Regulation of expression of the rat CYP2B1 and CYP2B2 genes

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Abstract

CYP2B1 and CYP2B2 proteins are highly induced in rat liver by phenobarbital (PB). CYP2B1 promoter sequences from -179 to -347 bp and -348 to -451 bp were observed in gel shift assays to bind liver nuclear protein that was either more abundant or activated from PB-treated than untreated rats.

The DNA-binding activity of the protein bound to the sequence between -348 and -451 bp was enhanced when liver nuclear extracts from both untreated and PB-treated rats were treated with ATP prior to gel shift assays. While pre-treatment with either calf intestinal alkaline phosphatase (CIP) or 2-aminopurine (2-AP), a general protein kinase inhibitor inhibited complex formation. Thus, phosphorylation of this protein increases its binding to DNA and dephosphorylation inhibits binding. When primary hepatocytes or whole animals were treated with 2-aminopurine, it could totally suppress both basal and PB-induced CYP2B mRNAs expression in vitro but only partially suppress PB-induction of CYP2B mRNAs in vivo.

A PB-responsive element (PBRE) has been identified in the distal region of the CYP2B2 promoter. The homologous region in the CYP2B1 promoter between -2142 and -2301 bp was cloned into a reporter gene construct and was shown to confer PB-responsiveness to the luciferase gene when transfected either into primary rat hepatocytes or directly into rat liver tissues. A higher fold induction was observed with in vivo DNA transfection. The PBRE sequence could activate gene expression better
when the -348 to -451 bp sequence was included in the promoter region of the reporter construct.

The CYP2B2 promoter, between -183 and -199 bp, also bound more liver nuclear protein from PB-treated rats in gel shift assay. An octamer consensus oligonucleotide competed for protein binding to this region. An antibody which recognised the DNA-binding domain of Oct-1 and Oct-2 inhibited complex formation and an Oct-1 specific antibody supershifted the protein-DNA complex.
To my parents, Kok Meng and especially to Kia Joo who have all inspired more than this work
Acknowledgements

This research degree could not have progressed smoothly and be fulfilled without the support of a number of people whom I would like to extend my sincere gratitude to.

My parents who have been extremely supportive. They have always understood my desire to pursue my studies further and given me lots of encouragement throughout the years. I am also immensely proud of my brother, Kok Meng who has taken full responsibility in caring for our parents all these while. I love them very much and would like them to know that I have put the best into what I am doing.

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Dr Shaun Thomas for his kind gift of the Oct1/2 antibody and Dr Peter Shaw for his gift of the pBLCAT(3.9kb) construct containing the CYP2B1 promoter sequence up to 3.9 kb.

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<table>
<thead>
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<th>Description</th>
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</thead>
<tbody>
<tr>
<td>AGs</td>
<td>anti-glucocorticoids</td>
</tr>
<tr>
<td>AhR</td>
<td>Arylhydrocarbon receptor</td>
</tr>
<tr>
<td>ALAS</td>
<td>5-aminolevulinate synthase</td>
</tr>
<tr>
<td>2-AP</td>
<td>2-aminopurine</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>Arnt</td>
<td>AhR nuclear translocator protein</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenine triphosphate</td>
</tr>
<tr>
<td>ATPγ-S</td>
<td>Adenosine-5'-O-(3-thiotriphosphate)-Li$_4$</td>
</tr>
<tr>
<td>bHLH</td>
<td>basic helix-loop-helix</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CAR</td>
<td>Constitutively activated receptor</td>
</tr>
<tr>
<td>CAT</td>
<td>Chloramphenicol acetyltransferase</td>
</tr>
<tr>
<td>C/EBP</td>
<td>CCAAT/Enhancer binding protein</td>
</tr>
<tr>
<td>CDI</td>
<td>1-Cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-$\beta$-toluene sulfonate</td>
</tr>
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<td>CIP</td>
<td>Calf intestinal alkaline phosphatase</td>
</tr>
<tr>
<td>CoCl$_2$</td>
<td>Cobalt chloride</td>
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<tr>
<td>CTF</td>
<td>CCAAT-transcription factor</td>
</tr>
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<td>Cytochrome P450</td>
</tr>
<tr>
<td>db-cAMP</td>
<td>Dibutylryl cAMP</td>
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<tr>
<td>DDT</td>
<td>1,1,1-trichloro-2,2-bis($\beta$-chlorophenyl)ethane</td>
</tr>
<tr>
<td>Dex</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>DME</td>
<td>Drug-metabolising enzymes</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
</tr>
<tr>
<td>dNTP</td>
<td>2' Deoxynucleoside 5'- triphosphate</td>
</tr>
<tr>
<td>DPBF</td>
<td>Dystrophin promoter bending factor</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDNA</td>
<td>Epstein-Barr virus nuclear antigen</td>
</tr>
<tr>
<td>EBSS</td>
<td>Earles-balanced salt solution</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra-acetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol-O, O'-bis(2-amino-ethyl)-N,N',N',N'-tetraacetic acid</td>
</tr>
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<td>EMSA</td>
<td>Electrophoresis mobility shift assay</td>
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<td>Ethidium bromide</td>
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<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
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<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>GRE</td>
<td>Glucocorticoid -responsive element</td>
</tr>
<tr>
<td>HAH</td>
<td>Halogenated aromatic hydrocarbon</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-Hydroxyethylpiperazine-N' 2-ethanesulphonic acid</td>
</tr>
<tr>
<td>HNF</td>
<td>Hepatocyte nuclear factor</td>
</tr>
<tr>
<td>hPAR</td>
<td>human peroxisome proliferator activator receptor</td>
</tr>
<tr>
<td>hsp</td>
<td>Heat-shock protein</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IP</td>
<td>Intra-peritoneal</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-Thiogalactopyranoside</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin responsive sequence</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>MAP</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-[N-Morpholino]propane-sulfonic acid</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide-adenine dinucleotide (reduced)</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide-adenine dinucleotide phosphate (reduced)</td>
</tr>
<tr>
<td>NaF</td>
<td>Sodium fluoride</td>
</tr>
<tr>
<td>NaN₃</td>
<td>Sodium azide</td>
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<tr>
<td>NE</td>
<td>Negative element</td>
</tr>
<tr>
<td>NF1</td>
<td>Nuclear factor 1</td>
</tr>
<tr>
<td>NP-40</td>
<td>Nonidet-40</td>
</tr>
<tr>
<td>Oct</td>
<td>Octamer</td>
</tr>
<tr>
<td>OK</td>
<td>Okadaic acid</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAH</td>
<td>Polycyclic aromatic hydrocarbon</td>
</tr>
</tbody>
</table>
PB  Phénobarbital
PBRE  PB responsive element
PBREM  PB response enhancer module
PBRU  PB response unit
PBS  Phosphate buffered saline
pBS  pBluescript
PCB  Polychlorinated biphenyl
PCN  Pregnenolone-16α-carbonitrile
PCR  Polymerase chain reaction
PDE  Phosphodiesterase
PE  Positive element
PEPCK  Phosphoenolpyruvate carboxykinase
PK  Protein kinase
PLB  Passive lysis buffer
PMSF  Phenylmethylsulfonyl fluoride
PNK  Polynucleotide kinase
PP  Peroxisome proliferator
PP1  Protein phosphatase-1
PP2A  Protein phosphatase-2A
PPAR  Peroxisome proliferator activator receptor
PPRE  Peroxisome proliferator response element
PR  Prolactin receptor
psi  pound per square inch
PXR  Pregnane X receptor
RARE  Retinoic acid response element
RP  Random prime
RT  Room temperature
RXR  Retinoid X receptor
SAPK  Stress-activated protein kinase
SDS  Sodium dodecyl sulphate
(Sp)-cAMPS  Phosphorothioate stereoisomer of cAMP
SRC-1  Steroid receptor coactivator-1
SRE  Serum response element
SRF  Serum response factor
STAT  Signal transducer and activator
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>STAT</td>
<td>Signal transducer and activator</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian virus 40</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate-EDTA</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-borate-EDTA</td>
</tr>
<tr>
<td>TCF</td>
<td>Ternary complex formation</td>
</tr>
<tr>
<td>TCPOBOP</td>
<td>1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>TEMED</td>
<td>(N,N,N',N')-tetramethylethylenediamine</td>
</tr>
<tr>
<td>TESS</td>
<td>Transcription element search software</td>
</tr>
<tr>
<td>TK</td>
<td>Thymidine kinase</td>
</tr>
<tr>
<td>(T_m)</td>
<td>Melting temperature</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>Tris</td>
<td>Trizma® Base (Tris[hydroxymethyl]amino-methane)</td>
</tr>
<tr>
<td>VDR</td>
<td>Vitamin-D receptor</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-Bromo-4-Chloro-3-indolyl-(\beta)-D-Thiogalactopyranoside</td>
</tr>
<tr>
<td>XRE</td>
<td>Xenobiotic-responsive element</td>
</tr>
<tr>
<td>YY1</td>
<td>Yin Yang-1</td>
</tr>
</tbody>
</table>
Chapter One

Introduction
1.1 Overview of xenobiotic metabolism

Each living organism, from microorganisms, plants to animals, are under constant exposure to many different kinds of foreign compounds. These are both naturally occurring and synthetic chemicals. Most of the xenobiotics, being lipophilic in nature, are easily absorbed by an organism. The very property, i.e. lipophilicity, which facilitates the absorption of these chemicals also makes them difficult to eliminate. If they are continually absorbed and not excreted from the organism fast enough, they would accumulate to eventually overwhelm and kill the organism. To prevent these chemicals from accumulating to toxic levels, organisms have evolved defense mechanisms to eliminate these chemicals. There are two main detoxification systems: one is a non-catalytic multidrug resistance system, where harmful compounds bind to P-glycoprotein and are then transported out of the cell (Endicott and Ling, 1989); the other is a metabolic process involving several enzyme systems. These enzymes are generally known as drug-metabolising enzymes and they eliminate foreign compounds basically by making them more water-soluble. The latter system is the major detoxification mechanism utilised by an organism and is the most complex one too.

Drug-metabolising enzymes are generally divided into two broad categories, 'Phase I' and 'Phase II' (Williams, 1971). Phase I (functionalisation) reactions involve hydrolysis, reduction and oxidation. They expose or introduce a functional group, such as a hydroxyl, to the parent compound rendering it more hydrophilic. The increase in hydrophilicity is
usually small and further Phase II (conjugation) reactions, whereby a conjugate such as glutathione is added to the functional group, are usually required to generate a product relatively hydrophilic that is readily excreted (Testa and Jenner, 1976, Nebert, 1994). Unfortunately, the detoxication process can sometimes produce intermediate or final products that are more toxic and/or carcinogenic than the parent compound. Examples of some drug-metabolising enzymes involve in Phase I or II reactions are listed in Table 1.1.

1.2 Cytochrome P450-dependent mixed function oxygenase system

Among the Phase I enzymes, cytochrome P450 is one of the most versatile biological catalysts because of the number of xenobiotics it detoxifies and activates to reactive intermediates (Guengerich, 1987, Waterman and Johnson, 1991). Cytochrome P450 is widely distributed in nature and has been found to be present in virtually all mammalian tissues examined, with the greatest abundance in the liver. With the exception of some soluble bacterial proteins, all known cytochrome P450s are membrane-bound and located predominantly in the endoplasmic reticulum. However, some cytochrome P450s have also been found in mitochondria (Hollis, 1990).

Cytochrome P450 is a monooxygenase and catalyses the incorporation of a single atom of molecular oxygen into a substrate with the
### Phase I enzymes

- Cytochrome P450
- Flavin-containing monooxygenase
- Aldehyde dehydrogenase
- Epoxide hydrolase
- NADPH-cytochrome P450 reductase
- Monoamine oxidase
- Carboxylesterase

### Phase II enzymes

- UDP glucuronosyltransferase
- Glutathione-S-transferase
- Acetyltransferase
- Methyltransferase
- β-glucuronidase
- Sulfotransferase

---

**Table 1.1 Examples of drug-metabolising enzymes involved in Phase I and Phase II reactions.** (adapted from (Nebert, 1994))
concomitant reduction of the other oxygen atom to water as represented in the following equation, where RH refers to the substrate:-

\[
\text{RH} + \text{O}_2 + \text{NAD(P)H} + \text{H}^+ \text{Cytochrome P450} \rightarrow \text{ROH} + \text{H}_2\text{O} + \text{NAD(P)}^+
\]

Cytochrome P450 cannot carry out the above reaction alone but requires other components in the cytochrome P450-dependent mixed function oxygenase system (Lu and Coon, 1968). The mammalian microsomal system is made up of cytochrome P450, NADPH-cytochrome P450 reductase and phospholipid. NADPH is the reducing co-factor that donates electrons to mammalian cytochrome P450 for oxidative reaction to occur, while NADH is the electron donor in bacterial systems. However, NAD(P)H is a two-electron donor and cytochrome P450 accepts one electron at a time. Cytochrome P450, therefore cannot interact directly with the co-factor. Instead, it receives the electrons via an accessory enzyme. In microsomal systems, the accessory enzyme is NADPH-cytochrome P450 reductase. This is a flavoprotein and possesses two flavin prosthetic groups. This enables it to accept the two electrons donated by NAD(P)H simultaneously and transfer one electron to each of two different cytochrome P450s. Because NADPH-cytochrome P450 reductase transfers electrons much faster than cytochrome P450 can use them, one NADPH-cytochrome P450 reductase provides electrons for more than one cytochrome P450. This may account for the low ratio of NADPH-cytochrome P450 reductase to cytochrome P450 in liver microsomes (e.g. one molecule of NADPH-

Another constituent of the cytochrome P450-dependent system is phospholipid. It is not required for soluble forms of cytochrome P450 but is particularly important for membrane-bound forms because it facilitates interactions between NADPH-cytochrome P450 reductase and cytochrome P450 in the endoplasmic reticulum. However, the actions by which phospholipid facilitates this interaction is not very clear (Parkinson, 1996, Josephy, et al., 1997).

Hepatic microsomes also contain another haemoprotein, cytochrome b$_5$. Although NADPH-cytochrome P450 reductase is absolutely required for cytochrome P450 activity, cytochrome b$_5$ can transfer the second of the two electrons required by cytochrome P450. Cytochrome P450 catalysed turnover of some substrates can be increased synergistically by electron transfer from cytochrome b$_5$. This is not always simply due to an increase in the rate of catalysis by cytochrome P450, cytochrome b$_5$ can also increase the apparent affinity with which certain cytochrome P450s bind their substrates. Some cytochrome P450s, including CYP3A, CYP2E1 and CYP2C9 have been demonstrated to require cytochrome b$_5$ for maximal catalytic activities in reconstituted monooxygenase systems (Shet, et al., 1995, Yamazaki, et al., 1996, Shimada and Yamazaki, 1998). A more detailed description of the oxygenation of a substrate by cytochrome P450 is presented in Fig. 1.1.

* (Parkinson, 1996)
Fig. 1.1 Catalytic cycle of cytochrome P450. (1) binding of substrate (RH), (2) reduction of ferric, substrate-bound enzyme to the ferrous form, (3) binding of oxygen, (4), (5) and (6) addition of second electron, released water and oxidized substrate and (7) releasing of oxidized substrate (ROH). (modified from Parkinson, 1996)
Apart from hydroxylation reaction, cytochrome P450 also catalyses a range of other reactions such as N-, O-, S-dealkylation, sulfoxidation, deamination and N-oxide reduction as shown in Fig. 1.2.

1.3 Discovery of cytochrome P450

In 1958, Garfinkel and Klingenberg while studying microsomal haem proteins by optical spectroscopy, observed a strong absorption band at 450 nm (Soret band) when pig and rat liver microsomes were treated with a reducing agent in the presence of carbon monoxide (Garfinkel, 1958, Klingenberg, 1958). This was a unique characteristic because many other haemoproteins that form a complex with carbon monoxide absorb light maximally at ~420 nm. The protein was later purified by Omura and Sato, who confirmed that the unique absorption spectra was indeed due to a new class of haem-binding protein and named it cytochrome P450, based on its atypical absorption maximum at 450 nm (Omura and Sato, 1964). With time, this terminology was found to be unsuitable since cytochrome P450 acts as an oxygenase rather than just an electron carrier. The term 'haem-thiolate protein' was suggested in replacement of 'cytochrome', however it did not gain favour and the name cytochrome P450 is still universally recognised (Nelson, et al., 1993).
Fig. 1.2 Examples of diverse activities catalysed by cytochrome P450s.  
(adapted from (Nebert and Gonzalez, 1987))
1.4 The cytochrome P450 gene superfamily

When cDNA and cloning techniques were introduced in the 1980s, more new forms of cytochrome P450 were isolated. Each laboratory involved in cytochrome P450 isolation began developing its own nomenclature system according to electrophoretic mobility, substrate specificity or maximal absorption wavelength making the situation very complex. There were cases where an enzyme was designated several different names. The fact that cytochrome P450s have a broad substrate specificity and catalyse different reactions make the classical method of naming an enzyme according to its function very difficult.

When amino acid sequence data was derived from DNA sequences, it made possible a naming system based on the amino acid sequence similarities. Table 1.2 lists a few examples of cytochrome P450s to illustrate the diversity of the previous nomenclature and how the new classification helped to overcome this complexity. Since it was first recommended in 1987 (Nebert, et al., 1987), there have been a few revisions (Nebert, et al., 1989, Nebert, et al., 1991, Nelson, et al., 1993, Nelson, et al., 1996). A cytochrome P450 gene is named by the italicised root symbol ‘CYP’ (‘Cyp’ for mouse and Drosophila) to denote Cytochrome P450, followed by an Arabic number for the family, a letter for the subfamily and another Arabic number for the individual gene, i.e. CYP2B1 (‘Cyp2b1’ in mouse). A pseudogene will have a ‘P’ (‘ps’ in mouse and Drosophila) after the gene number. The non-italicised form and all capital letters should be used for mRNA, cDNA and protein in all species including mouse and Drosophila (Nelson, et al.,
### Table 1.2 Diversity of nomenclature of some mammalian CYPs.

(adapted from (Paine, 1991, Soucek and Gut, 1992) and references therein for sources of nomenclature)

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Ryan</th>
<th>Guengerich</th>
<th>Waxman</th>
<th>Rabbit</th>
<th>Mouse</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A1</td>
<td>c</td>
<td>βNF-B</td>
<td>β-NF-B</td>
<td>LM6</td>
<td>P1,450</td>
<td>P1</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>d</td>
<td>βNF/ISF-G</td>
<td>ISF-G</td>
<td>LM4</td>
<td>P3,450</td>
<td>P1</td>
</tr>
<tr>
<td>CYP2A1</td>
<td>a</td>
<td>UT-F</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CYP2B1</td>
<td>b</td>
<td>PB-B</td>
<td>PB-4</td>
<td>LM2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CYP2B2</td>
<td>e</td>
<td>PB-D</td>
<td>PB-5</td>
<td>LM2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CYP2C6</td>
<td>k</td>
<td>PB-C</td>
<td>PB-1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CYP2C11</td>
<td>h</td>
<td>UT-A</td>
<td>2c</td>
<td>-</td>
<td>P450 16α</td>
<td>-</td>
</tr>
<tr>
<td>CYP2C12</td>
<td>i</td>
<td>UT-I</td>
<td>2d</td>
<td>-</td>
<td>P450 15β</td>
<td>-</td>
</tr>
<tr>
<td>CYP2D1</td>
<td>-</td>
<td>UT-H</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>db1</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>j</td>
<td>-</td>
<td>-</td>
<td>LM3a</td>
<td>-</td>
<td>j</td>
</tr>
<tr>
<td>CYP3A1</td>
<td>p</td>
<td>-</td>
<td>-</td>
<td>LM3</td>
<td>-</td>
<td>P450nf</td>
</tr>
<tr>
<td>CYP4A1</td>
<td>-</td>
<td>PB/PCN-E</td>
<td>PB-2a</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Members within the same family are defined as usually having >40% amino acid sequence identity and mammalian sequences within the same subfamily are always >55% identical. Although these definitions were made arbitrarily, they turned out to be very useful despite a few exceptions (reviewed in (Nelson, et al., 1993, Nelson, 1998)).

By 1996, 481 CYP genes were identified in 85 eukaryote and 20 prokaryote species, the number is still increasing (Nelson, et al., 1996). But how did CYP evolve to become such a superfamily of proteins? This superfamily is ancient and believed to have begun with only a few genes coding for CYP forms that were engaged in the metabolism of endogenous substrates important for cellular functions (Nebert, 1991, Soucek and Gut, 1992). The increase in the number of CYP genes, according to the evolutionary tree, arose during the past 400 million years. And 'animal-plant war-fare' is believed to be the driving force for the recent burst in new CYP genes, particularly in the CYP2 family (Nebert and Gonzalez, 1987, Gonzalez and Nebert, 1990). New genes encoding for new forms of CYP appear through increased frequency of gene duplications and conversions as the animal continues to encounter new types of foreign compounds, including drugs and pesticides of the present days.

The diversity of genes has evolved mainly in the CYP families 1 to 4. Hence, it is not surprising to find these four families more important in xenobiotic metabolism than the other CYP families which are involved mainly in the metabolism of endogenous substrates such as steroids, fatty acids and hormones (Table 1.3). Apparently, most of the CYPs involved in endogenous substrate metabolism are highly specific, while those involved
<table>
<thead>
<tr>
<th>Gene families</th>
<th>Occurrence and functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1</td>
<td>Vertebrates; dioxin-inducible; metabolism of polycyclic hydrocarbon, halogenated and heterocyclic hydrocarbon, and aromatic amines</td>
</tr>
<tr>
<td>CYP2</td>
<td>Vertebrates and invertebrates; metabolism of drugs and environmental chemicals</td>
</tr>
<tr>
<td>CYP3</td>
<td>Vertebrates; metabolism of drugs and environmental chemicals</td>
</tr>
<tr>
<td>CYP4</td>
<td>Vertebrates, fatty acid hydroxylases; invertebrates, unknown function(s)</td>
</tr>
<tr>
<td>CYP5</td>
<td>Vertebrates; thromboxane synthase</td>
</tr>
<tr>
<td>CYP6</td>
<td>Insects; metabolism of plant products and pesticides</td>
</tr>
<tr>
<td>CYP7A</td>
<td>Vertebrates; cholesterol 7α-hydroxylase</td>
</tr>
<tr>
<td>CYP7B</td>
<td>Vertebrates; unknown function(s)</td>
</tr>
<tr>
<td>CYP8</td>
<td>Vertebrates; prostacyclin synthase</td>
</tr>
<tr>
<td>CYP9</td>
<td>Insects</td>
</tr>
<tr>
<td>CYP10</td>
<td>Molluscs (mitochondrial enzyme)</td>
</tr>
<tr>
<td>CYP11</td>
<td>Vertebrates; cholesterol side-chain cleavage, steroid 11β-hydroxylase, and aldosterone synthase (mitochondrial enzyme)</td>
</tr>
<tr>
<td>CYP12</td>
<td>Insects (mitochondrial enzyme)</td>
</tr>
<tr>
<td>CYP13</td>
<td>Nematodes</td>
</tr>
<tr>
<td>CYP14</td>
<td>Nematodes</td>
</tr>
<tr>
<td>CYP15</td>
<td>Insects</td>
</tr>
<tr>
<td>CYP16</td>
<td>Nematodes</td>
</tr>
<tr>
<td>CYP17</td>
<td>Vertebrates; steroid 17α-hydroxylase</td>
</tr>
<tr>
<td>CYP18</td>
<td>Insects</td>
</tr>
<tr>
<td>CYP19</td>
<td>Vertebrates; aromatization of androgens</td>
</tr>
<tr>
<td>CYP21</td>
<td>Vertebrates; steroid 21-hydroxylase</td>
</tr>
<tr>
<td>CYP24</td>
<td>Vertebrates; steroid 24-hydroxylase (mitochondrial enzyme)</td>
</tr>
<tr>
<td>CYP27</td>
<td>Vertebrates; steroid 27-hydroxylase (mitochondrial enzyme)</td>
</tr>
<tr>
<td>CYP51</td>
<td>Animals, filamentous fungi, yeast and plants; sterol biosynthesis</td>
</tr>
<tr>
<td>CYP52</td>
<td>Yeast; alkane hydroxylase</td>
</tr>
<tr>
<td>CYP53 to CYP62</td>
<td>Fungi</td>
</tr>
<tr>
<td>CYP71 to CYP92</td>
<td>Plants</td>
</tr>
<tr>
<td>CYP73</td>
<td>Plants, cinnamic acid hydroxylase</td>
</tr>
<tr>
<td>CYP101 to CYP118</td>
<td>Bacteria</td>
</tr>
</tbody>
</table>

Table 1.3 Overview of CYP families and enzymes functions in various species. (reproduced from (Nelson, et al., 1996))
in xenobiotic metabolism exhibit broad and overlapping substrate specificities allowing them to deal with a wide range of foreign compounds.

1.5 Molecular mechanisms of CYP gene expression

The regulation of CYP gene expression is complex and governed by several different mechanisms. CYP expression can be tissue, strain and sex-specific and/or regulated at the level of development. Certain families of CYP, particularly those involved in xenobiotic metabolism, can also be induced in response to many foreign compounds (reviewed in (Bernhardt, et al., 1995; Gonzalez, et al., 1989). And more than one mechanism is usually involved in the regulation of any particular CYP.

Although the most common means of regulating CYP expression is at the level of transcription, some forms of CYP have been found to be regulated via post-transcriptional mechanisms, at the level of mRNA (Song, et al., 1987) or protein (Eliasson, et al., 1990) stabilisation as shown in Fig. 1.3 (reviewed in (Gonzalez, 1989, Okey, 1990)).

1.5.1 Xenobiotic inducible CYPs

It was recognised more than 20 years ago that many xenobiotics can induce their own metabolism and the metabolism of other compounds of similar structure (Conney, 1967). This happens because they induce the expression of one or more CYPs that are responsible for their metabolism. These CYPs are generally present in low amounts and are substantially
Fig. 1.3 Diverse mechanisms in the regulation of CYP expression.
(reproduced from (Porter and Coon, 1991) and references therein for the sources of different mechanisms)
induced only in response to the xenobiotics that they metabolised. Mammalian CYP inducers can be categorised into four distinct classes as shown in Table 1.4. Although each class of inducer is capable of inducing a number of CYP genes, a particular subfamily of CYP genes, most efficient in metabolising the inducers, are predominantly induced.

For instance, polycyclic aromatic hydrocarbons (PAHs) such as benzopyrenes and anthrenes induce CYPs belonging to the CYP1A subfamily (Whitlock, et al., 1996), chlorinated pesticides such as DDT and drugs like PB greatly induce proteins of the CYP2B subfamily (Waxman and Azaroff, 1992). While peroxisome proliferators and glucocorticoids such as dexamethasone predominantly induce members of the CYP4A (Reddy and Mannaerts, 1994) and CYP3A (Okey, 1990) subfamily respectively. The increase in the amount of these four families of CYPs is achieved mainly by transcriptional activation and a simplified model of the molecular mechanisms known so far is presented in Fig. 1.4 (Dogra, et al., 1998).

1.5.1.1 Steroid inducible CYP genes

Glucocorticoids, such as dexamethasone, activate the transcription of numerous genes in the liver, such as the rat CYP3A1 gene, via the classical glucocorticoid receptor-dependent mechanism. Members of the CYP3A subfamily are the most induced by these molecules. However, the involvement of the glucocorticoid receptor in the transcriptional activation of CYP3A genes is controversial (Fig. 1.4). In humans, CYP3A5 induction appears to require the glucocorticoid receptor (Schuetz, et al., 1996) while in
<table>
<thead>
<tr>
<th><strong>Inducer</strong></th>
<th><strong>CYP gene</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Some PAHs, HAhs, and PCBs</td>
<td>CYP1A1</td>
</tr>
<tr>
<td></td>
<td>CYP1A2</td>
</tr>
<tr>
<td></td>
<td>CYP1B1</td>
</tr>
<tr>
<td></td>
<td>CYP2A3</td>
</tr>
<tr>
<td>Barbiturates, DDT, dieldrin, some PCBs</td>
<td>CYP2A1</td>
</tr>
<tr>
<td></td>
<td>CYP2B1/2</td>
</tr>
<tr>
<td></td>
<td>Cyp2b10</td>
</tr>
<tr>
<td></td>
<td>CYP2H1/2</td>
</tr>
<tr>
<td></td>
<td>CYP2C1/6/7/11</td>
</tr>
<tr>
<td></td>
<td>CYP3A1/2</td>
</tr>
<tr>
<td></td>
<td>CYP6A1</td>
</tr>
<tr>
<td>Peroxisome proliferators</td>
<td>CYP2B1</td>
</tr>
<tr>
<td></td>
<td>CYP4A1/2/3/6/7</td>
</tr>
<tr>
<td>Dexamethasone, PCN, AGs</td>
<td>CYP3A1</td>
</tr>
<tr>
<td></td>
<td>CYP2B1/2</td>
</tr>
<tr>
<td></td>
<td>CYP2C6</td>
</tr>
</tbody>
</table>

Table 1.4 Specific CYP genes, whose expression is increased, by distinct classes of inducers. PAHs, polycyclic aromatic hydrocarbons; HAhs, halogenated aromatic hydrocarbons; PCBs, polychlorinated biphenyls; DDT, 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane; PCN, pregnenolone-16α-carbonitrile; AGs, anti-glucocorticoids. (reproduced and modified from (Denison and Whitlock, 1995))
Fig. 1.4 Simplified general model for the transcription activation of various xenobiotic inducible CYP genes by their respective prototype inducers. PAH, polycyclic aromatic hydrocarbon; PP, peroxisome proliferator; PB, phenobarbital; Dex, dexamethasone; AhR, arylhydrocarbon receptor; hsp90, heat-shock protein 90; PPARα, peroxisome proliferator-activated receptor; CAR, constitutively activated receptor; GR, glucocorticoid receptor; Arnt, AhR nuclear translocator protein; RXRα, retinoid X receptor; XRE, xenobiotic-responsive element; PPRE, peroxisome proliferator response element; GRE, glucocorticoid-responsive element. (adapted and modified from (Dogra, et al., 1998))
rat, CYP3A23 induction is not mediated through the classical mechanism. Instead, Schuetz et al (1998) (Schuetz, et al., 1998) and Lehmann et al (1998) (Lehmann, et al., 1998) have recently reported the activation of a novel orphan receptor, PXR (pregnane X receptor) by glucocorticoids, pregnanes and other compounds that induce CYP3A. These compounds also activate another nuclear receptor termed hPAR (human peroxisome proliferator activator receptor) which apparently regulates only human and not mouse CYP3A expression (Bertilsson, et al., 1998). It is possible that PXR and hPAR represent orthologous proteins from different species.

In rats, PXR heterodimerises with RXR (retinoid X receptor) and binds to a dexamethasone-responsive element in the promoter sequence of the CYP3A23 gene and is able to activate reporter gene transcription (Quattrochi, et al., 1998).

1.5.1.2 Peroxisome proliferator inducible CYP genes

Peroxisome proliferators comprise a variety of structurally dissimilar compounds including hypolipidemic drugs, industrial solvents and herbicides. As the name implies, they induce peroxisome proliferation and also lead to an increase in the oxidation of fatty acids through peroxisomal β-oxidation and microsomal ω-oxidation pathways (Lock, et al., 1989, Johnson, et al., 1996). The microsomal ω-oxidation reaction is catalysed by the CYP4A subfamily.

Specific members of the CYP4A subfamily are induced by peroxisome proliferators in different species (Kimura, et al., 1989, Bell, et al.,
The rat CYP4A1 and rabbit CYP4A6 are the most highly induced in the liver (Johnson, et al., 1996). A member of the nuclear receptor superfamily known as peroxisome proliferator activator receptor (PPARα) is involved in the transcriptional activation of CYP4A genes (Isseman and Green, 1990). However, its ability to activate transcription requires the binding of ligand. A number of exogenous peroxisome proliferators (Devchand, et al., 1996) as well as fatty acids (Keller, et al., 1993) have been observed to bind and activate PPARα. Because of the structural diversity of exogenous peroxisome proliferators, the idea of fatty acids being the true ligand for PPARα is more favourable. There is a possibility that peroxisome proliferators may lead to an accumulation of endogenous fatty acids which then activate PPARα resulting in transcriptional activation (Dogra, et al., 1998). The general molecular mechanism for the activation of CYP4A gene expression is shown in Fig. 1.4. A peroxisome proliferator responsive element (PPRE) has been identified in the promoter of genes responsive to this class of inducers. Apparently, PPARα has to heterodimerise with RXRα in order to bind to the PPRE and inducers activate transcription by enhancing the dimerisation between PPARα and RXRα (Palmer, et al., 1994).

1.5.1.3 Polycyclic aromatic hydrocarbon inducible CYP genes

Unlike chemicals that induce CYP4A or CYP2B subfamily members, high affinity ligands that greatly increase CYP1A subfamily members possess very
similar structures, i.e. uniformly planar and aromatic, which include dioxins, 3-methylcholanthrene and benzo(a)pyrene. Of all the xenobiotic inducible CYP subfamilies, the molecular mechanism regulating $CYP1A1$ gene expression is the most well-characterised to-date.

The inducer on entering the cell interacts with a protein known as arylhydrocarbon receptor (AhR) and dissociates the AhR from heat-shock protein 90 (hsp90) in the cytosol. The liganded AhR then translocates into the nucleus and heterodimerises with a nuclear protein called AhR nuclear translocator protein (Arnt) through the Helix-Loop-Helix (bHLH) and the PAS domains. The heterodimer then binds to specific DNA sequences known as xenobiotic-responsive elements (XRE) and activates transcription. Protein phosphorylation may be required for heterodimerisation (Chen and Tukey, 1996), XRE-binding (Pongratz, et al., 1991) and/or transcriptional activation (Li and Dougherty, 1997) to occur. The chromatin structure of the enhancer (XRE) and the promoter region of the $CYP1A1$ gene assumes a nucleosomal configuration in the uninduced state. Upon induction, the AhR/Arnt heterodimer binding to the XRE can disrupt a nucleosome. It then recruits general transcription factors, somehow disrupts the nucleosomal structure at the promoter region, and finally stabilises the binding of the general transcription factors to the promoter (reviewed (Whitlock, et al., 1996)). Fig. 1.5 shows the molecular mechanism in more detail than Fig. 1.4.
Fig. 1.5 Model for the induction of CYP1A1 transcription by polycyclic aromatic hydrocarbon (PAH). (adapted from (Whitlock, et al., 1996))
1.5.1.4 Phenobarbital (PB) inducible CYP genes

PB, an anti-epileptic drug, causes pleiotropic effects which include induction of numerous drug-metabolising enzymes and various cellular processes (Fig. 1.6 and (Honkakoski and Negishi, 1998b)). A wide variety of structurally dissimilar compounds such as pesticides, chlorinated biphenyls, organic solvents and drugs also induce a similar set of enzymes as PB and are generally known as ‘PB-like’ inducers (fig. 1.7).

The mechanism whereby PB induces gene expression was thought to be highly conserved when it was found to induce genes in both prokaryotes (bacteria) and eukaryotes (birds and mammals). However, this does not appear to be the case. PB regulation of certain genes such as CYP2B1, CYP2B2 and CYP102 is blocked by cycloheximide signifying that ongoing protein synthesis is required for their increased transcription (Bhat, et al., 1987, Waxman and Azaroff, 1992). For CYP3A, CYP2H1 and CYP2H2 genes, cycloheximide is observed to synergise with PB leading to ‘superinduction’ (Burger, et al., 1990, Hamilton, et al., 1992). The phenomenon seen in the latter case is deduced to be due to the loss of a labile repressor protein (Dogra, et al., 1993). Furthermore, the inductive response of different proteins to PB can differ enormously (e.g. 50- to 100-fold with CYP2B1 and CYP2B2 and 2- to 4-fold with CYP2A1 and CYP2C6). It therefore seems that PB might induce CYPs by more than one mechanism.

So far, the molecular mechanism of gene activation by steroids, peroxisome proliferators and polycyclic aromatic hydrocarbon has been shown to involve the binding of specific liganded receptors to specific DNA
Fig. 1.6 Pleiotropic effects of PB. (adapted from (Honkakoski and Negishi, 1998))
Fig. 1.7. Structure diversity of PB and PB-like inducers. (adapted from (Waxman and Azaroff, 1992))
regulatory elements. Thus, the idea of a receptor involved in PB mediated transcriptional activation is highly probable.

Recently, Honkakoski and co-workers (1998) (Honkakoski, et al., 1998b) have identified an orphan nuclear receptor known as constitutively activated receptor (CAR) that is involved in PB induction of the mouse Cyp2b10 and the human CYP2B6. CAR activates Cyp2b10 and CYP2B6 gene transcription by binding to nuclear receptor binding sites, i.e. NR1 and/or NR2 (see section 1.6.2 for more detail) within the PB-responsive enhancer module (PBREM, generally known as PB-responsive element (PBRE)) as a heterodimer with RXRα. CAR has previously been shown to dimerise with RXR and bind to a subset of retinoic acid response elements (RAREs) (Baes, et al., 1994). However, CAR appears to function differently from the conventional nuclear receptor pathway. Unlike classical nuclear receptors which are activated by their cognate ligands, CAR is a constitutive transcriptional activator (Choi, et al., 1997). Recently, androstane metabolites have been identified, in the mouse, as ligands for an isoform of CAR called CARβ. Instead of activating the receptor, these metabolites inhibit the constitutive activity of CARβ (Forman, et al., 1998). These androstane ligands are found to be examples of naturally occurring inverse agonists that can reverse the transcriptional activation by nuclear receptors (Klein, et al., 1996).

For the activation of classical receptors, ligand binding induces a conformational change in the receptor which promotes the recruitment of
transcriptional co-activators like steroid receptor coactivator-1 (SRC-1) for transcriptional activation (Onate, et al., 1995). On the other hand, CAR has been shown to act in an opposite manner and is speculated to adopt an active conformation in the absence of ligand. The binding of ligand to CAR has been shown to directly dissociate the interaction between CAR and SRC-1 by shifting the receptor to an inactive conformation (Forman, et al., 1998). However, the micromolar concentration of androstenol needed to inhibit CAR in in vitro studies is higher than that reported in the circulation of adult man (Gower and Ruparelia, 1993). Hence, it is still not entirely clear as to how CAR is repressed in the liver.

Sueyoshi et al (1999) (Sueyoshi, et al., 1999) demonstrated that the cotransfection of CAR with a reporter construct containing either the mouse or human PBREM sequence into HepG2 cells gave high levels of luciferase activity. The endogenous CYP2B6 mRNA, normally not expressed in HepG2 cells, were also detected upon transfection with CAR. Both the luciferase activity and the endogenous CYP2B6 mRNA expression were suppressed when CAR transfected cells were treated with androstenol. The subsequent addition of PB to androstenol-treated CAR-transfected cells overcomes the suppressive effect of androstenol and was observed to induce luciferase activity and endogenous CYP2B6 mRNA expression. Sueyoshi et al (1999) (Sueyoshi, et al., 1999) believe the transfection results suggested that PB induces CAR probably by displacing androstenol. However, there is no evidence at present indicating that CAR does bind PB. Furthermore, CYP2Bs can also be induced by many other structurally dissimilar ‘PB-like’ compounds. And so the mechanism whereby PB activates CAR is still not
clear. It is not known if PB and PB-like inducers act like peroxisome proliferators, leading to the accumulation of an endogenous substrate which then derepressed CAR resulting in transcriptional activation. An indication that this might be the case had been proposed by Shaw et al (1993) (Shaw, et al., 1993) when they observed that the antiprogestin-antiglucocorticoid, RU486 could block the increased expression of reporter constructs containing either the CYP2B1 or CYP2B2 5'-flanking sequences by PB. They proposed that PB acts indirectly to cause the accumulation of an endogenous steroid, which is the direct inducer of CYP2B genes.

1.6 PB-responsive regulatory elements in PB-inducible CYP genes

1.6.1 Elements proximal to the transcription start site

An element known as the ‘Barbie Box’ located within the promoter region of the CYP102 and CYP106 genes in Bacillus megaterium was initially identified and reported to be important for PB induction (He and Fulco, 1991, Liang, et al., 1995, Liang and Fulco, 1995). However recently, this sequence is found to be responsive to PB only in the more PB-inducible CYP102 and not the less PB-inducible CYP106 gene (Shaw, et al., 1998). But the ‘Barbie Box’ sequence is important in the negative regulation of both CYP102 and CYP106 genes in uninduced bacteria (Shaw, et al., 1998).

Liang et al (1995) (Liang, et al., 1995) has reported the presence of a Barbie-box like DNA sequence within many mammalian PB-inducible CYP genes such as the rat CYP2B1, CYP2B2, CYP3A2 and rabbit CYP2C1 and

Apart from the ‘Barbie Box’, liver nuclear protein from PB-treated rats has also been observed to bind more to *CYP2B2* promoter sequences from -31 to -72 bp and -183 to -199 bp (Shephard, *et al.*, 1994). Using an *in vitro* transcription system, Shervington (1998) (Shervington, 1998) recently reported that the sequence between -178 and -368 bp of the *CYP2B2* promoter enhances transcription in response to PB but also reported a negative regulatory region, between -2880 and -5600 bp that inhibits transcription.

There are also many reports indicating that the ‘Barbie Box’ sequence in *CYP2B* genes plays no role in gene regulation by PB. First of all, many laboratories did not detect any protein binding to the ‘Barbie Box’ or the PE sequences of *CYP2B1, CYP2B2* or *Cyp2b10* genes (Ramsden, *et al.*, 1993,
Shephard, et al., 1994, Honkakoski, et al., 1996, Luc, et al., 1996, Park and Kemper, 1996, Sommer, et al., 1996). Mutations in the ‘Barbie Box’ sequence of either CYP2B2 or Cyp2b10 have no effect on the transcription of reporter gene constructs transfected into hepatic cells (Honkakoski, et al., 1996, Park, et al., 1996). Furthermore, the ‘Barbie Box’ sequence of the mouse Cyp2b10 is disrupted by a 42 bp insertion (Honkakoski, et al., 1996). In fact, Ramsden et al (1993) (Ramsden, et al., 1993) showed that a CYP2B2 transgene containing the first 800 bp promoter sequence could not confer PB responsiveness while another transgene containing promoter sequences up to 19 kb could confer PB responsiveness in transgenic mice indicating that a PB regulatory element lies between -0.8 and -19 kb. The proposed proximal PB regulatory elements of the CYP2B genes are schematically represented in Fig. 1.8.

1.6.2 Elements distal to the transcription start site

A sequence between -2155 and -2318 bp of the CYP2B2 promoter region was observed to mediate PB induction in rat primary hepatocyte cultures (Trottier, et al., 1995) and in in situ transfection assays in rat liver (Park, et al., 1996) when attached to two different heterologous promoters. The equivalent region in the mouse Cyp2b10 5'-flanking sequence located between -2250 and -2426 bp can also induce gene transcription in PB-treated mouse primary hepatocytes (Honkakoski and Negishi, 1997). This region that confers PB-responsiveness is highly conserved in PB-inducible genes in both rat and mouse. It has been intensively dissected, and studied,
Fig. 1.8 Schematic representation of proposed PB regulatory elements in the proximal and distal regions of CYP2B promoters reported by various laboratories: (1) the ‘Barbie Box’ element found in numerous CYP genes, specifically located between -73 and -89 bp of the CYP2B1 promoter (He and Fulco, 1991); (2) the negative element (NE) between -126 and -160 bp and the positive element (PE) between -69 and -98 bp identified in the rat CYP2B promoter (Nirodi, et al., 1996, Prabhu, et al., 1995, Ram, et al., 1995, Upadhya, et al., 1992); (3) 2 regions from -199 to -183 bp and -31 to -72 bp were identified by Shephard and coworkers (Shephard, et al., 1994); (4, 5 and 6) the PBRU or PBREM containing a central NF1 (nuclear factor 1) core element (■) flanked on both sides by accessory elements (■) such as GRE (glucocorticoid response element), AF1 (accessory factor 1) (Stoltz, et al., 1998), RE (named here as repressor element) (Liu, et al., 1998), NR1 and NR2 (nuclear receptor 1 and 2) (Honkakoski, et al., 1998) respectively and (7) an AP1 binding site (Roe, et al., 1996).
and found to be a multicomponent enhancer whereby multiple recognition sites bound by different regulatory proteins are essential for maximal PB inducibility. This region, previously known as a PB-responsive element (Trottier, et al., 1995) has now been redesignated as the PB response unit (PBRU) in the rat (Liu, et al., 1998, Stoltz, et al., 1998) and PB-responsive enhancer module (PBREM) in the mouse (Honkakoski, et al., 1998a).

A central core element containing a NF-1 motif is determined to be required for PB induction but not sufficient on its own for PB responsiveness and sequences flanking either upstream or downstream of this core element are necessary. In both rat and mouse, mutations of the NF-1 motif or sequences flanking either upstream or downstream of the NF-1 motif independently of one another were observed to reduce but do not completely abolish PB responsiveness (Honkakoski, et al., 1998b, Liu, et al., 1998, Stoltz, et al., 1998).

In the rat CYP2B2 promoter, a glucocorticoid-like response element upstream of the NF-1 motif and a downstream region known as AF1 are reported to contribute to PB responsiveness (Stoltz, et al., 1998). However, Liu et al (Liu, et al., 1998) observed that a region within the AF1 is involved in repressing basal expression. Two putative nuclear receptor binding sites, designated NR1 and NR2, are found in the flanking region on each side of the NF-1 core element of the mouse Cyp2b10 (Honkakoski, et al., 1998b). A region highly homologous to the mouse NF-1, NR1 and NR2 region is also found within the PBRU of the rat CYP2B1 and CYP2B2 and the human CYP2B6 genes. A CAR/RXR heterodimer has been reported to bind to the NR1 of the mouse Cyp2b10 (Honkakoski, et al., 1998b) as well as both
NR1 and NR2 of the human CYP2B6 (Sueyoshi, et al., 1999) (see section 1.5.5). In PB-treated mice, binding of both CAR and RXR increases rapidly and occurs before the increase of Cyp2b10 mRNA (Honkakoski, et al., 1998b). The fact that the PBREM of a PB-noninducible mouse Cyp2b9 gene, is mutated and non-functional, further indicates that this region is important for PB inducibility (Honkakoski and Negishi, 1997).

The binding of liver nuclear protein from PB-treated rats to an AP-1 site located at -1441 bp of the CYP2B2 gene is also found to be increased (Roe, et al., 1996). Sequences that confer PB responsiveness have also been reported in the distal region between -1.1 and -5.9 kb of the chicken CYP2H1 promoter (Hahn, et al., 1991). However, reporter constructs containing this domain fused with its own proximal promoter sequences showed only a weak induction response when transfected into PB-treated chick embryo hepatocytes (Hahn, et al., 1991). This, according to May and co-workers, is due to the high basal expression of the CYP2H1 proximal promoter sequences in in vitro systems as compared to an almost undetectable basal expression of CYP2H1 in vivo (Dogra and May, 1997). They deduce a mechanism similar to CYP1A1 gene expression for the repressed basal expression of the CYP2H1 gene. They suggested that in the native CYP2H1 gene, the promoter is normally not accessible due to the nucleosomal configuration but PB activation of a receptor complex leads to loss of nucleosomes from the enhancer which in turn initiates the removal of nucleosomes from the promoter region resulting in transcription (Dogra, et al., 1998). The importance of chromatin structure in the activation of the rat CYP2B genes has also been reported (Liu, et al., 1998). The possible distal
PB regulatory elements of the CYP2B genes are schematically represented in Fig. 1.8.

1.7 CYP2B1 and CYP2B2

Rat CYP2B1 and CYP2B2 are the most highly induced by PB of all the PB-inducible CYPs (Phillips, et al., 1981, Thomas, et al., 1981, Phillips, et al., 1983b) and are therefore the most extensively studied (Gonzalez, 1990). Their gene sequences have been deduced using recombinant DNA methods specifically by sequence analysis of cloned cDNA and genomic DNAs (Fujii-Kuriyama, et al., 1982). CYP2B1 and CYP2B2 gene sequences are >97% similar. Although they are so similar in sequence, analysis of genetic cross-experiments have indicated that they are non-allelic and are closely linked on chromosome 1 (Rampersaud and Walz, 1983, Rampersaud and Walz, 1987). Both genes have nine exons and eight intervening sequences. The overall molecular sizes of CYP2B1 and CYP2B2 are 23 kb and 14 kb respectively and the difference in size lies in intron 1, i.e. 12 kb in CYP2B1 and 3.2 kb in CYP2B2 (Suwa, et al., 1985). Of the forty base substitutions found in the exon sequences between the two genes, 15 of them result in 14 amino acid replacements. These replacements occur in relatively limited regions of the gene sequences. Most of them are found in exons 6, 7, 8 and 9, but most frequently in exon 7 (Mizukami, et al., 1983).
1.8 CYP2B1 and CYP2B2 5'-flanking sequences

The close sequence identity between the two genes is also found to be extended to the promoter region up to 2.3 kb (Shaw, et al., 1996). However, within the homologous region, there is a notable exception whereby an alternating purine-pyrimidine (-CA-) sequence is repeated a different number of times in the two genes: 5 times in CYP2B1, (CA)₅ and 19 times in CYP2B2, (CA)₁₉. This -CA- repeat sequence is located upstream from the CAP site beginning at about -255 bp and has been suggested to play a role in the regulation of gene expression due to its potential to form Z-helical DNA structure (Suwa, et al., 1985). The transcription initiation site is putatively about 30 bp upstream from the ATG translation initiation site. Both genes have the same modified TATA sequence, CATAAA, 20 bp upstream from the transcription initiation site (Mizukami, et al., 1983).

1.9 Other regulatory elements

In addition to the PB regulatory elements discussed in section 1.6, other regulatory sequences not pertaining to PB-inducibility have also been reported. Many laboratories have confirmed, using transient transfection assays and mutational studies, that a sequence between -45 and -65 bp in both CYP2B1 and CYP2B2 promoters binds members of the C/EBP (CCAAT/Enhancer Binding Protein) transcription factor family (Shephard, et al., 1994) and is essential for basal promoter activity (Luc, et al., 1996, Park and Kemper, 1996, Dell, 1997). Honkakoski et al (1996) (Honkakoski, et al., 1996) also made similar observations with the corresponding C/EBP region.
in the mouse Cyp2b10. There are also reports of a region involved in repressing CYP2B high basal level of expression further upstream (Ramsden, et al., 1993, Honkakoski, et al., 1996). A putative glucocorticoid receptor element (GRE) found between -1349 and -1335 bp has been shown to be functional and hence capable of conferring dexamethasone inducibility on a heterologous promoter (Jaiswal, et al., 1987, Shephard, et al., 1994).

1.10 Regulation of CYP2B gene expression

1.10.1 Tissue-specific expression

Liver is the major organ involved in detoxification and a large number of CYPs are expressed in this tissue. However, CYPs have also been found in many extrahepatic tissues and virtually all tissues examined so far contain one or more forms of CYP.

The rat CYP2B1 and CYP2B2 may be 97% identical in their amino acid sequences but their expression profiles in hepatic and extrahepatic tissues are very different. CYP2B1 mRNA is barely detectable in the liver while CYP2B2 mRNA is constitutively expressed. Both mRNAs are highly induced upon PB treatment from 20- up to >100-fold in the liver (Christou, et al., 1987, Yamazoe, et al., 1987). In extrahepatic tissues, CYP2B1 is constitutively expressed but not inducible by PB in lung and testis while CYP2B2 is absent from these tissues regardless of PB treatment (Omiecinski, 1986, Christou, et al., 1987). In small intestine, only CYP2B1 is PB-inducible (Gonzalez, 1989, Traber, et al., 1990, Elia, 1996). Using
antibodies against both CYP2B1 and CYP2B2, these two proteins were detected in the brain, particularly in the Bergmann glial cells of the cerebellum (Warner, et al., 1988). PB is also able to induce both CYP2B1 and CYP2B2 in the adrenal glands (Christou, et al., 1987). Another member of the CYP2B subfamily, CYP2B3 is constitutively expressed in the liver and not inducible by PB (Labbe, et al., 1988). However, CYP2B3 is not detectable in other tissues such as lung, kidney and prostate. In breast tissue, CYP2Bs have been detected in pregnant rats. Apparently, a decrease was observed during lactation which then greatly increased in 3-week post-lactating rats (Hellmold, et al., 1995).

Even within a single organ, distinct regions or cell-types may be differentially responsive to PB. The PB induction of CYP2B1 occurs predominantly in the enterocytes of the small intestines (Traber, et al., 1988) and induction is found to be greater in the proximal region than the distal region of the small intestines (Traber, et al., 1990). There is also zonal distribution of CYP2B inducible expression by PB in the liver. Both CYP2B1 and CYP2B2 mRNA were observed to be expressed uniformly across the centrilobular and mid-zonal regions of the hepatic lobule in PB-induced liver except for a small band of cells immediately surrounding the periportal tract that is refractory to PB-induction (Chianale, et al., 1986, Hassett, et al., 1989, Traber, et al., 1989). Therefore, it seems that the genes encoding for the rat CYP2B subfamily contain tissue-specific regulatory and inducer control elements. However, very little is known about these regulatory mechanisms.
1.10.2 Developmental-specific expression

When utilising standard blotting and solution hybridisation assays, PB-inducible CYP2B mRNAs were not detectable in foetal rat liver before gestational day 21 (Giachelli and Omiecinski, 1987). These CYP mRNAs were found to be PB-inducible in foetal rat liver from day 15 of gestation with the use of the Polymerase Chain Reaction (PCR) technique (Omiecinski, et al., 1990). And the induction levels increase with increasing developmental age, reaching maximal levels approximately 3 weeks postpartum (Giachelli and Omiecinski, 1986). Studies done by Agrawal et al (1996) showed that animals of younger age exert a more inhibitory effect than mature animals (Agrawal and Shapiro, 1996). It was observed that CYP2B1 and CYP2B2 expression was suppressed more in young adults (65 days of age) than mature adults (150 days of age). And young rats tend to show the greatest suppression because they produce a more elevated growth hormone pulse amplitude that results in greater inhibition of PB induction of CYP2B1 and CYP2B2 (Agrawal and Shapiro, 1996). The effects of growth hormone on CYP2B expression are given in more detail in section 1.10.3 that follows.

1.10.3 Gender-specific expression

Several CYPs are expressed in rat liver in a sex-specific manner, where they are subjected to complex developmental regulation and endocrine control (Zaphiropoulos, et al., 1989). However, gonadal hormones do not directly regulate the sex-specific pattern of CYP expression.
in the liver but mediate their effects via the gonadal-hypothalamic-pituitary axis and its sex-dependent regulation of pituitary growth hormone secretory patterns (Waxman and Chang, 1995). Growth hormone is secreted by the pituitary gland and its secretory profile is sexually differentiated in many species but the difference between the sexes are most prominent in rodents (Eden, 1979). Male rats have an intermittent plasma growth hormone pulse while females show a more continuous profile. The sexual dimorphism in the plasma growth hormone profile not only regulates the constitutive sex-specific CYPs but also the xenobiotic-inducible CYPs.

Growth hormones and thyroid hormones have been shown to suppress both the basal and PB-inducible expression of CYP2B1 and CYP2B2 in vivo as well as in hepatocyte culture in vitro and this regulation is at the level of transcription (Yamazoe, et al., 1987, Yamazoe, et al., 1989a, Murayama, et al., 1991, Shapiro, et al., 1994, Shimada, et al., 1997). Studies done by Agrawal et al (Agrawal and Shapiro, 1996) indicated that the female gender was associated with the greatest PB-induction inhibition, i.e. both CYP2B1 and CYP2B2 expression were suppressed more in females as compared to their male counterparts. It is the continuous secretion characteristic of the females that is more suppressive to CYP2B induction than the pulse secretion characteristic found in males (Agrawal and Shapiro, 1996). And in the males, it is the height of the growth hormone pulse during each episodic burst that signals the extent of suppression (Shapiro, et al., 1994). In addition, Agrawal and Shapiro (1996) (Agrawal and Shapiro, 1996) observed that PB-induced CYP2B1 and CYP2B2 expression are differentially regulated between the sexes. The effect of gender on
CYP2B1 was at the mRNA level and CYP2B2 at the protein level, suggesting the effect to be pre-transcriptional or transcriptional for the former and post-transcriptional for the latter.

Although growth hormone has been shown to be the primary pituitary hormone in suppressing PB induction of CYP2Bs, the removal of growth hormone by hypophysectomy does not eliminate the difference in response of CYP2Bs to PB between the sexes. This leads to the suggestion of the possible involvement of a growth hormone-independent but sex-dependent regulatory factor (Shapiro, et al., 1994) or neonatal androgenic imprinting effects (Einarsson, et al., 1973). Also the findings that hypophysectomised rats showed more enhanced PB induction of CYP2Bs than monosodium glutamate- or methimazole-induced growth hormone-deficient rats indicating that other anterior pituitary hormones, adrenal and thyroid hormones may also be involved in altering PB induction (Yamazoe, et al., 1987, Yamazoe, et al., 1989a, Murayama, et al., 1991, Shapiro, et al., 1994, Shimada, et al., 1997).

The suppression of PB-induced CYP2B expression by growth hormone was shown to be a direct antagonising effect and not PB interfering with the normal secretory profiles of growth hormone that leads to altered expression of CYP2B1 and CYP2B2 (Schuetz, et al., 1990, Waxman, et al., 1990, Agrawal and Shapiro, 1996). Growth hormone is involved in many different cellular functions and apparently exerts its effects through the activation of various different signal transduction pathways (Maharajan and Maharajan, 1993). For example, the expression of the female-specific CYP2C12 may involve a phospholipase A2-dependent pathway (Tollet, et al.,

1.10.4 Strain-specific expression

A study carried out by Larsen et al (1994) (Larsen, et al., 1994) indicated an obvious female-linked strain polymorphism in the induced expression of a number of PB-responsive CYPs including CYP2B1 and CYP2B2. As mentioned previously in section 1.10.3, CYP2Bs are less inducible by PB in female than male rats. This sexual dimorphism in PB-induction is found to be much more evident in some strains than in others and extends to many structurally dissimilar chemicals that produce PB-like effects (Lubet, et al., 1992, Blouin, et al., 1993, Larsen, et al., 1994, Larsen and Jefcoate, 1995). The suppression of growth hormone secretion from the pituitary by suppressing the level of thyroxine and triiodothyronine via hypophysectomy or methimazole treatment reduce the strain differences in PB-induced CYP expressions not only among the females but between males and females too (Larsen, et al., 1994, Larsen and Jefcoate, 1995).
1.11 Factors affecting basal and/or PB-induced CYP2B gene expression

1.11.1 Cytokines

During inflammation or infection, an acute phase response is triggered to help maintain homeostasis (Schreiber, et al., 1989). The response is characterised by alteration in the synthesis of a number of hepatic proteins. But the principal homeostatic mediators of the acute phase response are cytokines such as TNFα, IL-1 and IL-6, of which IL-6 is thought to be the most important (Heinrich, et al., 1990). One of the effects mediated by these cytokines is a decrease in total hepatic CYP enzymes (Morgan, 1989). Studies into the effects of each of these cytokines on specific forms of CYPs have also been carried out both in vivo and in vitro. Although the results obtained by different laboratories are not all in agreement, the general observation is a decrease in many of the specific CYPs investigated (Wright and Morgan, 1991, Fukuda, et al., 1992, Abdel-Razzak, et al., 1993). There are reports suggesting that cytokines down-regulate CYPs by affecting their synthesis (Stanley, et al., 1988, Renton and Knickle, 1990, Wright and Morgan, 1990) while others indicated that cytokines might affect the degradation of CYPs (Cantoni, et al., 1991, Moochhala and Renton, 1991).

The induction of CYP2B1 and CYP2B2 and their enzyme activities by PB has been observed to be inhibited by IL-1β and in particular IL-6 in primary rat hepatocyte cultures (Williams, et al., 1991, Clark, et al., 1995, Clark, et al., 1996). Experiments carried out by Williams et al (1991)
(Williams, et al., 1991) and Clark et al. (1996) (Clark, et al., 1996) indicated IL-6 to inhibit PB-induction of CYP2B1 and CYP2B2 mRNA expression. There was suggestion that this inhibition is at the level of transcription (Williams, et al., 1991). The ability of IL-6 to block CYP2B1 and CYP2B2 induction by PB is specific because the induction of NADPH Cytochrome P450 reductase by PB is not affected (Clark, et al., 1996).

11.1.2 Insulin

The expression of members of certain CYP subfamilies such as CYP2B, CYP2E, CYP3A and CYP4A has been shown to be enhanced in both spontaneous and chemically-induced diabetic rats (Yamazoe, et al., 1989b, Shimojo, et al., 1993). The basal as well as PB-induced CYP2B1 and CYP2B2 expression are both higher in diabetic rats than normal rats (Yamazoe, et al., 1989b, Yoshida, et al., 1996). The increase in the expression of CYPs in diabetes was attributed to hyperketonemia (Barnett, et al., 1990a, Barnett, et al., 1990b) or decreased growth hormone and testosterone amounts (Yamazoe, et al., 1989b, Thummel and Schenkman, 1990) which are a few of the pathological events that normally occur in the diabetic state. Although the administration of insulin to diabetic rats could lower the amount of affected CYPs to that seen in normal animals, there was no evidence for the direct action of insulin. However, the expression of CYPs in a non-insulin-dependent diabetic rat was not affected (Barnett, et al., 1992).

The ability of insulin to directly down-regulate CYP2B1 and CYP2B2 expression has recently been shown in a rat hepatoma cell line, Fao cells
(De Waziers, *et al.*, 1995) and in primary rat hepatocyte cultures (Yoshida, *et al.*, 1996, Woodcroft and Novak, 1997). Insulin has been reported to alter the expression of a number of genes either at the transcriptional (O'Brien and Granner, 1991) and/or post-transcriptional levels (Flores-Riveros, *et al.*, 1993). Although a number of insulin responsive sequences (IRS), shown to mediate insulin response in the phosphoenolpyruvate carboxykinase (PEPCK) gene, are found within the proximal promoter region of both *CYP2B1* and *CYP2B2* genes, it is not known if these sequences are functional (Yoshida, *et al.*, 1996). Furthermore, there are indications that insulin suppresses the expression of *CYP2B1* and *CYP2B2* by accelerating mRNA turnover rate (De Waziers, *et al.*, 1995, Woodcroft and Novak, 1997).

### 1.11.3 Haem

Haem is known to regulate the levels of several haemoproteins and enzymes of haem metabolism (Maines, 1984, Padmanaban, *et al.*, 1989, Ades, 1990). However, reports on the role of haem in regulating *CYP* expression, in particular the rat *CYP2Bs* and chicken *CYP2Hs* have been controversial.

On one hand, haem has been implicated as the positive modulator of rat *CYP2B1* and *CYP2B2* gene transcription (Ravishankar and Padmanaban, 1983, Ravishankar and Padmanaban, 1985, Dwarki, *et al.*, 1987, Venkateswar and Padmanaban, 1991). The inhibition of haem synthesis by the administration of CoCl$_2$ or aminotriazole to PB-treated rats could block PB-induced transcription of *CYP2Bs* as monitored by nuclear
run-on transcription assays and RNase protection assays (Dwarki, et al., 1987, Rangarajan and Padamanaban, 1989, Srivastava, et al., 1990, Sultana, et al., 1997). A similar result was obtained in an in vitro cell-free transcription assay of a minigene construct containing exon I and the first 179 bp promoter region of the CYP2B2 gene using nuclei from rats treated with PB and CoCl₂ (Rangarajan and Padamanaban, 1989, Sultana, et al., 1997). Apparently, the inhibitory effect of CoCl₂ and aminotriazole could be overcome by the addition of exogenous haemin (Dwarki, et al., 1987, Rangarajan and Padamanaban, 1989, Sultana, et al., 1997). Other studies using succinylacetone, a more specific haem biosynthesis inhibitor than CoCl₂ and aminotriazole have reported no effect on PB-induced CYP2B1 and CYP2B2 mRNA expression both in primary rat hepatocyte cultures (Sinclair, et al., 1990) and in rat liver in vivo (Srivastava, et al., 1989). Furthermore, the addition of haem or δ-aminolaevulinate to cultured primary rat hepatocytes has no effect on basal as well as PB-induced CYP2B1 and CYP2B2 mRNA levels (Burger, et al., 1990).

There are studies indicating that the basal CYP2H1 mRNA and protein are increased by the administration of a haem biosynthesis inhibitor into chick embryo liver in vivo (Rifkind, 1979, Brooker, et al., 1983). The administration of δ-aminolaevulinate, to generate intracellular haem, to PB-treated chick embryos in vivo also resulted in a further increased in CYP2H1 mRNA levels (Ryan and Ades, 1989). On the contrary, Hamilton and co-workers (1988 and 1992) (Hamilton, et al., 1988, Hamilton, et al., 1992) showed that inhibitors of haem biosynthesis have no effect on both basal and inducible CYP2H1 and CYP2H2 expression by PB and PB-like inducers.

### 1.12 Inhibitors of protein synthesis

The requirement for *de novo* protein synthesis in the induction of CYPs by PB and PB-like compounds is also a subject that has been under intensive investigations. Unfortunately, studies carried out on the various PB-inducible CYPs have yielded contradicting results.

In the chicken model, cycloheximide generally enhances both CYP2H1 and CYP2H2 expression in hepatocytes *in vitro* and *in vivo*. However, cycloheximide was observed to affect only the basal expression in some studies (Hamilton, *et al.*, 1988, Hahn, *et al.*, 1991, Hamilton, *et al.*, 1992) while affecting both basal and PB-induced expression in others (Ryan and Ades, 1989, Dogra, *et al.*, 1993). The action of cycloheximide was shown to be at the level of transcription (Hansen and May, 1989, Hamilton, *et al.*, 1992, Dogra, *et al.*, 1993). The ability of PB to induce gene expression in the presence of cycloheximide as reported by some laboratories suggested that *de novo* protein synthesis is not necessary for PB induction of CYP2H mRNA. Instead, the enhanced CYP2H expression by cycloheximide led to the suggestion of the presence of a labile repressor protein.
In contrast, many studies have shown that PB-induced rat CYP2B1 and CYP2B2 expression in vivo and in vitro could be blocked by cycloheximide (Hardwick, et al., 1983, Bhat, et al., 1987, Chianale, et al., 1988, Burger, et al., 1990, Schuetz, et al., 1990). It was thus generally deduced that the regulation of CYP2B genes by PB or PB-like compounds requires ongoing protein synthesis. However, a recent study carried out by Sidhu and Omiecinski (1998) (Sidhu and Omiecinski, 1998) reported that cycloheximide did not block PB induction of CYP2B gene expression through inhibiting the synthesis of protein. They tested a range of protein synthesis inhibitors and showed that the potency of a compound to inhibit protein synthesis is not proportional to its ability to block PB-induction of CYP2B mRNA expression. Furthermore, an analogue of the protein synthesis inhibitor, puromycin, devoid of any protein synthesis inhibitory activity produced results similar to its active counterpart. Honkakoski et al (1996) (Honkakoski, et al., 1996) reported that PB-induced Cyp2b10 mRNA expression was not affected by cycloheximide in primary mouse hepatocyte cultures. It does appear as though the effects exerted by compounds that inhibit protein synthesis, in particular cycloheximide, on the regulation of PB-inducible CYP genes are different in different species. It is possible that in certain species, protein synthesis is a prerequisite while in others, alternative mechanism(s) may be utilised.
1.13 Protein kinases

1.13.1 Protein kinase A (PKA)

PB induces many CYPs by increasing the rate of transcription of their genes (Hardwick, et al., 1983, Pike, et al., 1985, Gonzalez, 1990). Unable to rule-out the need for de novo protein synthesis in PB-induction of CYP expression as discussed in section 1.12 above, the PB-inductive response may be mediated through post-translational modifications of pre-existing proteins. The regulation of transcription factors binding to DNA or their transactivation activity by phosphorylation has been described and it is one of the most important regulatory mechanisms for controlling gene expression (Hunter and Karin, 1992).

Further support was provided by Blankenship and Bresnick (1974) (Blankenship and Bresnick, 1974) when they reported that PB could stimulate the phosphorylation of acidic nuclear proteins in vivo. The administration of a cAMP analogue, db-cAMP was also observed to stimulate the phosphorylation of specific acidic nuclear proteins in rat liver (Johnson and Allfrey, 1972). The involvement of a cAMP-dependent protein kinase signalling pathway in PB-inducible gene expression has been extensively investigated, most probably because studies in vivo showed a stimulation of intracellular cAMP and PKA activity shortly after PB treatment (Byus, et al., 1976, Costa, et al., 1976). But conflicting observations have been reported.

A study done by Costa et al (1976) (Costa, et al., 1976) on total microsomal CYP content in rats indicated that increasing intracellular cAMP
by the administration of aminophylline and/or db-cAMP slightly decreased CYP concentration. But the administration of aminophylline and/or db-cAMP to rats treated with a PB-like inducer, Aroclor-1254, at a dose that on its own has no effect on CYP concentration, could elevate the concentration of CYPs.

Using primary rat hepatocyte cultures, Brown et al (1997) (Brown, et al., 1997) reported that specific inhibitors of PKA abolish the induction of CYP2B1, CYP2B2 and CYP3A23 mRNA by PB while cAMP analogues and PKA activator, (Sp)-cAMPS, only slight augment PB induction of these genes. Opposing results were reported by Sidhu and Omiecinski (1996) (Sidhu and Omiecinski, 1995) who also performed their study in primary rat hepatocytes under similar treatment conditions. They found that cAMP analogues, adenylate cyclase activators and PKA activator could all attenuate PB-induced CYP2B1 and CYP2B2 mRNA expression. The fact the inhibitors of phosphodiesterase (PDE) activity could potentiate the inhibitory effect of cAMP on PB-induced CYP2Bs mRNA and that hydrolysis products of cAMP have no effect, further indicated that cAMP itself is responsible for the inhibition observed (Sidhu and Omiecinski, 1995). Dell (1997) (Dell, 1997) found activators of PKA and cAMP analogues to inhibit both basal and PB-induced CYP2B1 and CYP2B2 mRNA in rat primary hepatocytes.

Investigations into the effects of cAMP on PB-inducible CYP expression in other species have shown that increased cAMP levels inhibit PB-induced CYP1A1 expression in primary rainbow trout hepatocytes (Sadar, et al., 1996) and CYP3A31 expression in primary hamster hepatocytes (Bani, et al., 1998) but enhances PB-induced CYP2H1 expression in primary chick hepatocytes (Giger and Meyer, 1981) and both
basal and PB-induced Cyp2a5 expression in primary mouse hepatocytes (Salonpää, et al., 1994). Furthermore, Honkakoski and Negishi (1998) (Honkakoski and Negishi, 1998a) reported that TCPOBOP-induced Cyp2b10 mRNA in primary mouse hepatocyte cultures was not affected by inhibitors of PKA. At present, the involvement of cAMP signalling pathway in mediating PB induction response is still questionable.

1.13.2 Other protein kinases

According to Jirtle and co-workers, PB reduction of EGF (epidermal growth factor) receptor expression in primary rat hepatocytes was found to be independent of protein kinase C (PKC) activation (Meyer, et al., 1989, Meyer and Jirtle, 1989). Their studies into the translocalisation of PKC activity in PB-treated rat hepatocytes further indicated that PB does not activate PKC (Jirtle and Meyer, 1991). Not only does PB not activate PKC, it was even reported to down-regulate PKC activity (Chauhan and Brockerhoff, 1987, Brockenbrough, et al., 1991). More recently, both inhibitors and activators of PKC were observed to have no effect on PB-induced chicken CYP2H1 (Dogra and May, 1996) and mouse Cyp2a5 mRNA (Salonpää, et al., 1994) and TCPOBOP-induced mouse Cyp2b10 mRNA but induces basal Cyp2b10 (Honkakoski and Negishi, 1998a) in primary hepatocytes in vitro. In contrast, PKC inhibitors were observed to attenuate PB-induction of CYP1A1 in primary rainbow trout hepatocytes (Sadar, et al., 1996).
Tyrosine kinases were reported not to be involved in PB mediated
induction in chicken CYP2H1 (Dogra and May, 1996) and mouse Cyp2a5

2-aminopurine (2-AP), a protein kinase inhibitor, could inhibit PB-
induction of chicken CYP2H1 mRNA (Dogra and May, 1996) and rat CYP2B1
appears to be a non-specific protein kinase inhibitor but it does show
preference for the interferon-inducible double-stranded RNA activated
protein kinase, PKR (Debenedetti and Baglioni, 1983) and seems to block
kinase activity by modifying serine and threonine residues (Tiwari, et al.,
1988). Preliminary results obtained by Dogra and May (1996) (Dogra and
May, 1996) indicated that the effect of 2-AP on PB-induced CYP gene
expression is most probably not via the inhibition of PKR.

Honkakoski and Negishi (1998) (Honkakoski and Negishi, 1998a)
also showed an inhibitor of Ca^{2+}/Calmodulin-dependent protein kinase to
exert no effect on TCPOBOP-induced Cyp2b10 mRNA in mouse primary
hepatocytes. However, an inhibitor of Ca^{2+}/Calmodulin-dependent protein
kinase could abolish the inhibitory effect that okadaic acid (OK) has on
TCPOBOP-induced Cyp2b10 mRNA (see section 1.14 on the effect of OK).

It appears that one or more phosphorylation steps, sensitive to 2-AP,
are important events leading to the activation of CYP2H1, CYP2B1 and
CYP2B2 gene transcription by PB. However, the effect of 2-AP on PB-
induced mouse Cyp2b10 is not known.
1.14 Protein phosphatases

More consensus observations were made in delineating which protein phosphatase(s) is involved. Okadaic acid (OK), a phosphoserine/threonine phosphatase inhibitor of PP1 and PP2A, is reported to inhibit PB-induction of CYP2B1, CYP2B2 and Cyp2b10 mRNAs (Nirodi, et al., 1996, Dell, 1997, Sidhu and Omiecinski, 1997, Honkakoski and Negishi, 1998a). The use of other inhibitors of PP1 and PP2A also yielded similar observations while inactive analogues of OK showed no inhibitory effect (Sidhu and Omiecinski, 1997, Honkakoski and Negishi, 1998a). Furthermore, inhibitors of tyrosine phosphatase were observed to have no effect on mouse Cyp2b10 induction by TCPOBOP (Honkakoski and Negishi, 1998a).

Sidhu and Omiecinski (1997) (Sidhu and Omiecinski, 1997) showed that PP1 and PP2A inhibitors could potentiate PKA activators in repressing PB-induction and is strongly supportive of a concerted PKA and PP1/2A modulatory mechanism for PB-induction. While Honkakoski and Negishi (1998) (Honkakoski and Negishi, 1998a) reported that the inhibitory effect of OK could be overcome by inhibitors of Ca\(^{2+}\)/Calmodulin-dependent protein kinase, although the latter inhibitors have no effect on their own. In addition, 2-AP sensitive protein kinase(s) also have a role to play in PB-mediated gene transcription (Dogra and May, 1996, Nirodi, et al., 1996) (see section 1.13.2).

The interpretation of results reported by different laboratories should be handled with care because of the species used and the different cell culturing conditions could dictate the phenotype of the cells, leading to one
signalling pathway dominating over another. The actual mechanism of PB-mediated induction may involve multiple kinases and/or phosphatases that act in several signalling pathways which could cross-talk with each other (Hunter, 1995).

1.15 Scope of this thesis

CYP2B1 and CYP2B2 are the CYPs most highly induced by PB in the rat liver. The previous sections have shown that the expression and PB-induction of both CYP2B1 and CYP2B2 are subjected to developmental, gender and tissue-specific regulation. In addition, the constitutive as well as PB-inducible expression of these two genes in the liver is also differentially regulated, in that CYP2B1 (50 to 100-fold) is induced more than CYP2B2 (20-fold). It is known that PB induces CYP2B1 and CYP2B2 mRNA by increasing the transcription of their genes (Pike, et al., 1985). Thus, to elucidate the molecular mechanisms whereby PB-mediates transcriptional activation, extensive studies have been carried out on the 5'-flanking sequence of the CYP2B2 gene to identify any cis-acting elements and trans-acting factors that are involved. Efforts were also put into dissecting the signal transduction pathway(s) involved in mediating PB-inductive response. Unlike the PAH induction of the CYP1A1 gene, relatively little is known about the PB induction of CYP2B genes.

At the time, most of the promoter studies were carried out on the CYP2B2 because the CYP2B1 promoter sequence was not available. Recently Dr Andrew Elia (1995) (Elia, 1996) in our laboratory cloned the
CYP2B1 promoter sequence up to 450 bp upstream and subsequently, Shaw and co-workers (Shaw, et al., 1996) determined the CYP2B1 promoter sequence up to 3.9 kb upstream.

The main aim of my research is to elucidate the mechanism(s) regulating the basal and PB induced gene transcription of CYP2Bs, in particular CYP2B1. I began by analysing the CYP2B1 promoter sequence that was cloned in our laboratory using in vitro cell-free systems to identify any cis-acting element(s) involved in PB-responsiveness and the transcription factor(s) binding to the element(s). When the binding of liver nuclear protein from PB-treated rats to specific regions of the CYP2B1 promoter was observed to be either in greater abundance or activated, I then analysed if PB treatment affected the phosphorylation status of the transcription factor(s) involved in binding since the DNA binding and/or transactivation ability of many transcription factors are dependent on phosphorylation status. Different methods used for transfecting reporter gene constructs into primary rat hepatocytes cultured under conditions that have been previously optimised in our laboratory was also performed to find the best method. The transfection in primary cultures was also compared to a new in vivo transfection method. During the course of this project, much progress have been made by other and there are now reports on the possible signalling pathways involved in PB-mediated transcriptional activation. Very recently, a nuclear receptor involved in PB induction has also been determined (Honkakoski, et al., 1998b).
Chapter Two

Materials
And
Methods
2.1 DNA cloning and sub-cloning

2.1.1 DNA digestion with restriction endonucleases

Materials

DNA: Vector DNA or recombinant DNA.

Restriction Enzymes: Obtained from several sources, e.g. Pharmacia Biotech Ltd (St. Albans, Herts, U.K.) and New England Biolab. (Beverly, MA, U.S.A.). Stored at -20°C.

Enzyme buffers: Supplied with each restriction enzyme. Used at manufacturers’ recommended working concentration for optimal activity of each enzyme. If enzymes from two different manufacturers were used in the same reaction, the Pharmacia’s One-Phor-All buffer plus (10X concentration contained 100 mM Tris-acetate, pH 7.5, 100 mM magnesium acetate and 500 mM potassium acetate) was used. Stored at -20°C or 4°C if in constant use.

Equipment: Grant water-bath.

Method

DNA, the appropriate restriction enzyme(s), enzyme buffer and sterile distilled water to make up the total volume were mixed in a 1.5 ml Eppendorf tube. The amount of restriction enzyme(s) added depended on the amount of DNA to be digested. A unit of enzyme was added for every microgram of DNA. However, the total volume of enzyme(s) should not constitute more than 10% of the total reaction volume. The reaction mixture was then
incubated at a temperature for optimal activity of the restriction enzyme(s) (usually 37°C) for at least 2 hrs or up to 24 hrs. A small portion of the digested DNA (approximately 0.1 to 0.5 μg) was electrophoresed on an agarose gel to assess if DNA digestion was complete (see section 2.1.2).

2.1.2 Agarose gel electrophoresis

Materials

*Agarose (Sigma Chemical Co., St. Louis, MO, U.S.A.):* Type I, Low EEO. Stored at RT.

*10X TBE buffer:* 0.89 M Trizma® base, 0.89 M boric acid and 20 mM EDTA, pH 8.0. Autoclaved and stored at RT.

*Electrophoresis buffer:* 1X TBE buffer.

*10 mg/ml Ethidium Bromide (EtBr, BDH Chemicals Ltd, Poole, England):* Used at a final concentration of 0.5 μg/ml in agarose gel or running buffer.

Stock solution was stored at 4°C.

*6X DNA loading buffer I:* 0.25% (w/v) bromophenol blue (Bio-Rad Laboratory, Hercules, California, U.S.A.), 30% (v/v) glycerol. Filter sterilised through a Millex®-HA 0.45 μm filter unit (Millipore (U.K.) Ltd, Watford, England) and stored in aliquots at -20°C. Used at 1X final concentration.

*DNA marker:* 1 kb DNA ladder (Gibco BRL, Life Technologies, Inc., Gaithersburg, U.S.A.)
Equipment: Mini-the-Gel Cicle™ submarine agarose gel unit, electrophoresis power pack and CHROMATO-VUE® UV transilluminator (Model TM-15).

Method

An agarose gel of the appropriate percentage for the separation of particular DNA fragment sizes was prepared. A higher percentage was used to separate smaller DNA size fragments. For example, to prepare a 1% (w/v) agarose gel in 1X TBE buffer: 1 g of agarose was added to 100 ml of 1X TBE buffer and the contents were boiled to dissolve the agarose. The solution was then cooled to ~45°C before 2 μl EtBr was added to give a final concentration of 0.5 μg/ml. The solution was mixed well, poured into a gel casting tray and allowed to solidify for at least 30 mins. The gel was then fully submerged in the gel electrophoresis tank containing 1X TBE buffer. DNA loading buffer I was added to the DNA sample to 1X final concentration. Samples were loaded into the wells of the gel, and electrophoresed at 100 to 120 V for a suitable length of time until the DNA fragments were well separated. The separation of the DNA fragment(s) was visualised on a UV transilluminator.

2.1.3 DNA purification from low melting point agarose

Materials

Agarose (Gibco BRL): UltraPURE Low Melting Point Agarose. Stored at RT.
**50X TAE buffer:** 2 M Trizma® base, 5.71% (v/v) glacial acetic acid and 0.05 M EDTA, pH 8.0. Autoclaved and stored at RT. Used at 1X working concentration.

**Electrophoresing buffer:** 1X TAE buffer.

**10 mg/ml EtBr:** see section 2.1.2.

**6X DNA loading buffer I:** see section 2.1.2.

**Spin-X Column (Corning Costar Corporation, Cambridge, MA, U.S.A.):** Costar 0.22 µm cellulose acetate centrifuge tube filters. Stored at RT.

**TE Buffer:** 10 mM Tris-HCl, pH 8.0 and 1 mM EDTA, pH 8.0. Autoclaved and stored at RT.

**Sodium Acetate:** 3 M aqueous solution, pH 5.2. Filter sterilised and stored at RT.

**Ethanol:** Absolute

**Equipment:** Mini-the-Gel Cicle™ submarine agarose gel unit, electrophoresis power pack, UV transilluminator and Eppendorf Centrifuge 5414S.

**Method**

According to the size of the DNA to be purified, an appropriate percentage of low melting point agarose gel was prepared in 1X TAE buffer (refer to method in section 2.1.2 for the preparation of gel). Digested DNA was mixed with DNA loading buffer I, loaded onto the solidified agarose gel and electrophoresed at 80 V until DNA fragments were well separated. The DNA band(s) of interest was carefully excised from the gel taking the
minimum amount of gel possible. The excised gel was then transferred to the top compartment of the Spin-X column. Each column should not contain more than 150 µl volume of gel. (If the volume of gel is more than 150 µl, it should be divided into two columns for purification.) The gel in the tube was allowed to freeze at -70°C and then centrifuged at 'high' speed (~13 000 rpm) for 10 mins at RT in a microcentrifuge. This freeze/spin step was repeated three times before 100 µl of TE buffer was added to the top compartment and the tube re-centrifuged as above. The top compartment was then discarded and 0.2 volumes of 3 M sodium acetate, pH 5.2 and 2 volumes of 100% ethanol was added to the filtrate to precipitate the DNA. The DNA was precipitated from 1 hr to overnight (~16 hrs) at -70°C depending on its size such that the DNA of larger size required a shorter time to precipitate. The DNA was then pelleted by centrifugation at ~13 000 rpm for 20 to 30 mins at 4°C in a Eppendorf centrifuge. The supernatant was carefully removed and the pellet dried briefly in vacuo. The DNA was dissolved in 10 to 20 µl of sterile distilled water and stored at -20°C.

2.1.4 Dephosphorylation of linearised plasmid DNA

Materials

Tris-HCl: 10 mM aqueous solution, pH 7.9. Autoclaved and stored at RT.

10X One-Phor-All buffer plus: see section 2.1.1.
**Calf Intestinal Alkaline Phosphatase (CIP; Pharmacia Biotech):** 1000 units/ml. Stored at -20°C.

**Phenol (Fischer Scientific U.K. Ltd, Loughborough, England)/chloroform:** 1:1 (v/v) ratio of Tris-equilibrated phenol and chloroform. Stored at 4°C wrapped with aluminium foil.

**Ethanol:** Absolute.

**Equipment:** Grant water-bath and Eppendorf Centrifuge 5414S.

**Method**

The volume of the digested plasmid DNA (see section 2.1.1) was increased to 300 µl. An equal volume of phenol/chloroform was added. The tube was vortexed and then centrifuged at ~13 000 rpm for 3 mins at RT in a microcentrifuge. The aqueous phase was transferred to a new Eppendorf tube and 2 volumes of 100% ethanol was added to precipitate the DNA. Since the size of plasmid DNA is large, 15 mins at -70°C is sufficient for precipitation. The DNA was pelleted by centrifuging at ~13 000 rpm for 20 mins at RT in a microcentrifuge. The supernatant was removed and the DNA dried briefly *in vacuo*. The DNA was resuspended in an appropriate volume of 10 mM Tris-HCl buffer, pH 7.9. One-Phor-All buffer plus was added to a final concentration of 1X and 1 unit of CIP was added for every 100 pmoles of 5' ends. The total reaction volume should be such that the CIP does not constitute more than 10% of the total reaction volume. The mixture was incubated for 45 to 60 mins at 37°C. After which, the volume was increased to 300 µl and the DNA was extracted with phenol/chloroform and precipitated...
with ethanol as described above. The dephosphorylated DNA was dissolved in 10 to 20 μl of sterile distilled water and stored at -20°C.

2.1.5 Phosphorylation of synthetic oligonucleotide 5' ends

Materials

10X ONE-PHOR-ALL buffer plus: see section 2.1.1

100 mM ATP, sodium salt (Pharmacia Biotech): Diluted to 5 mM in aqueous solution. Stored at -20°C.

T₄ Polynucleotide kinase (PNK; Pharmacia Biotech), FPLCpure®, Cloned: 9 500 units/ml. Stored at -20°C.

Method

The phosphorylation reaction containing 1X ONE-PHOR-ALL buffer plus, 1 mM ATP and 1 unit of T₄ PNK for every nanomole of synthetic oligonucleotide to be phosphorylated was set up in a sterile 1.5 ml Eppendorf tube. The mixture was incubated for 30 to 45 mins at 37°C. The total volume was increased to 300 μl and the DNA extracted with phenol/chloroform and precipitated with ethanol as described in the method of section 2.1.4. Due to the small size of the oligonucleotides, the precipitation time was increased to 1 hr or even overnight at -70°C. The phosphorylated oligonucleotides were dissolved in 10 to 20 μl of sterile distilled water and stored at -20°C.
2.1.6 Ligation reaction

Materials

*Linearised plasmid DNA*: pBluescript KS II, pUC19 plasmid or pGL3 vector series of plasmids were used.

*DNA inserts*: Either restriction enzyme digested DNA fragments (see section 2.1.1) or phosphorylated synthetic oligonucleotides (see section 2.1.5).

*10X One-Phor-AII buffer plus*: see section 2.1.1.

*T₄ DNA Ligase (Pharmacia Biotech)*: 5 500 Weiss units/ml. Stored at -20°C.

*100 mM ATP, sodium salt*: see section 2.1.5.

Method

2.1.6.1 Ligation of DNA with sticky ends

Both the linearised plasmid vector and the DNA insert should possess compatible ends for ligation to occur. The ligation mixture contained 50 ng of linearised plasmid DNA, 500 ng of DNA insert, 1X One-Phor-AII buffer plus, 1 mM ATP, 10 Weiss units of T₄ DNA ligase and water to a total volume of 20 µl. The contents were incubated for 4 hours to overnight at 16°C.

2.1.6.2 Ligation of blunt-ended DNA

The ligation mixture contained equal molar amount of both linearised plasmid DNA and DNA insert, 1X One-Phor-AII buffer plus, 0.5 mM ATP, 10
to 20 Weiss units of T₄ DNA ligase and water to a total volume of 20 μl. The content was incubated overnight at 25°C.

2.1.7 Transformation of bacterial cells

Materials

*Escherichia coli* strains: DH5α (*supE44supF58hsdS3(mB)dapD8lacY1glnV44Δ(gal-uvrB)47tyrT58gyrA29tonA53Δ(thyA57)* or JM109 (*recA1supE44 endA1hsdR17gyrA96relA1thiΔ(lac-proAB)* when the pGL3 vector series were used. Stored at -70°C.

50 mg/ml Ampicillin sodium BP (Beecham Research, Hertfordshire, U.K.): Prepared using sterile distilled water. Filter sterilised and stored as small aliquots at -20°C - enough to use one or two times. Used at 50 μg/ml final concentration in LB medium and 100 μg/ml final concentration in LB agar.

*LB-Agar Medium* (Bio101, Inc., La Jolla, CA, U.S.A.): Add LB-Agar capsules to distilled water according to the manufacturer’s instruction. The mix was autoclaved and stored at RT. Before use, the agar was melted completely in a microwave oven. The molten agar was cooled to 40°C before pouring onto Petri dishes (if necessary, antibiotic was added just before pouring). The agar was then allowed to solidify. The plates could be kept for up to a week at 4°C if sealed with Nesco film.
**LB medium** *(Bio101, Inc.)*: Add LB medium capsules to distilled water according to the manufacturer's instruction. The mix was autoclaved and stored at RT.

**SOB medium** *(Bio101, Inc.)*: Add SOB medium capsules to distilled water according to the manufacturer's instruction. The mix was autoclaved and stored at RT.

**FSB Transformation buffer, pH 6.4**: 10 mM potassium acetate, pH 7.5, 45 mM manganese chloride, 10 mM calcium chloride 2-hydrate, 100 mM potassium chloride, 3 mM hexamminocobalt chloride and 10% (v/v) glycerol. Filter sterilised through a 0.45 μm filter unit. The container was wrapped in foil and stored at 4°C.

**Dimethylsulphoxide (DMSO; Sigma Chemical)**: Stored at RT.

**Isopropyl β-D-thiogalactoside (IPTG; Gibco BRL)**: 0.1 M aqueous solution. Filter sterilised, aliquoted and stored at -20°C.

**5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal; Melford Laboratories Ltd, Suffork, England)**: 2% (w/v) solution in dimethylformamide. Filter sterilised, aliquoted and stored at -20°C. Stored tubes wrapped in aluminium foil.

**90 mm Polystyrene Petri dish (Bibby Sterilin Ltd, Staffordshire, U.K.)**: Sterile and disposable.

**Equipment**: UV/Visible spectrophotometer (CECIL 1000 Series; CECIL Instruments, Cambridge, England), Water-bath shaker-incubator (Gyrotory®,
Waterbath shaker Model G76; New Brunswick Scientific (U.K.) Ltd, Hatfield, England), bench-top centrifuge (MSE Centaur 2), 37°C incubator.

**Method**

**2.1.7.1 Preparation of fresh competent cells**

A glycerol stock of *Escherichia coli* was streaked onto a LB-agar plate and incubated for ~16 hrs at 37°C. A single colony was picked, inoculated into 10 ml of LB medium and grown for ~16 hrs at 37°C with vigorous shaking. When the optical density of the culture at 550 nm was between 0.6 and 0.8, it was transferred to a sterile 12 ml Falcon tube. The tube was placed for 10 mins on ice before it was centrifuged at 600 x g for 10 mins at RT to pellet the bacterial cells. The supernatant was swiftly decanted and the pellet was resuspended in 8 ml of ice-cold FSB transformation buffer. The suspension was placed on ice for another 10 mins and centrifuged using similar conditions as above. After the supernatant was decanted, the pellet was resuspended in 2 ml of FSB transformation buffer. 200 µl was aliquoted into each Eppendorf tube for use in the transformation reaction.

**2.1.7.2 Preparation of frozen competent cells**

All steps were carried out as in section 2.1.7.1 till the step where the bacterial pellet was resuspended in 2 ml of FSB transformation buffer. At this stage, instead of aliquoting 200 µl, 70 µl of DMSO was added to the 2 ml
of cell suspension. The cells were gently mixed by swirling and left for 15 mins on ice. Another 70 μl of DMSO was added, the cells were again gently mixed by swirling and placed on ice. The cell suspension was immediately dispensed into 200 μl aliquots and snap-frozen in liquid nitrogen. The competent cells were stored at -70°C. When required, an aliquot of cells was thawed slightly by holding it in the hand. The aliquot was transferred into ice just as it started to thaw and then left for 10 mins. The cells were then ready for transformation.

2.1.8 Transformation reaction

2μl and/or 4μl of the ligation reaction mix from section 2.1.6 was added to a 1.5 ml Eppendorf tube containing 200 μl of competent cells from either sections 2.1.7.1 or 2.1.7.2. Two additional tubes were set up: one containing only the cells, to confirm for the susceptibility of the cells to the antibiotic and another tube containing 50 ng of undigested plasmid vector to evaluate the transformation efficiency. A fourth tube containing 50 ng of linearised plasmid DNA can also be set up to confirm the dephosphorylation efficiency. All tubes were placed, for 30 mins, on ice and then heat shocked for exactly 90 secs at 42°C. 1 ml of SOB medium was added to each tube which was then incubated for 1 hour at 37°C with vigorous shaking. If the plasmid utilised in ligation supported the blue-white assay, the LB-agar plates were pre-coated with a mixture of 50 μl of X-gal and 10 μl of IPTG and allowed to dry just prior to the plating of cultures. 200
μl of cells with no added DNA was spread onto two LB agar plates - one plate contained 100 μg/ml ampicillin and the other no antibiotic. For transformed cells, 200 μl or 400 μl were spread onto LB agar plates containing 100 μg/ml ampicillin. The plates were incubated for ~16 hrs in a 37°C incubator. For plasmids that support the blue-white assay, transformed cells containing the recombinant plasmids were observed as white colonies while non-recombinant ones were blue.

2.1.9 Confirmation of recombinants

Bacterial colonies (white colonies if blue/white selection was possible) were picked at random and grown in 10 ml of LB medium containing 50 μg/ml ampicillin for ~16 hrs at 37°C with vigorous shaking. The plasmid DNA was isolated using the mini-scale preparation (section 2.2). The plasmid DNA isolated was digested with the appropriate restriction endonucleases (section 2.1.1) and electrophoresed on an agarose gel (section 2.1.2) to confirm clones.

2.1.10 Preparation of glycerol stocks

An equal volume of the bacterial culture containing the recombinant plasmid and LB medium containing 30% to 40% (v/v) glycerol were mixed
together and quick frozen on dry ice. The glycerol stocks were stored at -70°C.
2.2 Mini-scale preparation of plasmid DNA

Materials

*LB medium*: see section 2.1.7.

*50 mg/ml Ampicillin*: see section 2.1.7.

*Potassium Acetate*: 5 M solution, autoclaved and stored at RT.

*Solution I*: 50 mM glucose; 10 mM EDTA, pH 8.0 and 25 mM Tris-HCl, pH 8.0. Filter sterilised and stored at 4°C.

*Solution II*: 0.2 N NaOH and 1% (w/v) SDS. Prepared fresh.

*Solution III*: Mix together 60 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid and 28.5 ml of sterile distilled water. Stored at 4°C.

*Phenol/chloroform*: see section 2.1.4.

*Ethanol*: Absolute and 70% (v/v).

*90 mm Polystyrene Petri dish (Bibby Sterilin Ltd, Staffordshire, U.K.)*: Sterile and disposable.

*Equipment*: 37°C incubator, Waterbath shaker-incubator, Eppendorf Centrifuge 5414S.

Method

The bacterial colony was inoculated into 10 ml of sterile LB medium containing 10 µg/ml of ampicillin and was grown overnight in a 37°C waterbath shaker-incubator with vigorous shaking.

1.5 ml of the bacterial culture was transferred to a 1.5 ml Eppendorf tube and centrifuged for 1 min in an Eppendorf microcentrifuge to pellet the bacterial cells. The supernatant was promptly removed, leaving the cell
pellet as dry as possible. The pellet was fully resuspended in 100 µl of ice-cold solution I and incubated for 5 mins at RT. Then 200 µl of solution II was added, the content were mixed by inverting the tube rapidly for a few times and the tube was allowed to stand for 5 mins on ice. Finally, 150 µl of ice-cold solution III was added and the tube was gently inverted for 10 seconds before being placed for 5 mins on ice. The tube was centrifuged for 10 mins at RT in an Eppendorf microcentrifuge. The supernatant was carefully transferred to a new 1.5 ml Eppendorf tube, leaving the white precipitate behind. An equal volume of phenol/chloroform was added to the tube containing the supernatant. The tube was vortexed and centrifuged for 2 mins at RT. The top aqueous phase was transferred to a new Eppendorf tube and two volumes of 100% ethanol were added. The tube was vortexed and allowed to stand for 2 to 10 mins on ice to allow the DNA to precipitate. The precipitated DNA was recovered by centrifugation for 10 mins. The supernatant was decanted, the DNA pellet was washed with 1 ml of 70% ethanol and recentrifuged for 5 mins. The pellet was either air-dried or dried briefly in vacuo. The DNA was dissolved in 10 to 15 µl of sterile distilled water. The solvation of the DNA pellet was assisted by vortexing. The tubes were then incubated at 65°C for 5 mins to inactivate any endogenous nucleases present, and then placed at -20°C.
2.3 Maxi-scale preparation of plasmid DNA (Maxi Qiagen® plasmid kit, Cat. No. 12162/3)

Materials

Most solutions and Anion-exchange columns are provided in the Maxi Qiagen® Plasmid Kit except isopropanol and 70% (v/v) ethanol.

*LB-Agar Medium:* see section 2.1.7.

*LB medium:* see section 2.1.7.

*5. mg/ml Ampicillin:* see section 2.1.7.

*Equipment:* Sorvall® Centrifuge, Rotor types: GSA and SS34, waterbath shaker-incubator, Sorvall® Dry-Spin™ 250 ml Leakproof polypropylene centrifuge bottle with canted neck (Kendro Laboratory Products Ltd., Hertsordshire, England), siliconised 30 ml Corex tubes, 50 ml polypropylene centrifuge tubes with caps, 64 µm nylon mesh and UV/visible spectrophotometry.

Method

A loopful of recombinant cells from the glycerol stock was streaked onto LB agar containing 100 µg/ml of ampicillin or the appropriate selective antibiotic for the particular plasmid and allowed to incubate in the 37°C incubator for ~16 hours. A single colony was then picked and inoculated into 10 ml of LB medium containing 10 µg/ml of ampicillin or the appropriate selective antibiotic for the particular plasmid. The bacteria were cultured for
8 hours at 37°C under vigorous shaking in a waterbath shaker-incubator. Then, the 10 ml culture was transferred to 200 ml of LB medium containing 10 μg/ml of ampicillin or the appropriate selective antibiotic and was further grown for 16 hrs at 37°C under vigorous shaking as before. The bacterial cells were poured aseptically into Sorvall® Dry-Spin™ 250 ml Leakproof polypropylene centrifuge bottle with canted neck and harvested in a Sorvall® centrifuge at 6 000 x g for 10 mins at 4°C using the GSA rotor. The supernatant was decanted leaving the cell pellet as dry as possible. The cell pellet was resuspended in 10 ml of Buffer P1 and transferred to 50 ml polypropylene tube. Then, 10 ml of Buffer P2 was added and the contents in the tube gently mixed by inversion before being incubated for 5 mins at RT. 10 ml of pre-chilled Buffer P3 was added, the contents were gently mixed and incubated for 20 mins on ice. The tube was then centrifuged at ≥20 000 x g for 30 min at 4°C using the SS34 rotor. The supernatant containing the plasmid DNA was quickly filtered through a prewetted nylon cloth mesh into another fresh tube. This step was carried out to remove any suspended particulates before loading the supernatant onto the Qiagen-tip 500. A Qiagen-tip 500 was equilibrated with 10 ml of QBT buffer before the supernatant recovered from above was loaded. Thereafter, the Qiagen-tip 500 was washed with QC buffer twice at 30 ml buffer each wash. The plasmid DNA was then eluted with 15 ml of QF buffer and collected into a siliconised, sterile 30 ml Corex tube. The plasmid DNA was precipitated with the addition of 0.7 volumes of isopropanol. The contents of the tube
were mixed by inversion for a few times and centrifuged at ≥15 000 x g for 30 to 60 mins at 4°C using the SS34 rotor. The DNA pellet was washed with 5 ml of 70% ethanol and centrifuged again at ≥15 000 x g for 10 to 15 mins at 4°C. The DNA pellet was air-dried before approximately 0.15 to 0.3 ml sterile distilled water was added to dissolve the DNA which was stored at -20°C. The yield of DNA could be determined by UV/visible spectrophotometry at 260 nm.
2.4 Annealing of single-stranded DNA sequences

Materials

DNA: A pair of complementary synthetic oligonucleotide.

10X Annealing buffer: 0.1 M Tris-HCl, pH 8.0; 0.1 M MgCl₂, 0.5 M NaCl. Filter sterilised through a 0.22 µm filter unit (Sartorius AG, Goettingen, Germany) and stored at -20°C. Use at 1X final concentration.

Method

An equimolar amount of each of the single-stranded complementary DNA sequences was added to a 1.5 ml Eppendorf tube. Annealing buffer was included at a 1X final concentration. The required volume was made up with sterile distilled water and the annealing reaction carried out as follows according to the melting temperature ($T_m$).

2.4.1 Calculation of melting temperature

The temperature at which the two oligonucleotides dissociate can be determined using the following equation:-

\[ T_m = 2(A + T) + 4(G + C) \]

where $T_m$ = melting temperature (°C)

- $A/G$ = purines
- $C/T$ = pyrimidines
2.4.2 For $T_m$ above 25°C:

The mixture was heated to 65°C for 10 minutes and then allowed to slowly cool to RT. The annealed DNA was stored at -20°C.

2.4.3 For $T_m$ below 25°C:

The mixture was placed at a temperature at least 5°C below the calculated $T_m$ for 15 minutes and the temperature of the annealed DNA sequences were not allowed to rise above its $T_m$ for subsequent usage. The annealed DNA was stored at -20°C.
2.5 Radioisotope-labelling of DNA

2.5.1 DNA with a 5'-overhang

Materials

DNA: Either restriction enzyme digested DNA fragment or annealed double-stranded complementary synthetic oligonucleotides with at least one 5'-overhang end.

*Ultrapure dNTP set, 2'-deoxynucleoside 5'-triphosphate, sodium salt (Pharmacia Biotech)*: 100 mM dATP, 100 mM dGTP, 100 mM dTTP and 100 mM dCTP solution in water, pH 7.5. Dilute each to 10 mM with sterile distilled water. Stored at -20°C.

*Easytides™ Radioactive Isotope (NEN Research Products, Boston, MA, U.S.A.): (α-32P)dCTP, 10.0 mCi/ml, 3000 Ci/mmol. Stored at 4°C.*

*10X One-Phor-All buffer plus*: see section 2.1.1.

*FPLCpure® DNA Polymerase I, Klenow Fragment, cloned (Pharmacia Biotech)*: 6 435 units/ml. Stored at -20°C.

*Equipments*: Bench-top centrifuge (MSE Centaur 2), Geiger Counter mini monitor g.m meter type 5.10 (Mini-instruments Ltd, Essex, England).

Method

The labelling reaction contained 1X One-Phor-All buffer plus, 20 μCi of [α-32P]dCTP, 1 mM each of dATP, dGTP and dTTP, 100-300 ng DNA, 6.4...
units DNA polymerase I, Klenow fragment and water to make the volume to 20 μl. The mixture was incubated for 45 mins at 37°C.

The use of radiolabeled [α-32P]dCTP is not always suitable. This is because DNA polymerase I, Klenow fragment is not efficient at filling in the two most outer bases of a 5’-overhang. For example, filling-in a DNA fragment with a 5’-overhang (A↓AGCTT) generated from Hind III enzyme digestion, will be more efficient with radiolabeled [α-32P]dATP.

2.5.2 DNA with blunt ends

Materials

DNA: Generated from restriction enzyme digestion or annealed double-stranded complementary synthetic oligonucleotides.

10X One-Phor-All buffer plus: see section 2.1.1.

Easytides™ Radioactive Isotope (NEN Research Products): (γ-32P)ATP, 10.0 mCi/ml, 3000 Ci/mmol. Stored at 4°C.

T₄ PNK, FPLCpure®, Cloned: see section 2.1.5.

Method

The reaction mix contained 1X One-Phor-All buffer plus, 100-200 ng DNA, 20 μCi of (γ-32P)ATP, 9.5 units T₄ PNK and sterile water to make up volume to 20 μl. The mixture was incubated for 45 mins at 37°C.
2.5.3 Purification of radiolabeled DNA probe

2.5.3.1 Chroma Spin™ Column

Materials

Chroma Spin™-10 Columns (Clontech Laboratories Inc, Palo Alto, Ca, U.S.A.): Stored at RT.

Equipments: Bench-top centrifuge (MSE Centaur 2)

Method

The column was inverted numerous times to ensure that the resin was fully resuspended. The top and bottom cover were removed and the column was equilibrated by centrifugation at 600 x g for 5 mins at RT using the MSE Centaur 2 centrifuge. The volume of the radiolabeled mix from section 2.5.1 or 2.5.2 was increased to 50 µl before it was loaded onto the column and centrifuged using the same conditions as for equilibration. The labelled probe was collected in the eluate and stored at -20°C.

2.5.3.2 NucTrap® probe purification column

Materials

NucTrap® push column (Stratagene Ltd, Cambridge, England): Stored at 4°C

STE buffer: 100 mM NaCl, 20 mM Tris-HCl, pH 7.5, 10 mM EDTA, pH 8.0. Autoclaved and stored at RT.
Equipment: Push column beta shield device (Stratagene Ltd), 10 ml disposable, sterile polypropylene syringe (Becton Dickenson and Co., NJ, U.S.A.).

Method

The column was first washed by adding 70 µl of STE buffer and the buffer was pushed through the column by attaching a 10 ml syringe to the top of the column. The volume of the radiolabeled probe from sections 2.5.1 and 2.5.2 was increased to 70 µl before it was added and pushed through the column again. An additional 70 µl of STE buffer was added, and pushed through the column. The purified probe was collected at this stage. To ensure that all the labelled probe are eluted, air was pushed once more through the column.

2.5.4 Determination of radioactive counts of a radiolabeled probe

Materials

Chromatography Paper (Whatman International Ltd, Maidstone, England): 23 mm diam. DE81 (DEAE loaded) ion-exchange cellulose discs. Stored at RT.

\( \text{Na}_2\text{HPO}_4\cdot2\text{H}_2\text{O} \) (di-Sodium hydrogen orthophosphate 2-hydrate): 0.5 M aqueous solution, pH 7.0. Autoclaved and stored at RT.

Ethanol: 95% (v/v).
Scintillation liquid: Ecoscint™ A (National Diagnostics, Atlanta, Georgia, U.S.A.). Stored at RT.

Equipment: Scintillation vial inserts & caps (Hughes & Hughes Ltd, Somerset, England), Tri-Carb 1600TR Liquid Scintillation Analyzer (Canberra Rackard)

Method

One microlitre of unpurified radiolabeled DNA probe was dotted onto each of two pieces of DE81 ion-exchange paper. The procedure was repeated after the probe was purified as in section 2.5.2. One piece each of DE81 paper from before and after purification was washed in 0.5 M Na₂HPO₄.2H₂O, pH 7.0 for 12 mins at RT changing the solution every 2 mins. The papers were then washed twice for 1 min each in sterile distilled water and finally twice for 1 min in 95% ethanol. The four pieces of DE81 papers were dried and then placed into a scintillation vial containing 3 ml of Ecoscint™ A. Radioactivity was counted using the Tri-Carb 1600TR Liquid Scintillation Analyzer.

2.5.4.1 Calculation of percentage of incorporation

\[
\% \text{ incorporation} = \frac{\text{Purified wash count} \times 100}{\text{Total count}}
\]
2.6 Isolation and culturing of rat hepatocytes

2.6.1 Coating of tissue culture Permanox® plate

Materials

CDI (1-Cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluene sulfonate; Aldrich Chemical Company, Dorset, England): 130 μg/ml stock in aqueous solution. Filter sterilised and stored at 4°C

Vitrogen 100®, purified bovine dermal collagen (98%, Type I; Imperial Laboratories (Europe) Ltd, Hampshire, U.K.): Stored at 4°C. Concentration is stated on each bottle.

0.9% (w/v) NaCl: Autoclaved and stored at RT.

60 X 15 mm tissue culture dish, Permanox®, sterile (Nunc, Inc., Naperville, IL, U.S.A.)

Method

Each Permanox® tissue culture plate was coated with 3 ml of solution made up of 100 μg/ml Vitrogen 100® in 130 μg/ml CDI. The plate was gently swirled to ensure that the solution covered the entire inner plate surface and was incubated overnight at 37°C under 5% CO₂. Unbound Vitrogen/CDI solution was removed and the plate was washed twice with 3 ml of 0.9% (w/v) NaCl. Coated plates were stored in an air-tight box for up to a week at 4°C.
2.6.2 Isolation of primary rat hepatocytes

Materials

All solutions were prepared and used in a sterile class 2 biological hood.

Animal: Male Sprague-Dawley rats from UCL Biological Unit weighing between 220 and 250 gm on the day of the procedure.

Pentobarbital sodium: Prepared fresh a 60 mg/ml solution in sterile 0.9% (w/v) NaCl. Filter sterilised and use 60 μg/gm of rat body weight. Stored dry powder at RT.

25,000 U/ml heparin sodium, injection B.P. (AAH Pharmaceutical Limited, Middlesex, England): 5, 000 U/ml stock solution in sterile 0.9% (w/v) NaCl. The working solution is prepared by further diluting the stock to 200 U/ml using sterile 0.9% (w/v) NaCl. All solutions were stored at 4°C.

EGTA: 25 mM stock solution in 0.1 M NaOH. Autoclaved and stored at 4°C.

1 M CaCl₂: Filter sterilised and stored at 4°C for 1 to 2 weeks.

7.5% (w/v) NaHCO₃: Filter sterilised and stored at 4°C.

10X EBSS, without calcium and magnesium (Earle's Balanced Salt Solution; Gibco BRL): Store at 20°C. Prepared fresh a 1X concentration solution by mixing 100 ml 10X EBSS with 30 ml 7.5% (w/v) NaHCO₃ and sterile distilled water to make up to 2 L. Adjust pH to 7.5.

Perfusion Buffer I: Prepared fresh 1X EBSS containing 0.5 mM EGTA.

Perfusion Buffer II: 1X EBSS. For preparation, see the 10X EBSS solution above.
**Perfusion Buffer III:** Prepared fresh 1X EBSS containing 5 mM CaCl₂. Add 0.08 U/ml of collagenase H (from *Clostridium histoylticum*; *Boehringer Mannheim, Mannheim, Germany*) and a spatula of trypsin inhibitor (Type 1-5; from soybean) just prior to use.

**Dispersal buffer:** 10 mM HEPES, 142 mM NaCl and 7 mM KCl. Adjust pH to 7.5, autoclaved and stored at 4°C. Add 2.5% (w/v) BSA, fraction V just prior to use.

**L-15 Leibovitz medium with Glutamax™ and L-amino-acid:** Stored at 4°C. Add 2.5% (w/v) BSA, fraction V just prior to use and adjust pH to 7.5.

**Trypan blue:** 0.1% (w/v) in 0.9% (w/v) NaCl. Filter sterilised and stored at RT.

**Surgical instruments (Scientific Laboratory Supplies, Nottingham, U.K. unless otherwise stated):** Deschamps ligature needle, Mayo's scissor with blunt tips and curved blades, 125 mm stainless steel Halstead's mosquito artery clamps, 38 mm stainless steel Dieffenbach's straight bulldog artery clip, 26d gauge sterile needle, glass cannula, Mersilk* Black 3 ligature thread (Ethicon Ltd, Edinburgh, U.K.), home-made vein-lifter by flattening a 19 gauge needle and making a 90° bent approximately 7.5 mm from the end of the needle, spatula, 64 μm nylon mesh, toothed forcep.

**Method**

This is a modified method of the two-step collagenase perfusion of Seglen (Seglen, 1976). The rats were housed in the University College London Biological Services on hardwood bedding in plastic cages. They were fed with water and standard dried rat food diet *ad lib*. The animals'
quarters had a 12 hrs of light and dark cycle. The animal was anaesthetised by injecting 60 $\mu$g/gm rat body weight of sodium pentobarbital intraperitoneally. The animal’s state of unconsciousness was determined by the pedal reflex. When the animal was completely unconscious, an incision was made in the mid-ventral line of the abdomen through the skin layer only. The skin layer was opened first by cutting vertically along the mid-ventral line and then horizontally across the middle of the mid-ventral line before the skin was reflected and the flaps of skin were pulled back to reveal the abdominal musculature. The abdominal wall was swapped with 70% (v/v) ethanol before it was cut in a similar manner as that to the skin layer. The abdominal layer was lifted up using a toothed-forcep while dissecting to avoid damage to any of the internal organs. The sternum was clamped away using the 125 mm stainless steel Halstead’s mosquito artery clamps and the liver was carefully flipped upward so that it rested against the diaphragm. The stomach and intestines were all displaced outside of the abdominal cavity to the right so as to reveal the portal vein and the vena cava. Using the ligature needle and thread, two loose ligatures were tied around the portal vein at a distance of approximately 10 mm apart from each other. A third loose ligature was tied around the vena cava above the vena suprarenal vein. At this point, 150 U of heparin was injected into the inferior vena cava. Slight pressure was applied at the point of injection to minimise the bleeding. The lower ligature around the portal vein was tightened by making 2 knots. A flat cut was made on the portal vein just above the tightened ligature. The cut was kept open using the home-made vein lifter and the sterile glass cannula was inserted into the portal vein slowly and
carefully, making sure that it did not pierce through the vein. The cannula was kept in place by tightening the upper ligature. The position of the cannula was further secured by winding the remaining thread of the lower ligature round the cannula and tightening it. Finally, the ligature around the vena cava was tightened. At this stage, the liver should be removed from the animal as quickly as possible. This can be achieved by cutting the rib-cage and diaphragm. The connections underneath the diaphragm was then severed using a Mayo's scissor with blunt tips and curved blades. The liver was then slowly lifted up using the index and the second finger while the cannula was secured by gently pressing the thumb on it. The remaining connections to the liver was swiftly but carefully severed until the liver was entirely free from the animal. The liver was placed onto the platform of the perfusion unit in a laminal hood. The bubble trap of the perfusion unit should be 20 cm from the platform where the liver was placed. The unit was run for a couple of mins with perfusion buffer I just before the liver was ready for perfusion to ensure that there was no bubbles in the system. During perfusion, all three perfusion buffers were gassed with 95% air and 5% CO$_2$ and the heat exchanger unit ensured that the temperature of all the buffers were 37°C when it reached the liver. The liver was perfused for 3 mins with buffer I, 7 mins with buffer II and between 20 to 25 mins with buffer III. The liver was lightly massaged initially to assist perfusion and to ensure that the entire liver was perfused especially the major lobes. A properly perfused liver should generally looked yellow colour with a shiny surface and yielded when lightly pressed. When the perfusion procedure was completed, the cannula was removed and the liver was carefully transferred to a sterile petri
dish containing 30 to 40 ml of supplemented L-15 Leibovitz medium to completely submerged the liver. The liver was kept in place using a sterile pointed forcep at a place on the liver that would not damage the hepatocytes, the Glisson’s capsule enclosing each lobes of the liver was carefully peeled off. In a well-perfused liver, the capsule peeled off relatively easily. The liver was then gently shaken to release the hepatocytes which could be further assisted by gently scraping the tissue with a curved-end forcep. When all the hepatocytes were released, the cell suspension was filtered through a 64 μm nylon mesh into a sterile receptacle to separate membranous debris. The mesh was rinsed twice with 10 to 20 ml of L-15 medium to maximise cell yield. The cell suspension was left undisturbed for 15 mins to allow viable cells to settle to the bottom of the receptacle. The supernatant was removed with minimal disturbance to the settled cells. The cells were gently resuspended in dispersal buffer, transferred to sterile 50 ml Falcon tubes and centrifuged at 50 x g for 2 mins at 4°C. The supernatant was quickly decanted and the cells were finally resuspended in fully-supplemented medium with 10% foetal bovine serum (see section 2.6.3). The viability of the cells were assessed using trypan blue exclusion. Cells were plated when more than 75% were viable.
2.6.3 Culturing of hepatocyte

Materials

William’s E medium with NaHCO₃, without L-glutamine and phenol red (Gibco BRL): Stored at 4°C.

GlutaMAX™-I Supplement (Gibco BRL): Supplied as 200 mM solution in 0.85% (w/v) NaCl. Store at -20°C.

Antibiotics (Gibco BRL): 200 U/ml penicillin, 200 µg/ml streptomycin and 2.5 µg/ml amphotericin. Store at -20°C when reconstituted.

Insulin from bovine pancreas: To prepare a 10 mg/ml stock solution, reconstitute 100 mg lyophilised powder in 10 ml sterile deionised distilled water followed by 100 µl sterile glacial acetic acid. Mix gently until solution becomes clear. Store powder at 0°C and at 4°C when reconstituted.

8 mM dexamethasone phosphate (DBL (David Bull Laboratories), Warwick, U.K.): Prepare a working concentration of 0.1 µM in William’s E medium. Store all solutions at 4°C.

Foetal bovine serum (dialysed, 10,000 MW cut-off; Gibco BRL): Store at -20°C.

Fully supplemented culture medium: William’s E medium containing 2 mM GlutaMAX™-I, 200 U/ml penicillin, 200 µg/ml streptomycin, 2.5 µg/ml amphotericin, 1.7 µM insulin, 0.1 µM dexamethasone and 10% foetal bovine serum (FBS).
Method

Once the viability of the hepatocyte cell population was determined to be ≥75%, 2 ml of cells were dispensed into each Vitrogen-coated Permanox® plate giving a plating density of 4 - 5 X 10^6 cells/plate. The plate was swirled gently to ensure that the cell suspension covered the entire surface of the plate. The plates were incubated for 3 hours at 37°C under 5% CO_2 for the cells to adhere to the plate. The culturing medium was then discarded and the adhered cells were washed twice with 3 ml of William’s E medium. Three ml of fully supplemented culturing medium was added to each plate and cells were cultured for ~20 hours at 37°C under 5% CO_2 before either treatment experiments (see section 2.7) or transfection experiments (see section 2.17) were carried out.
2.7 Chemical treatment of primary rat hepatocytes

Materials

William’s E medium: see section 2.6.3.

Supplemented culture medium: Fully supplemented culture medium (see section 2.6.3) without 10% FBS.

2-aminopurine, nitrate salt: 100 mM stock solution in plain William’s E medium. Vortex to dissolve and stored at 4°C. If precipitate out of solution, warm up slightly and vortex. Filter sterilised just prior to use.

Phenobarbital sodium: Prepared fresh a 3 mM stock solution and filter sterilised. Stored dry powder at RT.

10X PBS: Diluted to 1X working concentration, autoclaved and stored at RT.

Equipment: Sterile cell scraper (Greiner Labortechnik Ltd, Gloucestershire, U.K.), and refrigerated centrifuge.

Method

2.7.1 2-aminopurine treatment

The hepatocytes were washed twice with plain William’s E medium before 3 ml of fresh supplemented culture medium were added. For cells treated with 2-aminopurine, 10 mM final concentration was included in the culture medium and incubated for an hour at 37°C under 5% CO₂. 0.1 mM final concentration of PB was added directly into the existing culture medium and the cells were further incubated for 6 hrs at 37°C under 5% CO₂. The cells were washed twice with 1X PBS and then detached using a sterile cell
scraper, pelleted by centrifuging at 1 800 x g for 5 mins and frozen at -70°C for the isolation of RNA (see section 2.16.1).

2.7.2 PB treatment

After the cells were plated, fresh medium was changed every 24 hours. When the cells were to be treated with PB, they were washed twice with 1X PBS and 3 ml of supplemented culture medium but containing 0.1 mM PB was added to each culture plate. The cells were further cultured for 24 hrs before they were harvested as in section 2.7.1.
2.8 Nuclear protein extraction

2.8.1 From freshly isolated rat liver (Sierra, 1990).

Materials

Animal: Male Sprague-Dawley rats weighing between 180 and 200 gm. Each group is made up of three animals.

0.9% (w/v) NaCl: see section 2.6.1.

PB: 0.1% (w/v) in ordinary tap water for consumption through oral route and 10 mg/kg animal body weight in 0.9% NaCl for IP injection.

Sodium dodecyl sulfate (SDS): 0.5% (w/v) solution. Autoclaved and stored at RT.

\((\text{NH}_4)_2\text{SO}_4\): 4 M saturated solution. Autoclaved and stored at RT. Chilled to 4°C just before use.

Phenylmethylsulfonyl fluoride (PMSF): 0.2 M stock solution in absolute ethanol. Stored at -20°C. Ensured that solution has no more crystal before used.

Dithiothreitol (DTT; BDH Chemicals Ltd): 1M stock solution. Filter sterilised and stored at -20°C.

Homogenisation buffer: 10 mM HEPES, pH 7.6, 15 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, pH 8.0, 2 M sucrose, 10% (v/v) glycerol. All stock solutions were sterilised before use to prepare this buffer and once made up should be kept at -20°C. Just prior to use, 0.5 mM DTT, 0.5 mM PMSF, 10 μg/ml aprotinin, 1 μg/ml antipain, 1 μg/ml pepstatin, 1
μg/ml leupeptin were added. The solution should be kept as cold as possible throughout the preparation of the nuclear protein.

Nuclear lysis buffer. 10 mM HEPES, pH 7.6, 100 mM KCl, 0.1 mM EDTA, pH 8.0, 10% (v/v) glycerol, 3 mM MgCl₂. Autoclaved and stored at 4°C. 1 mM DTT, 0.1 mM PMSF, 10 μg/ml aprotinin, 1 μg/ml antipain, 1 μg/ml pepstatin, 1 μg/ml leupeptin were added prior to use.

Dialysis buffer. 25 mM HEPES, pH 7.6, 0.1 mM EDTA, pH 8.0, 40 mM KCl, 10% (v/v) glycerol. Autoclaved and stored at 4°C. 1 mM DTT was added just before use.

8/32” Dialysis tubing (Scientific Industries International Inc. (U.K.) Ltd, Loughborough, U.K.): 3 to 5 dialysis tubings of 1 cm width were cut to approximately 15 cm in length. They were boiled twice for 5 mins in 2% (w/v) NaHCO₃ and 1 mM EDTA, with a change of fresh solution between boil. The tubings were then rinsed at least 3 times in distilled water by boiling for 5 mins each rinse. The tubings were finally placed in distilled water, autoclaved and stored at RT.

Beckman Tubes (Beckman Instruments, Inc., Palo Alto, CA, U.S.A.): 39 ml Beckman polyallomer tubes; 25 X 89 mm Beckman polycarbonate centrifuge bottle with cap assemblies.

Equipment: Citenco electric overhead homogeniser and matching rounded teflon pestle; Beckman ultracentrifuge and SW28 or 70Ti rotors; Hand-held Dounce homogeniser and matching pestle ‘A’; UV/Visible spectrophotometer; dissecting scissors, spatula, disposable Pasteur
pipettes, Denly Spiramix 10 (Denly Tech Ltd, Sussex, England) and MSE microcentrifuge.

Method

2.8.1.1 Treatment of animals

Prior to sacrifice, the rats were kept in the University College London Biological Services. They were housed on hardwood bedding in plastic cages. They were fed water and standard rat dried food diet ad lib. The animals' quarters had a 12 hrs of light and dark cycle. Untreated (i.e. Control) rats were starved overnight before sacrificed. While PB-treated animals were given 0.1% (w/v) PB water for four consecutive days, changing fresh PB-water everyday. On the fifth day, 10 mg/kg rat body weight of PB in 0.9% NaCl was administered to the animals through IP injection. They were then given ordinary drinking water and were starved overnight before sacrificed. All rats were sacrificed using the cervical dislocation method.

2.8.1.2 Isolation of nuclei

The procedure was carried out as in Sierra (Sierra, 1990). Below provides a basic methodology which is better described in Sierra. The experiment should be carried at 4°C. After the animals were sacrificed, the livers were immediately removed and pooled in a cold beaker on ice. The livers were weighed and were minced as finely as possible with a sterile dissecting scissor. Approximately 10 ml of homogenisation buffer was added to per gram of liver tissue. This ratio can be reduced according to the capacity of the centrifuge tubes. However, the amount of tissue should never
constitute more than 15% of the total volume. Initially, a third of the homogenisation buffer was added to the minced tissue. The minced tissue was homogenised using a Citenco electric overhead homogeniser fixed with a sterile rounded teflon pestle for 3 to 4 strokes at about 7 200 rpm. The remaining homogenisation buffer was then added to the homogenate which was layered onto 39 ml prechilled Beckman polyallomer tubes already containing 10 ml of homogenisation buffer. The tubes were centrifuged at 24 000 rpm for 60 mins at 4°C in a Beckman ultracentrifuge using the SW28 rotor. The solid plug of whole cells and membranes at the top of each tube was removed using a sterile spatula. The supernatant was decanted and excess fluid was allowed to drain by placing the tubes slightly inverted on an ice bucket. Ensure that the nuclei pellet was kept cold. Without disturbing the pellet, the sides of the tube were washed with sterile distilled water using a syringe with its needle tip bent at 90°. At this point, the nuclei could be frozen directly on dry ice and kept at -70°C for several weeks or be used immediately.

2.8.1.3 Preparation of nuclear extracts

The experiment was carried out at 4°C. Nuclei that had been frozen at -70°C should be allowed to thaw on ice. Once thawed, the nuclei were then resuspended in 5 ml of nuclear lysis buffer for per rat liver. The nuclei suspension was homogenised for several strokes in a hand-held Dounce homogeniser using an ‘A’ pestle. The DNA concentration was determined
by measuring the optical density of the homogenate using a UV/visible spectrophotometer at 260 nm. For measurement, a sample of the homogenate was diluted 50-fold with 0.5% (w/v) SDS by adding it dropwise to the SDS solution and mixed by vortexing vigorously to obtain a homogenous lysate. The homogenate was kept cold while the optical density measurement mentioned above was being done. According to the optical density measurement, nuclear lysis buffer was added to the homogenate to achieve a final DNA concentration of 0.5 mg/ml. The homogenate was then transferred to prechilled 39 ml Beckman polycarbonate tubes with caps and one-tenth volume of 4 M (NH₄)₂SO₄ was added. The mixture was rolled on a Denly spiramix for 30 mins at 4°C before the tubes were centrifuged in an ultracentrifuge at 35 000 rpm for 60 mins at 4°C using a 70Ti rotor. The supernatant was quickly transferred to fresh Beckman polycarbonate tubes and 0.3 g per ml of supernatant of solid (NH₄)SO₄ was added. The tubes were rolled for another 30 mins before being centrifuged at 35 000 rpm for 20 mins at 4°C in a Beckman ultracentrifuge using the 70Ti rotor. The supernatant was discarded and the pellet was either processed immediately or stored on ice overnight. Using the optical density measurement carried out on the homogenate previously, dialysis buffer was added to the pellet to achieve a final concentration of 10 mg/ml. When the pellet was totally dissolved, the solution was transferred to sterile dialysis tubings and allowed to dialyse in 100 volume of dialysis buffer for a total of 4 hrs changing to fresh dialysis buffer once. The dialysed solution was then transferred to sterile Eppendorf tubes and centrifuged at
~6 500 rpm for 5 mins at 4°C using a MSE microcentrifuge. The supernatant was finally aliquoted in appropriate volume (usually in 50 µl) to prevent repeatedly freezing and thawing. The aliquots were immediately frozen on dry ice and then stored at -70°C. Aliquots of nuclear protein could be stored up to a year.
2.8.2 *From primary rat hepatocyte culture* (Rosette and Karin, 1995)

**Materials**

*PB*: 0.1 mM solution. Prepared fresh and filter sterilised.

*Nonidet-P40 (NP-40)*: Stored at RT.

*Hypotonic Buffer*: 10 mM HEPES, pH 7.9, 10 mM KCl, 0.2 mM EDTA, 0.1 mM EGTA. Add 1 mM DTT, 0.5 mM PMSF, 2.5 µg/ml aprotinin, 2.5 µg/ml leupeptin and 1 µg/ml pepstatin just prior to use.

*High Salt Buffer*: 20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA and 0.02% (v/v) NP-40. Add 1 mM DTT, 0.5 mM PMSF, 2.5 µg/ml aprotinin, 2.5 µg/ml leupeptin and 1 µg/ml pepstatin just prior to use.

*Trypan blue*: see section 2.6.2.

*10X PBS*: see section 2.7.

*Equipment*: Sterile cell scraper, refrigerated microcentrifuge, Denly Spiramix 10.

**Method**

The hepatocytes were washed twice with 1X PBS and was manually scraped using a cell scraper. The cells were pelleted by centrifugation at 800 x g for 5 mins in a refrigerated centrifuge. For every $2.5 \times 10^7$ cells, 1 ml of hypotonic buffer was added. The cells were resuspended by vortexing and allowed to swell on ice for 15 mins. After which, NP-40 was added to a final concentration of 0.02% (v/v) before the cells were passed through a 25G gauge needle for at least 6 times or more to lyse the cells. The lysis
efficiency was assessed using trypan blue exclusion. The nuclei were then pelleted at 10 000 x g for 3 min at 4°C using a refrigerated microcentrifuge. The supernatant could be recovered as the cytoplasmic fraction. The pellet was then resuspended in 500 µl high salt buffer and rolled on a Denly spiramix for 30 mins at 4°C. Particulate material was removed by pelleting the suspension at 10 000 x g for 5 mins at 4°C using a refrigerated microcentrifuge. The supernatant containing the nuclear proteins was aliquoted. The aliquots were quick freeze on dry ice before being stored at -70°C.
2.9 Protein quantitation (Lowry, et al., 1951)

Materials

*Bovine Serum Albumin (BSA):* Prepared stock solution by adding a recommended volume of sterile distilled water. Aliquot and stored at -20°C.

*CuSO₄·5H₂O:* 1% (w/v) solution. Stored at 4°C.

*Potassium sodium tartarate:* 2% (w/v) solution. Stored at 4°C.

*Na₂CO₃:* Prepared fresh a 2% (w/v) solution in 0.1N NaOH.

*Lowry Reagent A:* Prepared fresh a solution containing 1% CuSO₄·5H₂O, 2% potassium sodium tartarate and 2% Na₂CO₃ in NaOH in the ratio of 1:1:100 respectively.

*Folin and Ciocalteau Reagent:* Stored stock solution at RT. Prepared fresh a 1 in 1.5 dilution in sterile water.

*Equipment:* 1 cm path-length plastic disposable cuvettes No. 67.742 (Sarstedt Ltd, Leicester, England), UV/visible spectrophotometer.

Method

BSA standards usually ranging from 10 μg/ml to 90 μg/ml were set up by diluting the stock solution with water. Samples can either be used neat or diluted with water. The final volume of all standards and samples should be 200 μl. Both standards and samples should be performed in triplicate whenever possible. A tube containing 200 μl of water was set up as the sample blank and was processed as all other tubes.
1 ml of Lowry Reagent A was added to each tube containing either 200 µl of standard or sample. The mixture was vortexed and incubated for 20 mins at RT. Then 100 µl of the diluted Folin and Ciocalteau Reagent was added and each tube was vortexed immediately before they were incubated for 45 mins at RT in the dark. The mixture was then transferred to plastic disposable cuvettes and the optical density was determined at 700 nm using the spectrophotometer which had been zeroed with the sample blank.

The concentration of the samples can then be determined by plotting a graph of absorbance at 700 nm vs BSA concentration and the absorbance readings of the samples interpolated.
2.10 SDS-PAGE gel electrophoresis of protein

Materials

Protogel (National Dignostics): 30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide, gas stabilised. Stored at RT for up to a year.

Buffer 6.8: 0.5 M Trizma® base and 0.4% (w/v) SDS. Adjust pH to 6.8. Autoclaved and stored at RT.

Buffer 8.8: 1.5 M Trizma® base, pH 8.0 and 0.4% (w/v) SDS. Adjust pH to 8.8. Autoclaved and stored at RT.

TEMED: Stored at 4°C.

10% (w/v) ammonium peroxodisulphate (BDH Chemicals Ltd): Prepared fresh in sterile distilled water but can be stored up to a week at 4°C.

10% Separating gel: A 40 ml gel was prepared by mixing 13.3 ml of Protogel, 10 ml buffer 8.8, 27.5 ml sterile water, 563 µl 10% ammonium peroxodisulphate and 30.7 µl TEMED. The solution was mixed briefly and poured into an already assembled gel apparatus up to 1 cm below the base of the comb. 0.1% (w/v) SDS was immediately layered onto the gel. Allowed to set for 15 to 20 mins before preparing the stacking gel.

3% Stacking gel: A 20 ml gel was prepared by mixing 2 ml Protogel, 5 ml buffer 6.8, 12 ml sterile water, 225 µl ammonium peroxodisulphate and 20 µl TEMED. The 0.1% SDS on top of the separating gel was remove and the stacking gel was quickly added above the separating gel. The gel was allowed to completely polymerise for at least 2 hours at RT.
Protein gel electrophoresis buffer: 0.025 M Trizma® base, 0.192 M glycine and 10% (w/v) SDS. Freshly prepared.

2X Protein loading buffer: 1% (w/v) SDS, 10 mM EDTA, pH 8.0, 10 mM sodium phosphate, pH 7.0, 1% (v/v) 2-mercaptoethanol, 15% (v/v) glycerol, 4 mM PMSF and 0.01% (w/v) bromophenol blue. Filter sterilised through a 0.45 µm filter unit and stored as aliquots at -20°C. Use at 1X final concentration.

2-mercaptoethanol (BDH Chemicals Ltd): Stored at 4°C.

Equipment: Vertical gel electrophoresis apparatus, LKB Bromma power-pack.

Method

10 to 20 µg of protein sample was diluted with water to an appropriate volume, and protein loading dye was added to 1X final concentration. The contents were mixed and boiled for 3 mins. Then, 2 µl of 2-mercaptoethanol was added to every 10 µl of protein sample. The tube was centrifuged briefly in a MSE microcentrifuge to bring all the contents to the bottom of the tube before the sample was loaded on the gel which had been pre-run at a constant current of 10 mA for 15 to 20 mins. A single gel should be electrophoresed at a constant current of 24 mA for stacking and 34 mA for separating. The gel was electrophoresed until the bromophenol blue dye was approximately 2 cm from the end of the gel.
2.11 Staining of SDS-PAGE gel

Materials

*Coomassie brilliant blue stain*: 0.2% (w/v) Brilliant blue 6, 45% (v/v) methanol and 10% (v/v) glacial acetic acid. Filtered through Whatman paper No. 1 (Whatman International Ltd) and stored at RT. Stain can be reused many times.

*Coomassie brilliant blue destain solution*: 45% (v/v) methanol and 10% (v/v) glacial acetic acid. Stored at RT.

*7% (v/v) glacial acetic acid*: Stored at RT.

*Equipment*: 3MM Whatman chromatography paper and Gel-dryer (Model 583; Bio-Rad Laboratory).

Method

The stacking gel was removed and the separating gel was placed in a container containing Coomassie brilliant blue stain. The gel should be stained for at least an hour or overnight at RT with gentle shaking on a platform shaker. The stain solution was then decanted and the gel was destained in Coomassie brilliant blue destain solution. The destain solution was changed frequently until the background of the gel became clear. The gel was placed in 7% acetic acid to allow it to swell gel back to its original size before it was photographed or dried onto 3MM Whatman chromatography paper using a gel dryer at 80°C.
2.12 Gel retardation assay

Material

*DNA*: Can either be restriction digested DNA fragments or synthetic oligonucleotides (double-stranded).

*Nuclear protein*: Can be from tissues or cells. For preparation, see section 2.8.1 for tissues and section 2.8.2 for cells.

*Protogel*: see section 2.10.

*TEMED*: see section 2.10.

10% (w/v) *ammonium peroxodisulphate*: see section 2.10.

*10X TBE buffer*: see section 2.1.1.

4% Polyacrylamide gel: To make a 50 ml gel, add 2.5 ml 10X TBE, 6.67 ml Protogel, 40.5 ml distilled water, 300 µl 10% ammonium peroxodisulphate and 50 µl TEMED. Mix briefly and add to already assembled gel apparatus. Allowed to completely polymerised for at least 2 hours or overnight.

5X *Gel-shift binding buffer*: 60 mM HEPES, pH 7.9, 20 mM Tris-HCl, pH 7.9, 300 mM KCl, 150 mM NaCl, 25 mM MgCl$_2$, 25 mM DTT, 62.5% (v/v) glycerol. Filter sterilised using a 0.22 µm filter unit, aliquoted and stored at -20°C. Used at 1X final concentration.

*Poly(dl.dC).poly(dl.dC) DNA copolymer (Pharmacia Biotech)*: 5 µg/µl stock solution in sterile 5 mM NaCl. Incubated for 5 mins at 45°C to ensure a double-stranded configuration in solution. Stored as aliquots at -20°C.

*Gel-shift electrophoresing buffer*: 0.5X TBE.

6X DNA Loading Buffers:
a) Buffer I: 0.25% (w/v) bromophenol blue, 30% (v/v) glycerol.

b) Buffer II: 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 30% (v/v) glycerol.

Filter sterilised using a 0.22 μm filter unit and stored as aliquots at -20°C.

Proteinase K (Boehringer Mannheim): 25 μg/μl stock solution in 50 mM Tris-HCl, pH 8.0, 1 mM CaCl₂. Incubated for 5 mins at 37°C. Stored as aliquoted at -20°C. Diluted to 5 μg/μl final concentration.

Equipment: Vertical gel electrophoresis apparatus, Gel loading tips (Greiner Labortechnik Ltd), power pack (Bio-Rad Model 3000/300), Gel-dryer, Model 583.

Method

The binding conditions were as described in Rosette et al. (Rosette and Karin, 1995) with minimum modifications. In a sterile 1.5 ml Eppendorf tube, 2.5 to 10.0 μg of nuclear protein, 1X gel-shift binding buffer, poly(dl.dC) and 1-2 X 10⁴ cpm/μl of radiolabeled DNA probe (see section 2.5 for radiolabelling DNA) was added with sterile water to a total volume of 20 μl. The content was gently mixed and incubated on ice for 30 mins. The amount of poly(dl.dC) added should be optimised for different DNA probes. In most cases, either equal or half the amount of poly(dl.dC) to nuclear protein was used. The nuclear protein was diluted to 1 μg/μl with sterile water just prior to use and the DNA probe was added last. A tube with no nuclear protein added was also set up. To assess genuine protein-DNA complex, a
standard binding reaction was set up and incubated as normal. Then, 5 μg of proteinase K was added and the tube was further incubated at 37°C for 10 min. After the incubation, DNA loading buffer was added to 1X final concentration before the entire content in each tube was loaded onto the polyacrylamide gel using gel loading tips. DNA loading buffer I was used for DNA fragment up to 80 bp long and DNA loading buffer II was used for fragment longer than 80 bp. The gel was electrophoresed in 0.5X TBE buffer at 10 V/cm until the free probe was approximately 1 cm from the end of the gel. For example, in a 4% gel, the bromophenol blue dye travels with the 80 bp fragment while the xylene cyanol co-migrates with the 350 bp fragment. The gel was transferred to 3MM Whatman paper and dried using a gel-dryer. The dried gel was autoradiographed for an appropriate length of time (usually overnight) at -70°C with intensifying screens. However, sharper image can be obtained by autoradiographing the gel for a few days at room temperature.

2.12.1 Competitive gel retardation assays

These were usually performed concurrently with the standard binding assays. Both radiolabeled probe and non-radiolabeled competitor DNA were added to tube containing 1X gel-shift buffer and poly(dl.dC). The nuclear protein was added last. Generally, 1 X 10^4 cpm is equivalent to 0.5 ng of DNA labelled at only one end. Subsequent steps were as section 2.12.
2.12.2 Use of antibodies to identify DNA-binding proteins

1 to 2 μl of antibody was added to the mixture without DNA probe. The tube was incubated on ice for 10 mins before the DNA probe was added and processed as in section 2.12.

2.12.3 Chemical treatment of nuclear protein

The nuclear protein was diluted to 2 μg/μl with sterile water and 10 μg was used per reaction. The appropriate chemicals was added to the diluted nuclear protein in a total volume of 25 μl. The tube was incubated at 37°C for 20 mins and then on ice for 2 mins. Afterwhich, 1X gel-shift binding buffer, poly(dl.dC), radiolabeled DNA probe and water to make up the total volume to 35 μl. Subsequent steps were as in section 2.12.
2.13. DNase I footprinting (SureTrack Footprinting Kit from Pharmacia)

Materials

Supplied in the kit:-

4% (v/v) Formic acid: Stored at -20°C.

1% (w/v) SDS: Stored at -20°C.

10 M Piperidine: Stored at -20°C. Freshly prepared 150 µl of a 1 M aqueous solution for every reaction.

Loading dye: 10 mM EDTA, 0.3% (w/v) bromophenol blue, 0.3% (w/v) xylene cyanol in deionised formamide. Stored at -20°C.

Calf Thymus DNA: 0.5 µg/µl in sterile water, sonicated. Stored at -20°C.

BSA: 10 µg/µl in sterile water. Stored at -20°C.

Ca\(^{2+}\)/Mg\(^{2+}\) solution: 10 mM MgCl\(_2\) and 5 mM CaCl\(_2\). Stored at -20°C.

10U/µl FPLCpure™ DNase I: Stored at -20°C.

4X DNase stop solution: 768 mM sodium acetate, 128 mM EDTA, 0.56% (w/v) SDS and 256 µg/ml yeast RNA. Stored at -20°C. A 1X concentration was prepared by heating the 4X DNase stop solution at 50°C with occasional mixing until completely dissolved. Then 1.9 ml of the 4X DNase stop solution was added to a 10 ml falcon tube containing 5.7 ml of sterile distilled water. The 1X DNase stop solution could be stored for up to 6 months at 4°C. The 1X solution was warmed for 5 mins at 37°C to dissolve the salts just prior to use and was kept at RT during the DNase I reaction.
Other materials:-

5X Gel-shift binding buffer: see section 2.12.


Ethanol: absolute and 95% (v/v)

Phenol/chloroform: see section 2.1.4.

TE buffer: see section 2.1.3.

n-butanol: Stored at RT.

SequaGei® Concentrate (National Diagnostics): Stored at RT.

SequaGei® Diluent (National Diagnostics): Stored at RT.

SequaGei® Buffer (National Diagnostics): Stored at RT.

10% (w/v) ammonium peroxodisulphate: see section 2.10.

TEMED: see section 2.10.

8% Sequencing gel: A 100 ml gel was prepared by mixing 32 ml SequaGei® Concentrate, 58 ml SequaGei® Diluent, 10 ml SequaGei® Buffer, 400 µl 10% (w/v) ammonium peroxodisulphate and 20 µl TEMED. The solution was mixed briefly and poured into an already assembled gel apparatus. The gel was allowed to completely polymerise for at least 2 hrs at RT or overnight at 4°C wrapped in Saran wrap.

10X TBE buffer: see section 2.1.1.

DNase 1 footprint electrophoresis buffer: 1X TBE buffer.

Equipment: Eppendorf microcentrifuge 5417R, Vertical gel apparatus, electrophoresis power pack, temperature strip (Bio-Rad), 3MM Whatman chromatography paper, gel dryer and Geiger counter.
Method

2.13.1 Radiolabelling of DNA

The radiolabelling of DNA was described in section 2.5. However, only one end of the double-stranded DNA should be radiolabeled for DNase I footprinting. This could be easily achieved by using a DNA fragment with a 5'-overhang at one end and either a 3'-overhang or a blunt end at the other.

2.13.2 G + A reaction

A mixture containing 3 to 6 ng of radiolabeled DNA (~1 X 10^5 cpm), 1 μg of calf thymus DNA and TE buffer to 10 μl was set up in a 1.5 ml Eppendorf tube. 1 μl of 4% formic acid was added and the mixture was incubated for 25 mins at 37°C. The tube was then placed on ice, 150 μl of freshly prepared 1 M piperidine was added and the tube was incubated for 30 mins at 90°C using a PCR machine. The tube was then placed on ice for 5 mins before 1 ml of n-butanol was added. The tube was vortexed and centrifuged at 13 000 rpm for 2 mins at RT using an Eppendorf microcentrifuge to pellet the DNA. The supernatant was carefully aspirated before 150 μl of 1% SDS and 1 ml of n-butanol was added to the pellet. The tube was vortexed and centrifuged using the conditions as above. The supernatant was carefully removed and the pellet was washed with 500 μl of n-butanol. The tube was centrifuged using the same centrifugation conditions as above. The pellet was dried in vacuo for 10 mins, dissolved in 7.5 μl of loading dye and could be stored at -20°C for up to 2 weeks. The
presence of the DNA pellet after each centrifugation was monitored using a Geiger counter.

2.13.3 DNase I titration

Five to six microlitres of the 10 U/μl DNase I stock solution was diluted to 3 U/μl with Ca$^{2+}$/Mg$^{2+}$ solution and was labelled as tube ‘A’. Three successive 1:9 dilutions using tube A were prepared as shown in table 2.1, keeping the enzyme on ice at all times.

<table>
<thead>
<tr>
<th>Ca$^{2+}$/Mg$^{2+}$</th>
<th>DNase I</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 μl</td>
<td>1 μl from Tube A</td>
<td>0.33U/μl (=Tube B)</td>
</tr>
<tr>
<td>8 μl</td>
<td>1 μl from Tube B</td>
<td>0.037U/μl (=Tube C)</td>
</tr>
<tr>
<td>8 μl</td>
<td>1 μl from Tube C</td>
<td>0.0041U/μl (=Tube D)</td>
</tr>
</tbody>
</table>

Table 2.1 Preparation of successive dilution of DNase I.

A mock DNA-protein binding reaction was set up using BSA in place of nuclear protein. A bulk reaction mix sufficient for 9 assays was prepared as shown in Table 2.2.

Mock binding reaction mix

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>5X Gel-shift binding buffer</td>
<td>100 μl</td>
</tr>
<tr>
<td>Poly dI.dC (5 μg/μl)</td>
<td>5-20 μl</td>
</tr>
<tr>
<td>Radiolabeled target DNA (1-2 X 10$^5$ cpm)</td>
<td>10-30 μl</td>
</tr>
<tr>
<td>BSA (10 μg/μl)</td>
<td>10-50 μl</td>
</tr>
<tr>
<td>Sterile water</td>
<td>350-425 μl</td>
</tr>
<tr>
<td>Total Volume</td>
<td>500 μl</td>
</tr>
</tbody>
</table>

Table 2.2 DNase I mock binding reaction mix.
The mock reaction mix was incubated for 30 mins on ice before 50 µl was dispensed at RT into each of 9 Eppendorf tubes labelled 1 to 9. 5 µl of Ca²⁺/Mg²⁺ solution was then added to each tube, the contents were gently mixed and incubated for 1 min at RT. An appropriate volume of DNase I was added to each tube as indicated in Table 2.3.

<table>
<thead>
<tr>
<th>Tube</th>
<th>DNase I</th>
<th>Final Enzyme Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3 µl of Tube A (3U/µl)</td>
<td>9U</td>
</tr>
<tr>
<td>2</td>
<td>1 µl of Tube A (3U/µl)</td>
<td>3U</td>
</tr>
<tr>
<td>3</td>
<td>3 µl of Tube B (0.33U/µl)</td>
<td>1U</td>
</tr>
<tr>
<td>4</td>
<td>1 µl of Tube B (0.33U/µl)</td>
<td>0.33U</td>
</tr>
<tr>
<td>5</td>
<td>3 µl of Tube C (0.037U/µl)</td>
<td>0.11U</td>
</tr>
<tr>
<td>6</td>
<td>1 µl of Tube C (0.037U/µl)</td>
<td>0.037U</td>
</tr>
<tr>
<td>7</td>
<td>3 µl of Tube D (0.0041U/µl)</td>
<td>0.012U</td>
</tr>
<tr>
<td>8</td>
<td>1 µl of Tube D (0.0041U/µl)</td>
<td>0.0041U</td>
</tr>
<tr>
<td>9 (Control)</td>
<td>3 µl of Ca²⁺/Mg²⁺ Solution</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2.3 Titration of DNase I in footprint assay.

The contents were gently mixed and incubated for exactly 1 min at RT. The reaction was stopped by adding 140 µl of 1X DNase stop solution, vortexed briefly and stored aside at RT until all the other tubes were processed. Then, 200 µl of phenol/chloroform was added to each tube, vortexed and centrifuged at 13 000 rpm for 4 mins in an Eppendorf microcentrifuge. The aqueous phase was transferred to a new Eppendorf tube and 400 µl of
absolute ethanol was added. The tube was vortexed and stored for 10 mins on dry ice to precipitate the DNA. The DNA was pelleted by centrifuging at 13 000 rpm for 10 mins in an Eppendorf microcentrifuge. The supernatant was carefully removed and the pellet was washed with 150 μl of 95% ethanol before centrifuging for another 5 mins. The supernatant was carefully removed and the pellet was dried in vacuo. The pellets could either be stored overnight at -20°C or used immediately. 3 μl of loading dye was added to resuspend each DNA pellet. At this stage, the gel was pre-run for 30 mins at constant power of 40W until the temperature of the gel was about 50°C as indicated by the temperature strip on the glass plate. The samples were heated for 2 mins at 80°C and immediately placed on ice for 2 mins before they were loaded onto the pre-heated gel. The gel was electrophoresed at constant power, keeping the voltage at approximately 1250 V throughout the entire electrophoresis process (gel temperature at 50°C). The electrophoresis was stopped when the bromophenol blue dye reached the end of the gel. The gel was transferred onto 3MM Whatman paper and dried at 80°C for approximately 1 hr using a gel dryer. The dried gel was autoradiographed for an appropriate time (usually 48 hrs) at -70°C in a cassette with an intensifying screen.
2.13.4 Binding and footprinting reaction

The binding reaction was set up as for the gel shift assay in section 2.12. A control tube containing BSA instead of nuclear protein was set up in parallel. The amount of BSA added was equivalent to the amount of nuclear extracts used in the other tubes. After the binding reaction was complete, the tubes were placed at RT and 5 μl of Ca\(^{2+}/\text{Mg}^{2+}\) solution was added to each tube. The samples were then processed as in the DNase I titration as in section 2.13.3. The DNase I was diluted in Ca\(^{2+}/\text{Mg}^{2+}\) solution just before used, to a concentration as determined in the titration assay to be optimal and placed on ice. The gel was pre-run for 30 mins at a constant power of 40W until the temperature of the gel was about 50°C. Before loading the samples and the G+A reaction tube were heated for 2 mins at 80°C and immediately placed on ice. The gel was electrophoresed at constant power, keeping the voltage at approximately 1250 V throughout the entire electrophoresis process until the bromophenol blue dye reached the end of the gel. The gel was transferred onto 3MM Whatman chromatography paper and dried at 80°C for approximately 1 hr using a gel dryer. It was then autoradiographed for 48 hrs or longer at -70°C in a cassette with intensifying screen.
2.14 Partial nuclear protein purification using cellulose chromatography column

2.14.1 Pre-cycling of P11 phosphocellulose resins

Materials

P11 cellulose phosphate (Whatman International Ltd): Stored at RT.

NaOH: 0.5 N solution. Stored at RT.

HCl: 0.5 N solution. Stored at RT.

P11 Storage buffer: 25 mM HEPES, pH 7.6, 0.1 mM EDTA, pH 8.0, 40 mM KCl, 10% (v/v) glycerol.

Equipment: Retort stand, filter funnel, Whatman paper No. 1.

Method

An appropriate amount of P11 cellulose phosphate was weighed. It was stirred into 25 volumes of 0.5 N NaOH and was left for not more than 5 mins at RT. The supernatant was then filtered off through Whatman paper No. 1 using a filter funnel and the resin washed with distilled water until the pH of the filtrate was at or below 11. After which, 25 volumes of 0.5 N HCl was added to the washed resin and left again for not more than 5 mins at RT. The supernatant was filtered off and the resin washed with distilled water until the pH was at or just above 3. The resin could be stored in storage buffer for up to a week at 4°C.
2.14.2 Equilibrating column and fractionation of nuclear proteins

Materials

*Low salt buffer*: 25 mM HEPES, pH 7.6, 0.1 mM EDTA, pH 8.0, 40 mM KCl, 10% (v/v) glycerol. Filtered through 0.45 μm filter unit. 1 mM DTT, 0.1 mM PMSF, 10 μg/ml aprotinin, 1 μg/ml antipain, 1 μg/ml pepstatin, 1 μg/ml leupeptin were added prior to use. Stored at 4°C.

*High salt buffer*: As low salt buffer except for 1 M KCl.


Method

A column (1 cm diameter by 2 cm height) was packed with resin. After the pre-cycled phosphocellulose resin was gently dispersed in the storage buffer, it was slowly poured into the column and left to settle. The eluent was allowed to run to waste. The volume of resin added to the column could be calculated using the diameter of the column and the height of the packed resin. When a desirable volume of resin had been packed, ensuring no air-bubbles are trapped in the resin, the top end of the column was inserted. Low salt buffer was then run through the column at a flowrate of 0.3 ml/min for 15 to 30 mins to equilibrate the column. The liver nuclear protein sample (1 to 3 mg) was then loaded into the column at the same flow rate. Once all the sample had been loaded, the column was washed with low salt buffer to remove any proteins that did not bind to the column and were collected in 5
ml fractions until the chart recording showed that all unbound proteins had been washed out. The bound proteins were eluted from the column using an increasing salt concentration gradient by mixing 0% up to 100% of the high salt buffer with the low salt buffer. During the elution, 1 ml fractions were collected until the chart recording showed that no more protein eluted. The run was terminated and 250 to 500 µl of the fractions collected were concentrated on Microcon-10 according to manufacturer's instructions. All eluates were stored at -70°C. It should be mentioned here that the column for purification can be scale-up for the purification of larger amount of nuclear proteins.
2.15 Protein purification by magnetic DNA affinity

2.15.1 Preparation of DNA for coupling to Dynal® magnetic beads

Materials

*Tether DNA*: A short double-stranded DNA of length 6 to 10 bp with one of the 5'-ends biotinylated and preferably a suitable 5'-overhang at the other end of the sequence. In this case, it is a 5'-overhang that is compatible to the ends created by Sal I and Xho I.

*Oligo 1*: 5' Bioteg - GGAGGG - 3'

*Oligo 2*: 5' - GATCCCCTCC - 3'

*DNA containing binding site of interest*: DNA should preferably be concatamerised before use if the sequence is not longer than 50 bp. One end of the DNA should have a compatible 5'-overhang to the tether DNA for subsequent ligation. The other end should be dephosphorylated to minimise further uncontrolled concatamerisation occurring.

*10X Annealing buffer*: see section 2.4.

*10X One-Phor-All buffer plus*: see section 2.1.1.

*(γ-32P)ATP, Easytides™ Radioactive Isotope*: see section 2.5.2.

*5 mM ATP*: see section 2.1.5.

*T₄ PNK, FPLCpure®, cloned*: see section 2.1.5.

*T₄ DNA Ligase*: see section 2.1.6.

Method

2.15.1.1 Phosphorylation of Oligo 2

Oligo 2 was phosphorylated at the 5'-end using T₄ PNK as described in section 2.1.5. In order to assess subsequent ligation and coupling events, a small amount of [γ³²P]ATP was included in the reaction. After the phosphorylation reaction, the contents were extracted with phenol-chloroform and precipitated with ethanol as described in method of section 2.1.4. Due to the short sequence of the oligonucleotide, it was precipitated with ethanol on dry ice overnight and then recovered by centrifugation at 13 000 rpm for 20 mins. The presence of the DNA pellet was assessed using a Geiger counter to detect radioactivity. The DNA was finally dissolved in sterile distilled water.

2.15.1.2 Annealing of tether DNA

The annealing reaction was carried out essentially as described in section 2.4.2 for temperatures below 25°C. Since, the Tₘ of the oligonucleotides were below 25°C due to their short sequences. The contents should be added to the tube on ice and the annealing reaction carried out at 16°C for 15 mins. After annealing, the temperature of the annealed oligonucleotides should not be allowed to rise above 25°C.
2.15.1.3 Preparation of DNA containing binding site of interest

The tether DNA when annealed gave one end with a 5'-overhang with bases that were compatible with either a Sal I or Xho I cut. Therefore, the DNA to be ligated should possess either a Sal I or Xho I cut at one end. The DNA was usually cloned into a plasmid vector which should first be linearised at the end that was not to be ligated to the tether DNA. The linearised DNA was then dephosphorylated with calf intestinal alkaline phosphatase as described in section 2.1.4. Finally, the DNA was digested with Sal I and Xho I, electrophoresed on a low melt agarose gel and purified on a Spin-X column as described in section 2.1.3.

2.15.1.4 Ligation of DNA to tether

The annealed tether was ligated to the DNA obtained in section 2.15.4 in a molar ratio of 2:3 respectively to ensure that all the annealed tether was utilised. The ligation reaction was carried out as described in section 2.1.6.1.

2.15.2 Preparation of DNA-coated magnetic beads

Materials

Dynabeads® M-280 Streptavidin (Dynal A.S., Oslo, Norway): Supplied as a suspension containing 6.7 X 10^8 Dynabeads/ml (10 mg/ml) in PBS, pH 7.4 in 0.1% BSA and 0.02% NaN₃. Stored at 4°C.
**PBS Tablets:** Prepared according to manufacturer’s instruction. Autoclaved and stored at 2 to 8 °C.

1% (w/v) BSA in PBS: Stored at 4°C.

1 M NaCl in PBS: Stored at RT.

**TE buffer:** see section 2.1.3.

**Equipment:** Denly Spiramix 10, Dynal® MPC-E magnetic separator (Dynal A.S.), siliconised pipette tips, Eppendorf tubes and Sorenson tubes with screw caps.

**Method**

2.15.2.1 *Preparation of M-280 Dynabeads® for coupling*

Dynabeads were fully resuspended before an appropriate amount of beads required for coupling were taken. The beads were placed in a Sorenson tube with a screw cap. For every 1 μl of beads used, 1 μl of 1% BSA in PBS solution was added. The beads were gently mixed and the tube was placed in a Dynal® MPC-E magnetic separator for ~1 min to concentrate the beads to one side of the tube. The supernatant was then carefully removed ensuring that minimal beads were being taken. The tube was removed from the magnetic separator and the beads were again resuspended in 1% BSA in PBS. The beads were washed twice with 1% BSA in PBS and then twice with 1 M NaCl in PBS and finally twice with TE buffer. The beads were either stored at 4°C or used immediately for DNA coupling.
2.15.2.2 Coupling of biotinylated DNA to pre-washed M-280 Dynabeads®

Upon removal of the TE buffer in which the beads were stored, the ligated DNA from section 2.15.5 was added to the beads. Approximately 50 to 100 pmole of buffer to a volume equivalent to the initial volume of the beads used. The beads were gently resuspended and then rolled on the Denly spiramix at RT for 30 to 45 mins. The unbound DNA was then removed by washing the beads twice with TE buffer. The beads were finally resuspended and stored in TE buffer at 4°C. The coupling of DNA to the beads could be followed using a Geiger counter since the tether DNA was weakly radioactive.

2.15.3 Binding, washing and elution conditions for protein purification

Materials

5X Gel-shift binding buffer: see section 2.12. Used at 1X final concentration.

1X Wash buffer: 12 mM HEPES, pH 7.9, 4 mM Tris-HCl, pH 7.9, 60 mM KCl, 100 mM NaCl 5 mM MgCl₂, 5 mM DTT, 12.5% (v/v) glycerol. Stored at -20°C.

1X Elution buffer: Prepare as a 1X wash buffer but a range of NaCl concentrations from 1.0 M to 2.0 M are needed. Stored at -20°C.


Dialysis buffer: 1X gel-shift binding buffer containing no NaCl and 10% glycerol.

6X DNA Loading Buffer II: see section 2.12.
Equipment: 0.1 to 0.5 ml Slide-A-Lyzer cassette (Pierce & Warreiner (U.K.) Ltd, Chester, U.K.)

Method

An amount of nuclear protein was added to DNA-coated magnetic beads such that binding occurred at saturating conditions. The binding reaction was carried out in 1X gel-shift binding buffer containing 150 mM NaCl as in gel-shift reactions. Binding was allowed to occur for 30 mins at 4°C on a Denly spiramix. After which, the unbound proteins were removed as the supernatant fraction and the beads were washed twice with 1 µl of 1X wash buffer containing 5X excess of poly(dl.dC) with respect to the amount of DNA used for every µl of beads. These were then collected as wash fractions. The bound nuclear proteins were eluted from the DNA by washing the beads with a series of elution buffers each containing increasing concentrations of NaCl, 1 µl of elution buffer was used for every 1 µl of beads. Initially, the elution buffer containing 1.0 M NaCl was added to the protein bound beads and elution was allowed to occur for 10 mins at 4°C on a Spiramix. The elution buffer was then removing using a magnetic separator, and placed in siliconised 1.5 ml Eppendorf tubes on ice. The elution step was repeated with elution buffer containing 1.5 M NaCl and then with buffer containing 2.0 M NaCl. The elution fractions were dialysed using a Slide-A-Lyzer in an appropriate volume of dialysis buffer at 4°C for an hour while the beads were regenerated as described in section 2.15.10. The
volume of dialysis buffer to use was calculated according to the following formula:-

\[
\frac{\text{Volume of eluate}}{\text{Volume of Dialysis buffer + Volume of eluate}} \times \text{Initial [NaCl]} = \text{Final [NaCl]}
\]

The dialysed eluate was then used in gel shift assay to assess which NaCl concentration was optimal for the elution of the bound protein from the DNA.

**2.15.4 Determination of optimal elution conditions**

The different fractions collected were used in gel shift assays (see section 2.12) to assess the progress of the purification. A DNA sequence identical to that coated onto the Dynabeads was radiolabeled and used as the probe. The supernatant, wash and dialysed elution fractions were mixed with the radiolabeled probe. The mixture was incubated for 30 mins on ice before 1X DNA Loading buffer II was added and the sample was electrophoresed on a 4% polyacrylamide gel.

**2.15.5 Regeneration of used DNA-coated magnetic beads**

The used DNA-coated magnetic beads were washed 3 times in 1X elution buffer containing 2.0 M NaCl and then washed 3 times with TE buffer. The beads were resuspended in TE buffer, stored at 4°C and could be reused up to 10 times.
2.16 *Northern blot hybridisation*

2.16.1 *Isolation of total RNA*

**Materials**

*Ultraspec™-II RNA Isolation System (Total RNA Isolation kit; Biotecx Laboratories, Inc., Houston, U.S.A.)*: Stored in the dark at 4°C. The reagent may partially crystallised at 4°C. If so, bring solution to RT and shake well before use.

*Chloroform*: Stored at RT.

*Isopropanol*: Stored at RT.

*Ethanol*: 80% (v/v).

*Equipment*: Polytron homogeniser (The Northern media supply Ltd., Hull, England), 2 ml safe-lock Eppendorf tubes, bench-top refrigerated microcentrifuge 5417R and UV/visible spectrophotometer.

**Method**

For tissue samples, 10 to 100 mg were homogenised in 1 ml of Ultraspec™ RNA reagent using a polytron homogeniser. For cell pellets, 5 to 10 X 10⁶ cells were resuspended and lysed by vortexing in 1 ml of Ultraspec™ RNA reagent. The lysate was immediately transferred to 2 ml sterile Eppendorf tubes and left on ice for 5 mins. For every ml of Ultraspec™ RNA reagent used, 0.2 ml of chloroform was then added. The sample was shaken vigorously for 15 secs and left on ice for further 5 mins before centrifugation at 12 000 x g for 15 mins at 4°C in a microcentrifuge.
The top aqueous layer was transferred to a new Eppendorf tube, care was taken not to disturb the interface. An equal volume of isopropanol was added to the aqueous phase and the tube was vortexed. The sample was left on ice for 10 mins to precipitate the RNA. The RNA was pelleted by centrifuging at 12 000 x g for 15 mins at 4°C. The supernatant was removed and the RNA pellet was washed twice with 80% ethanol. One ml of ethanol was added for every ml of Ultraspec™ reagent used, the tube was vortexed and centrifuged at 12 000 x g for 5 mins at 4°C. After the final wash, as much ethanol was removed as possible and the pellet was air-dried briefly before it was dissolved in 10 to 30 μl of sterile distilled water. The solvation of the RNA was enhanced by vortexing the sample for 1 min. Samples were stored at -20°C. The yield and purity of RNA was determined by measuring the optical density at 260 nm and 280 nm using a UV/visible spectrophotometer. The integrity of RNA was observed by electrophoresing 1 μg on a 1% (w/v) agarose gel (see section 2.16.2).

2.16.2 Northern blotting

Materials

Agarose: see section 2.1.2.

Formaldehyde (Sigma Chemical): 37% solution (Formalin), ACS Reagent. Stored at RT.
10X MOPS/EDTA: 0.2 M MOPS (3-[N-Morpholino]propane-sulfonic acid), 80 mM sodium acetate and 10 mM EDTA. Adjust pH to 7.0. Autoclaved and stored at RT. The solution was kept in the dark by wrapping the container with aluminium foil.

Deionised formamide: For deionisation, 5 g of mixed bed ion-exchange resin (Bio-Rad AG501-X8 or X8(D) resin) was added to every 100 ml of formamide. The contents were stirred for 1 hr at RT and filtered through Whatman No. 1 filter paper twice. It was then filter sterilised through a 0.45 µm filter unit and stored at -20°C.

RNA Loading buffer: 0.75 ml deionised formamide, 0.15 ml 10X MOPS/EDTA, 0.24 ml 37% formaldehyde, 0.1 ml 100% (v/v) glycerol, 0.08 ml 10% (w/v) bromophenol blue and 0.1 ml RNase-free deionised water. The contents were thoroughly mixed, aliquoted and stored at -20°C.

RNA electrophoresis buffer: 1X MOPS/EDTA buffer. Prepared fresh.

10 mg/ml EtBr: see section 2.1.2.

20X SSPE (sodium salt phosphate EDTA): 3.6 M NaCl, 200 mM di-sodium hydrogen orthophosphate and 20 mM EDTA. Adjust pH to 7.4. Autoclaved and stored at RT.

Equipment: Blotting membrane - optimised nylon (BDH Electran®), QuickDraw™, Extra Thick, Blotting paper (Sigma Chemical), Horizontal gel electrophoresis system, Grant BT1 block thermostat (Grant Instruments (Cambridge) Ltd, Cambridge, England), UV Stratalinker® 1800 (Stratagene Ltd., Cambridge, England) and Heraeus Oven (Kendro Laboratory Products Ltd.).
Method

2.16.2.1 Preparation of agarose gel for RNA electrophoresis

The apparatus for preparing and electrophoresing the gel was thoroughly rinsed with 70\% (v/v) ethanol and then with sterile distilled water prior to use. To prepare a 1\% (w/v) gel, 1 g of agarose was added to 100 ml of 1X MOPS/EDTA. The agarose was dissolved by boiling. When the solution was cooled to 50°C, 5.1 ml of 37\% formaldehyde was added. The mixture was poured into the gel casting tray and allowed to solidify for at least 1 hr.

2.16.2.2 Preparation and electrophoresis of RNA

The isolation of RNA was described in section 2.16.1. Samples were prepared by adding sterile distilled water to either 10 or 20 μg of RNA to 5 μl total volume. Then, 25 μl of RNA electrophoresis dye was added to each sample before the mixture was heated to 65°C for 15 mins in a block thermostat. One microlitre of a 1 mg/ml of EtBr solution was added to each sample. The contents were mixed and loaded on the gel. The gel was electrophoresed at a constant voltage of 70 V until the bromophenol blue dye was ~5 cm from the bottom of the gel. The RNA in the gel could be visualised using a short wave UV transilluminator (302 or 254 nm).
2.16.2.3 Blotting of RNA

After electrophoresis, the gel was washed briefly in 5X SSPE at RT. A piece of optimised nylon membrane and several pieces of QuickDraw™ blotting paper of dimensions slightly larger than that of the gel were cut. The membrane was pre-wetted in 10X SSPE. The RNA in the gel was transferred to the optimised nylon membrane using capillary action for at least 6 hrs, preferably overnight in 10X SSPE. The capillary action was enhanced with the use of several sheets of QuickDraw™ blotting paper. The RNA was fixed onto the membrane by UV auto-cross-linking in a Stratalinker® with the RNA-side facing the UV source before it was baked dry in a 80°C oven. The membrane could be kept wrapped in Saran wrap at RT.

2.16.3 Radiolabelling by random priming

Materials

DNA: Either restriction enzyme digested fragments purified in low melting point agarose or synthetic oligonucleotides.

DTT: see section 2.8.1.

10X RP (Random prime) buffer: 600 mM Tris-HCl, pH 7.8, 100 mM MgCl₂ and 100 mM 2-mercaptoethanol. Filter sterilised and stored at -20°C.

3.33 mg/ml Random primers (RP; hexanucleotide): Diluted to 75 ng/μl concentration in sterile distilled water. Aliquoted and stored at -20°C.
Ultrapure dNTP set, 2'-deoxynucleoside 5'-triphosphate, sodium salt: see section 2.5.1.

dNTPs mix: Dilute each deoxynucleoside 5'-triphosphate to 5 mM. An equal amount of each dNTP was mixed together. Stored at -20°C.

(α-32P)dCTP Easytides™ Radioactive Isotope: see section 2.5.1.

FPLCpure® DNA Polymerase I, Klenow Fragment, cloned: see section 2.5.1.

Method

Two 1.5 ml Eppendorf tubes, labelled tube I and II, were set up. Tube I contained 20 mM DTT, 10 mM dNTPs mix, 1 X RP buffer and 60 μCi [α-32P]dCTP. Tube II contained 300 ng DNA, 112.5 ng RP and sterile distilled water was added such that the total volume would be 90 μl when the two tubes were mixed together. Tube II was boiled for 5 mins and immediately placed on ice for 3 mins before it was added to tube I. Then, 6.4 units of Klenow fragment was added. The contents of the tube were mixed well and incubated for 3 hrs at 37°C. The radiolabeled probe was purified as described in section 2.5.3.1. The purified probe should have a specific activity of 5 X 10^9 dpm/μg (calculation as shown below).

2.16.4 Calculation of percentage incorporation, probe yield and specific activity of the radiolabeled probe

It is necessary to calculate the probe yield because during random prime labelling, there is a net synthesis of radiolabeled DNA while the initial
DNA template remains non-radiolabeled. However, both populations of DNA can participate in hybridisation.

For calculation of percentage incorporation, see section 2.5.4.

Probe yield = ng initial DNA + ng synthesised DNA

and ng synthesised DNA = \[
\frac{\mu Ci \text{ incorporated} \times 309 \times 4}{3000}
\]

whereby 309 is the average molecular weight of a nucleoside monophosphate in DNA, 4 denote the four nucleotides whereby only one is radiolabeled and 3000 is Ci/mmole of the radiolabeled nucleotide.

Once the probe yield has been calculated, the specific activity could be determined as follows:-

\[
\text{Specific activity (dpm/\mu g)} = \frac{\text{total activity incorporated (dpm)}}{\text{probe yield (\mu g)}}
\]

Taking 1 \( \mu Ci = 2.2 \times 10^6 \) dpm

2.16.5 Hybridisation of RNA blot

Materials

100X Denhardt's reagent: 2% (w/v) BSA, Fraction V, 2% (w/v) Ficoll (MW 400 000) and 2% (w/v) polyvinylpyrrolidone (MW 400 000). Filter sterilised and stored at -20°C.
Salmon sperm DNA (ssDNA), denatured and sheared: 10 mg/ml in aqueous solution. Upon addition of water to the DNA, it was rotated on a spiramix overnight at RT to completely dissolve the DNA. The DNA was then sheared by passing it 20 times rapidly through a 17G gauge needle. It was then aliquoted and stored at -20°C. Just prior to use, the DNA was boiled for 5 mins to denature the DNA and immediately cooled on ice for 5 mins to keep the DNA in the denatured state.

Prehybridisation buffer. 5X SSPE, 50% (v/v) formamide, 5X Denhardt’s reagent, 0.1 - 0.5% (w/v) SDS. The solution was freshly prepared, filtered sterilised through a 0.45 μm filter unit and 100 μg/ml of denatured ssDNA was added just prior to use.

Hybridisation buffer. 5X SSPE, 50% (v/v) formamide, 5X Denhardt’s reagent, 0.2% (w/v) SDS and 10% (w/v) dextran sulphate. The solution was freshly prepared, filter sterilised through a 0.45 μm filter unit and 100 - 200 μg/ml of denatured ssDNA was added just prior to use.

RNA wash buffer A: 2X SSPE and 1% (w/v) SDS. Stored at RT.

RNA wash buffer B: 1X SSPE and 1% (w/v) SDS. Stored at RT.

RNA wash buffer C: 0.1X SSPE and 1% (w/v) SDS. Stored at RT.

RNA strip buffer: 0.1% (w/v) SDS. Stored at RT.

Equipment: Heat sealable bag, Audion Elektro Sealboy (Type 235A; A1 Packaging Ltd, London, U.K.), platform shaker, Tracker tape™ (Amersham International plc., Buckinghamshire, England), Fuji medical X-ray film (Fuji Photo Film Co. Ltd., Tokyo, Japan), cassettes with intensifying screen and water bath with shaking platform.
Method

The membrane with the RNA as prepared in section 2.16.2.3 was placed in a heat-sealable bag. For every square area of membrane, 0.25 ml of prehybridisation buffer was added. The membrane was prehybridised for at least 1 hr at 42°C. The radiolabeled probe was added to hybridisation buffer (0.25 ml/cm²) such that the probe concentration was not more than 20 ng/ml and its activity should be 1 to 5 X 10⁸ cpm/ml. The hybridisation buffer containing the probe was boiled for 10 mins to denature the probe and immediately chilled on ice for another 10 mins. After the prehybridisation buffer was completely removed from the bag, the hybridisation buffer containing the denatured probe was then added. The membrane was hybridised for 16 to 24 hours at 42°C.

The membrane was washed once with RNA wash buffer A for 15 mins at RT on a platform shaker. It was further washed with RNA wash buffer B for 30 mins at RT changing new buffer B after 15 mins. The membrane was then washed with RNA wash buffer C for 15 mins at 50°C. The hot wash step may be repeated a few more times if necessary until the background radioactivity on the membrane is no longer detected. The temperature of the hot wash step could also be increased up to 65°C if required. For synthetic oligonucleotide probes, the hot wash step was omitted. Finally, the membrane was wrapped with Saran wrap ensuring that there was no air-bubbles trapped between the membrane and Saran wrap. The membrane should not be allowed to dry if it is required for rehybridisation. The membrane was exposed to X-ray film for an appropriate
length of time, usually overnight to 3 days with the RNA-side contacting the film. A small piece of tracker tape was stucked at the bottom left hand corner of the membrane. This phosphoresces results in a permanent image on the film which provides easy identification of the orientation of the film to the membrane.

2.16.6 Stripping of hybridised membrane

The bound radiolabeled probe can be removed from the membrane by washing it for 30 mins in boiling RNA strip buffer, changing the solution after 15 mins. The membrane could be exposed to X-ray film after stripping to ensure that all the probe had been stripped. The membrane can then be stored dry in a heat-sealable bag ready for the next hybridisation.
2.17 Transient Transfection of rat primary hepatocyte

Four different transfection protocols were assessed for their suitability for used in primary hepatocyte culture. The firefly reporter plasmid and the Renilla reporter plasmid were used in a ratio of 10:1 respectively in all the transfection protocols. Plasmid DNA was prepared using the Maxi Qiagen plasmid kit as described in section 2.3. The quality of the DNA was assessed by agarose gel electrophoresis to ensure that at least 90% of the DNA was supercoiled. Transfections were carried out on primary hepatocytes that had been cultured for 24 hrs.

2.17.1 Transfection Using calcium phosphate (CaPO₄)

Materials

250 mM CaCl₂·4H₂O Suprapur® (Merck, Darmstadt, Germany): Filter sterilised and stored at 4°C.

2X HeBS: 0.28 M NaCl, 10 mM KCl, 1.5 mM Na₂HPO₄, 42 mM HEPES, 2% (w/v) glucose. Adjust pH to 7.1.

HeBS-glycerol: 10% (v/v) glycerol in 1X HeBS.

Hank’s solution: 137 mM NaCl, 5.4 mM KCl, 0.4 mM MgSO₄·7H₂O, 0.5 mM MgCl₂·6H₂O, 0.35 mM Na₂HPO₄·2H₂O, 0.44 mM KH₂PO₄ and 2 mM HEPES. Adjust pH to 7.4.

William’s E medium: see section 2.6.3.

Fully supplemented culture medium: see section 2.6.3.

Supplemented culture medium: see section 2.6.4.
Method

2.17.1.1 Preparation of CaPO$_4$/DNA precipitate

The DNA for use in transfection can be diluted in 250 mM CaCl$_2$ the day before and stored at 4°C. The CaPO$_4$/DNA precipitate should be prepared 30 mins before addition to the cells. For every 60 mm diameter culture dish, 343 µl of CaPO$_4$/DNA precipitate containing 4.1 µg of total DNA was required. This was prepare by adding dropwise 171.5 µl of 250 mM CaCl$_2$ containing 4.1 µg of DNA to 171.5 µl of 2X HeBS in a Falcon tube using a glass Pasteur pipette. During addition, the solution was mixed by bubbling air from the bottom of the tube using another glass Pasteur pipette and a mechanical pipettor. The addition of DNA should take 1 min. The mixture was then vortexed at maximum speed for 2 to 3 secs and incubated for 25 to 30 mins at RT.

2.17.1.2 Transfection

Just before transfection, existing medium was removed from the 60 mm diameter culture dish and 2.57 ml of supplemented medium was added. The CaPO$_4$/DNA precipitate was mixed again with a 1 ml Gilson pipette just prior to the addition to the cells and 343 µl was added to the fresh medium. The plate was swirled gently during addition of the CaPO$_4$/DNA mixture. It is essential that the cells are not left at RT for more than 6 mins during the addition of the CaPO$_4$/DNA precipitate. The precipitate was left on the cells for 5 hrs in a 37°C incubator under 5% CO$_2$.
The cells were washed twice with Hank’s solution. Then, 860 µl of HeBS-glycerol was added to each plate of cells and incubated for exactly 2 mins at RT. The HeBS-glycerol was promptly removed, the cells washed twice with Hank’s solution and washed once with William’s E medium. The cells were further cultured in 3 ml of fully supplemented medium without FCS for 24 hrs. The cells were washed once with William’s E medium and new fully-supplemented medium without FCS was added. At this stage, cells that were to be treated with PB, it was added to the medium at a final concentration of 0.1 mM. The cells were cultured for an additional 24 hrs before they were lysed for the dual luciferase reporter assay as described in section 2.19.

2.17.2 Transfection using TransFast™ Transfection Reagent (Promega Corporation)

Materials

Supplied in kit:-

TransFast™ Transfection Reagent: Stored at -20°C. Dried reagent was stable for 6 months and hydrated reagent was stable up to 2 months.

Nuclease-free water: Stored at -20°C.

Other materials:-

William’s E medium: see section 2.6.3.

Fully supplemented culture medium: see section 2.6.3.

Supplemented culture medium: see section 2.6.4
Method

The procedure was carried out according to the manufacturer's instruction. The day before transfection, the TransFast™ reagent was resuspended by adding 400μl of nuclease-free water at RT, vortexing for 10 secs. This was frozen at -20°C. The charge ratio of TransFast™ reagent to DNA used was 2:1. That required 6 μl of TransFast™ reagent for every μg of DNA. 4.1 μg of DNA was used to transfect every 60 mm culture dish. The transfection of each dish required a total volume of DNA, medium and TransFast™ reagent of 2 ml. The plasmid DNA was added to William's E medium first before the TransFast™ reagent was added and the mixture was vortexed immediately. The DNA mixture was incubated for 10 to 15 mins at RT. The existing culturing medium was removed from the cells and the cells were washed once with William's E medium. The DNA mixture was vortexed after incubation and added to the cells. The cells were incubated with the DNA in a 37°C incubator with 5% CO₂ for an hour before 4 ml of fully supplemented culture medium was gently layered onto the cells which was further incubated for 24 hrs. The cells were washed once with William's E medium and new supplemented culture medium was added. At this stage, cells that were to be treated with PB, it was added to the medium at a final concentration of 0.1 mM. The cells were cultured for an additional 24 hrs before they were lysed for the dual luciferase reporter assay as described in section 2.19.
2.17.3 Superfect™ Transfection Reagent (Qiagen Ltd)

Materials

Supplied in kit:-

3 mg/ml Superfect™ Transfection Reagent: Stored at -20°C.

Other materials:-

William’s E medium: see section 2.6.3.

Fully supplemented culture medium: see section 2.6.3.

Supplemented culture medium: see section 2.6.4

10X PBS: see section 2.7.

Method

The procedure was carried as described in the Qiagen Superfect™ Transfection Reagent Handbook. Briefly, to transfec a 60 mm culture dish, 4.1 μg plasmid DNA was diluted (the day before transfection) with William’s E medium to a total volume of 150 μl (DNA concentration should not be less than 0.1 μg/μl) and stored at 4°C. On the day of transfection, 30 μl of Superfect™ transfection reagent was added to the DNA solution and mixed by vortexing for 10 secs. The mixture was incubated for 5 to 10 mins at RT to allow complex formation. At this point, the hepatocyte cells were washed once with William’s E medium. Then, 1 ml of fully supplemented culture medium was added to the DNA complexes after incubation. The content was mixed by pipetting up and down for 2 times and the entire volume was immediately dispensed onto the cells. The cells were incubated with the
DNA complexes in a 37°C incubator with 5% CO₂ for 2 to 3 hrs. New culture medium was added to the cells after the cells had been washed once with William’s E medium and then left to incubate in the 37°C incubator with 5% CO₂ for another 24 hrs. The cells were washed once with William’s E medium and new supplemented culture medium was added. At this stage, cells that were to be treated with PB, it was added to the medium at a final concentration of 0.1 mM. The cells were cultured for an additional 24 hrs before they were lysed for the dual luciferase reporter assay (see section 2.19).

2.17.4 Effectene™ Transfection Reagent (Qiagen Ltd)

Materials

Supplied in kit:-

1 mg/ml Effectene™ Transfection Reagent: Stored at 4°C.

1 mg/ml Enhancer: Stored at 4°C.

Buffer EC: Stored at 4°C.

Other materials:-

William’s E medium: see section 2.6.3.

Fully supplemented culture medium: see section 2.6.3.

Supplemented culture medium: see section 2.6.4

10X PBS: see section 2.7.
Method

The procedure was carried out as described in the Effectene™ Transfection Reagent Handbook. Briefly, to transfec a 60 mm culture plate, 1 μg of plasmid DNA was diluted in Buffer EC to a total volume of 150 μl (DNA concentration should not be less than 0.1 μg/μl) the night before and stored at 4°C. 8 μl Enhancer was added to the DNA, the tube was vortexed briefly and incubated at RT for 2 to 5 mins. 25 μl Effectene™ Transfection Reagent was added, the tube vortexed for 10 secs and incubated at RT for 5 to 10 mins. At this stage, the existing medium on the hepatocytes were aspirated and the cells washed once in William’s E medium. 2 ml of fully supplemented culture medium was added to the cells. One ml of fully supplemented culture medium was added the DNA complex. The contents were mixed by pipetting up and down two times before they were added immediately drop-wise to the culture. The cells were incubated with the DNA mixture for 24 hrs in the 37°C incubator with 5% CO₂ before the cells were washed once with William’s E medium. Supplemented culture medium was then added either in the absence or presence of a final concentration of 0.1 mM PB and the cells were cultured for a further 24 hrs. The cells were then lysed for the dual luciferase reporter assay (see section 2.19).
2.18 In vivo gene transfer using the Helios™ Gene Gun

System (Bio-Rad)

The system includes the gene gun, the tubing prep station, tubing cutter, a special helium regulator and hose assembly.

2.18.1 Cartridge Preparation

Materials

Spermidine: 0.05 M in aqueous solution. Filter sterilised and stored at -20°C.

1.0 micron Gold microcarriers: Stored at RT.

DNA: Supercoiled plasmid DNA isolated using Qiagen Maxi kit (see section 2.2). Stored at -20°C.

Polyvinylpyrrolidone (PVP; MW 360,000): 20 mg/ml solution in absolute ethanol. Stored at -20°C. Used at 0.05 mg/ml final concentration.

Ethanol: Absolute. A newly opened container was aliquoted to sterile 50 ml tubes and stored desiccated. Use every aliquot only once after opening.

CaCl₂: 1 M in aqueous solution. Filter sterilised, aliquoted and stored at -20°C.

Equipment: Balance, sonicator (Model FS100B; Decon® Laboratory Ltd, East Sussex, England), Tefzel tubing (1/8" OD X 2/32" ID), Tubing Prep Station, Nitrogen tank and regulator, 10 ml syringe, tubing cutter, 1 ml Sorenson tubes with screw caps, desiccant pellets, cartridge storage vials.
Method

2.18.2 Precipitation of DNA onto gold microcarrier

The procedure was carried out according to the manufacturer's instructions. Briefly, 25 mg of 1 \( \mu \)m gold particles were weighed into a 1 ml Sorenson tube with screw cap and 100 \( \mu \)l of 0.05 M spermidine was added. The contents were vortexed and then sonicated for 3 to 5 secs. The plasmid DNA, up to 100 \( \mu \)g (volume should not be more than 100 \( \mu \)l), was added and the tube vortexed for 5 secs. Then, 100 \( \mu \)l of 1 M CaCl\(_2\) was added dropwise whilst the contents were vortexed on moderate speed. The tube was left for 10 mins at RT before it was centrifuged at ~13,000 rpm for 15 secs at RT in an Eppendorf microcentrifuge. The supernatant was removed and the pellet was washed 3 times with 1 ml of 100% ethanol each wash. 10 \( \mu \)l of 20 mg/ml PVP was premixed with 4 ml of 100% ethanol in a 15 ml Falcon tube. The washed DNA-coated gold microcarriers were resuspended initially in 200 \( \mu \)l of the PVP/ethanol mixture. The suspended gold microcarriers were transferred to a new 15 ml Falcon tube. The gold remaining in the 1 ml Sorenson tube was washed out with more PVP/ethanol mixture. Finally, the PVP/ethanol mixture was added to the 15 ml tube containing the DNA-coated gold microcarrier to a total volume of 3.5 ml. The suspension could be stored at -20°C at this stage sealed with Nesco film.
2.18.3 Loading DNA-coated gold microcarrier into tubing using the tubing prep station

The procedure was carried out according to the manufacturer’s instructions. Briefly, the entire Tefzel tubing was initially purged with nitrogen gas via the tubing prep station for 15 mins to ensure that the tubing was completely dry. A tubing of length ~75 cm was cut for every 3 ml of DNA-coated suspension. The suspension was vortexed and then swiftly drawn into the cut tubing using a 10 ml syringe with a rubber adapter tubing attached to the other end of the Tefzel tubing. The tubing was immediately placed into the tubing support cylinder of the prep station. The tubing was left untouched for 3 to 5 mins to allow the DNA-coated gold microcarriers to settle. The ethanol was removed from the tubing using a 10 ml syringe attached to the free end of the tubing, drawing the fluid at a fairly constant rate of 0.5 to 1.0 /sec. which usually takes about 30 to 45 sec. The tubing support cylinder holding the tubing was rotated 180° immediately upon the withdrawal of ethanol and was left for 3 to 4 sec. Then, the prep station was switched on to begin rotating the tubing support cylinder continuously to allow the gold microcarrier to smear the inner lining of the tube. After rotating for 20 to 30 sec, the valve on the flowmeter was opened to allow 0.35 to 0.4 LPM of nitrogen gas to flow into and dry the tubing while the cylinder continue to rotate. This was left for 3 to 5 mins before the prep station was switched off, followed by closing the valve on the flowmeter. The tubing could now be removed from the tubing support cylinder, cut to 0.5” cartridges using a tubing cutter and stored in a cartridge storage vial containing a
desiccant pellet. The vial was further sealed with Nesco film and stored at 4°C.

### 2.18.4 Particle delivery using Helios™ gene gun

**Materials**

**Animals:** Male Sprague-Dawley rat weighing between 230 and 250 gm.

**Ketamine (Sigma Chemical):** Stored desiccated at 4°C.

**Xylazine (Sigma Chemical):** Stored desiccated at -20°C.

**Anaesthetic solution:** Mixture of 100 mg/kg body weight of ketamine and 10 mg/kg body weight xylazine in water. Prepared fresh.

**Surgical instruments (Scientific Laboratory Supplies, unless otherwise stated):** Spatula, toothed forcep, scissors, 61/4” Gillies scissor/needle holder (Rocket of London Ltd., Watford, U.K.), Surgical blades No. 23 (Swann-Morton Ltd., Sheffield, England), Wella CONTURA Clipper (International Market Supply, Cheshire, U.K.).

**Sutures:** Sterile black Mersilk® braided silk (3/0) with round bodies 16 mm 1/2 c (RB-1) needle and sterile blue polyamide 6 monofilament non-absorbable suture (3/0) with 26 mm 3/8 c reverse cutting (FS) needle (ETHICON Ltd.).
Method

The animals was injected with 0.25 ml of the anaesthetic solution. The animal’s state of unconsciousness was checked by pedal reflex. It was then laid on its back, and the fur on the abdominal area, just below the sternum, was shaved using a CONTURA clipper. The skin layer was swabbed with 70% ethanol before a vertical slit, of 2 to 3 cm in length, was cut along the mid-ventral line using a surgical blade. The muscle layer was lifted up using a toothed forcep and a slit of similar length was cut carefully through this layer with a dissecting scissor making sure that the internal organs especially the liver were not damaged. Then, using a spatula, a lobe of the liver, usually the middle lobe, was partially brought out from the inner cavity and carefully laid on a piece of paper towel. The lobe then was ready to be shot. The Helios gene gun had to be calibrated and activated before used according to manufacturer’s instruction. Briefly, the gun should first be calibrated to the appropriate pressure for shooting. The loaded cartridge holder was then positioned in the gun and the gun was activated by pressing the cylinder advance lever. The gun could only be fired if both the safety interlock switch and the firing trigger were pressed simultaneously. To deliver the DNA-coated microcarriers, the spacer of the barrel liner was touched onto the target area on the liver with the gun perpendicular to the target. After firing the gun, there would be a 5 sec delay for charging before it may be fired again. While charging, the cartridge holder was advanced by squeezing the cylinder advance lever. The gun was now ready for the next shot. A number of shots could be made on various regions of each exposed liver depending on the size of the lobe being brought out. Usually, two shots
were made using the helium gas pressure of 250 psi. The positions of the shots were marked by making a cut at the edge of the liver lobe between the area of the shots. When all the shots were made, the liver was very carefully manoeuvred back into the abdominal cavity and the muscle layer was closed up using the 61/4" Gillies scissor/needle holder with the sterile black Mersilk\* braided silk (3/0) with round bodies 16 mm 1/2 c (RB-1) needle and the skin layer was closed using sterile blue polyamide 6 monofilament non-absorbable suture (3/0) with 26 mm 3/8 c reverse cutting (FS) needle. The animal was allowed to recover for 24 hours before being sacrificed by cervical dislocation. The targeted area on the liver could normally be visualised as a yellow patch due to the gold particles and a fixed area was excised using a steel punch. The section was quick frozen on dry ice. The assay for luciferase activity was performed as described in section 2.19.
2.19 Dual-Luciferase™ reporter assay system (Promega Corporation)

Materials

Supplied in the kit:-

5X Passive lysis buffer (PLB): Diluted to 1X working concentration with sterile distilled water. May be stored at 4°C for up to a month. Prepared sufficient amount of working solution for each experiment. Stored 5X stock solution at -20°C.

Luciferase assay buffer II: 10 ml. Stored at -20°C.

Luciferase assay substrate: Resuspend lyophilised product with 10 ml of Luciferase assay buffer II. Stored as 1 ml aliquots at -20°C. Aliquots were labelled as LAR II (luciferase assay reagent II). Before used, thawed the reagent in a water bath at RT and mixed well by gentle vortexing.

Stop & Glo® substrate solvent: 250 µl and stored at -20°C.

Stop & Glo® substrate: Dissolved content in 200 µl of Stop & Glo® substrate solvent to obtain a 50X concentrated stock solution. Stored at -20°C.

Stop & Glo® buffer.

Stop & Glo® Reagent: Dilute 5X Stop & Glo® substrate in Stop & Glo® buffer to 1X final concentration in a siliconised glass tube. Prepared fresh.

Other materials:-

10X PBS: see section 2.7.
Equipment: sterile cell scraper, siliconised 30 ml Corex tube, Platform shaker, TD-20/20 luminometer DLReady (Turner Design Instruments, CA, U.S.A.) and luminometer tubes.

Method

2.19.1 Lysis of cultured cells

For every 60 mm diameter culture dish, 800 µl of 1X PLB was added to the primary hepatocytes that was washed once with 1X PBS. The lysis of the cells were assisted by placing the cultures on a orbital shaker with moderate shaking. The cultures were shaked for 15 mins or longer at RT depending on the extent of cell lysis visualised under the microscope. The cells were further scraped from the culture plates and the lysate was transferred to an Eppendorf tube. It was then centrifuged for 30 secs at top speed in a refrigerated microcentrifuge. Lysate may be kept at -70°C for long term storage.

2.19.2 Lysis of whole tissues

Tissue obtained was flash-frozen in liquid nitrogen immediately and kept at -70°C. Without thawing the tissue, it was grinded using a pestle and mortar. Then it was transferred to a hand-held homogeniser and homogenised in 1X PLB. For every gram of tissue, 1 ml of 1X PLB was added for homogenisation. The tissue was homogenised with at least 6 strokes or more clumps of tissue could still be seen. The homogenate was transferred to an Eppendorf tube and centrifuged for 30 secs at top speed in
a refrigerated microcentrifuge. Lysate could be kept at -70°C for long term storage.

2.19.3 Dual-Luciferase Assay

100 μl of LAR II was predispensed into luminometer tube. 20 μl of lysate was added to the LAR II and content was mixed by pipetting 2 or 3 times. The tube was placed in the luminometer for the first luminescence reading. The tube was removed from the luminometer and 100 μl of 1X Stop & Glo® Reagent was added. The content was mixed by pipetting 2 or 3 times and the second luminescence reading was taken.

Note: All experiments were performed at least two times, usually three times except for the in vivo 2-AP treatment experiment in section 3.2 and the in vivo gene gun experiment as stated in the text of section 3.3.2 which were done only once.
Chapter Three

Results
And
Discussion
Section I

Analysis of the 5′-flanking sequence of CYP2B1 and CYP2B2 genes using in vitro systems
3.1.1 Sub-cloning of the CYP2B1 proximal promoter region

The first 450 bp promoter sequence of the rat CYP2B1 gene was previously isolated by Dr Andrew Elia of our laboratory (Elia, 1996) using the PCR technique. Hind III sites were introduced to both ends of the product during amplification. The product was subsequently cloned into the Hind III site of the plasmid pUC19 and this recombinant was called Clone 27. Fig. 3.1.1, lane 2 shows the restriction digestion of Clone 27 with Hind III to generate a 450 bp insert and a 2686 bp linearised pUC19 plasmid.

To carry out gel shift studies in this promoter region, the 450 bp was restriction digested to generate smaller fragments. Unique restriction enzyme sites for Xba I and Stu I are located at positions -348 and -179 bp respectively upstream of the transcription initiation site of CYP2B1 as indicated in Fig. 3.1.2. Digestion of the 450 bp fragment with these two enzymes generates three sub-fragments: -1 to -179 bp (Hind III/Stu I), -179 to -348 bp (Stu I/Xba I) and -348 to -451 bp (Xba I/Hind III) of lengths 178 bp, 169 bp and 103 bp respectively. Each of these fragments was then sub-cloned into pBluescript KSII plasmid and designated pBS(-179), pBS(-348) and pBS(-451) according to the distal position of the clone fragment with respect to the transcription initiation site. Fragments, -1 to -179 bp and -179 to -348 bp, that have a Stu I site at one end were cloned using the Sma I site of pBluescript KSII as this plasmid has no Stu I site. Thus, these two fragments could only be released from the plasmid using enzymes whose sites are either next to or close to the Sma I site on the plasmid, i.e. Bam HI and Hind III for the clone containing fragment -1 to -179 bp and Pst I and Xba
I for the clone containing fragment -179 to -348 bp. The correct clones were identified by restriction enzyme digestion and subsequent agarose gel electrophoresis as shown in Fig. 3.1.1, lanes 3 to 5.
Fig. 3.1.1 Agarose gel electrophoresis of restriction digestion of Clone 27 and subfragments of the first 450 bp promoter sequence of CYP2B1 cloned into pBluescript KSII. Lane 2: Clone 27 digested with *Hind* III; Lane 3: pBS(-179) digested with *Bam* HI and *Hind* III; Lane 4: pBS(-348) digested with *Pst* I and *Xba* I; Lane 5: pBS(-451) digested with *Hind* III and *Xba* I and Lanes 1 and 6 are 1 Kb DNA molecular weight ladder (Gibco BRL).
Fig. 3.1.2 Comparison of nucleotide sequences of the proximal promoter region between CYP2B1 (top strand) and CYP2B2 (bottom strand) genes. Differences between the two promoters are depicted either in red or by dashes (-). The position of nucleotide bases with respect to the transcription initiation site of the CYP2B1 gene are given with vertical arrows indicating the corresponding base. The positions where Stu I and Xba I cut the CYP2B1 promoter sequence for subsequent subcloning are also shown. The CYP2B1 and CYP2B2 promoter sequences were taken from (Shephard, et al., 1994) and (Elia, 1996) respectively.
3.1.2 Multiple proteins including members of the C/EBP transcription factor family bind to the CYP2B1 proximal promoter sequence

Hoffmann et al (1992), using an in vitro transcription assay, determined that the first 177 bp of the 5'-flanking sequence of the CYP2B2 gene is the minimal promoter region (Hoffmann, et al., 1992). Sommer et al arrived at a similar conclusion for both the CYP2B1 and CYP2B2 genes when they transfected HepG2 and COS-1 cells with CAT reporter constructs containing progressive 5'-deletions of either CYP2B1 or CYP2B2 5'-flanking sequences (Sommer, et al., 1996). Studies previously carried out in our laboratory on the CYP2B2 promoter showed that the sequence between -31 and -177 bp could bind more protein from liver nuclear extracts of PB-treated than untreated rats in gel shift assays. Competitive gel shift assays refined the region responsible for the difference in abundance of protein binding to -31 and -72 bp (Shephard, et al., 1994). Computer analysis of the shorter fragment revealed a potential binding site for C/EBP at -44 to -67 bp (Shephard, et al., 1994, Dell, 1997). Sequence comparison of this putative binding site with the C/EBP consensus sequence showed that 6 out of 8 bases matches exactly (Fig. 3.1.3). Supershift assays with antibodies specific to different members of the C/EBP protein family revealed that C/EBPα and β were involved in binding to the putative site (Dell, 1997).

Initial studies were done mainly on the CYP2B2 promoter because the CYP2B1 5'-flanking sequence was not available. As mentioned in
**CYP2B2 promoter**

-67 bp ACATCTGAAGTTGCATAACTGAGT

C/EBP Consensus

TGCAGATTGCGCAATCTGCA

Fig. 3.1.3 Sequence comparison of the CYP2B promoters and the C/EBP consensus binding site. (Top) Sequence of the CYP2B2 promoter region between -44 and -67 bp which contains a putative C/EBP binding site. This region of the promoter is identical between CYP2B1 and CYP2B2. The numbers indicate the position of the nucleotides with respect to the transcription initiation site. (Bottom) The sequence of the consensus binding site for the C/EBP family of transcription factors taken from Santa Cruz Biotech, Inc. Vertical lines indicate bases of the CYP2B2 promoter that match with the C/EBP consensus binding site exactly while mismatches are shown in red.
section 3.1.1, our laboratory had obtained the first 450 bp 5'-flanking sequences of the CYP2B1 gene. This was digested into three smaller fragments which were subcloned into pBluescript plasmid. One of these clones, pBS(-179), contains the CYP2B1 5'-flanking sequences from -1 to -179 bp and gel shift analysis was carried out on this sequence. Although sequence comparison on the first 177 bp 5'-flanking sequence of the CYP2B1 and CYP2B2 showed that they are highly similar (see Fig. 3.1.2), it was still interesting to see if results obtained with the CYP2B2 promoter were also observed with the CYP2B1 promoter.

Fig. 3.1.4 shows the gel shift assay of the -1 to -179 bp sequence with various amounts of liver nuclear protein from either untreated or PB-treated rats. Several protein-DNA complexes were observed to bind to this sequence. At least seven protein-DNA complexes, designated as complex 'a' to 'g', were discernible. Complexes ‘a’, 'b', 'c' and 'e' appeared to bind proteins that were either in greater abundance or activated in liver nuclear extracts from PB-treated rats while the reverse seemed to apply for complexes ‘g’ and ‘h’. These complexes were interactions of protein with DNA because the addition of 5 μg of proteinase K to the binding reaction abolished the formation of all the complexes (Fig. 3.1.4, lane 8). These protein-DNA interactions were also specific because the addition of a 100-fold molar excess of cold self probe could compete for all the complexes as shown in Fig. 3.1.5, lane 3.

Competitive gel shift assay with a 100-fold molar excess of the CYP2B2 -44 to -67 bp sequence as a competitor could compete for some of the complexes as shown in Fig. 3.1.5, lane 4. However, a 100-fold molar
Fig. 3.1.4 Gel shift analysis of the CYP2B1 promoter sequence from -1 to -179 bp. Various amounts of liver nuclear proteins from either untreated (U; lanes 1, 3 and 5) or PB-treated rats were used (lanes 2, 4 and 6). The reaction in lane 7 was further treated with 5 μg of Proteinase K at 37°C for 10 mins. Lane 8 contains no nuclear protein.
Fig. 3.1.5 Competitive gel shift analysis of the CYP2B1 promoter sequence between -1 to -179 bp. 5 μg of liver nuclear protein from either untreated (U) or PB-treated rats was used. Unlabelled self probe (lane 3), CYP2B2 -44 to -67 bp (lane 4) and an oligonucleotide to the C/EBP consensus sequence (lane 5) were added as competitor DNA at a 100-fold molar excess with respect to the labelled probe. Lane 6 contains no nuclear protein.
excess of an oligonucleotide to the C/EBP consensus sequence did not compete for protein binding as efficiently as the -44 to -67 bp sequence (Fig. 3.1.5, lane 5). The remaining complexes that were not competed by the CYP2B2 -44 to -67 bp sequence could be proteins binding to other regions on the -1 to -179 bp fragment. There are several indications that sequences outside -44 to -67 bp do bind protein. A footprinting analysis of the -1 to -179 bp sequence showed a footprint not only at -44 to -72 bp but also at -117 to -136 bp and -138 to -146 bp (Fig. 3.1.6). Shephard et al (1994) (Shephard, et al., 1994) previously reported that three protein-DNA complexes were observed when the CYP2B2 promoter sequence between -86 and -177 bp was incubated with liver nuclear extracts. Since then, several laboratories have also reported that regions between -116 and -143 bp (Park and Kemper, 1996, Sommer, et al., 1996) and -153 to -158 bp (Sommer, et al., 1996) of both the CYP2B1 and CYP2B2 promoters were protected in DNase I footprinting assays.

Fig. 3.1.7 shows the supershift assay of -1 to -179 bp sequence and liver nuclear extracts with specific antibodies to C/EBPα, β and δ. Supershift was observed with the antibody to C/EBPβ only (lane 4). No obvious supershift was observed with the antibody to C/EBPα, apart from complex 'b' which appears to be slightly less visible. Subsequently, other laboratories confirmed the binding of both C/EBPα and β to the proximal promoter regions of both CYP2B1 and CYP2B2 genes with supershift assays (Park and Kemper, 1996) and trans-activation assays whereby CYP2B promoter constructs were co-transfected with either C/EBPα or β expression vectors.
Fig 3.1.6 DNase I footprint analysis of the template strand of CYP2B1 promoter between -1 and -179 bp. The fragment was incubated with either 10 (lanes 3 and 4) or 15 μg (lanes 5 and 6) of liver nuclear protein from untreated (U) rats before digestion with either 0.5 (lanes 3 and 5) or 0.8 (lanes 4 and 6) units of DNase I. The Maxam and Gilbert (G+A) sequencing reaction of the fragment is shown in lane 1. The vertical line indicates the regions of footprint.
Fig. 3.1.7 Supershift analysis of the CYP2B1 promoter sequence between -1 and -179 bp. 5 µg of liver nuclear proteins from either untreated (U) or PB-treated rats were used. 1 µl of antibody to each of the different C/EBP family members was added in lane 3 to 5. Lane 6 contains no nuclear protein. The arrow on the right depicts the supershifted band.
(Luc, et al., 1996, Park and Kemper, 1996). The supershift obtained with the C/EBPα antibody was observed not to be very clear even when the sequence between -1 and -110 bp of the CYP2B1 promoter was utilised in supershift assay. Hence, the inability to observe a supershift complex with the antibody to C/EBPα in the -1 to -179 bp sequence could be due to the presence of multiple protein-DNA complexes which might have obscured the supershift signal. All other reports of a supershift with the C/EBPα antibody used DNA fragments which encompassed the putative C/EBP binding site that were shorter than 178 bp used in this study as the probe in their supershift assays.

The results obtained using both gel shift and supershift analysis on the -1 to -179 bp sequence agreed with the results reported by several laboratories, including ours, indicating that members of the C/EBP transcription factor family bind to the CYP2B1 and CYP2B2 proximal promoter. While the identities and significance of proteins binding to the other two regions of this sequence as observed in the footprint analysis will require further investigations.
3.1.3 CYP2B1 promoter sequence between -179 and -348 bp
binds more nuclear protein from rats treated with PB

The -179 to -348 bp fragment, of the CYP2B1 promoter, from Clone 27 was subcloned as described in section 3.1.1, and then analysed for protein binding using gel shift assays.

When gel shift assays were performed on this sequence, using nuclear extracts from either untreated or PB-treated rats, most of the DNA probe was retained in the well of the gel except for a single complex of a relatively high molecular weight as shown in Fig. 3.1.8. Nuclear extract from the livers of PB-treated rats formed a complex which was of greater abundance than that with extracts from untreated rats (see Fig. 3.1.8; lane 1 and 2). The treatment of the binding reaction with 5 μg proteinase K abolished complex formation which indicated that the complex was a DNA-protein interaction (lane 3). The complex formed was specific as it could be completely competed by the addition of a 100-fold molar excess of cold self-probe (Fig. 3.1.9; lane 3).

To refine the region within the -179 to -348 bp sequence which is involved in this high molecular weight complex formation, competitive gel shift assays were carried out using DNA fragments that spanned different regions within this sequence (Fig. 3.1.9). Fragments -177 to -219 bp (lane 5), -179 to -245 bp (lane 7) and -207 to -272 bp (lane 4) were not able to compete completely at 100-fold molar excess while a similar fold molar excess of the fragments -179 to -318 bp (lane 8), -245 to -348 bp (lane 6) and -272 to
**Fig. 3.1.8** Gel shift analysis of the *CYP2B1* promoter sequence between -179 and -348 bp. 5 μg of liver nuclear proteins from untreated (U; lane 1) and PB-treated (lane 2) rats were used. After the binding reaction was carried out, proteinase K was added with further incubation at 37°C for 10 mins (lane 3). Lane 4 contained no nuclear protein.
Fig. 3.1.9 Competitive gel shift analysis of the CYP2B1 promoter sequence between -179 and -348 bp. 5 µg of liver nuclear protein from either untreated (U; lane 1) or PB-treated (lanes 2 to 9) rats were used. Competitor DNAs were added at 100-fold molar excess and lane 10 contained no nuclear protein.
-348 bp (lane 9) were able to compete (see Fig. 3.1.10). Therefore, the region that is involved in protein binding seems to lie between -272 and -318 bp.

As a gel shift assay was not performed using the sequence between -272 and -318 bp as a probe, no clear conclusion could be drawn as to whether the sequences within this region are sufficient to bind protein and give rise to the difference in the abundance of the protein bound between untreated and PB-treated animals.
Fig. 3.1.10 Schematic diagram showing different subfragments that were either able or not able to compete for protein(s) binding to the CYP2B1 promoter sequence between -179 and -348 bp. ■ shows the entire -179 to -348 bp sequence. Subfragments that are able to compete are shown as ■, those that are unable to compete are shown as ■. The numerals depict the positions of the fragments with respect to the transcription initiation site.
3.1.4 CYP2B1 5'-flanking sequence between -348 and -451 bp also binds more nuclear protein from rats treated with PB

The -348 to -451 bp fragment, of the CYP2B1 promoter, from Clone 27 was subcloned as described in section 3.1.1, and then analysed for protein binding using gel shift assays.

Fig. 3.1.11 showed the binding using different amounts of liver nuclear protein with the CYP2B1 5'-flanking sequence between -348 and -451 bp. Multiple protein-DNA complexes (at least 4) could be clearly observed as indicated by the arrows in Fig. 3.1.11 and they were designated 'a' to 'd'. Treatment of the binding reaction with 5 µg of proteinase K abolished the formation of all complexes as shown in Fig. 3.1.11, lane 7 which meant that the complexes were indeed protein-DNA interactions. However, the addition of cold self probe up to 300 fold molar excess only partially competed for protein binding to the radiolabeled probe as shown in Fig. 3.1.12, lanes 2 to 4. To be more precise, only complexes 'a' and 'b' were competed whilst complex 'd' appeared to increase in intensity. The inability of excess cold self probe to compete for all the complexes formed still cannot be explained at present.

Furthermore, complex 'a' was formed only with liver nuclear extract from PB-treated rats. This suggested the appearance of a protein in the nucleus of PB-treated rats that is otherwise absent from the nucleus of untreated animals. Recently, a study done in chick embryos indicated that PB affects the expression of a large number of genes in the chick liver (about 50 or more) and of the 27 genes identified, 16 were up-regulated in
Fig. 3.1.11 Gel shift analysis of the CYP2B1 5'-flanking sequence between -348 and -451 bp. Various amounts of liver nuclear protein from either untreated (U; lanes 1, 3 and 5) or PB-treated (lanes 2, 4, and 6) rats were used. Binding reaction in lane 7 was further digested with 5 µg of proteinase K and lane 8 contained no nuclear protein.
Fig. 3.1.12 Competitive gel shift analysis of the CYP2B1 5'-flanking sequence between -348 and -451 bp. Binding assay was performed using 5 µg of liver nuclear extracts from PB-treated rats. Lanes 2 to 10 indicate the different competitor DNAs used.
their expression (Frueh, et al., 1997). Roe et al (1996) (Roe, et al., 1996) reported that PB enhanced the binding of the transcription factor, AP1 to its cognate DNA sequence. Thus, the expression of the protein involved in the formation of complex 'a' might well be induced by PB. Besides acting at the level of transcription, PB has also been shown to act at the post-translational level. PB has been reported to stimulate the phosphorylation of acidic nuclear proteins in vivo (Blankenship and Bresnick, 1974). And Baffet et al (Baffet and Corcos, 1996) also observed a transient increased in the phosphorylation of a 34 kDa nuclear protein in primary rat hepatocytes as well as in the liver of whole animals treated with PB. Therefore, could the protein in complex 'a' be expressed in both untreated and PB-treated rats and PB treatment somehow alter the conformation of this protein which enables it to bind DNA? However, it is unclear at this stage if PB directly or indirectly alters the binding of the protein in complex 'a'.

The observation of an additional complex (i.e. complex 'a') in gel shift assays with liver nuclear extracts from rats treated with PB is intriguing and prompted further investigations. The first thing to do would be to refine the region within the -348 to -451 bp sequence that is involved in the formation of complex 'a'. This sequence was initially put through a transcription factor binding site database known as TESS which searches for potential binding sites. The search showed numerous potential transcription factor binding sites of which a few seemed to be worth examining. They were sites for the transcription factors such as GLI at -354 to -361 bp, Octamer at -384 to -391 bp, Vitamin D receptor (VDR) at -424 to -438 bp, glucocorticoid receptor (GR), prolactin receptor (PR) and androgen receptor (AR) at -424 to -429 bp
and Hepatocyte nuclear factor 1 (HNF-1) and 3 (HNF-3) at -421 to -431 bp as shown in Fig. 3.1.13. Oligonucleotides to regions -347 to -368 bp, -379 to -404 bp and -419 to -444 bp which covered all the potential binding sites mentioned above were synthesised. In addition, the -348 to -451 bp was also digested with a selection of restriction enzymes as shown in Fig. 3.1.13 to generate a series of smaller fragments. This would allow the analysis of regions outside those of the oligonucleotides. Both the sub-fragments and the oligonucleotides were then used as competitors in gel shift assays as shown in Fig. 3.1.12, lanes 5 to 10 and Fig. 3.1.14, lanes 2 to 4 respectively. In Fig. 3.1.12, fragments -348 to -379 bp (lane 6), -348 to -404 bp (lane 7) and -404 to -451 bp (lane 11) were not able to compete for protein involved in the formation of complex 'a' while fragments -379 to -451 bp (lanes 8 and 9) and -394 to -449 bp (lane 10) could compete for binding. Oligonucleotide sequences from -347 to -368 bp (lane 3) and -419 to -444 bp (lane 4) were not able to compete off complex 'a' even at 350-fold molar excess while oligonucleotide -379 to -404 bp (lane 2) was observed to compete complex 'a' slightly when added at a 150-fold molar excess in Fig. 3.1.14. The competitive gel shift analysis suggested that sequences upstream of -379 bp are important and the sequences, -379 to -404 bp, around the putative octamer consensus binding site, was not sufficient.

Thus, several more oligonucleotides of different lengths, spanning the region between -379 and -428 bp were synthesised and then used as competitors in gel shift assays as shown in Fig. 3.1.14, lanes 5 to 10. Oligonucleotides coding for regions -379 to -428 bp, -370 to -414 bp and -379 to -414 bp compete for protein binding and abolished complex 'a'
Fig. 3.1.13 Potential transcription factors binding sites within the CYP2B1 5'-flanking region between -348 and -451 bp. Open boxes highlight the regions where oligonucleotides were synthesised. Transcription factors consensus sequences are in ■ and the abbreviation in brackets indicates the respective factors that bind. Red letters highlight the mismatch nucleotides from that of the consensus sequences. Vertical arrows pin-point the sites where restriction enzymes would cut. The numerals indicate the positions of the sequence with respect to the transcription initiation site.
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</tr>
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**Fig. 3.1.14** Competitive gel shift analysis of the CYP2B1 5'-flanking sequence between -348 and -451 bp. 5 µg of liver nuclear proteins from PB-treated rats were used. Oligonucleotides synthesised to different regions within the radiolabeled probe were added as competitors at various fold molar excess.
formation while the region -370 to -404 bp could not compete. The results obtained for all the competitive gel shift assays carried out on -348 to -451 bp are summarised schematically in Fig. 3.1.15 and the minimum sequence that is required to completely abolish complex ‘a’ appears to be localised between -379 and -414 bp. Only the putative octamer binding site is contained within this sequence. It is not clear if an octamer protein is involved. But the other potential binding sites do not appear to be involved. Fragments that were able to compete for protein binding were further analysed and the results presented in section 3.1.7.
Fig. 3.1.15 Schematic diagram showing different subfragments that were either able or not able to compete for protein(s) binding to the CYP2B1 promoter sequence between -348 and -451 bp. ■ shows the entire -348 to -451 bp sequence. Subfragments that are able to compete are shown as ■, those that are unable to compete are shown as ■. While the fragment indicated as ■ could only compete partially. The numerals depict the positions of the fragments with respect to the transcription initiation site.
3.1.5 DNase I footprinting analysis of the CYP2B1 proximal promoter region

As mentioned in sections 3.1.3 and 3.1.4, a greater abundance of nuclear protein from the livers of PB-treated than untreated rats bound to the CYP2B1 promoter from -179 to -348 bp and -348 to -451 bp in gel shift assays (see figs. 3.1.8 and 3.1.11 for the respective results). It is thus of interest to locate the specific bases within these two regions which bind transcription factors and which result in the difference in the abundance of liver nuclear protein binding from untreated and PB-treated animals. This was to be achieved using a DNase I footprinting assay.

Initially, footprint analysis was carried out on the region spanning between -179 and -451 bp as this covered the two fragments that were used for the gel shift assays mentioned above. Analysis was carried out on both strands of this sequence. To analyse the template strand, the 272 bp sequence was excised from Clone 27 (see section 3.1.1) using HindIII and StuI restriction enzymes. It could then be radiolabeled at the -451 bp end, which was cut by HindIII. For radiolabelling at the -179 bp end for analysis of the sense strand, the HindIII and StuI digested fragment had to be subcloned. It was cloned into the HindIII and SmaI sites of the pBluescript KSII plasmid. It was then excised using SpeI and ApaI restriction enzymes and radiolabelling at the end cut with SpeI would allow analysis of the sense strand.

Analysis performed on the sense strand did not reveal any distinct footprint with liver nuclear extracts from either untreated or PB-treated
*Because a similar experiment was not performed with poly dI.dC in the absence of nuclear protein, thus it is not confirmed at this stage if the potential footprints observed were due to binding of nuclear proteins.*
animals (Fig. 3.1.16). To rule out the possibility that the lack of a footprint(s) was not due to insufficient amount of nuclear protein resulting in the presence of excess unbound radiolabeled probe in the reaction, more protein was used (Fig. 3.1.16, lane 5 and 6). The addition of more nuclear protein did not reveal any footprint either. However, a slight reduction in the intensity of all the bands along the entire lane was observed. There was also a DNase I hypersensitivity site (HS) located at position -328 bp which was more susceptible to DNase I digestion with increasing amount of nuclear protein. With respect to lane 2 of Fig. 3.1.16, where no nuclear protein was added to the reaction, there were several regions of the DNA spanning approximately 10 bases in length which did not seem to be cleaved by DNase I. They were located at -181 to -191 bp, -215 to -224 bp, -247 to -257 bp, -278 to -288 bp and -292 to -300 bp as indicated in Fig. 3.1.16.

Fig. 3.1.17a shows the analysis of the template strand of the -179 to -451 bp sequence. Five micrograms of liver nuclear protein from either untreated (lane 3) or PB-treated (lane 4) rats were used and no obvious footprint was detected but hypersensitivity sites were observed at location -294 bp (HS1) and -281 bp (HS2). Nucleotides in the regions from -412 to -396 bp, -335 to -327 bp and -294 to -281 bp were found to be resistant to DNase I digestion. The amount of non-specific competitor DNA, polydI.dC was also varied. And it was observed that upon the addition of more non-specific competitor, the nucleotide at position -294 bp became more sensitive to DNase I and possible footprints may be seen from -412 to -396 bp and -335 to -294 bp (Fig. 3.1.17b; lane 2 to 4).*
Fig. 3.1.16 DNase I footprint analysis of the sense strand of the CYP2B1 promoter between -179 and -451 bp. DNA was incubated with either 5 or 7.5 µg of liver nuclear proteins from untreated (U; lane 3 and 6) or PB-treated (lane 4 and 7) rats. The Maxam and Gilbert (G+A) sequencing reaction of the fragment is in lane 1. HS depicts DNase I hypersensitivity site. Vertical lines indicate the regions of the DNA that appear to be resistant to DNase I digestion and their positions with respect to the transcription start site are shown.
Fig. 3.1.17 DNase I footprint analysis of the template strand of the CYP2B1 promoter between -179 and -451 bp. (a) DNA was incubated with liver nuclear protein from either untreated (U; lane 3,5 and 6) or PB-treated (lane 5) rats and then digested with 1 unit of DNase I. Lane 1 is the Maxam and Gilbert (G+ A) sequencing reaction of the fragment. HS depicts DNase I hypersensitivity sites. Dotted lines indicate regions of the DNA that seem difficult to be digested by DNase I. (b) DNA was incubated with 5 µg of liver nuclear protein from untreated rats in the presence of various amount of poly dl.dC before subjected to DNase I digestion (1 unit). Vertical lines indicate regions of possible footprints.
Footprint analysis was also carried out on the sense strand of a shorter DNA sequence between -348 and -451 bp (Fig. 3.1.18) because the result with the fragment, -179 to -451 bp could not separate the DNA sequences upstream of -328 bp very well when the sense strand was studied (Fig. 3.1.16). No distinct footprint was observed with 10 µg of liver nuclear extracts from either untreated (Fig. 3.1.18, lane 3) or PB-treated (lane 4) rats. When 50 µg of liver nuclear extracts (lane 5 and 6) were added, the intensity of all the bands along the entire lane were greatly reduced but there was still no footprint. However, there were regions in this DNA sequence that appeared to be resistant to DNase I activity: -432 bp to -424 bp, -423 to -439 bp which were more visible in Fig. 3.1.21 than Fig. 3.1.18.

The inability to produce any distinct footprint with the sequences analysed above could be due to problems with the technique. Thus, the technique was challenged by first carrying out the assay using the DNA sequence and nuclear protein that were provided with the footprinting kit. Following the assay conditions given, a footprint was observed as expected on the EBNA-1 (Epstein-Barr virus Nuclear Antigen-1) DNA sequence (Fig. 3.1.19). Analysis was also carried out on another region of the CYP2B1 promoter between -1 and -179 bp using the same liver nuclear extract from untreated rats as for the analysis of the CYP2B1 promoter sequences mentioned above. 10 to 15 µg of nuclear protein was sufficient for the formation of three distinct footprints (see Fig. 3.1.20). One of the footprints was observed between -44 and -72 bp, the same region to footprint assays done in other laboratories (Luc, et al., 1996, Park and Kemper, 1996,
Fig. 3.1.18 DNase I footprint analysis of the sense strand of the CYP2B1 promoter between -348 and -451 bp. DNA was incubated with 10 and 50 μg of liver nuclear protein from either untreated (U; lanes 3 and 5) or PB-treated (lanes 4 and 6) rats. The Maxam and Gilbert (G+A) sequencing reaction of the fragment is in lane 1.
Fig. 3.1.19 DNase I footprint analysis of the EBNA-1 sequence. DNA was incubated with 2 μg of EBNA-1 extract and then subjected to varying amounts of DNase I (lanes 3 to 5). Lane 1 shows the Maxam and Gilbert G+A sequencing reaction of the fragment. Lane 2 contains no protein. The vertical line indicates the region of the footprint.
Probe CYP2B1 -1 to -179 bp

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<td>DNase I (units)</td>
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Fig 3.1.20 DNase I footprint analysis of the template strand of CYP2B1 promoter between -1 and -179 bp. The fragment was incubated with either 10 (lanes 3 and 4) or 15 µg (lanes 5 and 6) of liver nuclear protein from untreated (U) rats before digestion with either 0.5 (lanes 3 and 5) or 0.8 (lanes 4 and 6) units of DNase I. The Maxam and Gilbert (G+A) sequencing reaction of the fragment is shown in lane 1. The vertical line indicates the regions of footprint.
Sommer, et al., 1996), including ours (Shephard, et al., 1994). This region was shown to be bound by the transcription factor, C/EBP (see section 3.1.2). The other two footprints were observed at -117 to -136 bp and -138 to -146 bp.

There is at present no clear explanation as to why no distinct footprint was observed when the sequence between -179 and -451 bp of the CYP2B1 promoter was analysed. The possibility of problems with the technique could be ruled out since positive results were obtained with other DNA sequences analysed as shown in Fig. 3.1.19 and 3.1.20. However, gel shift assays performed on the CYP2B1 promoter sequence between -348 and -451 bp in section 3.1.10 showed that pre-treatment of liver nuclear proteins from both untreated and PB-treated rats with MgCl$_2$ before binding reaction decreased the complex formation of the high MW band (Fig. 3.1.33). It was realised subsequently that most divalent ions, such as Mn$^{2+}$ and Ca$^{2+}$ also had similar effects on the binding of protein(s) to this DNA sequence. It was noted that DNase I activity requires the presence of both Mg$^{2+}$ and Ca$^{2+}$. It requires 1 mM of Mg$^{2+}$ and 0.5 mM of Ca$^{2+}$ for optimal activity. According to gel shift assays in section 3.1.10, as little as 1 mM of MgCl$_2$ alone already had some effect on protein binding. Therefore, footprinting assays were performed using a lower Mg$^{2+}$ and Ca$^{2+}$ concentration. The instruction booklet that accompanied the footprinting kit indicated that the Mg$^{2+}$/Ca$^{2+}$ concentrations may be up to 5 times less than that for optimal DNase I activity as mentioned above without compromising on the activity of DNase I too much. Thus, a 3-fold and a 5-fold dilution of the Mg$^{2+}$ and Ca$^{2+}$ were used and the footprinting result is shown in Fig. 3.1.21 (lanes 3 and 4). The
Fig. 3.1.21 DNase I footprint analysis of the sense strand of the CYP2B1 promoter between -348 and -451 bp using varying concentrations of Ca\(^{2+}\) and Mg\(^{2+}\). Lanes 2 to 4 are DNA incubated with 20 \(\mu\)g of liver nuclear protein from PB-treated rats which were then subjected to DNase I digestion at various concentrations of Ca\(^{2+}\)/Mg\(^{2+}\). Lanes 7 to 10 were DNA incubated with 20 or 30 \(\mu\)g of liver nuclear proteins from either untreated (U) or PB-treated rats and were digested with DNase I in the presence of 0.2 mM Mg\(^{2+}\) and 0.1 mM Ca\(^{2+}\). The Maxam and Gilbert (G+A) sequence reaction is in lane 5.
use of a lower concentration of Mg$^{2+}$ and Ca$^{2+}$ also did not reveal any distinct footprint but the intensity of bands along the entire lane were marginally reduced. This may probably be due to DNase I working at a sub-optimal condition. Another assay was further performed on 20 and 30 µg of liver nuclear protein from both untreated and PB-treated rats using 5-fold dilution of Mg$^{2+}$ and Ca$^{2+}$ (Fig. 3.1.21; lanes 7 to 10). This gel was autoradiographed for a longer period of time in order to achieve darker bands. Unfortunately, decreasing Mg$^{2+}$ and Ca$^{2+}$ concentrations and increasing the amount of nuclear protein still did not generate any footprint. A 5-fold dilution of the Mg$^{2+}$ and Ca$^{2+}$ would bring their final concentrations down to 0.2 mM and 0.1 mM respectively. Gel shift assays had shown that Mg$^{2+}$ concentration at 0.2 mM did not affect the binding reaction but the effect of both Mg$^{2+}$ and Ca$^{2+}$ present together in binding assay was not assessed. However, it is highly unlikely that such low concentration of divalent ions would be able to disrupt protein binding.

Another more reasonable explanation for the lack of footprint could be due to the nature of DNA-protein binding. Multiple bands were observed when gel shift assays were performed on the sequence between -348 and -451 bp (Fig. 3.1.11 of section 3.1.4) and a large proportion of the DNA probe of -179 to -348 bp was retained in the wells in gel shift (Fig. 3.1.8 of section 3.1.3). This usually indicates the binding of multiple proteins to the DNA sequence. And when several proteins are involved in binding to a target DNA fragment, that DNA is not the best candidate for footprint assays. This seems the most probable reason for the failure of the footprint assays performed.
3.1.6 Yin Yang-1 can bind to the CYP2B1 5'-flanking sequence between -348 and -451 bp

Once the region between -379 to -414 bp was determined to be the minimum sequence required to compete for protein binding with the -348 to -451 bp sequence in gel shift assays (section 3.1.4), it was studied more carefully to find out what transcription factors do bind to this sequence. A closer look revealed a potential binding site for the Yin Yang-1 (YY1) transcription factor at -400 to -410 bp and the comparison of this sequence with a YY1 consensus sequence and a YY1 oligonucleotide sequence from Santa Cruz Biotechnology is shown in Fig. 3.1.22.

Although YY1 was reported to be widely expressed (Shrivastava and Calame, 1994), it was not clear from the literature if it is expressed in the liver. Therefore, a gel shift assay was carried out using Santa Cruz YY1 oligonucleotide as the probe with liver nuclear extracts as in Fig. 3.1.23. YY1 was shown not only to be expressed in the liver, it could also bind to the CYP2B1 5'-flanking sequence between -348 to -451 bp as shown in Fig. 3.1.23; lanes 3 and 4. The addition of a 100-fold molar excess of the -348 to -451 bp completely abolished the binding of any protein to the YY1 oligonucleotide. When the YY1 consensus binding site was used as a competitor DNA in the gel shift assay with the -348 to -451 bp sequence as the radiolabeled probe, only complex 'd' was abolished (Fig. 3.1.24; lanes 3 and 4). Therefore, the formation of complex 'd' is probably due to the binding of the transcription factor, YY1 to the -348 to -451 bp sequence.
YY1 Oligo
CGCTCCGGCCATCTTGGCGGCTGGT

5'  | | | | | 3'

CYP2B1 promoter
-410 bp CCTCCATAATTAAAGA -400 bp

| | | | |

YY1 repressor consensus
CCATNTTNNA

YY1 activator consensus
CGGCCATCTTGNCCTG

Fig. 3.1.22 Sequence comparison of the CYP2B1 5'-flanking sequence with YY1 consensus binding sites. (Top) The sequence of the YY1 oligonucleotide taken from Santa Cruz Biotech, Inc. followed by the CYP2B1 5'-flanking sequence between -400 and -410 bp. The bottom two sequences are the YY1 repressor and activator consensus sequences (Shrivastava and Calame, 1994). The bases of the CYP2B1 5'-flanking sequence which match exactly with either the YY1 oligo or the YY1 repressor consensus site are indicated by vertical lines and green letters depict bases that match with the YY1 activator consensus site.
Fig. 3.1.23 Gel shift analysis of the YY1 consensus oligonucleotide sequence. 5 μg of liver nuclear proteins from either untreated (U; lanes 1 and 3) or PB-treated (lanes 2 and 4) rats were used. A 100-fold molar excess of the CYP2B1 5'-flanking sequence between -348 and -451 bp was added as a competitor DNA in lanes 3 and 4. Lane 5 contains no nuclear protein.
Fig. 3.1.24 Competitive gel shift assay of the CYP2B1 5'-flanking sequence from (a) -348 to -451 bp and (b) -379 to -428 bp with YY1 oligonucleotide sequence. 5 μg of liver nuclear extracts from PB-treated rats were used. YY1 oligonucleotide from Santa Cruz Biotech was added as a competitor DNA at either 300 or 100-fold molar excess. Arrows indicate the complex that was formed due to the binding of YY1 protein to the probes.
To further determine if YY1 was indeed binding to the putative binding site as mentioned earlier, a gel shift assay was performed using a smaller sequence between -379 and -428 bp as the probe and then adding the YY1 oligonucleotide as a competitor DNA. Again, the formation of one of the complexes was abolished as indicated by the arrow in Fig. 3.1.23, lane 6 which confirmed that YY1 was indeed binding to the putative YY1 binding site identified between -400 and -410 bp.

YY1 is a zinc finger protein belonging to the GLI-Kruppel family (Shi, et al., 1991). YY1 is a multifunctional factor which can activate transcription, repress transcription or initiate transcription of many different cellular and viral genes indicating YY1 as an important transcription regulator ((Shrivastava and Calame, 1994) and references therein). It is observed to interact with numerous transcription factors including SP1, c-Myc, p300, TAFII 55, ATF/CREB, TFIIB and histone deacetylase HDA2/mRPD3 ((Shrivastava and Calame, 1994, Galvagni, et al., 1998) and references therein). The interaction of YY1 with these factors can either activate transcription in a synergistic manner (i.e. SP1) or initiate transcription (i.e. TFIIB and TAFII 55). There has been a general observation that YY1 acts as an activator in genes that are expressed ubiquitously and acts as a repressor in highly regulated genes that are mostly expressed in a tissue-specific manner (Shrivastava and Calame, 1994). Apparently, in genes where YY1 represses transcription, YY1 sites often overlap or occur near an activator-binding site and the increased expression of the activator relieves YY1-dependent repression ((Shrivastava and Calame, 1994) and references therein; (Galvagni, et al., 1998)).

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Although YY1 has been determined to bind to a putative YY1 site within the -348 to -451 bp sequence of the CYP2B1 promoter, YY1 does not appear to be in greater abundance in the nuclear extracts of PB-treated rats and it does not appear to be involved in formation of complex 'a' in gel shift assay where -348 to -451 bp was used as the probe. A comparison of the CYP2B1 putative YY1 binding site with the consensus site compiled by Shrivastava and Calame (1994) (Shrivastava and Calame, 1994) showed the putative YY1 binding site to be more identical to the YY1 repressor consensus site (Fig. 3.1.22). There is no evidence at present to support this speculation. It seems as though the expression of YY1 is not affected by PB treatment. However, it is not known at this stage if YY1 is involved in the expression of CYP2B1 gene transcription in vivo, in particular basal expression. It would be interesting to determine if YY1 plays a role in vivo and what function, if any, it plays in CYP2B1 gene transcription.
3.1.7 CYP2B1 5'-flanking sequence from -379 to -414 bp is not as efficient in binding nuclear protein from PB-treated rats as the -348 to -451 bp sequence

The -379 to -414 bp sequence of the CYP2B1 gene was deduced to be the minimum sequence required to compete for protein binding to the -348 to -451 bp as analysed previously in gel shift assays (section 3.1.4). However, a difference in nuclear protein binding between untreated and PB-treated rats to the latter sequence was not seen when the -379 to -414 bp was used as the probe in gel shift assays (Fig. 3.1.25, lanes 9 and 10).

It was suspected that sequences flanking the -379 to -414 bp region might be necessary. DNA sequences spanning different regions within the -348 to -451 bp had been used as competitor DNA in gel shift assays with the -348 to -451 bp as the probe (see Figs. 3.1.12 and 3.1.14 in section 3.1.4). DNA fragments spanning sequence between -370 and -451 bp were then radiolabeled to use as probes in gel shift assays as shown in Fig. 3.1.25. Fragments, -379 to -428 bp (lanes 5 and 6) and -379 to -451 bp (lanes 1 and 2) could bind more liver nuclear protein from PB-treated than untreated rats indicating that sequences upstream of -414 bp are important. But neither sequences upstream or downstream of -414 bp were capable of resulting in a difference in the abundance of protein binding between nuclear extracts from untreated and PB-treated rats. In fact, the -394 to -449 bp sequence which efficiently competed for complex ‘a’ as shown in Fig. 3.1.12 of section 3.1.4 only showed a very faint high molecular weight complex (indicated by the arrow in Fig. 3.1.25) when it was used as a probe.
Fig. 3.1.25 Gel shift analysis using different regions within the CYP2B1 5'-flanking sequence between -348 and -451 bp as probes. 5 µg of liver nuclear proteins from either untreated (U; lanes 1, 3, 5, 7, 9, 11 and 13) or PB-treated (lanes 2, 4, 6, 8, 10, 12 and 14) rats were used. The free probe is absent from the gel shift of lanes 7 and 8. The arrow on the right indicates the complex that is referred to as the high molecular weight complex in the text.
in the gel shift assay (lanes 3 and 4). On the other hand, sequences between -370 and -414 bp (lanes 7 to 14) generated very similar gel shift banding patterns and a distinct high molecular weight complex (indicated by the arrow in Fig. 3.1.25) could be observed.

These results put together suggested there might be a core binding site situated within -379 to -404 bp. And the protein binding to this site could recruit another protein that binds to an adjacent site upstream. According to the gel shift analysis of the -394 to -449 bp, the latter protein is unable to bind to DNA independently and somehow requires interaction with protein binding to the deduced core binding site. The requirement for cooperativity in transcription factors binding has been reported and one classic example would be the “Ternary Complex Formation” whereby the serum response factor (SRF) can interact and recruit factors such as TCFs which cannot bind DNA by themselves (Treisman, 1994). Apart from this hypothesis, there is still no clue with regards to the identity of any of the proteins involved in the complex formed with the -348 to -451 bp fragment.
3.1.8 The CYP2B1 promoter regions from -179 to -348 bp and -348 to -451 bp could cross-compete each other in gel shift assay using rat liver nuclear extracts

As shown in the previous two sections, the CYP2B1 promoter regions from -179 to -348 bp (section 3.1.3) and -348 to -451 bp (section 3.1.4) bind more liver nuclear protein from PB-treated than untreated rats.

It was also observed that the -348 to -451 bp sequence, when added as a competitor, was able to abolish complex formation in gel shift assays when the -179 to -348 bp was used as a probe (Fig. 3.1.26, lane 3). This competition was specific as another region of the CYP2B1 promoter between -1 and -179 bp was unable to compete when added as a competitor (Fig. 3.1.26, lane 4). When the -179 to -348 bp sequence was added as a competitor, it could abolish the formation of complex ‘a’ in a gel shift assay where -348 to -451 bp was the probe as shown in 8, lane 3. Just like the self competitor, the -179 to -348 bp fragment only competed the formation of complex ‘a’ as shown by the arrow in Fig. 3.1.27. In section 3.1.3, it has been deduced that the region within -179 to -348 bp that is involved in protein binding most probably lies between -272 and -318 bp. When DNA fragments that correspond to different regions within the -179 to -348 bp were used as competitors in gel shift assay of the -348 to -451 bp sequence, it was observed that those competitor DNAs that encompass the region between -272 and -318 bp were able to compete (Fig. 3.1.27; lanes 5 to 7) while competitor fragments that lie outside that region could not (Fig. 3.1.27; lane 4). Of all the competitor DNA
Fig. 3.1.26 Competitive gel shift analysis of the CYP2B1 promoter sequence between -179 and -348 bp. 5 μg of liver nuclear protein from either untreated (U; lane 1) or PB-treated (lanes 2 to 5) rats were used. Competitor DNAs were added at 100-fold molar excess.

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<td>Lane 5</td>
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<tr>
<td>-348/-451</td>
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<td>-1/-179</td>
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- U: Untreated
- PB: PB-treated
- self: Self-competition DNA

DNA concentrations: 5 pg of DNA were used.
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<tr>
<th>Probe</th>
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Fig. 3.1.27 Competitive gel shift analysis of the CYP2B1 promoter between -348 and -451 bp. 5 μg of liver nuclear extract from PB-treated rats were used. Competitor DNAs were added at 100-fold molar excess.
subfragments that could compete, the -179 to -318 bp was observed to abolish the formation of a number of other complexes apart from complex 'a' and an additional low molecular weight complex was formed (Fig. 3.1.27, lane 6). It is not clear at present as to why the sequence from -179 to -318 bp was able to compete off more complexes than the longer -179 to -348 bp sequence. However, competitive gel shift assays performed with this DNA subfragment indicated that the region involved in binding more protein from liver nuclear extracts of PB-treated rats in -179 to -348 bp (i.e. -272 to -318 bp) is responsible for competing the protein involved in formation of complex 'a' in gel shift where -348 to -451 bp was used as the probe. It is interesting to note here that the binding of the protein involved in formation of complex 'a' is also enhanced when liver nuclear extracts from PB-treated rats were used. Again, the sequence between -1 and -179 bp was unable to compete when added as a competitor (Fig. 3.1.27, lane 8).

Because the CYP2B1 promoter regions from -179 to -348 bp and -348 to -451 bp could compete with each other for the protein involved in the formation of the high molecular weight complex in each case, it is highly probable that the two regions are bound by the same protein(s). However, a sequence comparison of the two regions shows that they are highly dissimilar. There is no evidence at this stage to rule out the possibility of the same protein binding to the two regions. The inability to find any consensus binding site, even remotely, could suggest a second possibility that the two regions be bound by different proteins. Possibly these sequences compete for protein binding because they bind different proteins that interact via protein-protein interactions. At what stage, protein-protein
interaction occurs is not known but perhaps the first protein has bound to DNA as in Fig. 3.1.28a or the two proteins may have already established interaction with each other before even binding DNA as in Fig. 3.1.28b.

It was noticed that it is the largest complex that was competed by both sequences. The formation of a high molecular weight complex can imply a number of possibilities. The DNA is bound by a single large size protein, more than one protein is involved or the protein binding can induce DNA bending leading to a complex of slow mobility. All these scenarios are possible and they may not be mutually exclusive. It would be interesting to determine if the same protein or different proteins actually binds to each of the two sequences.
Fig. 3.1.28a Schematic diagram of a possible scenario whereby the CYP2B1 promoter from -179 to -348 bp and -348 to -451 bp could cross compete with each other for nuclear protein binding in gel shift assays. ‘X’ and ‘Y’ represent different proteins present in a crude liver nuclear extract. Double stranded DNA sequences (probe and competitor) are shown as double lines. The * indicates radiolabeled DNA probes. The -179 to -348 bp sequence is in blue and the -348 to -451 bp sequence is in black. The arrows indicate the order in which events might occur.
Fig. 3.1.28b Schematic diagram of a possible scenario whereby the CYP2B1 promoter from -179 to -348 bp and -348 to -451 bp could cross compete with each other for nuclear protein binding in gel shift assays. 'X' and 'Y' represent different proteins present in a crude liver nuclear extract. Double stranded DNA sequences (probe and competitor) are shown as double lines. The * indicates radiolabeled DNA probes. The -179 to -348 bp sequence is in blue and the -348 to -451 bp is in black. The arrows indicate the order in which events might occur.
3.1.9 Excessive homogenisation of liver tissue from untreated rats can alter the abundance of nuclear protein binding to -348 and -451 bp 5'-flanking sequence of the CYP2B1 gene

The isolation of nuclear protein from rat liver was performed using the sucrose homogenisation method as described in section 2.8.1 which involves an initial homogenisation step of the liver tissue in sucrose homogenisation buffer. When gel shift assays were performed on the -348 to -451 bp sequence, one of the complexes (complex ‘a’) was observed to form only with liver nuclear extracts from PB-treated rats and all results and discussions that follow will be with reference to this particular protein.

When different batches of nuclear proteins extractions were studied, the results obtained were not always reproducible. In some of the batches, a similar complex to complex ‘a’ could be observed with liver nuclear extracts from untreated rats. And the abundance of the nuclear protein from untreated rats as compared to PB-treated rats seemed to vary among different batches of nuclear protein preparations. This lack of reproducibility could not be explained at the time. However, it was noticed that the increase in nuclear protein binding from untreated rats seemed to occur at the same period a decision was made to homogenise the liver tissue for a longer time, i.e. until the tissue was completely homogenised with no visible clumps. This was carried out in an effort to increase the yield of liver nuclei.

To test if prolonged homogenisation was the cause of the problem, the homogenisation step of the nuclear protein extraction was closely
monitored whereby not more than 4 strokes (2 ups and 2 downs) were performed regardless of complete homogenisation as in the case of liver nuclear proteins batch no. 4. The gel shift showed that complex 'a' was absent from nuclear extract of untreated animals as observed in the majority of the previous batches (Fig. 3.1.29; lanes 7 and 8). Hence, excessive homogenisation of the liver tissue seemed to mimic the effects of PB. It was evident from in vitro treatment experiments of liver nuclear proteins as discussed in section 3.1.10, that the ability of the protein to bind to the -348 to -451 bp sequence is dependent on the phosphorylation status of the protein. It can bind when phosphorylated and could not bind when dephosphorylated. Thus, in those batches where complex ‘a’ was observed to form when liver nuclear extracts from untreated rats were used in binding assays, the protein appears to be in the phosphorylated form which renders it able to bind to DNA.

How does prolonged homogenisation of the tissue lead to protein phosphorylation? One possibility could be the generation of heat. The process of homogenisation can produce a certain amount of heat which is why the entire nuclear protein preparation was performed at 4°C. Prolonged homogenisation might therefore have resulted in the generation of more heat, raising the temperature of the homogenate. Although the change in temperature may not be drastic, it could still have an effect on the cells. This might have activated a signalling cascade leading to the phosphorylation of the protein concerned. An example of a protein affected by heat is heat-shock factor (HSF). HSF binds to DNA only when cells are heat treated (Morimoto, 1993). Homogenisation could also possibly cause stress...
**Fig. 3.1.29** Gel shift analysis of the CYP2B1 5'-flanking sequence between -348 and -451 bp with different batches of liver nuclear extracts. 5 μg of liver nuclear extracts from either untreated (U; lanes 1, 3, 5 and 7) of PB-treated (lanes 2, 4, 6 and 8) rats from four different batches of nuclear protein isolations were used.

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to the cells by the shearing apart of the cells through the use of mechanical forces. Furthermore, cell lysis could also lead to the release of radicals and oxidants which cause cells to undergo oxidative stress. There are examples of numerous kinases such as the stress-activated protein kinases (SAPKs) (Cohen, 1997), the ROS-sensitive tyrosine kinases (i.e. Src and syk) and even transcription factors like NF-κB which can be activated in cells in response to such stress signals. The fact that the response take place vary rapidly (in a matter of minutes), it is also possible that the phosphorylation of the protein leads to its enhance translocation from the cytosol to the nucleus. A number of studies have shown that transcription factors themselves can be selectively re-located from the cytoplasm to the nucleus in response to extracellular stimuli (Hunter and Karin, 1992). For example, the transcription factor, FNIL-6, which binds to the serum response element in the c-fos promoter in response to cAMP, is normally situated in the cytoplasm of untreated cells. Upon stimulation of the cells, FNIL-6 becomes phosphorylated and was then found to be predominantly in the nucleus (Metz and Ziff, 1991). Apart from transcription factors, protein kinases themselves, such as PKA catalytic subunit, Rsk protein kinase, MAP/ERK protein kinases can also be translocated into the nucleus in response to stimulus (review (Hunter and Karin, 1992) and reference therein). However, the translocation of a protein kinase is unlikely in this case due to an observation made in section 3.1.11.

Seeing that the CYP2B1 promoter between -179 and -348 also binds more protein from extracts of PB-treated rats. And this sequence was also shown to cross-compete with the -348 to -451 bp for protein binding. It
would be interesting to find out if the same occurs to the protein binding to the -179 and -348 bp sequence.
3.1.10 Phosphorylation enhances and dephosphorylation inhibits liver nuclear protein binding to the CYP2B1 5'-flanking sequence from -348 to -451 bp

The PB induction of CYP2B subfamily mRNA has been demonstrated by several laboratories to involve signal transduction pathways that are sensitive to inhibitors of protein kinases or protein phosphatases. The induced expression of mRNA encoded by the rat CYP2B1 and CYP2B2, the mouse cyp2b10 and the chicken CYP2H1 and CYP2H2 by PB were reported to be suppressed in primary hepatocyte cultures, of the appropriate species, that had been treated with either kinase (Sidhu and Omiecinski, 1995, Dogra and May, 1996) or phosphatase (Sidhu and Omiecinski, 1997, Honkakoski and Negishi, 1998a) inhibitors. An influence of PB treatment on kinase and phosphatase activities was shown by the transient increase in the phosphorylation of a 34 kDa nuclear protein in PB-treated rats and primary rat hepatocyte cultures (Baffet and Corcos, 1996). PB has also been reported to stimulate the phosphorylation of acidic nuclear proteins in vivo (Blankenship and Bresnick, 1974). Nirodi et al (1996) (Nirodi, et al., 1996) reported the phosphorylation of a nuclear protein, from PB-treated rats, binding to the rat CYP2B1 promoter sequence (-69 to -98 bp). In this present investigation, when liver nuclear proteins from PB-treated rats were observed to bind more to the -348 to -451 bp sequence of the CYP2B1 gene than proteins from untreated rats (section 3.1.4), it seemed probable that this difference was due to post-translational modifications, such as phosphorylation and dephosphorylation of the nuclear protein.
To investigate this possibility, liver nuclear extracts from both untreated and PB-treated rats were treated with calf intestinal alkaline phosphatase (CIP) and ATP. The general and non-specific action of CIP makes it a good choice for use. However, there is no kinase that phosphorylates protein in a general and non-specific manner. As protein kinase and phosphatase activities are known to be retained in nuclear protein preparations, ATP was added as a phosphate source for endogenous kinases. The treatments were initially performed according to that described in Kwast-Welfeld et al (1993) (Kwast-Welfeld, et al., 1993). Fig. 3.1.30 shows the gel shift assay of nuclear extracts that have been pre-treated with either 10 units of CIP (lanes 5 and 6) or 1 mM of ATP (lanes 7 and 8) in the presence of 4 mM MgCl₂. Both treatment groups resulted in partial inhibition in the formation of complex ‘a’. However, the protein-DNA complex was noticed to migrate slower when nuclear extracts were pre-treated with ATP (lanes 7 and 8). Because MgCl₂ had been included in the nuclear protein treatments carried out so far, it was necessary to find out if MgCl₂ alone could affect the binding of the protein to DNA. Interestingly, the pre-treatment of nuclear extracts with MgCl₂ alone partially inhibited complex ‘a’ formation, and its effects appeared to be more profound in the extract of PB-treated rats Fig. 3.1.30, lanes 3 and 4. The effect of MgCl₂ was not understood but the possibility of Mg²⁺ ions interacting with the phosphate backbone of the DNA, thus obstructing protein-DNA interactions has not been ruled out.

Having observed that MgCl₂ alone could inhibit protein-DNA interaction, the effects seen previously in nuclear extracts pre-treated with CIP and ATP in the presence of MgCl₂ might be due solely to MgCl₂. Thus,
Fig. 3.1.30 The effects of different chemicals on liver nuclear protein binding to the CYP2B1 5'-flanking sequence between -348 and -451 bp. 10 μg of liver nuclear extract from either untreated (U) or PB-treated rats were used. Nuclear protein from lanes 3 to 14 had been treated with various chemicals prior to gel shift assay. The numeral that precedes the chemical name in lanes 13 and 14 refers to the sequence in which the chemicals were added while chemicals used in lanes 5 to 8 were added together. (MgCl$_2$, magnesium chloride; ATP, adenosine triphosphate; CIP, calf intestinal alkaline phosphatase).
another set of treatments with CIP and ATP in the absence of MgCl₂ as shown in Fig. 3.1.30; lanes 11 and 12 and lanes 9 and 10 respectively was carried out. CIP alone could partially inhibit complex ‘a’ formation while ATP was seen to enhance the protein binding to DNA. The migration of complex ‘a’ from nuclear extract treated with ATP was still observed to be retarded. Although there is no explanation for the retardation, results obtained so far highly suggest that phosphorylation and dephosphorylation by ATP and CIP respectively of the nuclear protein altered its DNA binding affinity. This prediction was confirmed when liver nuclear extract was first treated with ATP followed by CIP (lanes 13 and 14). The initial phosphorylation of nuclear proteins by endogenous kinase when ATP was added were dephosphorylated by the subsequent addition of CIP, generating a gel shift banding pattern similar to that of CIP treatment alone.

To further verify that ATP was indeed acting as a phosphate source for endogenous kinase in the nuclear extracts, another compound, ATP-γ-S was used. ATP-γ-S has a very similar chemical structure to ATP except for the replacement of the oxygen atom at the terminal phosphoric residue by a sulphur atom thus it acts as an ATP analogue which can substitute for ATP in various kinase reactions. However, the thiophosphorylated protein becomes resistant to subsequent dephosphorylation by protein phosphatases. ATP-γ-S did not mimic the effect of ATP as initially predicted. On the contrary, the formation of complex ‘a’ was completely abolished with the addition of 0.48 mM ATP-γ-S (Fig. 3.1.31; lanes 3 and 4). The subsequent addition of ATP to nuclear extracts that had already been treated
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Fig. 3.1.31 The effects of different chemicals on liver nuclear protein binding to the CYP2B1 5'-flanking sequence between -348 and -451 bp. 10 µg of liver nuclear extract from either untreated (U; lanes 1, 3, 5, 7 and 9) or PB-treated (lanes 2, 4, 6, 8 and 10) rats were used. Prior to the binding reaction, nuclear extracts were incubated for 20 mins at 37°C either in the absence (lanes 1 and 2) or in the presence of chemicals. The numeral that precedes the chemical name refers to the sequence in which the chemicals were added to the extracts. (ATP-γ-S, adenosine-5'-O-(3-thiotriphosphate; ATP, adenosine triphosphate)
with ATP-γ-S could not restore the formation of complex ‘a’ indicating the thiophosphate group that had been transferred onto the proteins was indeed resistance to dephosphorylation, ATP added later could no longer acts as a source of substrate. When the sequence of the addition of ATP and ATP-γ-S was reversed (lanes 7 and 8), a small amount of protein binding still remained, presumably left over from protein that was phosphorylated with ATP. The inability of the thiophosphorylated protein to bind DNA may be due to a change in the conformation of the protein such that it no longer can bind DNA. Auto-phosphorylation could be another possible reason for this observation. The transfer by the kinase of the phosphate group from ATP-γ-S to itself may have caused it to be inactivated.

A range of phosphatase inhibitors were used to pretreat the nuclear protein in an effort to elucidate the particular phosphatase involved. A cocktail of general, non-specific phosphatase inhibitors consisting of sodium fluoride, β-glycerophosphate and sodium orthovanadate as described by Grenfell et al (1996) (Grenfell, et al., 1996) was tried initially and gel shift results in Fig. 3.1.32, lane 3 showed that the inhibition of phosphatase activities enhanced protein binding mimicking the results of ATP treatment. However, when each of these inhibitors was added separately, sodium fluoride (lane 5) slightly inhibited protein binding, β-glycerophosphate (lane 6) did not have any effect and sodium orthovanadate (lane 4) enhanced protein binding. Sodium fluoride and β-glycerophosphate are known to inhibit serine and threonine phosphatase, type 1, 2A, 2B and 2C families. Sodium orthovanadate is normally utilised as a broad spectrum
**Probe**

<table>
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*Fig. 3.1.32 The effects of protein kinase and phosphatase inhibitors on the CYP2B1 promoter between -348 and -451 bp. 10 μg of liver nuclear extracts from PB-treated rats were used. Prior to binding reactions, nuclear extracts were incubated for 20 mins at 37°C either in the absence (lane 1) or in the presence of various chemicals. *The phosphatase inhibitor cocktail used in lane 3 consists of 50 mM NaF, 1 mM β-glycerophosphate and 1 mM Na<sub>3</sub>VO<sub>4</sub>. The concentrations of all other chemicals are given in the table. (ATP, adenosine triphosphate; Na<sub>3</sub>VO<sub>4</sub>, sodium orthovanadate; NaF, sodium fluoride; β-GP, β-glycerophosphate; Cyper, cypermethrin; OK, okadaic acid; Tauto, tautomycin; DeP, dephostatin; 2-AP, 2-aminopurine)*
* (Seargeant and Stinson, 1979, Boyd, et al., 1985)
protein tyrosine phosphatase inhibitor, although it is also known to inhibit acid and alkaline phosphatases and specific kinases such as adenylate kinase and phosphofructokinase. The opposing results seen with sodium fluoride and β-glycerophosphate was very puzzling since they both affect similar families of protein phosphatase. Thus, a range of more specific phosphatase inhibitors were used: cypermethrin is a potent inhibitor of the PP2B family, OK inhibits PP1/2A but is more potent for PP2A, tautomycin also inhibits PP1/2A and dephostatin is a protein tyrosine phosphatase inhibitor. As shown in Fig. 3.1.32, lanes 7 to 11, no effect on protein binding was observed with any of the specific phosphatase inhibitors tested. As the treatment of nuclear proteins with dephostatin did not cause an effect, the involvement of a protein tyrosine phosphatase seemed unlikely at this stage. Thus, the enhanced protein binding observed with nuclear extracts that were treated with sodium orthovanadate might be due to the other inhibitory effects of the chemical and not due to the inhibition of tyrosine phosphatases. Unable to elucidate the specific phosphatase involved, a kinase inhibitor, 2-aminopurine (2-AP) was tested. 2-AP had been shown to suppress PB increased CYP2H gene expression in chicken primary hepatocyte cultures (Dogra and May, 1996) and CYP2B gene expression in the rat both in vivo and in vitro (Nirodi, et al., 1996). 2-AP appeared to significantly inhibit protein binding (Fig. 3.1.32, lane 12) suggesting a 2-AP sensitive protein kinase to play a role either directly or indirectly in phosphorylating the protein binding in complex ‘a’. At this stage, no specific protein kinase or phosphatase had been pin-pointed. However, the slight retardation of complex ‘a’ with nuclear extract treated with ATP suggested that the complex
had become larger. Could phosphorylation events lead to the recruitment of yet another protein to the complex? More than one protein binding in complex ‘a’ had been suspected, and it is not clear at present whether the gel shift results observed are due to the phosphorylation and dephosphorylation of a single protein.

The idea that Mg\(^{2+}\) ions interacting with the DNA phosphate backbone disrupted protein-DNA interactions non-specifically was shown to be unlikely. This was because nuclear protein binding to the CYP2B2 between -183 and -199 bp was not affected by treatment with MgCl\(_2\) (data not shown). To determine if MgCl\(_2\) is the only compound that can cause such an effect, a range of monovalent and divalent salts were added to the nuclear protein prior to gel shift assays as in Fig. 3.1.33. Lithium chloride and sodium acetate did not affect protein binding while different magnesium salts affected binding to varying extents in the order of inhibition of MgSO\(_4\) > MgCl\(_2\) > Mg(OAc)\(_2\). Manganese chloride was observed to inhibit to a similar extent as magnesium sulphate and calcium chloride could completely abolish protein binding. The conclusion drawn from this gel shift was that divalent cations appeared to inhibit protein binding more than monovalent cations. It is known that Mg\(^{2+}\) can act as a cofactor in enzyme reactions and Mn\(^{2+}\) can act as an activator of various enzymes, it is a possibility that their presence might have stimulated the activities of certain protein kinases and phosphatases activities.

The treatment of nuclear protein already bound to DNA revealed some interesting findings. In Fig. 3.1.34, the treatment of protein pre-bound to DNA with MgCl\(_2\) (lane 2) or CIP (lane 3) appeared to have little effect on the
Fig. 3.1.33 The effects of different salts on liver nuclear protein binding to CYP2B1 5'-flanking sequence between -348 and -451 bp. 10 µg of liver nuclear extract from untreated (U) rats were used. Prior to binding reaction, nuclear extracts were incubated for 20 mins at 37°C either in the absence (lane 1) or in the presence of different salts. All salts were added at 4 mM final concentration except MnCl₂ which was added at 5 mM. (Mg(OAc)₂, magnesium acetate; NaOAc, sodium acetate; MgCl₂, magnesium chloride; MgSO₄, magnesium sulphate; LiCl, lithium chloride; MnCl₂, manganese chloride; CaCl₂, calcium chloride)
Fig. 3.1.34 The effects of chemicals on liver nuclear protein already bound to the CYP2B1 5'-flanking sequence between -348 and -451 bp. 10 μg of liver nuclear extract from PB-treated rats were used. The binding reaction was carried out first before the reaction mixture was incubated at 37°C for 20 mins in the absence (-) or presence of chemicals. The free probe had electrophoresed out of the gel. (MgCl₂, magnesium chloride; CIP, calf intestinal alkaline phosphatase; ATP, adenosine triphosphate)
binding of the protein while ATP (lane 4) resulted in a slight retardation of complex ‘a’ as observed with binding of ATP pre-treated nuclear extracts (Fig. 3.1.30, lanes 9 and 10). This result suggests that phosphorylation of protein is possible even when the protein is bound to DNA but dephosphorylation can not occur in the same situation. It is not clear at this stage if the protein kinase and phosphatase are actually acting on two different sites of the same protein target or acting on different proteins. If the protein kinase and phosphatase are acting on different proteins then it might explain the inconclusive results obtained with the protein kinase and phosphatase inhibitors. Furthermore, if MgCl$_2$ is indeed activating the activity of certain enzymes, it appears possible that it may be a phosphatase since MgCl$_2$ is also unable to affect the protein binding activity of the protein involved in complex ‘a’ once it has bound to DNA just like in the case of CIP (fig 3.1.34; lane 2).
3.1.11 Nuclear protein binding to the CYP2B1 5'-flanking sequence between -348 and -451 bp with rat liver protein appears to be lost in primary rat hepatocyte cultures

Many studies have shown that the induction of CYP2B mRNA by PB is suppressed by chemicals that are either inhibitors of protein kinase (Dogra and May, 1996, Nirodi, et al., 1996) or phosphatase (Nirodi, et al., 1996, Sidhu and Omiecinski, 1997, Honkakoski and Negishi, 1998a) as well as activators of protein kinase (Sidhu and Omiecinski, 1995). The difference in the abundance of liver nuclear protein binding from either untreated or PB-treated rats to the -348 to -451 bp sequence of the CYP2B1 gene (see section 3.1.4) could depend on whether the protein is phosphorylated or dephosphorylated (see section 3.1.10). Treatment experiments with the chemicals mentioned above are easier to carry out using in vitro systems such as primary rat hepatocyte cultures. Also, performing in vitro rather than in vivo treatment experiments rules out the influence of other factors such as hormones. And the results obtained relate directly to the specific cell-type under investigation.

Due to limited starting material from primary hepatocyte cultures, the nuclear protein extraction method used for whole liver tissue is not suitable and an alternative nuclear protein extraction method had to be devised. The majority of the nuclear extract isolation methods from cultured cells involves two lysis steps, lysing of the plasma membrane followed by lysing of the nuclear membrane. The method described by Rosette and Karin (1995) (Rosette and Karin, 1995) was used first on a hepatoma cell line, FAZA 967.
to determine if this method was suitable for small quantities of cells. Nuclear protein was extracted from FAZA 967 cells cultured on three different tissue culture plastics (i.e. Permanox®, Nunc™ and Falcon). The Permanox® plate was coated with Vitrogen to allow the cells to grow in an environment similar to that of primary hepatocyte cells. The gel shift results of FAZA 967 cell nuclear extracts, from the three different conditions, with the -348 to -451 bp sequence are shown in Fig. 3.1.35; lanes 2 to 5. Although protein-DNA complexes were observed with FAZA 967 nuclear extract, 20 µg of FAZA 967 extracts showed significantly less protein binding than 10 µg of liver nuclear extracts (lane 1). This was most probably due to cytosolic protein contamination which is inevitable with this protocol and results in the underestimation of the nuclear protein content.

The Rosette and Karin extraction method was then used to isolate nuclear proteins from primary rat hepatocyte cultures that were untreated or PB-treated. According to the gel shift results in Fig. 3.1.36; lanes 3 and 4, the protein involved in complex ‘a’ and ‘b’ appear to be lost and an extra low molecular weight complex was observed. The nuclear proteins were unlikely to be degraded because complex ‘d’ was observed in the gel shift with all samples. From section 3.1.6, it has been shown that the transcription factor YY1 is involved in the formation of complex ‘d’ and apparently, YY1 is prone to proteolytic degradation. Furthermore, distinct protein bands could be visualised on an SDS-polyacrylamide gel as in Fig. 3.1.37. An additional very low molecular weight complex could be observed as depicted by the arrow on the right of Fig. 3.1.36; lanes 3 and 4. As the protein involved in complex
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Fig. 3.1.35 Gel shift analysis of the CYP2B1 5'-flanking sequence between -348 and -451 bp with FAZA 967 cell lines. Nuclear proteins from either the liver of untreated (U) rats and untreated (U) or PB-treated FAZA 967 cell line were used. FAZA 967 cells were grown on different kinds of culture plastic: vitrogen-coated Permanox® plate (lane 2) as that use in primary hepatocyte cultures, Nunc™ (lanes 3 and 4) or Falcon (lane 5).
Fig. 3.1.36 Gel shift analysis of nuclear protein isolated from primary rat hepatocytes on the CYP2B1 5'-flanking sequence between -348 and -451 bp. 5 µg of nuclear extract from either untreated (U) or PB-treated rat livers (lanes 1 and 2) and primary rat hepatocyte cultures (lanes 3 to 6) were used. The nuclear proteins used in lanes 5 and 6 had sodium orthovanadate included in all the solutions used in the nuclear protein extraction. (* The batch of liver nuclear extract whereby there is no difference in protein binding between untreated and PB-treated rats was used in this case. See section 3.1.9 for detail).
Fig. 3.1.37 SDS-polyacrylamide gel electrophoresis of nuclear extracts isolated using different methods. (a) Extraction from liver tissues using the method by Sierra, (1990) and (b) from primary hepatocyte cells cultured for 48 hrs using the method by Rosette and Karin, (1995) which was either untreated (U; lanes 2 and 5) or PB-treated (lanes 3 and 6). Lanes 1 and 4 are mid-range protein molecular weight markers range between 14.4 and 97.4 kDa from Promega Corporation.
'a' was known not to bind DNA when dephosphorylated, the loss of complex 'a' in the gel shift might be due to the activation of protein phosphatases during the process of nuclear protein extraction. From section 3.1.10, sodium orthovanadate, a protein phosphatase inhibitor was able to inhibit the specific protein phosphatase that dephosphorylates the protein binding in complex 'a'. Thus sodium orthovanadate was included in all the solutions used in the extraction protocol, to inhibit the action of phosphatases from the moment the cells were lysed. However, the problem did not appear to be due to the activation of protein phosphatases as complex 'a' was still not observed (Fig. 3.1.36; lanes 5 and 6) in nuclear protein extracted in the presence of sodium orthovanadate.

There was some concern regarding the storage of the nuclear proteins in the high salt buffer (420 mM NaCl) used in this extraction method as described in section 2.8.2. An experiment was set up such that 5 μg of liver nuclear protein isolated from rat livers were added into 200 μl of high salt buffer and left overnight at -70°C before it was concentrated through a microconcentrator. A set of nuclear extracts from primary hepatocyte cells was also concentrated. Gel shift assays in Fig. 3.1.38 confirmed that prolonged storage in the high salt buffer did not affect the ability of the protein to bind DNA (lanes 3 and 4). And the concentration of nuclear extracts from primary hepatocyte cells did not seem to make any difference (lanes 5 and 6).

SDS-polyacrylamide gel electrophoresis of the nuclear proteins isolated using the method for hepatocytes cells and liver tissues showed different population of proteins (Fig. 3.1.37). Therefore, two other nuclear
Fig. 3.1.38 Gel shift analysis of the CYP2B1 5'-flanking sequence between -348 and -451 bp. 5 μg of nuclear extracts from PB-treated rat livers were used in lane 1. In lane 2, 5 μg of liver nuclear extract from PB-treated rat was added to 200 μl of high salt buffer and 5 μl of the concentrated fraction was used. 5 μl of concentrated nuclear protein from primary rat hepatocyte cultures were added in lane 3.
extraction methods (Andrew and Faller, 1991, Jost and Saluz, 1991) were tested. The nuclear extracts obtained were used in gel shift assays not only with the -348 to -451 bp sequence but also with DNA sequences from other regions of either the CYP2B1 or CYP2B2 5'-flanking regions and the result is shown in Fig. 3.1.39. This gel shift indicated that nuclear protein isolated using the protocol of Rosette and Karin generated gel shift results most resembling that of nuclear protein from rat livers, despite the difference in the abundance of different transcription factors (lanes 6 and 9).

Another very possible reason for the loss of the protein binding to the -348 to -451 bp region could be due to the loss of gene expression during culture. The hepatocyte cells harvested for nuclear protein isolation had been in culture for 48 hours. The expression of many genes, including transcription factors, are known to reduce continuously from the moment the liver is perfused and throughout the entire culturing period (Padgham, et al., 1993). To find out if the protein binding to the -348 to -451 bp sequence was one of the transcription factors whose mRNA declines in culture, nuclear proteins were extracted from cells harvested at different time intervals ranging from just before culture and during culture. From Fig. 3.1.40, it was surprising to notice that the protein of interest was absent from the moment the hepatocytes were isolated (i.e. 0 hr), even before being plated. A profile of the expression pattern of a number of transcription factors was investigated by Penny Smirlis in our laboratory with the use of oligonucleotides containing transcription factor consensus binding sites. NFκB was observed to be highly induced early in culture and the expression then slowly declined. AP1 was also activated early in culture and its level
Fig. 3.1.39 Comparison of three methods of isolating nuclear protein from primary rat hepatocytes. Gel shift analysis of the CYP2B 5'-flanking sequences. 5 μg of liver nuclear extracts from PB-treated rat were used in lanes 1, 5 and 8. Nuclear proteins were extracted from primary hepatocytes using 3 different methods designated 1, 2 and 3. Protocol 1 is the method by Rosette and Karin, (1995), protocol 2 is by Andrew and Faller, (1991) and protocol 3 was obtained from Jost and Saluz, (1991).
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<th>Probe</th>
<th>CYP2B1 -348 to -451 bp</th>
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<tr>
<td>Source</td>
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<td></td>
<td>Primary Hepatocyte</td>
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<td>Treatment</td>
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<td>Trolox</td>
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<td>Time (hrs)</td>
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<td>Lane</td>
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Fig. 3.1.40 The effect of trolox on nuclear protein from primary rat hepatocytes at various time-points upon seeding. 10 µg of nuclear extracts from either untreated rat livers (lane 1) or primary rat hepatocyte cultures were used. Hepatocyte cells harvested at various time points with respect to the time when the cells were isolated. In lanes 6 to 10, 300 µM of trolox was included in all buffers used in perfusion and media used in culturing.
remained relatively stable throughout culture. On the other hand, SP1 and SRE were more highly expressed before culture and the expression level dropped to near zero during culture. The expression of C/EBP and octamer were low both before and during culture (Smirlis, P., personal communication). The study into the expression pattern of a handful of transcription factors already indicates that changes occur rapidly once the liver is perfused. The cells undergo stress from very early on as the expression of NFkB, a factor associated with stress, was observed to be induced very early in culture. In fact, NFkB induction was known to occur as early as 20 mins upon the perfusion of the liver (Paine, A., unpublished results). Thus, could the exposure of cells to stress have led to the rapid loss of the protein binding to the -348 to -451 bp sequence. To test out this possibility, 300 μM trolox (an antioxidant and a vitamin E derivative), was included in all the buffers and medium used in the perfusion of the rat liver and the culturing of the hepatocytes. Still, the gel shift result (Fig. 3.1.40; lanes 6 to 10) was similar to that of cells that had not been exposed to trolox (lanes 2 to 5).

All the experiments performed so far had indicated that the loss of the protein as assessed by gel shift assays is probably not be due to degradation, the activation of sodium orthovanadate-sensitive protein phosphatases by or the hepatocytes response to stress. To completely rule out the possibility that the loss of the protein under investigation is due to the extraction method, the Rosette and Karin method was used to isolate nuclear proteins from liver tissues. Surprising, complex 'a' was not observed in a gel shift
performed with liver nuclear protein isolated using the Rosette and Karin method (Fig. 3.1.41, lanes 1 and 2). As mentioned earlier, the contamination of the nuclear protein fraction with cytosolic proteins was unavoidable when extracting nuclear protein using this method. Hence, it is possible that the nuclear protein binding in complex ‘a’ was interacting with some cytosolic factor and was inhibited from binding DNA. There are examples of numerous transcription factors which are present in an inactive form (i.e. interacting with other factors) in the cytosol. Upon stimulation, the transcription factor then becomes dissociated from the accessory factor, translocates to the nucleus and activates gene expression. Two typical examples would be NFκB and the glucocorticoid receptor (GR). In the cytosol, NFκB is kept in an inactive form due to its binding to IκB. This complex dissociates upon the phosphorylation of IκB which then allows NFκB to translocate to the nucleus to bind to the promoter of genes containing the NFκB response element and activate transcription of these genes (Schmitz and Baeuerle, 1995, Schreck, et al., 1997). GR is also known to be kept in an inactive state by its association with heat-shock protein 90 (hsp90) in the cytosol ((Sanchez, et al., 1985, Baniaahmad, et al., 1997) and references therein). The binding of glucocorticoid hormone to the GR dissociates the complex from hsp90 which then translocates to the nucleus to activate transcription via glucocorticoid receptor response element (GRE). To determine if there is really a factor in the cytosol that could interact with the protein under investigation, nuclear proteins isolated from liver tissues using the Sierra method should have been added to the
Fig. 3.1.41 Gel shift analysis of the CYP2B1 5'-flanking sequence between -348 and -451 bp with liver nuclear extract isolated using the Rosette and Karin method (Rosette and Karin, 1995). 10 μg of liver nuclear extracts from either untreated (U; lane 1) or PB-treated (lane 2) rats were used. YY1, Yin Yang-1.
nuclear proteins isolated from hepatocyte cells using the Rosette and Karin method.

Until that experiment is carried out, there is at present no reasonable explanation to account for the loss of the nuclear protein binding from the extracts of hepatocyte cultures to the -348 to -451 bp sequence, the initial idea to treat hepatocyte cultures with different chemicals and to study the effects of these chemicals on the protein binding to the -348 to -451 bp sequence could not be further pursued.
3.1.12 DNA affinity purification using Dynabeads® of liver nuclear protein binding to the CYP2B1 5'-flanking sequence between -348 and -451 bp

When nuclear extracts from PB-treated rats bound more to the -348 to -451 bp sequence of the CYP2B1 gene than untreated rats as previously described in section 3.1.4 and that the protein(s) binding was dependent on its phosphorylation and dephosphorylation state (section 3.1.10), there was interest to identify the protein(s). Unfortunately, the use of a various protein kinase and phosphatase inhibitors did not generate many clues as to the identity of the protein. Thus, the challenge of purifying the protein was undertaken.

DNA affinity purification technique is based on the ability of protein to bind to specific DNA sequences. The protocol was a combination taken from Ren et al (1994) (Ren, et al., 1994) and Gabrielsen and Huet (1993) (Gabrielsen and Huet, 1993) and is presented schematically in Fig. 3.1.42. Briefly, the DNA sequence containing the binding site of interest is ligated to a tether sequence. The tether is a short double-stranded DNA sequence with biotin tagged to the 5'-end of one of the strands and a designed Sal I restriction enzyme overhang at the 5'-end of the other strand as detailed in section 2.15.1. The pBS(-451) clone (see section 3.1.1) was digested with Xba I and Xho I restriction enzymes. Xho I generates a compatible 5'-overhang that allows ligation to the tether DNA. The ligated product was then mixed with streptavidin-coated magnetic Dynabeads to allow coupling of the DNA to the beads. Liver nuclear protein extract was then added to the DNA.
Fig. 3.1.42 Schematic diagram of DNA affinity purification of liver nuclear protein with Dynabeads. (adapted and modified from (Gabrielsen and Huet, 1993, Ren, et al., 1994))
coupled beads and the binding reaction was allowed to take place. The binding reaction should take place at near-saturating conditions and non-specific competitor DNA, poly dl.dC, should not be included at this point. The unbound proteins in the extract were removed as the supernatant fraction. The bound beads were then washed in the presence of poly dl.dC to dissociate any non-specific proteins that had bound to the DNA. The washing step was performed 2 to 3 times and wash fractions were also collected. Finally, the specific proteins bound to the DNA were dissociated with increasing salt concentrations beginning from 1 M up to 2 M. The eluate fractions were then dialysed to desalt the fractions.

Fig. 3.1.43 shows the gel shift results of different fractions collected from the purification procedure. Excessive binding could be observed with nuclear extract of the supernatant fraction (lane 2) indicating a lot of unbound proteins. The entire lane looked black because the X-ray film had been overexposed deliberately to allow for any weak band in the eluate fractions to show up (lanes 3 to 5 and 8 to 10). The gel shift shows the results of two purification experiments. Lanes 3 to 5 show the results from the first purification. No protein binding was observed for fractions eluted with 1 M (lane 3) or 2 M (lane 5) salt concentrations except for a very low molecular weight complex. The fraction eluted with 1.5 M salt (lane 4) showed some protein binding. Nuclear protein corresponding to complex ‘c’ and ‘d’ were selectively purified. When the experiment was repeated using similar conditions to the previous set of extracts. The results obtained with the eluate fractions were the same as before. Wash fractions were included in the gel shift assay and showed that some proteins were dissociated from
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Fig. 3.1.43 Gel shift analysis of CYP2B1 5'-flanking sequence between -348 and -451 bp with liver enriched nuclear extracts from PB-treated rats. (S/N, supernatant; W, wash and Concentration refer to the concentration of NaCl)
the DNA during the washing steps as observed in lanes 6 and 7. Elution using NaCl at 1.0 M concentration could not dissociate the protein from the DNA while 1.5 M concentration could efficiently dissociate all proteins from the DNA as no binding was observed when elution was further carried out with 2.0 M salt concentration. Although some degree of protein purification had occurred, the protein(s) involved in complex ‘a’ did not seem to have been purified. Complex ‘d’ which had been previously identified to contain the transcription factor, YY1 (see section 3.1.6) was very intense. In order to increase the chance of purifying the protein(s) binding in complex ‘a’, the YY1 binding site oligonucleotide was included in the binding reaction as a competitor to mop up YY1 protein. The YY1 complex was indeed mopped up by the YY1 oligonucleotide as complex ‘d’ was not observed in the eluate fraction any more (Fig. 3.1.44, lane 3). However, protein involved in binding to complex ‘a’ was still not purified.

The inability to purify the protein(s) under investigation could be due to the limited amount of DNA coupled to the Dynabeads because too much excess nuclear protein was observed in the supernatant fraction. To follow the coupling progress of DNA to the beads, the post-coupling supernatant was electrophoresed in an agarose gel and very little DNA should be left behind. However, a significant amount of DNA still remained in the post-coupling supernatant. These two observations together indicated that the amount of DNA bound to the beads might have been over-estimated. Because the amount of DNA bound to the beads was limited, leading to limited the number of sites available for protein to bind, yield of the protein purified decreased.
Fig. 3.1.44 Gel shift analysis of CYP2B1 5'-flanking sequence between -348 and -451 bp with DNA affinity enriched liver nuclear extracts from PB-treated rats. The binding reaction was performed in the presence of YY1 oligonucleotide as a competitor DNA followed by subsequent purification (W, wash).
At least three improvements should be made to the purification protocol: to use a shorter DNA sequence, to partially purify the crude nuclear extracts and to omit the ligation step of tether to the DNA of interest. The first suggested improvement applied particularly to this case, the sequence between -348 and -451 bp is 103 bp in length which is a considerably large fragment for use in purification. Most references recommend short oligonucleotides which may be concatamerised (Kadonaga and Tjian, 1986, Larson and Verdine, 1992). Although protein binding to this sequence was reported to be complex and the site involved in binding could not be precisely pin-pointed. The shorter sequence, between -379 and -428 bp, which had been found to be the minimum region to produce similar gel shift result as the parent sequence, could be used instead. The use of partially purified nuclear extracts, suggested by Gabrielsen and Huet (1993) (Gabrielsen and Huet, 1993) could greatly reduce the binding of non-specific proteins to the DNA sequence. Fig. 3.1.45 shows the gel shift of a crude liver nuclear extract partially purified through a phosphocellulose column. Bound proteins were eluted from the column using an increasing salt gradient. The collected fractions were desalted using microconcentrators and then used in gel shift assays. Proteins corresponding to different complexes were eluted at different salt concentrations, proteins involved in complexes ‘b’ and ‘c’ were eluted mainly in fraction 2, complex ‘a’ was mainly eluted in fractions 3, 4 and 6 while complex ‘d’ was preferentially eluted in fraction 9. Hence, fractions 3 and 4 would be the best fractions for subsequent use in the DNA affinity purification. The third suggested improvement would be to do away with the ligation step. This is because a substantial amount of DNA
probe CYP2B1 -348 to -451 bp

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Fig. 3.1.45 Gel shift analysis of CYP2B1 5' flanking sequence between -348 and -451 bp with partially purified liver nuclear extracts from PB-treated rats.
is lost and ligation does not only occur between tether and DNA but tether/tether and DNA/DNA ligation takes place as well. Instead of ligating a biotinylated DNA tether with a DNA of interest, a biotinylated deoxyribonucleotide could be directly incorporated to one end of the DNA. To reduce steric hindrance during the binding of the nuclear protein to the DNA, the binding site on the DNA should not be too near to the end of the DNA that is involved in direct interaction with the beads. Since only biotinylated dATP and dUTP are available commercially, the choice of restriction enzymes that generate suitable ends for filling-in becomes limited. If the three factors mentioned above could be incorporated into future purifications, the rate of successfully purifying the protein may increase. And once the purification conditions have been optimised, the protocol can then be scaled up in order to purify enough protein such that it can be visualised on a SDS-polyacrylamide gel stained with Coomassie blue dye. The protein would then be transferred to a PVDF membrane and the amino-terminal sequence could be determined by microsequencing. The sequence can then be compared to sequences in a protein database to identify the protein purified.
3.1.13 Oct-1 binds to the CYP2B2 promoter between -183 and -199 bp

Previous studies carried out in our laboratory had shown that the promoter sequence between -183 and -199 of the CYP2B2 gene bound more liver nuclear proteins from PB-treated than untreated rats (Shephard, et al., 1994). Within this 17-bp sequence, there is a region very similar to the consensus binding site for the octamer family of homeodomain transcription factors except for a single base mismatch from ‘C’ to ‘T’ as shown in Fig. 3.1.46. It is of interest to investigate if the protein binding to the CYP2B2 promoter region, -183 to -199 bp, is indeed a member of the octamer protein family.

Fig. 3.1.47 shows the DNA-protein complex formed with the CYP2B2 probe, -183 to -199 bp, and nuclear protein from the liver of PB-treated rats (Lane 1). Unlabelled -183 to -199 bp oligonucleotide added as a competitor at 100 to 300 fold molar excess could completely abolish the formation of all complexes as shown in Fig. 3.1.47, lanes 2 to 4. This indicates that the protein-DNA complexes formed are specific. The addition of an oligonucleotide containing the consensus binding site for the octamer family of protein as a competitor was able to gradually diminish formation of the complex ‘a’ with increasing molar excess (Fig. 3.1.47, lanes 5 to 7). However, the formation of complex ‘b’ was observed to increase with increasing molar excess of the octamer consensus oligonucleotide added. This competitive gel shift result shows that complex ‘a’ is most probably an octamer protein while complex ‘b’ does not appear to be an octamer protein.
**Fig 3.1.46** Sequence comparison of the CYP2B promoter regions between -183 and -199 bp with the Octamer consensus binding site. Open boxes highlight the putative octamer binding site. The letters in red indicate the bases that are different between CYP2B1 and CYP2B2 promoters. The vertical lines indicate bases within the putative octamer binding site of the CYP2B1 and CYP2B2 promoters that match with the octamer consensus binding site shown in the middle.
Fig. 3.1.47 Gel shift analysis of the CYP2B2 promoter region between -183 and -199 bp. 5 μg of liver nuclear protein from PB-treated rat was used. Unlabeled -183 to -199 bp (lanes 2 to 4) or octamer consensus (lanes 5 to 7) oligonucleotides were added as competitor DNA in 100- to 300-fold molar excess. Lane 8 contains no nuclear protein.
* While protein-DNA complex formation with the CYP2B2 probes was partially or completely abolished when 1 µl or 5 µl respectively of a 1 in 10 dilution of the antibody was used (Fig. 3.1.48b, lanes 6 and 7).
This seems to indicate that more than one protein is able to bind to the -183 to -199 bp of the CYP2B2 promoter and they may be competing to bind to an identical or an overlapping site. The protein involved in the formation of complex ‘a’ appears to have a higher DNA binding affinity than the protein involved in complex ‘b’. The addition of excess octamer consensus oligonucleotide as competitor sequester the octamer protein leaving the site on the -183 to -119 bp available for the binding of the protein in complex ‘b’.

An antibody which recognises the DNA-binding domain of Oct-1 and Oct-2 proteins, when added to the binding reaction, abolished complex formation of liver nuclear proteins with both the octamer consensus (Fig. 3.1.48a, lane 2). * The inhibition of complex formation by the Oct-1/2 antibody was specific as the addition of an unrelated antibody of the same isotype, p107 was not able to abolish or supershift the protein-DNA complex (Fig. 3.1.48a, lane 3 and Fig. 3.1.48b, lane 8). The use of an antibody that recognises Oct-1 specifically supershifted the protein-DNA complex formed between liver nuclear extract and the octamer consensus oligonucleotide as indicated in Fig. 3.1.49a, lane 2. This antibody was also able to supershift complex ‘a’ formed between liver nuclear protein and the CYP2B2 sequence (Fig. 3.1.49b, lane 6). The supershift complexes observed in both gel shifts were specific as the addition of an unrelated antibody (C/EBPα antibody), of the same isotype, did not supershift the complex (Fig. 3.1.49a, lane 3 and Fig. 3.1.49b, lane 7). The Oct-1 was also shown not to bind to DNA (Fig. 3.1.49b, lane 8).

The results of the competitive gel shift analysis with the octamer
Fig 3.1.48 Antibody interference analysis of (a) octamer consensus oligonucleotide and (b) -183 to -199 bp of the CYP2B2 promoter sequence. 5 μg of liver nuclear protein from PB-treated rat was used. In (a), 1 μL of either the human Oct-1/2 antibody (lane 2) or p107 antibody (lane 3) were added. In (b), 1 μL (lane 6) and 5 μL (lane 7) of a 1 in 10 diluted concentration of the human Oct-1/2 antibody was added. 1 μL of undiluted p107 antibody was added in lane 8. Lanes 4 and 9 contain no nuclear protein.
Fig 3.1.49 Supershift analysis of (a) Octamer consensus oligonucleotide and (b) -183 to -199 bp of the CYP2B2 promoter sequence as the probe. 5 μg of liver nuclear protein from PB-treated rats were used. 1 μl of antibody against Oct-1 (lanes 2, 6 and 8) or C/EBPα (lanes 3 and 7) were added. Lanes 4, 8 and 9 contain no nuclear protein and the arrow indicates the supershifted band.
consensus oligonucleotide suggests that one of the proteins binding to the
CYP2B2 promoter between -183 and -199 bp is related to the octamer
family of proteins. The antibody interference assay further indicates that the
protein involved in formation of complex ‘a’ could either be Oct-1 or Oct-2.
Finally, the fact that an antibody specific to Oct-1 could supershift complex ‘a’
confirms that Oct-1 protein binds to this region of the CYP2B2 promoter.
These results also confirm that the protein involved in the formation of
complex ‘b’ is not related to the octamer family of protein and is most
probably not Oct-1 or Oct-2 protein. The identity of this protein is still
unknown.

There is interest to study this region of the CYP2B2 promoter not only
because the DNA-protein complex formed with liver nuclear extracts from
PB-treated rats is more abundant but it was also reported previously in our
laboratory that no protein-DNA complex was observed when nuclear extract
from small intestines of rats was used in gel shift assay with the CYP2B2
probe (Elia, 1996). A low constitutive amount of CYP2B2 mRNA is known to
be expressed in rat liver and the expression is inducible by PB (Gonzalez,
1989). However, CYP2B2 is neither expressed nor PB-inducible in the
intestinal mucosa (Traber, et al., 1990). To observe Oct-1 binding to the
CYP2B2 promoter between -183 and -199 bp in the liver but not in the small
intestines may indicate that the Oct-1 protein is involved in the tissue-
specific expression of the CYP2B2 gene in the liver.

This is further supported by the fact that within the same region in the
CYP2B1 promoter, there is a 3-base change from ‘TAA’ to ‘GGG’ within the
putative octamer binding site (Fig. 3.1.46). This region in the CYP2B1 5'-
flanking sequence was shown not to bind protein (Shephard, et al., 1994).

And CYP2B1 expression is inducible by PB in both the liver and the small intestines (Traber, et al., 1990).
Section II

Effect of 2-aminopurine on basal and PB-inducible expression of CYP2B mRNAs
3.2 2-Aminopurine down-regulates both basal and PB-inducible expression of CYP2B genes

Unlike induction of the CYP1A1 gene by PAH which is well-understood, very little is known about the mechanism whereby PB activates CYP2B gene expression. However, numerous research studies have shown the requirement for phosphorylation/dephosphorylation steps in PB signalling. The involvement of phosphatases and kinases in PB signalling has been reported and a number of possible signal transduction pathways have been proposed.

Sidhu and Omiecinski (1995) had shown that the increased intracellular cAMP level leading to the activation of PKA activity could abolish PB-induced CYP2B1 and CYP2B2 mRNA expression in primary rat hepatocytes. They later showed that the treatment of primary rat hepatocytes with okadaic acid, an inhibitor protein phosphatase 1 and 2A could also lead to a complete inhibition of the increased expression of CYP2B1 and CYP2B2 mRNA by PB (Sidhu and Omiecinski, 1997). Furthermore, the treatment of hepatocytes with both chemicals together markedly augmented their suppressive effects on PB induction (Sidhu and Omiecinski, 1997). Therefore, they proposed that the PB signalling pathway involves the concerted interaction of cAMP-dependent PKA and PP1/2A.

However, treatment of primary rat hepatocytes with specific inhibitors of PKA have also been shown to abolish PB induction of CYP2B1, CYP2B2 and CYP3A23 mRNA (Brown, et al., 1997). The requirement for
phosphorylation events in PB signalling has been suggested in some other cases too. Dogra and May (1996) (Dogra and May, 1996) showed that the protein kinase inhibitor, 2-AP could completely inhibit the induction of CYP2H1 gene expression by PB but with no effect on basal expression in chicken embryo hepatocytes. According to them, the 2-AP sensitive protein kinase does not appear to be PKC or tyrosine kinases. Furthermore, Nirodi et al (1996) (Nirodi, et al., 1996) also found the PB-induced CYP2B1 and CYP2B2 mRNA to be inhibited by 2-AP in an in vivo experiment. For reasons unknown, they observed OK, a protein phosphatase inhibitor to exert a similar effect as 2-AP, a protein kinase inhibitor on the PB-induced expression of CYP2B1 and CYP2B2.

Recently, Honkakoski and Negishi (1998) (Honkakoski and Negishi, 1998a) studied the effect of a range of different protein kinase and phosphatase inhibitors on the basal as well as PB-induced expression of Cyp2b10 mRNA in primary mouse hepatocytes. They reported the increased transcription of Cyp2b10 by PB to be suppressed by OK and calyculin A, inhibitors of protein phosphatases 1 and 2A and that this suppression could be overcome by an inhibitor of Ca^{2+}/Calmodulin-dependent protein kinase but not by inhibitors of PKA, PKC or tyrosine kinases.

It is shown in the present study (see section 3.1.10) that the transcription factor(s) binding to specific regions of the CYP2B1 promoter is dependent on its phosphorylation status. Because 2-AP, a guanine analogue, has been observed to affect transcription factor binding to DNA, the effect of 2-AP on the expression of CYP2B genes both in vivo and in vitro were also investigated.
In primary rat hepatocyte cultures, each treatment was performed on three plates of cells derived from the same batch which were harvested into a single cell pellet for RNA extraction. The concentration of 2-AP used to treat the cells was according to that of Dogra and May (1996) (Dogra and May, 1996). Hepatocytes were pre-treated with 10 mM 2-AP for an hour before the cells were further incubated in the absence or presence of 0.1 mM PB for 6 hrs. The hepatocytes were then harvested and total RNA was extracted as described in section 2.16.1. Northern blot analysis was then carried out to determine the amounts of CYP2B1/2 mRNAs using a cDNA probe homologous to 1400 bp region of the CYP2B1/2 transcript (Phillips, et al., 1983a) and the result is as shown in Fig. 3.2.1. This experiment was done on two different batches of primary cells and both yielded similar results. One set of results is presented. Upon PB-treatment, CYP2B1/2 mRNA was increased by 4.4-fold. Treatment with 2-AP not only suppressed the increased of CYP2B1/2 mRNA in PB-treated cells (Fig. 3.2.1a; lanes 4 and 8), the basal expression was also greatly down-regulated (Fig. 3.2.1a; lanes 3 and 7). It is unlikely that the result observed with 2-AP was due to toxicity or irreversible cell damage because the total RNA extracted showed no degradation when electrophoresed on an agarose gel (result not shown) and the cells look fine in culture. The 28S rRNA of these samples were also analysed as a control and their amount was shown not to be altered by 2-AP (Fig. 3.2.1a, bottom panel). In addition, the same concentration of 2-AP used in chick embryo hepatocyte cultures did not affect the amount of GAPDH mRNA and the basal expression of CYP2H1 and ALAS (the first and rate limiting enzyme in haem biosynthesis) mRNAs.
Fig. 3.2.1 The effect of 2-AP on CYP2B mRNA in primary rat hepatocytes. (a) Northern blot analysis of total RNA from hepatocytes. 10 µg (lanes 1 to 4) and 20 µg (lanes 5 to 8) of RNA from cells treated in the absence (-) or presence (+) of 2-AP and PB were electrophoresed and blotted onto optimised nylon membrane. The blot was probed first with The CYP2B1/2 cDNA (top panel), stripped and then probed with a 28S rRNA oligonucleotide (bottom panel). (b) Graphical presentation of the relative abundance of CYP2B1/2 mRNA normalised with 28S rRNA of the Northern blot result obtained with 20 µg of total RNA shown in (a).
In animals, 5 mg/100 g body weight of 2-AP was injected (IP) 1 hr before administration of PB (IP) at 100 mg/ kg body weight. The rats were sacrificed 6 hrs later and RNA was extracted from the liver as described in section 2.16.1. Northern blot analysis of the total RNA isolated from the rat livers is shown in Fig. 3.2.2. It is observed that PB-treatment increased CYP2B1/2 mRNA by 9.6-fold and 2-AP could partially attenuate the PB-induction (Fig. 3.2.2a; lane 4). The basal expression was also found to be inhibited by 2-AP (Fig. 3.2.2a; lane 3) but not totally abolished as observed in primary hepatocytes.

From the result above, 2-AP is observed to have a general inhibitory effect on both basal as well as PB-induced expression of CYP2B1 and CYP2B2 in vivo and in vitro. The latter result agrees with that reported by Dogra and May (1996) (Dogra and May, 1996) in chick embryo hepatocytes and Nirodi et al (1996) (Nirodi, et al., 1996) in rat liver but both laboratories observed 2-AP to have no effect on basal expression. Dogra and May (1996) (Dogra and May, 1996) showed that 2-AP blocked the increased in CAT activity of a construct containing a region of the CYP2H1 5'-flanking sequence that confers PB-responsiveness on a heterologous promoter by PB while having no effect on the CAT activity of another construct containing the first 500 bp of the CYP2H1 5'-flanking sequence. The first 500 bp of the CYP2H1 5'-flanking sequence has been reported to drive CYP2H1 basal expression and not the response to PB (Hahn, et al., 1991). This would most probably explain why 2-AP did not affect the basal expression of CYP2H1. Although members of the CYP2H subfamily in the chicken are highly induced by PB and thought to be the orthologous genes to the rat
Fig. 3.2.2 The effect of 2-AP on CYP2B mRNA in rat liver in vivo. (a) Northern blot analysis of total RNA isolated from rat livers. 10 μg of RNA from rat liver treated in the absence (-) or presence (+) of 2-AP and PB probed with CYP2B cDNA (top panel) or 28S oligonucleotide (bottom panel). (b) Graphical presentation of the relative abundance of CYP2B1/2 mRNA normalised with 28S rRNA of the Northern blot result shown in (a).
CYP2B1 and CYP2B2, it is possible that PB mediated gene activation in the rat and chicken is via different regulatory mechanisms. For instance, cycloheximide was found to enhance the transcription of chicken CYP2H1 and CYP2H2 (Dogra, et al., 1993) while having an inhibitory effect on rat CYP2B1 and CYP2B2 expression (Schuetz, et al., 1990). It is also not known if an element similar to the PB-responsive element reported in the rat is present in chicken. On the other hand, Nirodi et al (1996) demonstrated that PB mediated increase in the transcription of a minigene construct containing exon I and the first 179 bp of the promoter region of CYP2B2 could be inhibited by 2-AP in an in vitro cell-free system (Nirodi, et al., 1996). And the 5'-flanking sequence of the CYP2B1 and CYP2B2 from the transcription initiation site to 177 bp immediately upstream has been determined to be the minimal promoter region (Sommer, et al., 1996). Therefore, it appears that 2-AP is affecting the transcription factor(s) binding to the proximal promoter of CYP2B1 and CYP2B2 resulting in the inhibition of basal expression observed here in vitro and in vivo (Figs. 3.2.1 and 3.2.2 respectively). 2-AP inhibits CYP gene expression in both chicken and rat could be at the transcriptional or post-transcriptional level. However, the exact molecular mechanism is still unclear.

2-AP is a protein kinase inhibitor which has been shown to be a highly selective inhibitor as it affects a very limited subset of phosphoproteins in the cell (Mahadevan, et al., 1990). It could inhibit the double-stranded RNA-dependent protein kinase (PKR) (Zinn, et al., 1988), the haem-regulated protein kinase (Kaufman and Murtha, 1987) and the ERK/MAP kinases implicated in a variety of cell-signalling pathways.

According to Nirodi *et al* (1996) (Nirodi, *et al.*, 1996), 2-AP inhibited the binding of nuclear proteins to the CYP2B2 promoter region as assessed by gel shift analysis. Because they utilised liver nuclear protein from a rat treated with 2-AP, it was not clear if 2-AP acted directly on the transcription factor binding to the DNA sequence. However, the latter case cannot be ruled out because the ability of 2-AP to directly affect the binding of nuclear protein to DNA has been observed in section 3.1.10 when 2-AP was added to liver nuclear extracts *in vitro* prior to gel shift assays. The ability of 2-AP to block PB-induction of chicken CYP2H1 by exerting a direct effect on transcription factors binding to the 5'-flanking sequence has not been studied (Dogra and May, 1996). However, Dogra and May (1996) showed that the 2-AP sensitive protein kinase is unlikely to be PKC or tyrosine kinases.

In contrast, activation of PKA activity was reported to block PB-induction of CYP2B1 and CYP2B2 mRNA in primary rat hepatocytes. The
result has been supported by observations made in our laboratory indicating cAMP analogues and PKA activators inhibit PB-induced CYP2B1 and CYP2B2 mRNA and protein amounts in primary rat hepatocytes (Muangmoonchai, R., personal communication; Dell, 1997). Because the actual target of 2-AP in inhibiting PB-induction of CYP genes is not known, the most probable explanation for the opposing results would be the involvement of multiple signalling transduction pathways in PB-mediated gene transcription. This would also be a likely reason for the observation of the less suppressive effect by 2-AP on CYP2B1 and CYP2B2 expression in vivo as compared to in vitro. It is possible that one of the several PB-mediated signalling pathway is inactivated in isolated hepatocytes cultured in vitro due to the lack of influence from extra-hepatic mediators. Could this also explain the fact that the PB-inductive response is always less in primary rat hepatocyte cultures than in the liver of whole animals?
Section III

Analysis of the 5'-flanking sequence of CYP2B1 gene using transient transfection assays
3.3.1 Preparation of luciferase reporter constructs

The Promega pGL3 vector series containing the firefly (Photinus pyralis) luciferase as the reporter gene was used in transfection studies. Three different vectors were utilised: pGL3-Basic (pGL3B) which has neither SV40 (simian virus) promoter nor enhancer, pGL3-Promoter (pGL3P) which has only the SV40 promoter and pGL3-Control (pGL3C) which has both SV40 promoter and enhancer as shown diagramatically in Fig. 3.3.1. And the pRL-tk vector containing the Renilla reniformis luciferase gene and herpes simplex virus thymidine kinase promoter was used as an internal control (Fig. 3.3.1).

3.3.1.1 Preparation of luciferase reporter constructs containing various CYP2B1 proximal promoter regions

Various lengths of the CYP2B1 proximal promoter ranging from -1 to -451 bp were cloned into the pGL3-Basic vector as shown in Fig. 3.3.2. To clone the promoter sequence from -1 to -179 bp, Clone 27 (see section 3.1.1) was digested with Hind III and Stu I restriction enzymes and then inserted into the Hind III and Sma I site of the pGL3-Basic vector. The luciferase reporter plasmid containing the CYP2B1 promoter from -1 to -348 bp was prepared by first linearising Clone 27 with Xba I and the ends were then blunted with DNA polymerase I, Klenow fragment. The linearised Clone 27 was subsequently cut with Hind III and the 272 bp fragment containing sequences from -1 to -348 bp was gel purified and cloned into the Hind III
Fig. 3.3.1 Schematic diagram of the pGL3 and pRL luciferase vector maps. Features common to all the pGL3 luciferase vectors are the luc+ gene (cDNA encoding the modified firefly luciferase); Amp′ (gene conferring ampicillin resistance in E. coli); f1 ori (origin of replication derived from filamentous phage); ori (origin of plasmid replication in E. coli). Arrows within boxes indicate the direction of transcription in luc+ and Amp′ gene or the direction of ssDNA strand synthesis in f1 ori. Restriction enzyme sites and the positions they cut on each vector are also shown. (adapted from Promega pGL3 luciferase reporter vectors technical manual, Part # TM033)
*♦ depicts the \textit{CYP2B1} promoter sequences and the numerals below the boxes indicate the sequence with respect to the transcriptional initiation site. ♦ represents the SV40 promoter sequence, ♦ represents the \textit{CYP2B1} PBRE sequence from -2142 to -2301 bp and ♦ represents the \textit{CYP2B1} 5'-flanking sequence between -348 and -451 bp. The arrow within the red and yellow boxes indicate the orientation and the number of copy at which the sequences were inserted into the constructs. Luc+ refer to the firefly luciferase gene.
Fig. 3.3.2 Schematic diagram of reporter gene constructs for transient transfection experiments. Cloning strategies are as outlined in section 3.3.
and Sma I sites of the pGL3-Basic vector. As for the cloning of the -1 to -451 bp sequence, it was excised from Clone 27 using Hind III and cloned directly into the Hind III site of the pGL3-Basic vector, ensuring the orientation of the insert was such that the -1 bp was proximal to the luciferase gene as illustrated in Fig. 3.3.2. The reporter constructs were designated pGL3B(-179), pGL3B(-348) and pGL3B(-451) according to the distal positions of the cloned fragment with respect to the transcription initiation site.

3.3.1.2 Heterologous promoter constructs

The fragment between -2142 to -2301 bp (159 bp) containing the PBRE was obtained by restriction digestion of the 3.9 kb fragment of the CYP2B1 promoter with Sau 3AI and then cloned into the Bam HI site of the pGL3-Promoter vector which has compatible ends with Sau 3AI. The clone containing one copy of the insert was called pGL3P(159).

3.3.1.3 Preparation of luciferase reporter constructs containing proximal and distal 5'-flanking regions of the CYP2B1 gene

The CYP2B1 5'-flanking sequence from -2142 to -2301 bp was to be inserted into the clones pGL3B(-179), pGL3B(-348) and pGL3B(-451) obtained as described in section 3.3.1.1. This was achieved by digesting the clone pGL3P(159) (see section 3.3.1.2) with Xba I and Sal I to obtain a 427 bp fragment. This releases the sequence -2142 to -2301 bp together with the vector’s SV40 late (poly(A)) signal (see Fig. 3.3.1). The recipient clones were also digested with Xba I and Sal I to generate two fragments of 268 bp
and ~4550 bp. The SV40 late (poly(A)) signal in the recipient clones was removed in the 268 bp fragment. So the 4550 bp fragment does not contain the SV40 late (poly(A)) signal but will be replaced when the insert is cloned. The 4550 bp fragment from each of the three clones was gel purified and the Xba I/Sal I fragment from pGL3P(159) was then ligated to each of the three recipient clones as shown in Fig. 3.3.2. Either one or multiple copies of the CYP2B1 promoter sequence between -348 and -451 bp was also to be cloned into pGL3B(-179) vector. The insert was obtained by Hind III and Xba I restriction digestion of the pBS(-451) (see section 3.1.1). The ends were filled-in using DNA polymerase I, Klenow fragment and then inserted into the Bam HI site of the recipient vector which had also been blunted with DNA polymerase I, Klenow fragment. The clone containing one copy of the -348 to -451 bp sequence was designated pGL3B(-179)/(103). The other two clones were found, by sequencing, to contain 2 copies or 1 and a half copies of the -348 to -451 bp sequence in the orientation as shown in Fig. 3.3.1.2. They were called pGL3B(-179)/(103X2) and pGL3B(-179)/(103X1.5) respectively.
3.3.2 Determination of promoter and enhancer properties of CYP2B1 5'-flanking sequences using in vitro and in vivo transient transfection systems

When liver nuclear proteins from PB-treated rats were observed to bind more to the CYP2B1 5'-flanking sequences from -179 to -348 bp and -348 to -451 bp in gel shift assays, there was interest to determine the role played by these sequences in the PB-inducible transcription of the CYP2B1 gene in rat hepatocytes.

Due to the lack of a PB-responsive hepatoma cell line and the lack of technology in transfecting liver tissues at the time, transient transfection in non-proliferating, primary hepatocyte cultures was the best option since it most resembles the differentiated state of whole liver. As the method of transfection using calcium phosphate had previously been used with success in our laboratory, initial experiments were performed using this method. The firefly luciferase vector series (pGL3) was selected for use because these plasmids can be co-transfected with Renilla luciferase reporter constructs (pRL) and the luciferase activities of sets of both vectors can be measured sequentially from a single sample using the Dual-Luciferase™ Reporter Assay. However, their suitability for use needed to be assessed first. The luciferase readings from three pGL3 vectors were normalised against the pRLtk vector and presented as relative luciferase activities in Fig. 3.3.3. The pGL3-Control vector containing both SV40 promoter and enhancer showed high luciferase activity. The pGL3-Promoter containing only the SV40 promoter had about a third the activity of the control
**Fig. 3.3.3** Transient luciferase activity of pGL3 reporter constructs transfected either into (a) primary rat hepatocyte cultures (*in vitro*) or (b) rat livers (*in vivo*). The relative luciferase light unit of pGL3-Promoter and pGL3-Basic vectors were expressed as a percentage of the corresponding relative light unit of pGL3-Control vector which had been arbitrarily defined as 100% in both untreated and PB-treated cultures. *In vitro*, the normalised activity of pGL3-Control varied from 85 to 200. The results shown in (a) are the mean of two independent experiments done in quadruplicate. *In vivo*, the normalised activity of pGL3-Control varied from 82 to 120. The results shown in (b) are the mean of one experiment done in duplicates.
vector while the pGL3-Basic vector with no promoter or enhancer hardly showed any activity. The expression of all three vectors was not affected by the treatment of hepatocytes with PB. It was also observed that the activity of the pGL3-Control vector varied considerably from one experiment to another but it always gave the highest activity. Hence, its relative luciferase reading was arbitrarily set at 100 and the relative luciferase activities of other vectors were expressed as a percentage of the activity of the pGL3-Control vector transfected within the same experiment. The percentage activity of both pGL3-Promoter and pGL3-Basic vectors are shown above each column in Fig. 3.3.3a. With reference to the results obtained in Fig. 3.3.3a, this vector series was determined to be suitable for the study of \textit{CYP2B1} 5'-flanking sequences in primary hepatocyte cells based on the following. The pGL3-Control exhibited a relatively high level of expression and could be used as a positive control and the pGL3-Basic showed almost zero expression and was used as a negative control. The pGL3-Promoter vector having a moderate promoter strength was suitable for determining enhancer elements within the \textit{CYP2B1} 5'-flanking sequence.

The pGL3-Control and pGL3-Promoter vectors were also transfected into HeLa cell cultures and the data obtained was similar to that reported in the Promega technical manual (Part# TM033). When compared to primary hepatocytes, the pGL3-Control vector showed a very similar level of luciferase activity while the pGL3-Promoter appeared to be more active in primary hepatocyte cells (see Fig. 3.3.4). It was not surprising that the same promoter element exhibits different transcription strengths in different cell
Fig. 3.3.4 Transient luciferase activity of pGL3 reporter constructs transfected into either primary rat hepatocyte cultures or HeLa cell cultures. The transfection of both cell cultures was performed at the same time using the same lot of DNA and transfection reagents. The experiment was done only once and the results shown are the mean of quadruplicates done within that one experiment. Standard deviations are indicated by error bars.
types and such observations have been previously reported both in vitro (Ponder, et al., 1991, Wenger, et al., 1994) and in vivo (Cheng, et al., 1993).

Transfections performed so far using the calcium phosphate method had been giving variable results as assessed by the pGL3-Control vector. Because this method of transfection was also tedious, time-consuming and had to be performed in the absence of FBS, a number of other transfection reagents were tested. These reagents allowed transfection to be carried out in the presence of FBS and the procedure was less time consuming. The first reagent tested was the TransFast™ reagent from Promega which comprised a mixture of synthetic cationic and neutral lipids. The second was Superfect™ reagent from Qiagen, it is a highly-branched polycation of defined shape and diameter. The third was Effectene™ reagent, also from Qiagen, a non-liposomal lipid which is highly recommended for transfecting primary cells. When the pGL3-Control and pGL3-Basic vectors were transfected into primary hepatocyte cells using the different transfection methods as in Fig. 3.3.5, the pGL3-Control vector gave significantly higher activity when transfected with Superfect™ and Effectene™ reagents while the expression of pGL3-Basic vector was low for all methods. Due to the ease of transfection technique and the high pGL3-Control activity obtained, Effectene™ reagent was chosen for use in all subsequent transfections. Within each experiment, parallel cultures were transfected with pGL3-Control as the positive control and pGL3-Basic as the negative control. The relative luciferase activities obtained for all constructs are expressed as a percentage of the pGL3-Control activity determined within that experiment.
pGL3 vector series

Fig. 3.3.5 Comparison of four different transfection methods of transfecting the pGL3 reporter constructs into primary rat hepatocyte cultures. Transfections using calcium phosphate (CaPO₄) and TransFast™ were performed at the same time on one batch of hepatocytes while transfections using Superfect™ and Effectene™ were performed at the same time on another batch of hepatocytes. The pGL3-Control and pGL3-Basic vectors are indicated here as Control and Basic respectively. Data shown are the mean of quadruplicates from one experiment except Superfect was from a single luciferase reading. Standard deviations are indicated by error bars.
Fig. 3.3.6a shows that data obtained with the constructs, pGL3B(-179), pGL3B(-348) and pGL3B(-451), containing different lengths of the CYP2B1 proximal promoter. Numbers are with respect to the transcription initiation site as detailed in section 3.3.1. In untreated primary hepatocytes cultures, pGL3B(-179) exhibited a very low level of expression (i.e. <10% of pGL3-Control activity) as expected since the basic promoter region of both CYP2B1 and CYP2B2 genes had been reported to be contained within the most proximal 177 bp sequence (Sommer, et al., 1996). The expression of this construct was not affected by PB-treatment. There has been a controversy regarding the ability of the first 179 bp promoter region of both CYP2B1 and CYP2B2 genes to play a role in their induction by PB (see section 1.6.1). The results obtained here further suggested that this sequence alone does not confer PB-responsiveness. The other two constructs, pGL3B(-348) and pGL3B(-451) also showed a low level of expression in both untreated and PB-treated cells despite the fact that the promoter in pGL3B(-348) contain the sequence -179 to -348 bp and the promoter in pGL3B(-451) contain sequences -179 to -348 bp and -348 to -451 bp. And these sequences had been reported in section 3.1.3 and section 3.1.4 to bind more liver nuclear protein from PB-treated rats. However, the involvement of these sequences in PB-induction of gene expression still cannot be completely ruled out. It is possible that nuclear protein from PB-treated animals binding to these sequences might need to cooperate with proteins binding to other 5′-flanking regions for transcriptional activation, i.e. the PB-Responsive Element (PBRE).
Fig. 3.3.6 Transient transfection of pGL3 constructs containing CYP2B1 proximal promoter sequences transfected either into (a) primary rat hepatocyte cultures (in vitro) or (b) rat livers (in vivo). Data show the mean of triplicates for in vitro and duplicates for in vivo, both taken from a single experiment. Standard deviations are indicated by error bars. Data were expressed as a percentage of the corresponding pGL3-Control activity which had been arbitrarily defined as 100% in both untreated and PB-treated cultures and animals.
The PB-response element was first reported by Trottier and co-workers in the CYP2B2 5'-flanking sequence at -2155 to -2318 bp as mentioned in section 1.6.2 (Trottier, et al., 1995). The PBRE element has been shown to confer PB-responsiveness not only on its own promoter but on other heterologous promoters as well, such as the HSV tk promoter (Trottier, et al., 1995), the CYP2B1 and CYP2C1 promoters (Park, et al., 1996). However, the PBRE activates transcription to different degrees when attached to different promoters. For example, in vivo transfection of a construct containing a copy of the CYP2B2 PBRE attached to the CYP2C1 promoter resulted in double the fold induction than when attached to the CYP2B1 promoter in response to PB (Park, et al., 1996). It was also observed to induce 1.4-fold more when attached to a construct containing a longer CYP2B2 promoter sequence from -1 to -1700 bp (Trottier, et al., 1995) than when attached to a shorter CYP2B1 promoter sequence from -1 to -110 bp (Park, et al., 1996). Therefore, it seems that sequences upstream of -110 bp together with the PBRE are needed for increased PB-responsiveness or decreased basal expression. The presence of certain transcription factors binding to promoter sequences has been shown to enhance or stabilise the formation of the basal transcription complex leading to increased transcription (Tjian and Maniatis, 1994, Wolffe, 1994, Galvagni, et al., 1997).

Could the proteins binding to the -179 to -348 bp and -348 to -451 bp sequences synergise with the PBRE to further activate gene expression in response to PB? To find out if this is so, the PBRE region was cloned into the constructs, pGL3B(-179) and pGL3(-348) to generate two new
constructs designated, pGL3B(-179)/(159) and pGL3B(-348)/(159) as detailed in section 3.3.1. However, the PBRE sequence is not obtained from CYP2B2 but instead from the homologous region of CYP2B1, located between -2142 and -2301 bp. PB treatment increased the expression of the pGL3B(-179)/(159) resulting in a 2.1-fold increase in luciferase activity as shown in Fig. 3.3.7a. A similar fold induction in luciferase activity of the pGL3B(-348)/(159) construct was also observed indicating that the sequence -179 to -348 bp exhibits no marked synergistic effect with the PBRE in activating gene expression. Unfortunately, the construct pGL3B(-451)/(159) was not available at this time for transfection. Hence, it was not known if the sequence from -348 to -451 bp could synergise with the PBRE to activate gene expression in vitro.

For every transfection experiment, the expression and PB-induction of the endogenous CYP2B mRNAs was also determined in parallel. All batches of primary hepatocytes were responsive to PB and the fold induction of the endogenous CYP2B mRNAs was found to be about 10-fold with little variation.

Although primary hepatocyte culture is the next best option to in vivo for transfection studies, the two systems are quite different. When hepatocytes are isolated and cultured, the expression of numerous CYPs declines very rapidly. The expression of many other genes including transcription factors also decline continuously throughout culture (Padgham, et al., 1993). Furthermore, the increased expression of both CYP2B1 and CYP2B2 genes by PB is generally 10-fold less in primary hepatocytes than in liver tissue (Schuetz, et al., 1988). Therefore, applying
Fig. 3.3.7 Transient transfection of the CYP2B1 promoter fusion constructs transfected either into (a) primary rat hepatocyte cultures (in vitro) or (b) rat livers (in vivo). Data show the mean of triplicates for in vitro from 2 independent experiments and duplicates for in vivo from a single experiment. Standard deviations are indicated by error bars. Data were expressed as a percentage of the corresponding pGL3-Control activity which had been arbitrarily defined as 100% in both untreated and PB-treated cultures or animals. N.D., not determined.
the results obtained from *in vitro* cultured cells to *in vivo* tissues should be interpreted with caution.

When a Helios Gene Gun System for performing *in vivo* transfection became available for use, the decision to switch to using the *in vivo* system was made. The use of *in vivo* transfection systems to study the 5'-flanking sequence of the CYP2B genes has been reported (Park, *et al.*, 1996). This method involved the direct injection of reporter constructs into the rat liver. Although DNA transfected into rat liver by this method has been shown to be taken up by hepatocytes and expressed (Malone, *et al.*, 1994), there are two major shortcomings to this system which is not found in the Helios Gene Gun System. Firstly, a small amount of DNA (about 3 to 4 μg) is required for a single transfection while the transfection by injection method requires between 300 and 500 μg of DNA. The treatment of rats with dexamethasone prior to and after transfection is necessary as an anti-inflammatory agent that increases the efficiency of expression of the injected genes by the liver (Malone, *et al.*, 1994) which is also not required in the Gene Gun system. The latter is particularly important since dexamethasone has been reported to both increase or decrease PB induction of CYP genes (Waxman and Azaroff, 1992, Ram, *et al.*, 1995).

The normalised luciferase activity of the pGL3-Control and the pGL3-Basic vectors transfected into rat livers are shown in Fig. 3.3.3b. PB treatment seems to increase the luciferase activity of pGL3-Control. However, this result needs to be further clarified since they are taken from a single experiment. Besides, the pGL3-Control vectors has been transfected numerous times into primary rat hepatocytes and PB does not appear to
alter the luciferase activity of this construct. Thus, as in primary hepatocytes, the pGL3-Control and pGL3-Basic vectors were transfected in parallel in each experiment to serve as positive and negative controls respectively and the relative luciferase readings of constructs under investigation were expressed as a percentage of the pGL3-Control activity obtained in the same experiment.

As shown in Fig. 3.3.6b, when the three constructs, pGL3B(-179), pGL3B(-348) and pGL3B(-451) were transfected into whole liver, a low level of expression was observed in the untreated rats similar to results obtained with untreated primary hepatocytes cells. The treatment of rats with PB slightly up-regulates the expression of the luciferase gene in all three constructs. The fold induction for each construct is shown in brackets in Fig. 3.3.6b. When the PBRE sequence is included into the pGL3B(-179), pGL3B(-348) and pGL3B(-451) constructs to generate pGL3B(-179)/(159), pGL3B(-348)/(159) and pGL3B(-451)/(159). The expression of the luciferase gene from all three constructs was increased in response to PB with the luciferase activity from the pGL3B(-451)/(159) construct showing the greatest enhancement (Fig. 3.3.7b) It, therefore, appears that the sequence between -348 and -451 bp can cooperate with the PBRE in further activating gene expression. The PBRE was also demonstrated to confer PB-responsiveness on a heterologous promoter (SV40) as shown in Fig. 3.3.7b. The latter result has also been recently reported by Kocarek and co-workers in primary rat hepatocytes (Kocarek, et al., 1998).

Because the -348 to -451 bp sequence seems to show some degree of synergism with the PBRE and the fact that liver nuclear protein from PB-

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treated rats binding to this sequence were activated in response to PB suggested involvement of this sequence in PB inducible gene expression. Bienz and Pelham (1986) (Bienz and Pelham, 1986) had demonstrated that a heat-shock consensus element located 62 bp upstream of the start site for transcription of the *Drosophila hsp70* gene could function as an enhancer when multiple copies were placed at a position well upstream of the transcription start site. To test if this property applied to the *cis*-acting element within -348 to -451 bp, one or multiple copies of this sequence was cloned far upstream of the transcription start site of the pGL3B(-179) construct. The construct containing one copy of this sequence (pGL3B(-179)/(103)) showed a similar result to the pGL3B(-451) whereby PB treatment only marginally induced gene expression (Fig. 3.3.8). While the construct containing two copies of this sequence, pGL3B(-179)/(103X2) showed a 1.7-fold PB induction. Although the fold induction was only slightly greater than that of the pGL3B(-179)/(103) and was not very significant, the percentage of activity of the pGL3B(-179)/(103X2) construct in PB-treated rats was almost as high as that of the pGL3B(-179)/(159). But a higher basal expression level of the pGL3B(-179)/(103) construct caused it to show a lower fold induction. However, the response of constructs containing even more copies of the -348 to -451 bp region should be further investigated. It is possible that a weak PB-responsive element may lie within this sequence. The construct, pGL3B(-179)/(103X1.5) containing one and a half copies of the 103 bp sequence in the reverse orientation (i.e. one complete copy and another half a copy from -348 to -390 bp) showed an almost 7-fold PB induction. This was due to the fact that it had a very low basal level of
**In vivo**

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<tr>
<th>Construct</th>
<th>Untreated</th>
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<tr>
<td>pGL3B(-179)/(103X1.5)</td>
<td>6.8X</td>
<td>1.7X</td>
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<tr>
<td>pGL3B(-179)/(103X2)</td>
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Fig. 3.3.8 Transient transfection of *CYP2B1* promoter fusion constructs into rat livers *in vivo*. Data show the mean of duplicates from a single experiment and are expressed as a percentage of the corresponding pGL3-Control activity which has been arbitrarily defined as 100% in both untreated and PB-treated cultures and animals.
expression but the percent activity of this construct in PB-treated rats was actually lower than the 2-copy construct (Fig. 3.3.8). The significance of the latter result is not known and the role played by this sequence in basal gene expression is also unclear. Fig. 3.3.9 summarises the fold induction, in response to PB, of luciferase activity observed when different constructs were used in vivo and in vitro.

In order to ensure that the different fold increase in luciferase activity from the different constructs is not due to a difference in the animals response to PB, the expression of the endogenous CYP2B mRNAs from every animal that has been transfected were also assessed in parallel. The fold induction of the CYP2B mRNAs in these animals in response to PB were all found highly similar apart from the animal that has been transfected with the pGL3B(-451) construct which was induced to a lesser extent (data not shown). Until the transfection experiment with the pGL3B(451) construct is repeated, it is not clear at present if the CYP2B1 promoter sequence from -1 to -451 bp can actually confer PB-responsiveness.

The constructs pGL3B(-179)/(159) and pGL3B(-348)/(159) which have been transfected into both primary hepatocytes as well as rat liver, showed luciferase activity to be lower in vitro than in vivo. This may be attributed to the fact that the increase in CYP2B mRNAs in response to PB in primary hepatocytes was determined to be lower than in the liver of whole animals.
Fig. 3.3.9 Fold induction of luciferase activity upon PB treatment as a consequence of different CYP2B1 promoter constructs. Experiments were carried out either in vitro or in vivo. N.D., not determined.
Chapter Four

General Discussion
General Discussion

Since the induction of rat CYP2B1 and CYP2B2 gene expression by PB was determined to be at the level of transcription (Pike, et al., 1985), extensive investigations into the molecular mechanism leading to transcriptional activation and the identification of cis-acting elements and trans-acting factors involved have been carried out.

For a long time, efforts to elucidate the molecular mechanism have been hindered by the lack of a PB-responsive hepatoma cell line and also the inability to maintain suitable levels of expression and inducibility of PB-inducible genes in primary hepatocytes. Hence, many studies were carried out to establish in vitro systems that maintain the PB response. Also the CYP2B1 promoter sequence was not available until recently and results obtained have been mainly from studies of the 5'-flanking sequences of the CYP2B2 gene.

When the first 450 bp promoter sequence of the CYP2B1 was isolated and cloned in our laboratory, it was analysed using in vitro assays. The regions, -179 to -348 bp and -348 to -451 bp when used as probes in gel shift assays were found to bind more protein from liver nuclear extracts of PB-treated than untreated rats as detailed in sections 3.1.3 and 3.1.4 respectively. The nuclear protein(s) binding to the -348 to -451 bp was observed to be altered by phosphorylation status of the protein(s) and PB treatment appears to result in the phosphorylation of the transcription factor(s) resulting in enhanced protein binding (see section 3.1.10). Padmanaban and co-workers (Upadhya, et al., 1992, Prabhu, et al., 1995, Ram, et al., 1995) have also reported the phosphorylation of a transcription
factor which upon PB treatment leads to the phosphorylated protein preferentially binding to the rat CYP2B2 promoter between -69 and -98 bp [designated as the positive element (PE)]. Shephard et al (1994) (Shephard, et al., 1994) have also identified two sequences, located at -183 to -199 bp and -31 to -72 bp of the CYP2B2 promoter, to bind liver nuclear extracts that are enriched or activated from rats treated with PB. Although the transcription of the construct containing the CYP2B2 promoter sequence from -178 to -4 bp did not increase with nuclear extract from PB-treated rats in an in vitro transcription assay, the other two constructs containing the CYP2B2 promoter sequences from -368 to -4 bp and -984 to -1 bp did enhance transcription in response to PB (Shervington, 1998). It is interesting to note that the two CYP2B1 promoter regions shown to bind more protein from PB-treated rats lie within the CYP2B2 promoter regions of the two constructs used in in vitro transcription assays. The sequences between CYP2B1 and CYP2B2 within this region are highly homologous except for the -CA-repeats. There is a possibility that the proximal promoter region may play a role in PB regulation of CYP2B1 and CYP2B2 gene expression. Further upstream from the transcription initiation site, Roe et al (1996) (Roe, et al., 1996) found that PB treatment increased protein binding to an AP-1 site at -1441 bp in the CYP2B2 gene. Although there have been a few reports on the difference in the abundance of nuclear protein binding from untreated and PB-treated rats to various regions of the CYP2B2 promoter, the work reported in this thesis is the first observation of increased nuclear protein binding from extracts of PB-treated rats to the CYP2B1 5'-flanking sequences from -179 to -348 bp and -348 to -451 bp.
Intensive research has been put into developing a primary hepatocyte culture that is responsive to PB. The general conclusion is that the use of extracellular matrix and modified culture media in primary hepatocyte cultures can maintain a longer-term cell viability with retention of several CYP activities as well as a better inductive response of specific CYPs to PB (Schuetz, et al., 1988, Waxman, et al., 1990, Sidhu, et al., 1993, Sidhu and Omiecinski, 1996).

The development of PB-responsive primary rat hepatocyte culture systems then led to the identification of a PB responsive element (PBRE), located at about -2.3 kb on the 5'-flanking sequence of the CYP2B2 gene (Trottier, et al., 1995). The CYP2B2 PBRE (Trottier, et al., 1996) and its homologous regions in CYP2B1 (Park, et al., 1996, Kocarek, et al., 1998) and mouse Cyp2b10 (Honkakoski and Negishi, 1997) have been shown in several independent reports to confer PB-responsiveness both in vivo and in vitro. However, the fold-induction of the CAT or luciferase activity in response to PB was not very great, generally about 3 to 6-fold. Although the extent of increase in endogenous CYP2B1 and CYP2B2 mRNA upon PB-induction was not reported in these studies, it should be expected to be at least 10-fold or more in primary hepatocytes and >20-fold in vivo. Hence, the induction of the reporter gene expression appears to be relatively low.

In this project, the PBRE from the CYP2B1 has been demonstrated to confer PB responsiveness when attached to a luciferase reporter construct containing either the CYP2B1 promoter or the SV40 promoter in both transfected primary rat hepatocytes (section 3.3.2; Fig. 3.3.7a) and rat livers (section 3.3.2; Fig. 3.3.7b) upon PB treatment. But, the fold-induction of
luciferase activity in response to PB both in vivo and in vitro was lower than that of the endogenous CYP2B mRNA expression. One possible reason for the lower fold-induction in the luciferase expression of reporter constructs may be attributed to the shorter half-life of the luciferase mRNA, i.e. 6 hrs (Thompson, et al., 1991) or protein, i.e. 3 hrs (Thompson, et al., 1991, Bronstein, 1994) as compared to the CYP2B mRNA, i.e. 15.7 hrs (Woodcroft and Novak, 1997) and protein, i.e. 37 hrs (Shiraki and Guengerich, 1984). The higher turnover rate of either the luciferase mRNA or protein may lead to the detection of a lower activity at the time of assay. However, this is unlikely to be the situation because a transfection carried out in vivo for 6 hrs compared to 24 hrs showed no increase in luciferase activity in response to PB although the endogenous CYP2B mRNA had already induced by about 11-fold upon PB-induction for 6 hrs (Muangmoonchai, R., personal communication). A second possible reason could be due to the competition between the reporter gene construct and the promoter of the endogenous CYP2B gene for the same transcription factor(s) which may be limiting in numbers. However, this also appears not to be the case. Kocarek et al (Kocarek, et al., 1998) observed a 4.4-fold PB-induction of luciferase activity in primary rat hepatocytes of a construct containing the CYP2B1 PBRE attached to the SV40 promoter. They showed also that the luciferase activity of a construct containing 2.4 kb of the 5'-flanking sequence of the CYP2B1 gene was induced to ~20-fold, almost 5 times more than the former construct indicating that transcription factors are not limiting. A similar observation was made with the CYP2B2 promoter but the fold difference is lower possibly because CYP2B2 is known to be less inducible than
CYP2B1 (Trottier, et al., 1995). Thus, the presence of sequences downstream of the PBRE appear to be necessary for PB-induction of gene transcription. In addition, the luciferase activity of reporter constructs containing 1.4 kb proximal promoter region, which do not include the PBRE, of either CYP2B1 or CYP2B2 when transfected into FGC4 cells, a rat hepatoma cell line were reported to increase by 3-fold in response to PB treatment (Shaw, et al., 1993). These reports together with the results of the in vitro transcription assays carried out by Shervington (1998) (Shervington, 1998) seem to indicate that the PBRE alone, although able to confer PB-responsiveness, is not sufficient to cause the large PB response observed in vivo for the CYP2B1 and CYP2B2 genes. It seems very likely that other promoter sequences may be required for full PB responsiveness. However, the precise promoter region(s) responsible for PB induction was not determined in the two reports above. In contrast, Trottier et al (1995) (Trottier, et al., 1995) showed that reporter constructs containing different lengths or combinations of different regions of the CYP2B2 5'-flanking sequence in the absence of the PBRE region did not increase CAT activity in response to PB.

Very recently, two nuclear receptor sites located within the PBRE have been determined to be bound by a CAR/RXR heterodimer (Honkakoski, et al., 1998b, Sueyoshi, et al., 1999). The fact that CAR mRNA is expressed preferentially in the liver (Baes, et al., 1994, Choi, et al., 1997) can not explain the ability of PB to induce CYP2B1 mRNA expression in the small intestine (Traber, et al., 1990, Elia, 1996) and the constitutive expression of CYP2B1 mRNA in the lung and testis (Omiecinski, 1986, Christou, et al., 1987). In addition, CYP2B2 is neither constitutively expressed nor inducible.
in all these tissues. This further supports the finding that cis-acting element(s) other than the PBRE may be important in the PB-induction of CYP2B1 and CYP2B2 gene expression. Shephard et al (1994) (Shephard, et al., 1994) has identified a region between -183 and -199 bp within the CYP2B2 promoter that confers tissue-specific expression to the CYP2B2 gene. However, it will be interesting to determine what role the PBRE plays in regulating CYP2B1 gene expression in the intestinal cell.

From the transfection assays in section 3.3.2, the reporter gene construct containing the CYP2B1 promoter sequence from -451 to -1 bp alone is not responsive to PB but this sequence was observed to exhibit a certain degree of synergism with the PBRE giving rise to a greater fold increase in luciferase activity upon PB induction. Although the promoter sequence on its own does not have the ability to confer PB-responsiveness, the involvement of this promoter region in PB induction cannot be completely ruled out.

The gel shift results in section 3.1.8 showed that the transcription factors binding to the CYP2B1 promoter sequences from -179 to -348 bp and -348 to -451 bp can interact with each other. Since these two sequences lie adjacent to each other, the interaction of protein binding to each of these sequences may possibly induce a bend in the DNA sequence. There are also other indications that DNA bending may play a role in transcription of the CYP2B genes. For instant, YY1 is shown to bind to the sequence between -440 and -410 bp in gel shift assay (see section 3.1.6) and YY1 has been reported to have the ability to bend DNA (Natesan and Gilman, 1993). It is interesting to note that YY1 competes with the
dystrophin promoter bending factor (DPBF) for a regulatory element within the dystrophin promoter. Apparently, YY1 and DPBF bend DNA in the opposite orientation resulting in the negative or positive regulation respectively of the dystrophin gene by alternatively organising the DNA structure. Another intriguing thing is that DPBF promoter binding acts as an architectural component that alters the promoter structure and enhances dystrophin transcription by facilitating the interactions of other regulatory proteins with components of the basic transcription machinery (Galvagni, et al., 1997). Other examples have been cited of enhancer elements that might not be involved in direct interaction with the transcription machinery, but instead may stabilise a certain DNA conformation to potentiate interactions between the promoter and other enhancer sequences (Tjian and Maniatis, 1994, Wolffe, 1994). Rippe et al (1995) (Rippe, et al., 1995) showed, using a model, that induced DNA curvature by proteins bound to the near upstream promoter element site can serve to impose constraints on local DNA conformations and create specific three-dimensional arrangements that favour the interaction of proteins bound to distal elements with the transcriptional machinery. As cellular DNA is organised into chromatin, the differential bending induced by certain factors could play a role in promoter accessibility as well as bringing distal enhancer elements in close proximity with the basic transcription machinery.

As suggested in section 3.1.6, the putative YY1 binding site within the CYP2B1 promoter matches more closely with the YY1 repressor consensus sequence. It has been reported that in genes where YY1 represses transcription, YY1 sites often overlap or occur near an activator-binding site.
and the increased expression of the activator relieves YY1-dependent repression (Shrivastava and Calame, 1994). There is also some indication that the YY1 activator site may be a tighter binding site than the repressor sequence since the former is more GC-rich (Satyamoorthy, et al., 1993, Javahery, et al., 1994, Shrivastava and Calame, 1994). This would appear to be of intrinsic importance because the lower binding affinity of YY1 to the repressor site allow it to be displaced by activators. Since the amount of YY1 protein in liver nuclear extract does not appear to be altered by PB, it may be involved in repressing the basal expression of CYP2B1. Upon PB treatment, the protein(s) involved in the formation of complex ‘a’, in the gel shift where -348 to -451 bp is the probe, is seen to increase in abundance or activity. Accordingly, the protein(s) involved in complex ‘a’ formation have been deduced to bind to the CYP2B1 promoter sequence around the vicinity of the putative YY1 binding site. Thus, it may be possible that the protein(s) binding in complex ‘a’ is activated in response to PB and displaces YY1 binding to its putative binding site just like the DPBF displacing YY1 in the dystrophin promoter in response to differentiation. However, it is not known if DNA bending is involved in this particular case. The proteins binding to -179 and -451 bp also behave very much like a protein such as the DPBF. This would most help to explain why the region between -179 to -451 bp of the CYP2B1 promoter was observed to bind more liver nuclear protein from PB-treated rats yet unable to transactivate a reporter gene construct on its own.

With regards to DNA bending, it is also interesting to mention that one of the significant differences between the first 2.3 kb sequence of the CYP2B1 and CYP2B2 promoters is the number of -CA- repeats (Suwa, et
al., 1985). Regions of alternating purines and pyrimidines have a tendency to result in DNA of the Z-form. Z-DNA is the only left-handed helix and because it contains more base pairs per turn than any other duplex form, it has the least twisted structure (Lewin, 1994). It is not known if Z-DNA occurs in vivo and whether its less twisted structure would result in less flexibility. However, it is hard to ignore the fact that \textit{CYP2B2}\ has more -CA- repeats than \textit{CYP2B1} and \textit{CYP2B2} is less PB-inducible than \textit{CYP2B1}. The greater number of -CA- repeats in the \textit{CYP2B2} promoter may inhibit gene looping and hence gene activation by preventing the efficiency of protein interactions between the PBRE region at -2.3 kb and/or other distal elements with the basic transcription machinery (Rippe, \textit{et al.}, 1995). There is no evidence at this stage to prove that the -CA- repeats indeed play a role in the difference in PB-inducibility between \textit{CYP2B1} and \textit{CYP2B2}.

Kim and Kemper (1997) (Kim and Kemper, 1997) have shown that PB can alter the chromatin structure within the PBRE region of the \textit{CYP2B1} and \textit{CYP2B2} promoters using an in vivo footprinting assay. However, in vivo footprinting assays have not been performed on other regions of the \textit{CYP2B} promoters, thus it is not known if the binding of transcription factors to other regions are also affected by PB treatment. It will be interesting to find out if the proteins binding within -179 to -451 bp are altered in vivo by PB treatment. It is also interesting to test if these proteins are able to recognise the DNA organised in nucleosomal structures and facilitate the access of different regulatory factors by exposing the DNA on the nucleosome.

In conclusion, the PBRE does not seem to be the whole answer to PB induced transcription of the \textit{CYP2B1} and \textit{CYP2B2} genes. There may be
other unidentified PB-responsive elements that can synergise with the PBRE to activate transcription. Although the CYP2B1 promoter sequence between -179 and -451 bp does not appear to have the characteristic of a PB-response element, the possibility that this region may still play a role in PB-mediated transcriptional activation in vivo cannot be ruled out.
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