT is a water soluble tetrazolium salt; the mitrochondrial dehydrogenases present inside living cells reduce MTT, converting it to a water-insoluble, purple, formazan derivative. This formazan derivative is clearly visible when the cells are examined under the microscope. The insoluble formazan derivative is then solubilised in acidic isopropanol and measured spectrophotometrically at 570nm. Dead cells do not cause this conversion. This assay has the advantage that it is performed relatively quickly and that the decrease in formazan formation occurs earlier than cytosolic enzyme leakage. This assay, if available, would have given a method for determining the incidence of cytotoxicity that was more relevant to the hepatocytes viability rather than an assay

which provides a physical measurement of membrane integrity.

Section 4.3.1.3 Effect of DNA concentration

All subsequent experiments were carried out using the optimised DOTMA/DOPE concentration of 20μg per flask. The next parameter to be examined was the effect of variations in DNA concentration in the transfection protocol. Hepatocytes were transfected with varying concentrations (0-8μg per flask) of β-gal DNA, whilst keeping the concentration of DOTMA/DOPE constant (20μg per flask) for 5 hours and the expression of β-gal determined after 48 hours. The data presented in Figure 3.23 demonstrates the effect of altering the β-gal DNA concentrations whilst keeping the DOTMA/DOPE concentration constant. Here the immediate effect of altering DNA concentration is demonstrated (Figure 3.23) with very low transfection efficiencies (1-4%) being recorded at 1-2μg of DNA per flask. At very low quantities of DNA the signal produced by the transfected cells may be at the threshold of detection for the assay, thus giving very low transfection efficiencies. As the concentration of DNA is increased there is a corresponding increase in transfection efficiency (from 2-13%), with maximal transfection occurring at 6μg per flask. Here the importance of obtaining the correct ratio of DOTMA/DOPE to DNA is demonstrated. A bell shaped distribution is obtained as the concentration of DNA is increased which is in agreement with many groups findings (Felgner *et al.*, 1994; Hofland *et al.*, 1996). At lower concentrations the ratio of DOTMA/DOPE to DNA may be too low for effective formation of complexes. It is believed that as the DNA concentration increases the ratio between DOTMA/DOPE and DNA increases until the correct molar ratios are achieved for complex formation. At the higher concentrations the ratio of DNA to DOTMA/DOPE may be such that complex formation is inhibited. Figure 3.23 also shows that beyond the optimal DNA concentration (above 6μg per flask), transfection efficiency is reduced by almost 50%. This reduction is not due to cytotoxicity, as the monolayers of these transfected cells still look healthy unlike the monolayers at the higher concentrations of DOTMA/DOPE. The reduction in transfection efficiencies at

higher concentration of DNA may be occurring due to inhibitory factors or contaminants present in the DNA. It is possible that these inhibitory factors or contaminants present in the DNA have no effect on the transfection protocol at lower DNA concentrations as they are at too low a level to have an effect. However, as the DNA concentration is increases so too does the inhibitory factors or contaminants which then may affect the transfection procedure resulting in the observed reduced transfection efficiency.

The data presented above demonstrates that transfection efficiency is affected by the concentration of DNA used in the complex formation step. Figure 3.23 shows that 6μg of DNA and 20μg of DOTMA/DOPE per flask of reporter DNA is optimal for this transfection protocol. The data also shows the importance of obtaining the correct molar ratios of DNA and DOTMA/DOPE in order to achieve high transfection efficiencies.

Section 4.3.1.4 Effect of exposure to the DNA/DOTMA/DOPE complex over time

The transfection protocol had been optimised for both DNA and DOTMA/DOPE concentrations. The final parameter to be examined was the length of time for which hepatocytes were exposed to the DNA/DOTMA/DOPE complex. Hepatocytes were transfected with 6μg of β-gal DNA and 20μg of DOTMA/DOPE per flask for between 1-24 hours and the expression of β-gal determined after 48 hours. Figure 3.24 shows how alterations in this parameter can affect the transfection efficiency of the system. The data demonstrated that there is very little difference between the transfection efficiencies obtained from hepatocytes exposed to the complex for an hour and that of hepatocytes exposed for 24 hours. There is a slight increase from 4.5% at 2 hours to 7% at 4 hours, but after this time-point the number of cells transfected remains constant. The data demonstrates that the interaction between the complex and the monolayers occurs within an hour of exposure and that prolonged exposure to the complex does not increase the initial level of transfection significantly. The concentration at which DOTMA/DOPE reagent was

used in these transfection experiments was sub-toxic hence there was no cytotoxic risk to the hepatocytes during the transfection protocol. Results presented in Figure 3.22 indicate that the DOTMA/DOPE reagent can be cytotoxic to hepatocytes at high enough concentrations. As no data was available on the long-term effects of exposure of hepatocytes to the DOTMA/DOPE reagent in this system, it was decided to limit the length of time for which the hepatocytes were exposed to the DOTMA/DOPE reagent during the transfection protocol to 5 hours.

A transfection protocol has been developed in this thesis so that reporter constructs containing the PBRE can be used to examined the mechanism of PB-mediated induction of the CYP2B2 gene. This transfection system has been optimised for both DNA and DOTMA/DOPE concentrations. Transfection efficiency was found to be optimal when 6μg of β-gal DNA and 20μg of DOTMA/DOPE were used per flask. The transfection protocol was affected adversely by the presence of foetal calf serum, poor quality DNA, salt and ethanol. The transfection protocol was unaffected by the temperature at which the complex was allowed to form at and the length of time hepatocytes were exposed to the complex. However all subsequent transfections were carried out at room temperature, with a 5-hour exposure of the hepatocytes to the complex so as to standardise the technique. The results of Figures 3.22-24 demonstrate how effective this transfection protocol is, they also show how variable the transfection efficiency is with the number of blue cells fluctuating from 23% to 7% between experiments.

Section 4.4 Functional testing of the PBRE by reporter assay

This optimised transfection system could now be used in conjunction with the primary rat hepatocyte culture system to test the functionality of the constructs discussed in section 4.2.3.1. The assays used in this study were carried out as described by various workers, β-gal (Sanes *et al.*, 1986), CAT (Seed and Sheen, 1988) and firefly luciferase (DeWet *et al.*, 1985, 1987). The spectrophotometric assay for β-gal was 100 times less sensitive than the differential extraction

method for CAT reporter activity and 100000 times less sensitive than the bioluminescent assay of firefly luciferase reporter activity. For this reason a luciferase reporter was used to assess the activity of the PBRE and a CAT reporter was used to normalise the transfection protocol. Initial experiments were carried out using the original firefly luciferase assay protocol as described by DeWet *et al*, 1987. However due to technical problems with the luciferase assays, a commercial assay kit was used to measure reporter gene activity.

Experiments were carried out on both the 1kb-Pluc and the 163bp-Pluc reporter constructs to determine if PB-mediated induction of the PBRE could be monitored with the reporter assays (Figure 3.26). Both the 1Kb-Pluc and the 163bp-Pluc constructs were examined to determine the effects of varying the length of the 5' flanking region of the CYP2B2 gene. Hepatocytes were transfected with 3μg of either 1Kb-Pluc or 163bp-Pluc and 3μg of the normalising reporter pCAT and the expression of the reporters measured after 30 hours. The results presented in Figure 3.26 showed an increase in the activity of the PBRE-containing reporter constructs although neither the 1Kb-Pluc nor the 163bp-Pluc constructs were not PB-responsive.

Figure 3.26 demonstrated that the two reporters had significantly different reporter activities with the 163bp-Pluc reporter expressing a 3-fold greater normalised signal than that of the 1Kb-Pluc reporter. The reduced activity of the 1Kb-Pluc reporter indicates the presence of a negative regulatory element located between -483 bp and -1,110 bp. The negative element was assumed to be within this area as -483 bp is the location of the start of the 3' section of the 1kb CYP2B2 fragment and -1110 bp is the location of the start of the 3' section of the 163bp-Pluc reporter. Negative regulatory elements have been reported in this area by Honkakoski *et al.* (1996) and Park and Kemper (1996). Honkakoski's group showed that a region between -755 bp and -971 bp of the mouse *Cyp*2b10 gene decreased basal activity in transfected primary mouse hepatocyte cultures. Park and Kemper suggested that HepG2 cells transfected with constructs containing up

to -1.4 kb of CYP2B2 DNA may contain negative regulatory elements at -550 bp or between -725 to -1,400 bp of the CYP2B2 gene. These experiments in conjunction with data presented in Figure 3.26 tends to suggest that a negative regulatory element is present somewhere between -483 and -1110 bp of the CYP2B2 5' flanking sequence.

The data presented in Figure 3.26 demonstrates that the two reporter constructs containing the PBRE have been transfected into primary rat hepatocytes and are functional. The presence of a negative regulatory element has been localised to a region of the 5' flanking sequence of the CYP2B2 gene between -483 bp and -1110 bp. The reason for the lack of PB-responsiveness in the two constructs was unknown and was further examined.

Section 4.4.1 Reporter assay optimisation

The results presented in Figure 3.26 indicated that no PB induction of the PBRE was occurring. This lack of PB-responsive may have been because the hepatocyte culture system had not been optimised for the activation of the PBRE. This was not thought to be likely as protection assays had previously demonstrated that a high level of PB-induced CYP2B2 activity was present in the hepatocyte culture system. The results presented in Figure 3.26 demonstrate that both the 1kb-Pluc and the 163bp-Pluc constructs were able to express firefly luciferase activity in this system but were unable to show any difference in their response to PB. It was thought that this was an artefact of some form of interaction between the two plasmids used in the reporter assay that was affecting the measurement of the PB-response.

One possible cause for the non-responsiveness of the PBRE containing reporter constructs may have been due to competition between the two promoters for the same general transcription factors which in turn would affect the level of expression measured (Natesan *et al.*, 1997). Here the amount of one reporter present in the hepatocyte may affect the level of expression of another reporter thus giving an artificially raised or lowered overall assay result. There have also been

reports that *trans* effects occurring between promoters on co-transfected plasmids could affect the outcome of reporter gene expression on either one or both of the reporters involved (Farr and Roman, 1991). Here different levels of transcription from the plasmid promoters may occur due to promoter cross-competition. Reporter constructs containing different promoters, exhibiting different promoter activities, were designed to test whether this would affect the PB response (section 3.7.2). No further experiments were carried out using the 1Kb-Pluc reporter, as the negative response element was irrelevant to the dissection of the PBRE mechanism. Before the PBRE mechanism could be analysed it was necessary to resolve the effects that were being observed due to promoter interactions.

Section 4.4.1.1 Titration effects of transcription factors upon PB induced reporters

Previous experiments had demonstrated that consistent reporter assay results could only be obtained when a commercial assay kit was used. In all subsequent experiments a commercial reporter assay kit (Promega Dual luciferase reporter assay, DLRA) was used to assay the expression of the reporter constructs. The DLRA was designed to measure the expression of two reporters in the same cell extract. In the reporter system used in this thesis, the experimental reporter (pGL-3-luciferase) containing the PBRE was attached to the firefly luciferase gene and the control reporter (used to normalise the PBRE activity) contained the *Renilla* luciferase gene (pRL-CMV). This new reporter assay system had to be optimised for the two reporters being used to determine if any of the promoter effects discussed above were affecting PBRE expression.

The effect of competition for general transcription factors was examined by altering the ratio of control pRL-CMV *Renilla* plasmid to that of the 163bp-D-Pluc plasmid. The concentration of the pRL-CMV control plasmid was altered from 0.1-0.00001μg per flask whilst the concentration of the 163bp-D-Pluc plasmid was kept constant at 6μg (Table 3.2). The results shown in Table 3.2 showed that there was an extremely high signal-to-noise ratio obtained with these

reporters. *Renilla* reporter activity could be measured even at 100pg per flask with 3-9000 relative light units (RLU) being measured. The RLU measured for *Renilla* increased as the concentration of *Renilla* plasmid in the transfection was increased. The *Renilla* reporter exhibited extremely strong constitutive expression in this hepatocyte culture system with 100pg of *Renilla* plasmid being detected and as little as 0.01μg per flask being outside the limits of detection for the luminometer. This experiment showed that altering the ratio of pRL-CMV reporter to that of the 163bp-D-Pluc reporter did not affect either the PB responsiveness of the 163bp-D-Pluc reporter or the level of its expression.

A further experiment was carried out to determine if altering the ratio of the 163bp-D-Pluc reporter to that of the control pRL-CMV *Renilla* reporter would affect the PB-responsiveness of the 163bp-Pluc reporter. The concentration of the 163bp-D-Pluc plasmid was also altered from 6-0.1μg per flask whilst the pRL-CMV plasmid was kept at a constant 0.1ng (Table 3.3). The 163bp-Pluc reporter was shown to have a narrow range of effective concentrations. The results of the experiments outlined in Table 3.3 also showed there are no difference in 163bp-D-Pluc expression between control and PB-treated hepatocytes. Unlike the previous experiment where altering pRL-CMV concentration altered the signal produced by the reporter, altering the concentration of the 163bp-Pluc reporter did not alter the signal produced by this reporter. There appears to be a slight increase in *Renilla* luciferase values as the 163bp-Pluc-reporter concentration is reduced from 6 to 3μg per flask but this increase was not tested for significance.

The values that were obtained in Table 3.2 and 3.3 demonstrate that the lack of PB-responsiveness in the 163bp-Pluc reporter was not due to competition for general transcription factors. These experiments also demonstrate the extremely high level of expression that is exhibited by the *Renilla* reporter in this hepatocyte culture system. To prevent the possibility of other promoter interactions affecting the PB-responsiveness of the 163bp-Pluc reporter, transfections

were carried out with the minimum concentrations of both firefly and *Renilla* luciferase reporters i.e. 1.5μg of firefly luciferase plasmid and 0.3ng of *Renilla* luciferase plasmid per flask.

Section 4.4.1.2 Effects of promoter interactions

The experiments carried out above demonstrate that there is no titration of general transcription factors occurring in the transfected hepatocytes. These experiments do not address whether there is any promoter cross-competition occurring in this system which could affect the transcriptional activity of either reporter. Experiments were carried out to examine whether promoter cross-competition has any affect on the PB-responsiveness of the PBRE reporter.

Previous experiments have been performed using an SV40 driven luciferase reporter and a CMV driven *Renilla* reporter. The reporters used in this transfection system had not been optimised for the possible interactions that may occur between their respective promoters. Therefore, various reporters were utilised so that a comparison between reporters containing different types of promoter could be made. It was believed that by altering the type of promoter present between the two reporters that a suitable promoter combination could be achieved. The 163bp-D-Pluc firefly luciferase reporter contains an SV40-driven promoter so another luciferase reporter was generated designated TK2B2-F-Luc which contained a TK-driven promoter. The normalising *Renilla* luciferase reporters used contained either a CMV-driven promoter, or a TK-driven promoter. These reporters would be referred to in future discussion by abbreviating the first letters of the promoter that drives each reporter. Hence SC would denote an SV40-driven luciferase reporter co-transfected with a CMV-driven *Renilla* reporter and TT would denote a TK-driven luciferase reporter co-transfected with a TK-driven *Renilla* reporter, other combinations would be abbreviated accordingly.

The PBRE containing luciferase reporter constructs were co-transfected with a combination of either the CMV-driven or the TK-driven *Renilla* reporters. The effect of switching between the

different promoters on the PB-responsiveness of the PBRE containing reporters can be seen in Figure 3.29. A complex picture is presented from this data. Firstly only the TK: TK promoter combination of firefly luciferase and *Renilla* luciferase reporter seems to give a 2-fold increase in PBRE activation when dosed with PB. All other combinations of promoter give rise to a higher signal being produced in the control samples. The effect of altering the promoter used in the normalising reporter can be seen when comparing the SC transfected hepatocytes to the ST transfected hepatocytes or the TC transfected hepatocytes to the TT transfected hepatocytes (Figure 3.29). Here the PBRE reporter has stayed the same but by simply altering the *Renilla* promoter there is a corresponding change in the level of normalised signal recorded. This is probably due directly to a lower signal being produced by the TK-driven *Renilla* reporter compared to the CMV-driven *Renilla* reporter. If the *Renilla* signal is low then this will result in an increased normalised luciferase signal and *visa versa*.

The results presented in Figure 3.29 demonstrate the dynamic interplay that occurs between the reporters present in the hepatocyte culture system when different promoters are used. The data shows that the overall luciferase assay signal is affected by how well the normalising reporter is expressed. The comparison made above between different normalising reporters and their effects on overall signal does not address promoter cross-competition but it does highlight the importance of choosing the correct reporter combination. The effect of promoter cross-competition is seen more clearly when ST transfected hepatocytes are compared with TT transfected hepatocytes. Here the *Renilla* reporter contains the same promoter in both treatment groups so the signal produced by the *Renilla* reporter will be similar. Thus the normalised value obtained for the transfection groups will not be complicated by the problems described above. However Figure 3.29 shows a marked difference between transfection groups. In the ST transfected hepatocytes there is a 2-fold reduction in the response of the PB-treated hepatocytes compared to the control group whereas, in the TT transfected hepatocytes there is a 2-fold

increase in the PB-treated hepatocytes over the control group. It is not clear whether this result can be ascribed to reporter promoter cross-competition.

Many other groups have used SV40-driven promoters on the reporters used in their studies (Hanh *et al.*, 1991; Stoltz *et al.*, 1998; Dogra and May, 1999). Trottier *et al.* (1995) have shown their PBRE containing reporters to be PB-responsive whether the reporter contains a TK or an SV40 promoter. Honkakoski and co-workers have not only varied the reporter the PBRE is attached to but also the combination of promoters used (Honkakoski *et al.*, 1998; Kawamoto *et al.*, 1999). The results published by these groups demonstrate that PB-mediated induction of the PBRE is possible with different combinations of reporter promoters and tends to suggest that both culture and transfection conditions have a role to play.

The results presented in Figure 3.29 show that when a PBRE containing TK-driven luciferase reporter is co-transfected with a TK-driven *Renilla* reporter a 2-fold induction is obtained with PB-treated hepatocytes. No other combination of reporter was able to achieve a PB-response. It was believed that further optimisation of this current combination of reporters may increase the level of induction achieved.

Section 4.4.1.3 Cytotoxic effects of the transfection protocol

The transfected PBRE reporter has been examined for the effects of both general transcription factor competition and promoter "cross talk" within the transfected hepatocyte system (Table 3.2, 3.3 and Figure 3.29). However, the decrease in response seen with PB-treated cells in Figure 3.29 may also suggest that some form of cytotoxicity is occurring when PB dosing is carried out subsequent to transfection. This was investigated by measuring the amount of lactate dehydrogenase (LDH) that was being lost from the cells both during the transfection protocol and afterwards during PB treatment.

Hepatocytes were transfected with 2μg of TK-2B2-Luc and 0.3ng of pRL-TK *Renilla*; the amount of intracellular LDH leakage was then measured at various time points. Figure 3.30 demonstrates that neither the transfection protocol nor the subsequent dosing of the hepatocytes with 1.0mM PB gave rise to any cytotoxic effects. A 3-fold increase in LDH leakage was seen during the time the cells were exposed to the lipid-DNA complex. This increase was most likely the after-effects of the hepatocyte isolation procedure, although the absence of serum during the transfection protocol and the presence of the transfection reagent itself cannot be ruled out as contributory factors. At time 0 hours the transfection reagent was removed and the cells dosed with 1.0mM PB. Cytotoxicity due to the transfection procedure would be indicated by an increase in LDH levels. However, at 0 hours a 50% decrease in LDH leakage is recorded. Beyond this time-point there was a general increase in the amount of LDH leakage occurring between non-transfected and transfected cells. This difference was considered not to be at a cytotoxic level as the LDH leakage of the hepatocytes was found to be < 30% of the total intra-cellular LDH content. This assay demonstrated that there was negligible difference between the amount of LDH leakage occurring between PB-dosed transfected hepatocytes and control transfected hepatocytes.

The LDH assay presented in Figure 3.30 demonstrates that the transfection protocol used on the primary rat hepatocytes is not cytotoxic. It also demonstrates that the dosing of hepatocytes with 1.0mM PB subsequent to the transfection protocol is not cytotoxic to the cells. This data indicates that the differences seen between the different reporter combinations in Figure 3.29 is not a consequence of cytotoxicity due to PB treatment.

There are multiple assays that can be used to monitor the extent of cytotoxicity occurring during various techniques. The LDH assay measures the amount of LDH leakage that occurs as an index of plasma membrane damage. As described in section 4.3.1.2 the MTT assay is useful for

investigating the effects on cellular functions by various techniques. Other methods include the use of the fluoresciein diacetate (FDA), which is used as a viability stain. Here, this non-fluorescent, non-polar molecular readily traverses the cell membrane where non-specific esterases capable of acetate hydrolysis rapidly convert FDA to fluorescein which causes viable cells to fluoresce green. Liver-specific markers such as secreated albumin or transferrin levels could also have been monitored (Schuetz *et al.*, 1988). Alternatively the expression of liver-specific transcription factors such as C/EBPα or HNF-1 could have been used as indicators of cell viability (Padgham *et al.*, 1993). Never the less the LDH assay used in this thesis, provided a rapid and easy assay to perform in order to determine the possiblity of cytotoxic effects brought about by the transfection protocol.

Section 4.4.1.4 Effect of endotoxin contamination of PBRE reporters

The reporter plasmids used in the transfection procedure sometimes contain contaminant endotoxins, which affect transfection efficiency and the expression of the reporter genes (Weber *et al.*, 1995). If a reporter containing the PBRE were contaminated with endotoxins then this would affect the PB-responsiveness of the reporter. The presence of endotoxin contamination was examined by purification of the plasmid.

Contaminant endotoxins are introduced into the reporter plasmids during the lysis step of plasmid preparation. These contaminants can be removed by further purifying Qiagen prepared plasmid DNA using two caesium chloride-ethidium bromide (CsCl-EtBr) ultracentrifugations (Weeks *et al.*, 1986). All of the plasmids used in the transfection procedures were of the highest purity. Plasmids suspected of contamination with endotoxins were purified by two CsCl-EtBr ultracentrifugations. Comparisons were made between plasmids prepared by Qiagen preparation and plasmids prepared by Qiagen preparation and CsCl-EtBr ultracentrifugations (Figure 3.34). The results presented in Figure 3.34 show that there was no endotoxin contamination of the

plasmids used in our transfection protocols.

Section 4.4.1.5 Time course of PBRE expression

The 2-fold induction seen in Figure 3.29 was achieved when specific combinations of luciferase and *Renilla* reporters were used. A time course of the activation of the TK2B2-F-Luc reporter by PB was carried out to see if the level of induction seen at 30 hours could be improved upon at another time point. The time course showed no difference in the expression of the TK2B2-F-Luc reporter between control and PB treated hepatocytes at 3 and 8 hours. The expression of the TK2B2-F-Luc reporter in PB treated hepatocytes was 1.6-fold above control hepatocytes at 24 hours. At 48 hours the ratio of firefly/*Renilla* luciferase activity had increased but this was not significantly above control levels.

The results presented above demonstrate that in this hepatocyte culture system PB-mediated induction of the PBRE is possible in the reporter constructs used in this thesis. A 2-fold induction above controls in PB treated hepatocytes is possible when a TK: TK combination of promoters is used in the reporters. These reporters are unaffected by the titration of general transcription-factors and endotoxin contamination. A study of the time course of the expression of the TK2B2-F-Luc reporter gives a maximal 1.6-fold induction of PB treated hepatocytes at 24 hours, this response can be measured up to 48 hours after the addition of PB.

Section 4.4.2 Comparison of TK2B2-Luc reporter activity to 163bpCAT activity

Many groups have used either CAT or luciferase reporter constructs containing the 5' flanking region of the CYP2B2 gene to examine the activation of the PBRE. Most of this work has been carried out on rat, mouse or chicken primary hepatocyte cultures or cell lines such as HepG2 cells (Stoltz *et al.*, 1998; Kawamoto *et al.*, 1999; Hahn *et al.*, 1991). Such studies have demonstrated the activation of CYP2B1/2, *Cyp*2b10 and CYP2H1 regulatory elements in response to compounds like PB and TCBOPOP. The type of reporter gene construct, cell line

or primary culture and culture conditions used varies between research groups. The response in PBRE activation obtained under these different conditions varies considerably, ranging from 2-3-fold (Hahn *et al.*, 1991) up to 9-fold (Honkakoski and Negishi, 1997).

The culture system used in this thesis has shown that PB treatment of hepatocytes containing PBRE reporter constructs gives a 1.3-1.6-fold induction above control samples. However, this level of induction is somewhat low in comparison to the 6-10-fold induction seen by Anderson's group (Trottier *et al.*, 1995). The reason for these differences are unknown. A comparison between the 163bp-F-Luc construct and the 163bpCAT Anderson construct was made to ascertain if the low PB-responsiveness of the 163bp-F-Luc reporter was due to differences in culture conditions or in the plasmids themselves. The 163bp-F-Luc construct may have been designed so that the PBRE was too close to the promoter of the luciferase reporter, which could affect the transcriptional activation of the reporter. By comparing the 163bp-F-Luc to the 163bpCAT reporters, differences in spacing between each reporter would be compensated for. The use of the 163bpCAT reporter would also highlight any enhancer-promoter interactions that may be occurring in this system. The 163bpCAT reporter may possess a mutation which causes a high expression, if this is so then this reporter would maintain its high actvity. Another possible cause for the low induction may be due to culture conditions, this possibility could also be resolved by a comparative study.

Results obtained from CAT assays on transfected PBRE-containing reporters (163bpCAT) provided by Alan Anderson showed similar amounts of activity in both the mock and 163bpCAT transfected hepatocytes. This indicated that there was a problem with the 163bpCAT plasmid or with the CAT assay (data not shown).

Section 4.4.1.4 deals with the possibility that an endotoxin contamination could be affecting the activity of the 163bpCAT plasmid. Another possibility is that the CAT assay used to measure the

activity of the 163bpCAT reporter required further optimisation. Crabb and Dixon (1987) and Ginot *et al.* (1989) have reported that heat inactivation of extracts from cultured cells is necessary before CAT activity can be measured using the CAT assay. These workers found that the cell extracts from rat hepatoma H4IIE C3 cells were capable of deacetylating acetylchoramphenicol and accelerating the rate of hydrolysis of the acetyl-CoA present in the assay. In the present study, extracts were taken from hepatocyte monolayers that had been transfected with the 163bpCAT and pGL3-control reporters. These extracts were examined for the effects of heat inactivation on the amount of CAT activity that could be measured by the CAT assay (Figure 3.35). This experiment demonstrated that without the heat inactivation step of the protocol, CAT activity was undetectable by the assay. This data suggests that the hepatocyte lysates that have not been heat inactivated may possess some deacetylating properties, which may affect the CAT assay. The necessity for heat inactivation is in agreement with work carried out by Sleigh (1986) who demonstrated that heat inactivation of cell extracts prepared from mouse embryonal carcinoma (F9) cells gave a 5-fold increase in CAT assay signal compared to untreated cells.

The results shown in Figure 3.35 demonstrated that the volume of cell extract used in the CAT assay was important for obtaining a good signal-to-noise ratio. The correlation between increased cell extract volume with CAT assay activity was further examined in Figure 3.36. Here the point at which the assay was unable to detect any difference between treated and untreated cells (minimum threshold) for the assay was found to be 5µl. A steady increase in CAT activity was noted, as the volume of cell extract used was increased. However, the amount of cell extract used in the assay is limiting, as the assay is only linear over a small volume range (10-60µl of cell extract). In this case, 50µl of extract would be used in all subsequent assays.

Once the CAT assay had been optimised for the 163bpCAT reporter, studies were carried out to compare the activation of CYP2B2 response elements in the TK2B2-F-Luc construct to that

of the 163bpCAT construct designed by Anderson's group (Trottier *et al.*, 1995; Stoltz *et al.*, 1998). Figure 4.1 depicts a schematic of the 5' flanking region of the CYP2B2 gene indicating where the PBRE is situated. This cartoon also shows three reporter constructs used during this thesis. Here 1kb-Pluc denotes the construct used in Figure 3.26 to determine the PB-responsiveness of the PBRE. The constructs 163bpCAT and TK2B2-F-Luc denote the two constructs used in Figure 3.37. These latter two constructs represent a construct provided by Alan Anderson's group designated in this thesis as 163bpCAT (Trottier *et al.*, 1995, designated here as Sa-Sa/Ev) and a construct designed in this thesis and designated TK2B2-F-Luc.

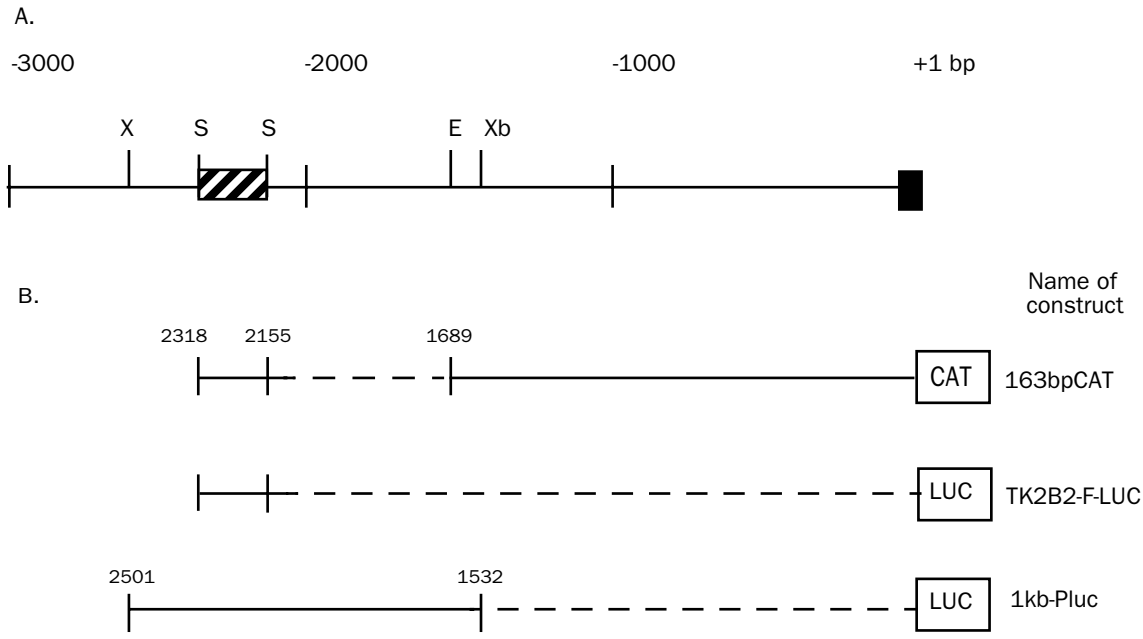

Figure 4.1 Schematic of CYP2B2 constructs containing the PBRE used in transfection studies on primary rat hepatocytes. A, Diagrammatic representation of the 5' portion of the CYP2B2 gene. The line represents the 5' flanking region, the solid box represents exon 1 of the gene and the hatched box represents the PBRE. Sequence co-ordinates are give in bp from the transcription start site at +1 to -3000 bp as shown. Restriction sites are labelled thus: Xb = Xba I, E = EcoRV, S = Sau3AI, X = Xho I. B, Diagram of constructs containing various lengths of the 5' flanking region (solid line) of the CYP2B2 gene inserted upstream of two reporter genes (open boxes). The dotted lines represent deleted fragments 5' of the CYP2B2 gene which have been removed to generate the CYP2B2 reporter constructs. The 163bpCAT reporter is the construct used in Anderson's work (Trottier *et al.*, 1995, designated here as Sa-Sa/Ev construct) and used in this thesis. TK2B2-F-Luc and 1kb-Pluc denote two reporters that were generated in this thesis. Diagram not to scale.

Work carried out by Anderson's group (Trottier *et al.*, 1995) showed that this 163bpCAT construct gave a 3.5-fold PB-induction when transfected into hepatocytes. In this thesis, this construct was shown to give levels of induction varying between 1.35 - 3.3-fold (Figure 3.37 and

Figure 3.40 respectively). By way of comparison the construct containing the PBRE designed in this thesis (TK2B2-F-Luc) was shown to give levels of induction varying between 1.37 - 2-fold (Figure 3.37 and Figure 3.29 respectively). However, in experiments comparing the PB-mediated induction of the two constructs, no difference in the levels of induction was found.

The conditions used when assessing the Anderson construct followed the original protocol as closely as possible (Trottier *et al.*, 1995). These experiments demonstrated that in our hands, hepatocytes transfected with the two PBRE-containing constructs (TK2B2-F-Luc and the 163bpCAT construct) show very little difference in the level of induction of CYP2B2 by PB (Figure 3.37). This provided further evidence to suggest that the lack of PB-induction of the TK2B2-F-Luc construct is not entirely a consequence of some attribute of the PBRE reporter itself or because of reporter promoter interactions.

The data presented above demonstrates that the 163bpCAT reporter is free from any endotoxin contamination. The optimised CAT assay conditions consist of 50µl of heat inactivated cell lysate extract incubated at 37^{o}C for 5 hours (Figure 3.35 and Figure 3.36). Using this CAT assay it was possible to demonstrate that the Anderson construct (163bpCAT) and the construct sub-cloned in this thesis (TK2B2-F-Luc) were able to show similar levels of PB-mediated induction of the PBRE containing reporters (Figure 3.37). Given this finding and the fact that the 163bpCAT construct has been demonstrated to mediate PB-induction in primary rat hepatocytes, it was decided that all subsequent analysis of the PBRE would be performed using the 163bpCAT construct. The low activity of the 163bpCAT reporter here does not agree with that observed by Trottier *et al.* (1995). This tends to suggest that differences in the culture conditions found between Anderson's group and the culture system used here may play a major role in determining whether a PB response was observed with these reporter constructs.

Section 4.4.2.1 PB-induction of the PBRE is unaffected by culture conditions

This primary rat hepatocyte culture system has already been shown to support CYP2B2 induction at the mRNA level in CL15 media. Chee's medium has been well documented as supporting PB-mediated induction of CYP2B2 (Waxman *et al.*, 1990; Trottier *et al.*, 1995). CYP2B proteins have also been shown to be PB-inducible in primary rat hepatocytes cultured in both CL15 and Chee's media in this system (Figure 3.14). Several recent studies have established that primary rat hepatocytes cultured on extracellular support matrix are able to support CYP2B2 induction by PB (Sidhu *et al.*, 1993; Hahn *et al.*, 1991, 1993; Honkakoski *et al.*, 1996). Anderson's group demonstrated that hepatocytes cultured with a Matrigel overlay in Chee's media showed a 6-10-fold induction of the 163bpCAT reporter in the presence of PB (Trottier *et al.*, 1995). It was the lack of extracellular matrix that was investigated as a possible cause for the reduced PB induction of the 163bp-CAT construct found in this thesis.

Primary hepatocytes were grown in the presence of plastic Falcon flasks, collagen type-I coated Falcon flasks (150μg/25cm^2 flask) or with Matrigel overlay (83.3μg/ml solution) and transfected with 5μg of 163bp-CAT, 0.1μg of pGL3-control luciferase, dosed with PB and harvested 48 hours post-transfection.

These investigations demonstrated that the PBRE-mediated induction of the 163bpCAT construct was unaffected by the conditions under which the hepatocytes were cultured (Figure 3.39). Here, a 1.5-fold increase in PB mediated induction of the 163bp-CAT construct was observed in hepatocytes cultured on plastic Falcon flasks. A similar level of induction was observed in hepatocytes cultured on Matrigel. Hepatocytes cultured on collagen coated flasks did not exhibit any difference between control and PB-dosed cells. There was no difference in the PB-mediated activation of the PBRE between hepatocytes cultured in a Matrigel overlay or on plastic flasks.

A post-transfection microscopic examination of the monolayers showed slight differences between control and PB treated hepatocytes. Here, PB-treated monolayers appeared more confluent than control monolayers (Figure 3.38). Conventional and Matrigel treated monolayers were very similar in their overall appearance (Figure 3.38 a, b and e ,f); but upon closer inspection Matrigel treated hepatocytes were found to be more spherical in appearance. Hepatocytes grown on collagen-coated flasks appeared to be more confluent and uniform with the cells appearing more flattened, there also appeared to be less cellular debris in the surrounding medium (Figure 3.38 c, d).

These results demonstrate that hepatocytes cultured under the conditions used in this thesis permit PB-mediated induction of the PBRE. A similar level of induction is observed with hepatocytes cultured with Matrigel overlay. This data confirms that the activity of the 163bpCAT reporter is not being inhibited by the conditions under which the hepatocytes are maintained. The difference in activity of the 163bpCAT reporter between hepatocytes cultured in collagen and hepatocytes cultured on plastic or Matrigel overlaid flasks is believed to derive from how the cells plate out. The hepatocytes grown on collagen-coated flasks are confluent and well spread out. It is thought that the cells' flattened shape means that there is minimal contact between them. Hepatocytes cultured with Matrigel overlay and to a lesser extent on plastic flasks are more spherical in shape and more clustered. This may lead to greater cell-cell contact between these hepatocytes, which is thought to be important in maintaining differentiated gene expression (Ben-Ze'ev *et al.*, 1988; Brown *et al.*, 1995).

Yoshida *et al.* (1996) have reported that the presence of insulin in culture medium could suppress the induction of CYP2B1/2 in primary rat hepatocytes. Experiments were carried out to test whether insulin would affect the PBRE response to PB in this system. The results of these experiments demonstrated that the PBRE response to PB was unaffected by the presence of

insulin (data not shown).

The hepatocytes transfected with the 163bpCAT reporter showed only a 1.5-fold induction in the presence of PB above controls (Figure 3.39). This relatively low level of induction was surprising, given that Trottier *et al.* (1995) showed the same reporter to give 6-10-fold induction in the presence of PB. An RNAse protection assay was carried out on hepatocytes that had previously been transfected with 5μg of 163bpCAT, 0.1μg of pGL3-control luciferase, dosed with PB and harvested 3 days post-transfection. This assay was performed to ascertain if the endogenous CYP2B2 mRNA expression was affected by the transfection protocol. The results shown in Figure 3.40 demonstrate that the CYP2B2 mRNA levels of the PB-dosed transfected hepatocytes were induced to a similar level to that of PB-dosed non-transfected hepatocytes. The results of the protection assay indicate that although PB-mediated induction of the PBRE is not occurring at as high a level as documented by Anderson's group (Trottier *et al.*, 1995), the transfected 163bp-CAT reporter did not affect the PB induction of the endogenous CYP2B2 mRNA present in the primary rat hepatocytes.

The data presented above demonstrates that the culture conditions used in this thesis, i.e. hepatocytes cultured in CL15 media containing 8.3 % foetal calf serum and insulin with no extracellular matrix, do not inhibit the PB-mediated induction of the 163bpCAT reporter. Using this culture system I was able to show that plastic flasks or Matrigel overlay are able to support PB-mediated induction of the 163bpCAT reporter. Moreover the transfection of hepatocytes with the 163bpCAT reporter was shown not to affect the endogenous CYP2B2 mRNA expression in this system.

Section 4.4.3 Optimising reporter concentrations

The reporters used in the experiments outlined above had not been examined for the effects of competition for general transcription factors (upon each reporter). This was investigated as it could be another possible cause for the low activity observed with the 163bpCAT reporter. The titration of general transcription factors was examined by altering the plasmid concentration ratio of both the PBRE containing reporter (163bpCAT) and the normalising reporter (pGL3-control). The concentration of the pGL3-control luciferase plasmid were altered from 1.0μg-0.01μg per flask whilst the concentration of the 163bpCAT plasmid was kept constant at 5μg (Figure 3.41 and 3.42).

Variation of time of transfection post-isolation of the hepatocytes was also examined. Many groups transfect 5 hours after isolation of the hepatocytes and begin dosing 4 hours subsequent to this (Honkakoski *et al.*, 1996; Trottier *et al.*, 1995). In this thesis all transfections were carried out 24-hours post-isolation of the hepatocytes. It was thought that this delay in transfection and dosing time might contribute to the reduced level of PB induction occurring in this system. Thus both the ratio of reporters and the transfection time were examined to determine if these variables had any relevance to the PB-mediated induction of the PBRE (Figure 3.41 and Figure 3.42).

The results presented in Figure 3.41 demonstrate the limits of the assay with regard to normalisation of 163bpCAT activity. If the concentration of the pGL3-control reporter is too high (above 0.1μg per flask) the signal produced by the control reporter "drowns out" the signal produced by the 163bpCAT reporter to the extent that it cannot be measured above the mock-transfected cells. Reducing the pGL3-control reporter concentration leads to an increase in normalised 163bpCAT signal and so a clearer analysis of the level of induction due to PB is achieved. The effect of time of transfection was also examined and the results of these experiments demonstrate that there is no difference between whether the hepatocytes were

transfected at 5 hours post-isolation (Figure 3.41a) or 24 hours post-isolation (Figure 3.41b). The difference in the level of induction between control and PB-dosed hepatocytes over both time and a range of concentration is shown more clearly in Figure 3.42. Here, the increase in normalised signal does not correspond to an increase in the level of induction of the 163bpCAT. This data shows that the effect of concentration over three orders of magnitude has little effect on the level of induction of the 163bpCAT reporter by PB at both time-points. However, the activity of the 163bpCAT-reporter shows a small increase in the level of induction when transfection is carried out at the 24-hour time point. This experiment demonstrated that altering the ratio of the pGL3-control reporter to that of the 163bpCAT reporter did not affect either the PB responsiveness of the 163bpCAT reporter or the level of its expression.

Further experiments were undertaken 24-hours post-isolation to determine the effect of altering the concentration of the 163bpCAT reporter (10μg-1μg per flask) whilst keeping the normalising reporter (0.01μg per flask) concentration constant (Figure 3.43a). This experiment demonstrates that as the concentration of 163bpCAT is increased, a corresponding increase in normalised 163bpCAT activity was observed. The experiment also demonstrates that the lowest concentration of transfected 163bpCAT reporter that can be measured under these conditions was 1μg. Figure 3.43 demonstrates that the normalised level of induction of the 163bpCAT reporter does not alter over the concentration range examined (approximately 1.25-fold), with 10μg being the only exception were a 1.65-fold induction was recorded.

The results presented in Figures 3.41-3 demonstrate that the 163bpCAT reporter is unaffected by competition for general transcription factors in this transfection system. Optimal 163bpCAT activity was obtained when hepatocytes were transfected 24 hours post-isolation with 10μg of 163bpCAT and 0.01μg of pGL3-control luciferase reporter. Under these conditions 163bpCAT and pGL3-control are in the correct molar ratio so that a 1.65-fold induction was achieved. Thus

the PB-mediated induction of a PBRE-containing reporter construct has been demonstrated with the use of a primary rat hepatocyte system and an optimised transfection system. This system could now be used to further elucidate the PB-mediated induction of the PBRE in the 163bpCAT reporter.

Section 4.4.4 Xenobiotic induction of the PBRE

The induction of the 163bpCAT reporter by various known CYP2B2 inducers was investigated using the transfection and reporter assays that had been optimised in this thesis. Three compounds were used to determine if the system could differentiate between various chemical structures, potencies and concentrations. Transfections were carried out using 10μg of 163bpCAT and 0.01μg of pGL3-control reporter to determine if the reporter system optimised in this thesis was capable of showing the PB-mediated induction of the CYP2B2 gene via the PBRE, using several different xenobiotics. The results of this experiment demonstrated that the 163bpCAT reporter containing the PBRE could be activated by these xenobiotics (Figure 3.44). Here, a significant 3.3-fold induction of the 163bpCAT reporter was demonstrated with 1.0mM PB. The response also occurred in a concentration-dependent manner, with less induction as the concentration of PB was decreased (Figure 3.44). This result was also mirrored, but to a lesser extent, with the potent CYP2B2 inducer, GWY (25μm and 10μm) and with CFA (1.0mM, 0.5mM).

These results demonstrate that I have established a primary rat hepatocyte culture system, which can be used to differentiate between different xenobiotics at different concentrations and which have different chemical structures and strengths. It is hoped that this reporter assay system could be used to identify the molecular mechanisms by which the PB-inducible transcription of the CYP2B2 gene occurs.

Section 4.4.5 Possible role of the constitutively active receptor in PB induction

Recent publications by Negishi's group have suggested a link between the transcription regulation of the PB-inducible *Cyp*2b10 genes and the nuclear orphan receptors CAR and RXR (Kawamoto *et al.*, 1999; Sueyoshi *et al.*, 1999; Honkakoski *et al.*, 1998). CAR binds to the PBRE NR sites as a heterodimer with the retinoid X receptor. This CAR-RXR-PBRE complex has been shown to be functional in gel shift assays (Sueyoshi *et al.*, 1999). However, this CAR-dependent transcriptional stimulation does not require the presence of PB to elicit a response in HepG2 cells. Androstane metabolites have been shown to inhibit CAR's transcriptional activity by dissociating the CAR-RXR-DNA complex from transcription co-activators like steroid receptor co-activator-1. It is this interaction that is thought to maintain the CAR receptor in an inactive conformational state (Forman *et al.*, 1998). PB and PB-like inducers have been shown to abolish the inhibitory binding of androstanes to CAR with the derepression of the receptor's activity.

An mCAR expression vector was generated to verify whether PBRE-dependent reporter gene activity in the 163bpCAT was possible in the absence of PB (Figure 3.46). This work was the final experiment in this thesis, and it was hoped that this construct would facilitate the determination of the role that androstane plays, if any, in this primary rat hepatocyte culture system.

Section 4.5 Summary

This project set out to study the molecular mechanism of the phenobarbital induction of the CYP2B2 gene in an *in vitro* primary rat hepatocyte culture system. A sensitive and specific RNAse protection assay was used to demonstrate that CYP2B2 mRNA was highly induced by PB in this hepatocyte culture system. The induction measured by protection assays was observed to occur in a dose and time-dependent manner with PB. This response has also been shown to occur in a dose-dependent manner with structurally dissimilar compounds that have been shown to be barbiturate type inducers. The system was able to discriminate between the different potencies of compounds used in the assay. Western blotting was also used to demonstrate the induction of CYP2B proteins by PB. Many workers have demonstrated that CYP2B2 induction was only possible with media such as Chee's medium. Western blotting was used to demonstrate that comparable levels of induction were achievable with the medium used (CL15) in this culture system.

Sequencing of the 5'flanking sequence of the CYP2B2 gene identified many putative regulatory elements, including the PBRE. The position of the PBRE was identified and truncated portions of the PBRE were sub-cloned into various reporter constructs. A transfection protocol was optimised during this thesis and this technique was utilised to transfect reporter constructs into primary rat hepatocytes. These PBRE-containing reporter constructs were shown to be functional in the hepatocyte culture system, although they did not show marked PB-mediated induction of the PBRE reporter. Many different variables were examined as to the reason why only a 1.3-1.6-fold induction of the PBRE reporter was possible. These included competition for general transcription factors between the two promoters present on the reporters as well as promoter cross-talk interactions. However, none of these were found to have an impact on the poor inducibility of this system.

It was not known if the low PB-inducibility of the 163bp-F-Luc reporter was due to a reporter interaction within the system or because of some unknown attribute of the reporter. It was possible that the reporter construct had been designed so that the position of the PBRE was too close to the promoter of the reporter. This may lead to some form of steric hindrance between the PBRE and the reporter promoter, thus interfering with the transcriptional activation of the reporter. Examination of the proximity of the PBRE to the promoter in the 163bp-F-Luc construct confirmed that this distance was comparable to that seen in a construct used by Anderson (Trottier *et al.*, 1995).

The work carried out by Anderson's group (Trottier *et al.*, 1995) demonstrated that with the 163bpCAT construct, a 3.5-fold induction was achievable when hepatocytes were exposed to PB. This level of induction was very similar to the maximal (optimised) 3.3-fold induction obtained in experiments carried out in Figure 3.44. However, in experiments which compared the 163bpCAT construct to the TK2B2-F-Luc construct (Figure 3.37), the level of induction of the 136bpCAT construct was lower (1.35-fold), but comparable to the TK2B2-F-Luc construct (1.37-fold). This difference in the level of induction between experiments highlights the fluctuations that occur sometimes between transfection experiments. The 163bpCAT construct provided by Anderson possesses an extra 1.6 kb of CYP2B2 5' flanking sequence, as demonstrated in Figure 4.1. A better comparison could have been made between the construct provided by Anderson and the TK2B2-F-Luc construct if the two constructs were to have contained just the PBRE alone. Anderson's group demonstrated that with such a construct, PB-mediated induction fluctuated between 2.7 - 8.8-fold depending upon the position and orientation of the PBRE (Trottier *et al.*, 1995). It has been demonstrated in this thesis that a comparison between the 163bpCAT reporter and the TK2BB-F-Luc reporter gave almost identical levels of induction in this system. This posed the question of the compatibility of the two culture systems. The conditions used in this thesis were changed to mirror the conditions

used by Anderson's group i.e. hepatocytes were cultured with a matrigel overlay. These experiments showed no difference in induction of the reporter between the culture conditions used this thesis and that of Anderson's group.

Another conclusion that may be drawn from this data is that there are some unknown attributes of the reporter or the culture system affecting the PB-responsiveness of the PBRE reporters. The simplest explanation for the lack of PB-responsiveness of the reporters is that expression in this system is not high enough for net induction to be detected in the hepatocyte culture system. Many of the reporters used in this thesis show poor induction with PB. In fact certain combinations of control and PBRE containing reporters showed a reduced expression in reporter activity in the presence of PB. These data suggest that the PBRE-containing reporters do not mediate PB induction. However, recent publications have demonstrated the importance of the PBRE in the PB-mediated induction of the CYP2B2 gene (Stoltz *et al.*, 1998; Park *et al.*, 1996; Honkakoski et al., 1997; Ramsden *et al.*, 1993). Ramsden et al. (1993) demonstrated that transgenes containing 19kb of the 5' flanking region of CYP2B2 (which included the PBRE) were responsive, whereas transgenes with only 800 bp (which did not contain the PBRE) were not. Both Negishi's and Anderson's groups have localised the PBRE to a 51 bp element in the 5' flanking sequence of the CYP2B2 gene (Honkakoski *et al.*, 1998; Trottier *et al.*, 1995). This PBRE has been shown to confer PB-inducibility to PBRE containing reporter genes. Site-directed mutagensis by these groups have demonstrated that certain nuclear receptor binding sites present in the PBRE are essential to confer maximal PB-inducibility. Experiments have shown that a 3.3-fold maximal PB induction is possible with PBRE-containing reporters in this thesis. Taken together with this, the data outlined above indicates that PB-mediated induction of the CYP2B2 gene occurs via the interactions of multiple regulatory proteins and *cis* acting elements which constitute the PBRE.

The RNAse protection and Western data both demonstrate that there is high-level induction with the endogenous CYP2B2 gene. This was further demonstrated when protection assays were carried out on hepatocytes that had previously been transfected with PBRE reporter constructs. Here the level of CYP2B2 mRNA was found to be unaffected by the transfection protocol (Figure 3.40).

In the presence of PB this optimised reporter system demonstrated a maximal 3.3-fold level of induction in constructs containing the PBRE. This result is comparable to data published by Anderson's group (Trottier *et al.*, 1995, Fig. 2) and does seem (given that the same construct gave a 1.35-fold induction in previous experiments) to be entirely dependent upon the successfulness of the transfection protocol. This response, although apparently maximal in this system, is still rather low in comparison to other workers. These results suggest that further optimisation of the reporter assay and hepatocyte culture system may be required. The implementation of this hepatocyte reporter assay and the localisation of the PBRE could be used to further the understanding of the mechanism of induction by PB. A main aim would be to develop a *trans*-activation assay for high-throughput drug screening to identify inducers of the CYP2B2 gene.

The validity of this system would depend entirely upon whether a reproducible, high-level response could be obtained with this reporter assay. Work carried out by Honkakoski's group has already demonstrated that once a high level of induction was obtained with these PBRE containing reporters, vast numbers of compounds could be screened for their ability to induce CYP2B2 (Honkakoski *et al.*, 1998b). It is anticipated that once optimised, that the current reporter assay system used in this thesis could be used for this purpose. The current system has great potential for use in high-throughput screening as the assay can be performed on a small number of cells in a 96-well format. However, given that the levels of induction obtained with this transfection protocol can vary between experiments, the day-to-day variations of this system

may result in this becoming a non-viable option. One possible solution to this "batch variability" may be the production of immortalised hepatocyte cell lines. Here viral oncogenes are transfected into the mammalian genome to create differentiated cell lines. Given the recent advances in genomic targeting vectors that have been developed, it may be possible to further augment these immortalised hepatocytes by the stable transfection of reporter genes containing the PBRE.

Section 4.5.1 Recommendations

The first aspect to be overcome in this thesis was whether the hepatocyte culture conditions used here could support CYP2B2 induction. The conditions used in this project were those that had already been set up by previous workers in this group to show the induction of other cytochrome P450 genes. Given that it has been demonstrated that CYP2B2 induction is difficult to reproduce in culture, I think that a better initial approach would have been to determine which type of culture media provided the best level of CYP2B2 induction. A comparison could then have been made to determine whether different forms of culture substratum could improve upon the level of induction obtained.

The quantities of RNA obtained by different extraction techniques varied considerably depending upon the method used and indeed whether RNA was extracted from tissue or from tissue culture material. I found that the technique used to extract RNA was a crucial discriminator between whether or not intact RNA was obtained, particularly when using tissue culture monolayers.

With the RNAse protection assay, I feel that a more robust argument could have been made for the specificity of the riboprobe if a sense probe had also been designed. This sense probe could have been shown to hybridise to the anti-sense probe with complete specificity thus showing that the spurious bands found in many of the protection autoradiographs were due to contaminated RNAse A or H as discussed in section 4.1.1.1. The specificity of the riboprobe could have been

demonstrated further still if a CYP2B1 specific riboprobe had been made. Experiments could then have been carried out to show that the CYP2B2 anti-sense probe could protect CYP2B2 sense transcripts but not CYP2B1 sense transcripts during treatment with RNAse A. This experiment would have provided further evidence for the specificity of the probe used in this thesis. The use of normalising probes such as GAPDH may also have strengthened the results obtained in the dose response assay.

Many of the techniques used during the course of this thesis were not established techniques in our laboratory. No prior experience was available within the group for both the transfection protocol and the reporter assays. This meant that a great deal of time was spent optimising many of the techniques. The use of an "in-house" transfection reagent meant that the transfection protocol was inexpensive although there was still the need for comparative studies with commercially available reagents.

Initially the reporter assays were carried out as described in the original papers (Seed and Sheen, 1988; DeWet *et al*, 1987). This proved to be extremely time consuming with four to five months spent trying purely to obtain consistent assay results, especially with the firefly luciferase assay. One of the main reasons for these inconsistencies may have been the method by which the hepatocytes were harvested. Three methods were tried, i.e. sonication, repeated freeze/thawing and the use of detergent such as Triton-X-100. Only the freeze/thaw method was shown to produce reasonable results. The decision was finally made to switch to a commercial luciferase assay in the interests of time. Although the experience of trying to optimise this assay improved my ability to design more effective experiments,. I feel that too much time was spent optimising a reporter assays that clearly was not going to yield reproducible results. Towards the end, a commercial kit enabled some headway to be made, which aided the development of the project.

A crucial point that must be made to any perspective researcher following on from this work

would be to be aware of the possible interplay which may occur between the various reporter vectors used in the transfection protocols.

During the course of this thesis I was unable to obtain levels of induction of CYP2B2 comparable to work of other groups. I therefore thought it prudent to include a positive control such as that afforded by the use of Anderson's 163bpCAT construct. I would suggest that comparison to a different Anderson construct, i.e. that containing the PBRE only, would have been a much more suitable experiment. This particular Anderson construct would have provided a perfect control reporter for my work. However this had not been donated.

Section 4.6 Further work to elucidate the mechanism of PB induction

Further work is needed to characterise the response elements identified by a number of different groups (Honkakoski and Negishi, 1998a; Stoltz *et al.*, 1998) by the use of the truncated PBRE reporters optimised in this thesis. An understanding of the importance of the response elements present within the 163 bp of the PBRE which were involved in PB-induction would further be identified if this element was to be delineated by sub-cloning or mutagenesis studies. Direct binding assays could then be used to assess the relevance of these response elements.

The levels of induction achieved with the PBRE reporters used in this thesis were low in comparison to other workers (Honkakoski and Negishi, 1998a). Recent data suggests that the composition or spatial orientation of proteins binding to the PBRE may play a role in the induction mechanism (Kim and Kemper, 1997), suggesting that chromatin structure is important in this process. Further work would be necessary to establish if a change in the chromatin structure due to acetylation of histones allows binding of regulatory factors or an alteration in their activity and activation, or the recuirtment of basal transcription factors and the RNA polymerase II complex to the RNA initiation site.

Recent publications have highlighted that PB induction is mediated by a constitutively active receptor (CAR) which is regulated by the PBRE (Honkakoski *et al.*, 1998c; Kawamoto *et al.*, 1999; Sueyoshi *et al.*, 1999). During the latter part of this thesis I attempted to sub-clone the mCAR receptor. This receptor and its heterodimer partner RXR have been identified as factors regulating the transcription of the PB-inducible *Cyp*2b10 genes. It is unknown if PB increases CAR DNA binding, which in turn increases the PBRE-dependent gene transcription by the translocation of CAR from the cytosol of the nucleus, or if CAR is constitutively localised in the nucleus and requires PB to activate the DNA-binding activity of the CAR-RXR heterodimer. Determining which of these two receptor-regulating mechanisms is correct would be a major step forward in elucidating the PB-response mechanism.

The transactivation of the PBRE has been reported to be repressed by androstane, and this repression has been shown to be derepressed by the presence of PB (Honkakoski *et al.*, 1998c; Kawamoto *et al.*, 1999; Sueyoshi *et al.*, 1999). A high concentration of androstane present in the hepatocyte culture system would lead to a direct binding to the CAR-RXR heterodimer. These high concentrations of androstane prevent the interaction between CAR and SRC-1, thus maintaining the CAR receptor in an inactive conformation. The addition of PB will abolish the inhibitory binding of androstane to CAR and thus a derepression of the receptor will lead to a high level of expression. It is believed that in this culture system the level of androstane is at a low concentration which leads to a constitutive repression of the PBRE present in the reporter constructs, although not of the genomic DNA PBRE's. This manifests itself in the low PB response of the PBRE-containing reporters, but in the high constitutive expression of the endogenous CYP2B2 mRNA (protection data, Figure 3.40). Therefore, a clear PB-induction of the PBRE reporter construct may be achieved if androstane is added to the culture system. This would have the effect of repressing the constitutive expression of the PBRE reporters. Thus the addition of PB to this system would result in the derepression of the PBRE, which would in turn

lead to a substantial increase in the CYP2B2 induction of the reporter constructs. It would be useful to determine whether androstane's interaction with CAR effects SRC-1 binding and how, or whether PB binds directly to CAR causing a conformational change which releases androstane's repressive hold on CAR.

It is thought that several proteins interact specifically with mCAR. One such protein is the small heterodimer partner SHP (Seol *et al.*, 1996). Its name is derived from its small size and ability to interact with many superfamily members. SHP proteins have been shown to possess ligand-binding and dimerisation domains that are well conserved, although these proteins lack a conventional DNA-binding domain. Unlike RXR, SHP inhibits transactivation by its interaction with such receptors as CAR, and so has a negative role in receptor-dependent signalling pathways. It may be possible that SHP is having an effect within the reporter system used in this thesis, and that SHP binds to CAR, repressing its activity which is manifested in the form of reduced PB-responsiveness.

Multiple growth/proliferation pathways are known to be regulated by a phosphorylation/dephosphorylation mechanism of specific protein receptors. These receptors, eg. for platelet-derived growth factor or epidermal growth factor are known as receptor protein tyrosine kinases. Of course, no such receptor for PB has been identified. However, activation of many such receptors culminate in similar intracellular effects. Most work to date, however helpful, has been to elevate intracellular events artificially with a view to observe any 2B-induction, or to inhibit enzymes (eg. specific phosphatases) involved in the signal transduction. There is some evidence to suggest that the cAMP-stimulated PKA pathway and protein phosphatases PP1 and/or PP2 may play a role in the modulation of PB signalling events (Sidhu and Omiecinski, 1995; Sidhu and Omiecinski, 1997; Honkakoski and Negishi, 1998d). No link as yet has been found between these cellular signalling and their impact on CAR's interaction with PB. No work was carried

out on this aspect of PB-induction during the course of this thesis but this area may provide an important new direction in determining the mechanism of PB induction.

Finally some further work must be carried out to evaluate the potential for the transfection protocol and reporter assays implemented in this thesis, to be developed for high-throughput screening studies.

Section 4.7 Conclusions

The main aim of this thesis was to implement an *in vitro* system that would support CYP2B2 induction in primary rat hepatocytes. This system could then be used to investigate the molecular mechanisms of CYP2B2 induction. It was hoped that this system would eventually enable the high-throughput screening of compounds to determine if they mediate CYP2B2 induction. Using RNAse protection assay analysis it was possible to demonstrate that the hepatocyte culture system used in this thesis was able to support the induction of CYP2B2 mRNA by PB. A transfection protocol was developed using an "in-house" transfection reagent and reporter assays were optimised so that the PB-mediated induction of the PBRE could be measured. This assay required a great deal of optimisation during all aspects of its implementation from the harvesting techniques to the types and combinations of promoters utilised in the different reporter constructs. The culmination of this work resulted in obtaining a construct (provided by Alan Anderson) so that a direct comparison could be made between the constructs designed in this thesis (TK2B2-F-Luc) and that of Anderson's (163bpCAT) construct. These experiments showed that no differences were apparent in the PB-responsiveness between the two constructs. It is hoped that these optimised reporter assay conditions could then be utilised to identify and characterise the molecular mechanisms involved in the PB-mediated induction of the CYP2B2 gene.

Chapter 5 References

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Chapter 6 Submitted abstracts

Mechanism of induction of the CYP2B2 gene.

Neill J. Horley, Maurice Dickins and David Bell.

Abstract submitted for British Toxicology Society Autumn meeting at University of York

Sept. 1998 awarded best Oral presentation.

Mechanism of induction of the CYP2B2 gene.

Neill J. Horley, Maurice Dickins and David Bell.

Abstract submitted for Pfizer Advances in Drug Metaoblism (poster symposium).

Summer meeting Aug. 1998.