THE REGULATION OF CYTOCHROME P450 2B GENE EXPRESSION BY THE ANTI-EPILEPTIC DRUG PHENOBARBITAL

by
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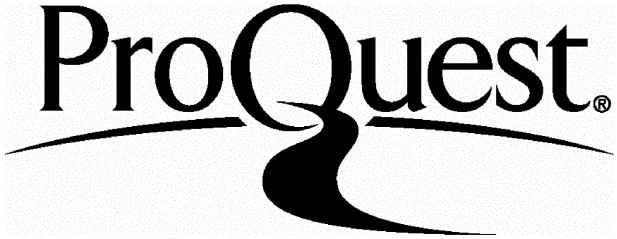

Abstract

Expression of the Cytochrome P450 *CYP2B2* gene is increased in rat liver following exposure to the anti-epileptic drug phenobarbital. The proximal 5' flanking sequence of this gene was studied to identify *cis*-acting DNA elements and *trans*-acting proteins which are involved in this induction mechanism. C/EBPα and C/EBPβ bind within the region from -34 to -86bp of the *CYP2B2* promoter. C/EBPβ is involved in complexes which increase in abundance in response to phenobarbital treatment of the animal. Neither C/EBPα or C/EBPβ increase in amount following phenobarbital treatment. However, formation of complexes containing C/EBPα and/or C/EBPβ is inhibited by alkaline phosphatase treatment of nuclear protein extracts. Competition gel retardations suggest that a GC box binding protein and AP1 are also involved in the complexes that bind to the -34 to -86bp region of the *CYP2B2* gene.

The study of the mechanism of phenobarbital induction of cytochromes P450 2B1 and 2B2 has suffered from the lack of an hepatocyte culture system or cell line capable of maintaining their expression and induction. Dr. G. Ciaramella set up a primary hepatocyte culture system that was capable of maintaining CYP2B1/2 mRNAs and their induction by phenobarbital for 4 days (Ph.D. Thesis, 1995). This system was characterised further and its suitability for use in studying the phenobarbital induction mechanism was demonstrated. Treatment of these cells with a cAMP analogue, a protein phosphatase inhibitor and a cAMP-dependent protein kinase activator inhibited the phenobarbital response at the protein level. The primary hepatocytes were transfected with constructs containing sections of the *CYP2B2* 5' flanking sequence attached to a luciferase reporter gene. Expression from the construct containing the -2915bp to +29bp region of *CYP2B2* increased slightly in response to phenobarbital treatment of the cells.

Contents

	Page
Abstract.	2
Contents.	3
Acknowledgements.	13
Abbreviations.	14

1. Introduction.

1.1 Drug metabolism: a molecular defence system.	19
1.2 The multi-function oxygenase system.	21
1.2.1 NADPH-dependent cytochrome P450 reductase (P450 reductase).	22
1.2.2 Lipid.	25
1.2.3 Cytochrome b5 and NADPH-dependent cytochrome b5 reductase.	25
1.3 Cytochromes P450.	25
1.3.1 Nomenclature, occurrence and evolution of cytochromes P450.	26
1.3.2 Regulatory mechanisms of cytochromes P450.	28
1.3.2a Induction by specific chemical agents.	29
1.3.2b Regulation of constitutive expression.	34
1.4 Phase II metabolism.	36
1.5 Glutathione-S-transferases (GSTs).	37
1.5.1 GST nomenclature.	39
1.5.2 Regulation of GSTs.	40
1.6 Hepatocyte culture.	43
1.6.1 The effects of soluble medium factors.	46

Page

1.6.2 The effects of extracellular matrix components. 47
1.6.3 The effects of cell-cell interactions. 49

1.7 Cytochromes P450 2B1 and 2B2. 50
1.7.1 Phenobarbital induction of *CYP2B1* and *CYP2B2* gene expression and the scope of this project. 53

2. Methods.

2.1 Bacterial cultures. 58
2.1.1 Preparation of glycerol stocks. 59
2.1.2 Preparation of agar plates. 59
2.1.3 Inoculation of cultures and chloramphenicol amplification of plasmid DNA. 59

2.2 Subcloning of DNA into a plasmid vector. 60
2.2.1 Ligation of cohesive ends. 61
2.2.2 Ligation of blunt ends. 61

2.3 Transformation of plasmid DNA into *E. coli* bacteria. 62

2.4 Preparation of plasmid DNA. 64
2.4.1 Small scale preparation of plasmid DNA. 64
2.4.2 Medium scale plasmid preparation. 65
2.4.3 Medium scale plasmid DNA preparation using Qiagen columns. 67

2.5 Measurement of DNA and RNA concentration. 68
2.5.1 The optical density method. 68
2.5.2 Using DNA DipStick™. 69

2.6 Restriction digests. 69

Page

2.7 Agarose gel electrophoresis of DNA. 70
2.7.1 Separation of DNA on an agarose gel. 70
2.7.2 Purification of DNA from a low melting point agarose gel. 72

2.8 Polymerase chain reaction. 73

2.9 Preparation of nuclear protein extracts from rat liver tissue. 75
2.9.1 Treatment of the animal. 75
2.9.2 Isolation of nuclei. 76
2.9.3 Method of nuclear protein extraction by Sierra (1990). 78
2.9.4 Method of nuclear protein extraction by Lavery and Schibler (1993). 80
2.9.5 Method of nuclear protein extraction by Jose-Estanyol *et al.* (1989). 81
2.9.6 One hour minipreparation of DNA-binding protein extracts. 82
2.9.7 Measurement of protein concentration by the Lowry method (1951). 83

2.10 The gel retardation assay. 84
2.10.1 Radioactive end-labelling of DNA. 84
2.10.1a 5' overhang labelling (filling). 84
2.10.1b Blunt end labelling. 85
2.10.1c Measurement of radioactive counts. 85
2.10.2 The gel retardation. 86
2.10.3 Competitive gel retardation. 88
2.10.4 Supershift gel retardation. 89

2.11 Preparation of RNA. 89
2.11.1 Preparation of total RNA from cell pellets using Ultraspec™ RNA solution. 89
2.11.2 Preparation of total RNA from cell pellets using guanidine thiocyanate. 90

2.12 Agarose gel electrophoresis of RNA. 92

Page

2.13 Slot blot analysis of RNA. 93
2.13.1 Preparation of the slot blot. 93
2.13.2 Preparation of the probe by the random priming method. 95
2.13.3 Hybridisation of the slot blot. 96

2.14 Preparation of whole cell homogenates from cell pellets. 98

2.15 Western blot analysis of proteins. 99
2.15.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE). 99
2.15.2 Staining of SDS-PAGE gels with Coomasie Brilliant Blue. 102
2.15.3 Transfer of the proteins to a nitrocellulose filter. 103
2.15.4 Probing the western blot. 104
2.15.5 Visualisation of the antibodies using the Bio-Rad Immun-Blot Assay Kit and the National Diagnostics HRPL kit. 105
2.15.6 Staining of a nitrocellulose filter using Amido Black. 105

2.16 Culture of primary rat hepatocytes. 106
2.16.1 Isolation of primary hepatocytes. 106
2.16.2 Culture of hepatocytes. 111
2.16.3 Transfection of the hepatocytes. 112
2.16.3a Preparation of DNA for transfection. 112
2.16.3b Preparation of $CaPO_4$/DNA precipitate for transfection. 113
2.16.3c Transfection of the hepatocytes. 114

2.17 Densitometry. 115

3. Results and Discussion.

Section I: Analysis of the 5' flanking sequence of the CYP2B2 gene.

	Page
3.1 Optimisation of gel retardation assay conditions.	118
3.1.1 Generation of the -34 to -86bp fragment of the *CYP2B2* gene.	118
3.1.2 The effect of different binding buffers.	120
3.1.3 Binding temperature.	121
3.1.4 Nuclear protein extraction.	124
3.1.5 Poly dI.dC concentration.	129
3.2 Analysis of the 5' flanking sequence of the *CYP2B2* gene.	131
3.2.1 Gel retardation assay with -44 to -67bp of the *CYP2B2* 5' flanking sequence.	131
3.2.2 Gel retardation with -6 to -38bp fragment of the *CYP2B2* gene promoter.	137
3.2.3 Gel retardation with the -34 to -86bp region of *CYP2B2* gene promoter.	139
3.2.4 Gel retardation with the -118 to -2bp fragment of the *CYP2B2* gene promoter.	147
3.2.5 Western blots of nuclear proteins using the anti-sera to C/EBPα and C/EBPβ.	154
3.2.6 Phosphatase treatment of the nuclear protein extracts.	156
3.3 Section I: Discussion.	160
3.3.1 Optimisation of the gel retardation assay.	160
3.3.2 Analysis of the 5' flanking sequence of the *CYP2B2* gene.	161
3.3.2a Binding at -44 to -67bp of the *CYP2B2* promoter.	161
3.3.2b Binding to the -118 to -67bp region of the *CYP2B2* promoter.	164
3.3.2c Binding of proteins at more distal regions of the *CYP2B2* gene.	168
3.3.2d Phenobarbital inducibility of the *CYP2B2* gene.	169

Section II: Use of a primary hepatocyte culture system to study the phenobarbital induction of the CYP2B2 gene.

Page

3.4 Characterisation of the primary hepatocyte culture system. 173

3.4.1 Expression of Glutathione-S-Transferases (GSTs) in primary hepatocyte culture. 176

3.4.1a Expression of GST 2-2/1-1 in primary rat hepatocytes. 176

3.4.1b Expression of GST 3-3/4-4 in primary rat hepatocytes. 181

3.4.1c Expression of GST 7-7 in primary rat hepatocytes. 186

3.4.2 Expression of NADPH dependent cytochrome P450 reductase (P450 reductase) in primary rat hepatocytes. 186

3.4.3 Expression of CYP2B1 and CYP2B2 in primary hepatocyte culture. 193

3.5 The effects of a cyclic AMP (cAMP) analogue, a cAMP-dependent protein kinase (PKA) activator and a protein phosphatase inhibitor on the phenobarbital-mediated induction of *CYP2B1/2* and P450 reductase. 198

3.6 Analysis of the 5' flanking sequence of the *CYP2B2* gene using the dual luciferase reporter gene assay. 207

3.6.1 Cloning for the luciferase assay. 207

3.6.2 Transient transfection of the hepatocytes with the luciferase constructs. 215

3.7 Section II: Discussion. 224

3.7.1 Characterisation of the hepatocyte culture system. 224

3.7.2 The effects of 8-CPT, (Sp) cAMPS and okadaic acid on the expression of CYP2B1/2 and P450 reductase, and on their induction by phenobarbital. 228

3.7.3 Transfection of the primary hepatocytes with constructs containing the luciferase reporter gene controlled by various fragments of the *CYP2B2* gene promoter. 231

3.8 General discussion. 234

Appendix.

	Page
Materials.	239
Bibliography.	244

List of figures.

1.1 Alternative routes of metabolism for bromobenzene.	20
1.2 The catalytic cycle of cytochrome P450.	23
1.3 Reactions catalysed by cytochromes P450.	24
1.4 Phenobarbital (PB) and 'PB-like' inducers.	30
1.5 Model of the transcriptional regulation of the *CYP1A1* gene by polyaromatic hydrocarbons.	32
1.6 Phase II metabolism.	38
2.1 Schematic diagram of perfusion apparatus.	108
3.1 P.C.R. amplification of the -34 to -86bp fragment of the *CYP2B2* promoter sequence.	119
3.2 The effect of different binding buffers at room temperature.	122
3.3 The effect of binding buffers on the gel retardation of -34 to -86bp *CYP2B2* at 4°C.	123
3.4 A comparison of different methods for the extraction of nuclear proteins.	125
3.5 SDS-PAGE of nuclear protein extracts.	126
3.6a Comparison of two different preparations of nuclear protein extracts.	127
3.6b Comparison of two different preparations of nuclear protein extracts.	128
3.7 The effect of altering the concentration of poly dI.dC.	130
3.8a Sequence from -44 to -67bp of the *CYP2B2* 5' flanking sequence showing putative C/EBP binding site.	132
3.8b Consensus C/EBP binding sequence.	132

Page

3.9a Gel retardation with the -44 to-67bp fragment of the *CYP2B2* 5'flanking sequence. 133
3.9b Competitive gel retardation of the -44 to -67bp fragment of the *CYP2B2* promoter. 135
3.10 Supershift gel retardation with the -44 to -67bp fragment of the *CYP2B2* promoter and anti-bodies to C/EBP. 136
3.11 Gel retardation with the -6 to -38bp fragment of the *CYP2B2* promoter. 138
3.12 Gel retardation with the -34 to -86bp fragment of the *CYP2B2* gene promoter. 140
3.13 Supershift gel retardation with the -34 to -86bp fragment of *CYP2B2* and anti-bodies to C/EBP. 143
3.14 Analysis of the -34 to -86bp fragment of the *CYP2B2* promoter by competitive gel retardation. 145
3.15a Oligonucleotide for the consensus binding sequence of Sp1. 146
3.15b Comparison of possible Sp1 binding sites in the proximal CYP2B2 promoter region to a consensus sequence. 146
3.15c The consensus oligonucleotide for the AP1 binding site. 146
3.16 Gel retardation of the consensus Sp1 binding sequence. 148
3.17 Analysis of the -118 to -2bp fragment of the *CYP2B2* gene promoter by competitive gel retardation. 150
3.18 Gel retardation with the AP1 consensus binding sequence. 153
3.19 Western blot to detect C/EBPα. 155
3.20 Western blot to detect C/EBPβ. 157
3.21 The effect of phosphatase treatment on the binding of nuclear proteins to the -2 to -118bp fragment of the *CYP2B2* promoter. 158
3.22 Summary of putative regulatory elements in the proximal *CYP2B2* promoter. 162
3.23 The molecular components of picrotoxin. 175
3.24 Western blot of whole cell homogenate from cultured primary hepatocytes probed with anti-GST 2-2/1-1. 178
3.25 Western blot of whole cell homogenate from cultured primary hepatocytes probed with anti-GST 2-2/1-1. 180
3.26 Western blot of whole cell homogenate from cultured primary hepatocytes probed with anti-GST 3-3/4-4. 183
3.27 Western blot of whole cell homogenate from cultured primary hepatocytes probed with anti-GST 3-3/4-4. 185

Page

3.28 Western blot of whole cell homogenates from primary hepatocytes probed with anti-GST 7-7. 187
3.29 Western blot of whole cell homogenates from primary hepatocytes probed with anti-GST 7-7. 188
3.30 Western blot of whole cell homogenate from cultured primary hepatocytes probed with anti-P450 reductase. 190
3.31 Western blot of whole cell homogenate from cultured primary hepatocytes probed with anti-P450 reductase. 192
3.32 Slot blot showing CYP2B1/2 mRNA from primary hepatocytes treated with varying amounts of phenobarbital. 194
3.33 Western blot of primary hepatocyte whole cell homogenates using anti-CYP2B1/2 serum. 196
3.34 Graph of the effect of changing phenobarbital concentration on the induction of CYP2B1/2 in primary hepatocytes. 197
3.35a Preparation of total RNA from primary hepatocytes using the guanidine thiocyanate method. 200
3.35b Preparation of total RNA from primary hepatocytes using Ultraspec solution. 200
3.36 Slot blot showing CYP2B1/2 mRNA from primary hepatocytes treated with 8-CPT, (Sp)cAMPS or okadaic acid. 202
3.37 Graph of the effect of 8-CPT, (Sp)cAMPS and okadaic acid on the amount of CYP2B1/2 mRNA. 203
3.38 Western blot to show the effect of 8-CPT, (Sp)cAMPS, and okadaic acid on the amounts of CYP2B1/2 and P450 reductase in primary heptocytes. 204
3.39 Graph of the effect of 8-CPT, (Sp)cAMPS and okadaic acid on the amount of P450 reductase. 205
3.40 The pGL3 enhancer vector map. 208
3.41 Partial restriction digests of the λP4502B2 clone with Nco I and Eco RI. 210
3.42a Cloning strategy to create luciferase reporter gene constructs. 211
3.42b Constructs made for use in the luciferase assay. 212
3.43a Agarose gel to show pGL-*CYP2B2* constructs. 213
3.43b Agarose gel to show the pGL -2915 clone. 216

Page

3.43c Table of the sizes of DNA fragments expected from restriction digests of the pGL-CYP2B2 constructs. 217
3.44 Luciferase activity in primary hepatocytes transfected with *CYP2B2* promoter constructs. 219
3.45 Graph of relative activity of the various constructs in transfected hepatocytes. 220
3.46 Graph of the effects of phenobarbital and/or okadaic acid treatment on the activity of firefly luciferase reporter gene in transfected hepatocytes. 222
3.47 Regulation of the expression and activity of DNA-binding proteins. 235

A.1 Molecular weight standards. 238

List of Tables.

1.1 Phase I and Phase II reactions. 20
1.2 Overview of the cytochrome P450 superfamily. 28
1.3 Rat cytosolic GST isoenzymes, subunit nomenclature and dimerisation possibilities. 41

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Abbreviations

ADP	Adenosine diphosphate
Ah receptor	Aromatic hydrocarbon receptor
Aldh 1	Aldehyde dehydrogenase
AMP	Adenosine monophosphate
AP1	Activator protein 1
ARE	Ah receptor response element
ATP	Adenosine triphosphate
b_5 reductase	NADPH-dependent cytochrome b_5 reductase
bp	Base pairs
BROD	Benzyloxyresorufin O-dealkylase
B.S.A.	Bovine Seurm Albumin
BTE	Basal transcription element
cAMP	Cyclic adenosine monophosphate
CAT	Chloramphenicol acetyl transferase
CDI	1-Cyclohexyl-3(2-morpholinoethyl)carbodiimide metho-*p*-toluene sulphonate
cDNA	Complementary DNA
CDNB	1-chloro-2,4-dinitrobenzene
C/EBP	CCAAT enhancer binding protein
Ci	Curies (unit of radioactivity)
cpm	Counts per minute
8-CPT	8-(4-chlorophenylthio)-cAMP
CYP	Cytochrome P450
dATP	Deoxyriboseadenosine triphosphate
DCNB	1,2-dichloro-4-nitrobenzene
dCTP	Deoxyribosecytosine triphosphate
DEPC	Diethylpyrocarbonate
dGTP	Deoxyriboseguanine triphosphate

DMEs	Drug metabolising enzymes
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleoside triphosphate
ds	Double-stranded
DTT	Dithiothreitol
dTTP	Deoxyribosethymine triphosphate
ECOD	7-ethoxycoumarin O-deethylase
E.coli	*Escherichia coli* bacteria
EDTA	Diaminoethanetetraacetic acid disodium salt
EGTA	Ethylene glycol-bis[β-aminoethyl ether]-N,N,N',N'-tetraacetic acid
F.C.S.	Foetal calf serum
g	Gravitational constant: $9.8 m/s^2$
GAR-HRP	Goat-anti-rabbit IgG conjugated to horseradish peroxidase
GH	Growth hormone
GRE	Glucocorticoid response element
GST	Glutathione-S-transferase
HEPES	N-2-Hydroxymethylpiperazine-N'-2-ethanesulphonic acid
HNF	Hepatic nuclear factor
HRPL	Horseradish peroxidase chemi-luminescence
Hsp 90	Heat shock protein 90
kb	Kilobase
1 KB	1 kilobase marker
Klenow	Klenow fragment of *E. coli* DNA polymerase
λ	Wavelength of light
LB medium	Luria-Bertani medium
MC	3-Methylcholanthrene
MOPS	[N-Morpholino]propane sulphonic acid
mRNA	Messenger RNA
M.W.	Molecular weight
NADH	Nicotinamide adenosine dinucleotide
NADPH	Nicotinamide adenosine dinucleotide phosphate

Nmo 1	NAD(P)H menadione oxidoreductase
OD	Optical density
Ok	Okadaic acid
P450 reductase	NADPH-dependent cytochrome P450 reductase
PAH	Polycyclic aromatic hydrocarbon
PAPS	3'-Phosphoadenosine-5'-phosphosulphate
PB	Phenobarbital
PBS	Phosphate buffered saline
PCN	Pregnenolone-16α-carbonitrile
P.C.R.	Polymerase chain reaction
PIPES	Piperazine N, N'- bis [2-ethane] sulphonic acid
PKA	cAMP-dependent protein kinase
PKC	Protein kinase C
PMSF	Phenylmethylsulphonyl fluoride
PPAR	Peroxisome proliferator activated receptor
PPRE	Peroxisome proliferator response element
psi	Pounds per square inch
Px	Picrotoxin
RNA	Ribonucleic acid
RNase	Ribonuclease
RP	Random priming
rpm	Revolutions per minute
RT-PCR	Reverse transcription P.C.R.
SAM	S-adenosylmethionine
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
ss	Single-stranded
STE buffer	Sodium tris.EDTA buffer
TAE buffer	Tris.acetate buffer
TAT	Tyrosine amino transferase
TBE buffer	Tris.borate EDTA buffer
TBS	Tris buffered saline
TCDD	2,3,7,8-Tetrachlorodibenzoparadioxin
TE buffer	Tris.EDTA buffer
TEMED	N, N, N', N'-tetramethyl ethyl diamine
TPA	12-*O*-tetradecanoylphorbol acetate

TTBS	Tween 20. Tris buffered saline
UDP-GT	Uridine diphosphoglucuronyl transferase
UV	Ultra-violet
v/v	Volume per volume
w/v	Weight per volume
XRE	Xenobiotic response element

Chapter One

Introduction

1.1 Drug metabolism: a molecular defence system.

All organisms continually undergo insult from toxic chemicals in the environment. Humans can consume up to 1.5g of natural pesticides each day in the form of plant phenols, flavinoids and others (Ames *et al.*, 1990a). Many of these compounds are rodent carcinogens, e.g. D-limonene, benzyl acetate, caffeic acid, all of which are widely found in foods and drinks (Ames *et al.*, 1990a, Ames *et al.*, 1990b). In addition to dietary components, the body produces highly reactive oxidizing species such as superoxide radicals and peroxide radicals in the course of respiration, exposure to ultraviolet light and inflammation (Nebert *et al.*, 1990). Consequently, various defence mechanisms have developed to protect the cell from a wide range of harmful molecules. Drug metabolism is the most versatile of these systems, which can deal not only with naturally occurring chemicals, but also a wide range of drugs, chemical carcinogens and other environmental contaminants (reviewed Porter and Coon, 1991; Nebert and Gonzalez, 1987; Gibson and Skett, 1994).

Drug metabolism is usually divided into two sequential phases - phase I (functionalisation) and phase II (conjugation) reactions (table 1.1). Phase I prepares the drug for phase II by producing a chemically reactive group on which the phase II reactions can occur. Phase II detoxifies the compound producing soluble, excretable products. Many phase I and phase II reactions are capable of being performed on the same compound, so that for a given compound there may be several routes of metabolism which would compete for the substrate. The regulation of the amounts of these enzymes relative to one another can therefore change the route of metabolism and the final product of metabolism. This can be significant as many phase I reactions produce highly reactive and damaging species (reviewed in

Phase I	Phase II
oxidation reduction hydrolysis hydration dethioacetylation isomerism	glucuronidation sulphation methylation acetylation amino acid conjugation glutathione conjugation fatty acid conjugation condensation

Table 1.1: Phase I and Phase II reactions. Reactions classed as phase I and phase II drug metabolism reactions (reproduced from Gibson and Skett, 1994).

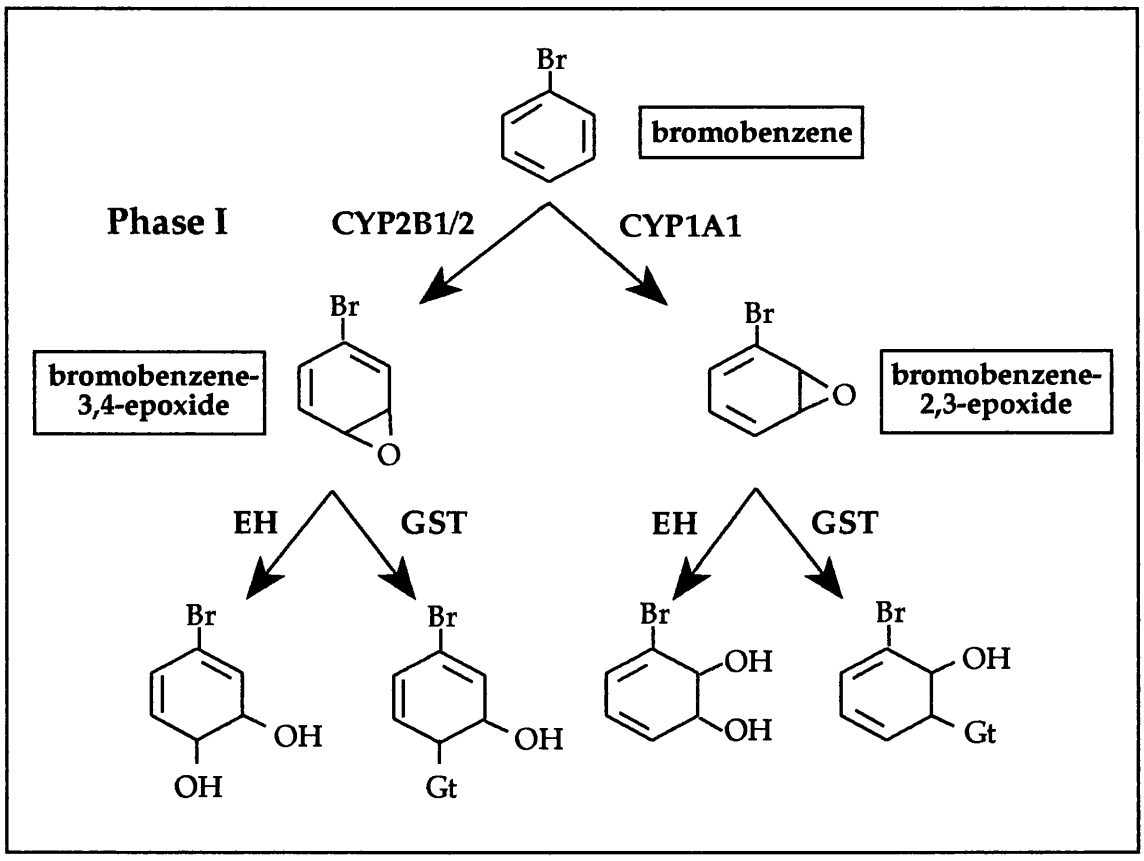


Figure 1.1: Alternative routes of metabolism for bromobenzene. Bromobenzene is metabolised by two different cytochrome P450 isoenzymes (CYP1A1 and CYP2B1/2) to two different epoxides. These are then metabolised further by epoxide hydrolase (EH) and glutathione-S-transferase (GST). Gt is a glutathione group. Reproduced from Gibson and Skett, 1994.

Guengerich, 1992). For example, during the metabolism of bromobenzene (figure 1.1), the phase I metabolites are two epoxides, both of which are hepatotoxic. However, bromobenzene-3,4-epoxide is substantially more toxic than bromobenzene-2,3-epoxide. Changing the ratio of CYP1A1: CYP2B1/2 would therefore change the route of metabolism to a more or a less toxic pathway.

Drug metabolising enzymes (DMEs) are also involved in the metabolism of numerous endogenous compounds such as steroids, bile acids, prostaglandins and retinoids. They consequently play an important role in maintaining the concentrations of ligands which regulate genes involved in growth, differentiation, apoptosis cellular homeostasis and neuroendocrine functions (reviewed in Nebert, 1994).

1.2 The multi-function oxygenase system.

Most phase I reactions are carried out by the mixed function oxygenase system (Lu and Coon, 1968). This is a membrane bound system found in the endoplasmic reticulum of many cells particularly in the liver, kidney, lung and intestine. The system consists of cytochromes P450, NADPH dependent cytochrome P450 reductase (P450 reductase), cytochrome b_5, NADPH-dependent cytochrome b_5 reductase (b_5 reductase) and lipid. All the oxidative reactions carried out by the mixed function oxygenase system involve the acceptance of electrons from NADPH via an interaction with either P450 reductase or b_5 reductase and the transfer of these electrons via cytochrome P450 or cytochrome b_5 to molecular oxygen. An atom of oxygen is introduced into the substrate and the second atom of oxygen is reduced to water (Gunsalus and Sligar, 1977). The generalised reaction is:

$$NADPH + H^+ + O_2 + RH \rightarrow NADP^+ + H_2O + ROH$$

where R is the substrate. The catalytic cycle, and the precise contribution of the cytochrome b_5 and b_5 reductase are not fully understood. One proposed mechanism is shown in figure 1.2. A subsequent rearrangement or decomposition of the product (ROH) may occur. The reactions involve numerous and structurally diverse substrates including endogenous compounds and a range of hydrophobic foreign compounds (xenobiotics). As well as hydroxylation the multi-function oxygenase system catalyses epoxidation, N-, O-, and S- dealkylation, oxidative deamination, N- and S-oxidation, and dehalogenation reactions (figure 1.3) (reviewed in Porter and Coon, 1991).

1.2.1 NADPH-dependent cytochrome P450 reductase (P450 reductase).

This enzyme is an essential component of the mixed function oxygenase system. The enzyme (M.W. ≈ 78 000) is closely associated with cytochrome P450 in the endoplasmic reticulum membrane. It contains one molecule of flavin adenine dinucleotide (FAD) and one molecule of flavin mononucleotide (FMN) per molecule (Iyanagi and Mason, 1973; Vermillon and Coon, 1974). It is one source for the reducing equivalents required during the catalytic cycle of cytochrome P450 (steps 2 and 5 figure 1.2). $NADPH + H^+$ is a 2 electron donor and cytochrome P450 is a 2 x 1 electron acceptor, so an intermediate is required between the two to accept the two electrons from NADPH and transfer them separately to cytochrome P450 (Lu *et al.*, 1969). The precise nature of the redox states of P450 reductase during the cytochrome P450 catalytic cycle is unclear as yet (reviewed in White, 1994).

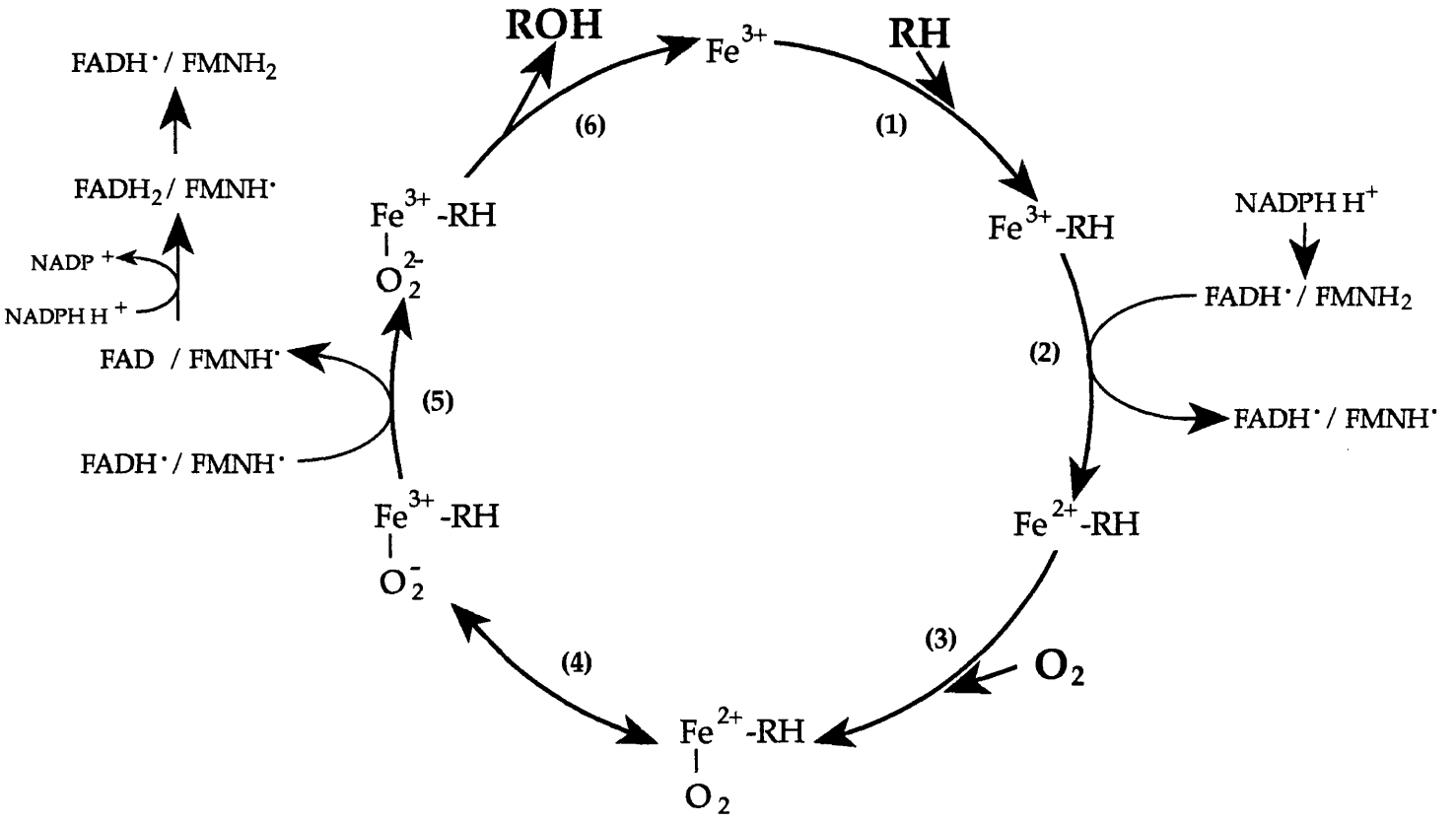


Figure 1.2: The catalytic cycle of cytochrome P450. RH represents the drug substrate and ROH the corresponding hydroxylated product. (1) The substrate is bound to the enzyme. (2) The ferric form of cytochrome P450 is reduced to the ferrous form. (3) Molecular oxygen is bound to the enzyme-substrate adduct. (4), (5) and (6) involve the introduction of the second electron, insertion of the oxygen into the substrate and release of the product, however they have not been fully elucidated as yet. Adapted from Gibson and Skett (1994).

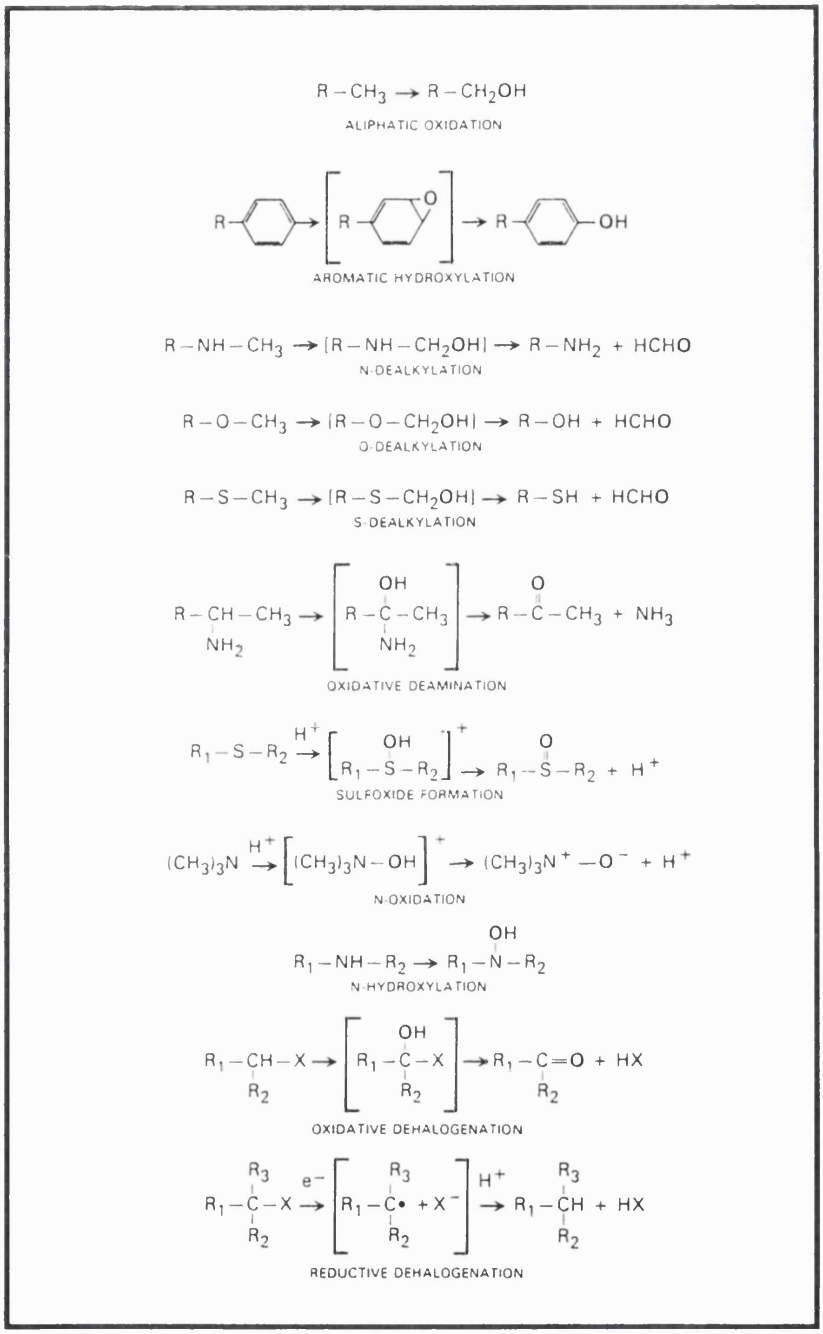


Figure 1.3: Reactions catalysed by cytochromes P450. Examples of the many and diverse reactions catalysed by cytochromes P450. Reproduced from Nebert and Gonzalez (1987).

1.2.2 Lipid.

Activity of the multi-function oxidase system requires a lipid component (Haugen *et al.*, 1976). Indications are that this is phosphatidylcholine, and that the fatty acid content of the lipid is crucial (Nelson and Strobel, 1988). It has been suggested that the lipid may be needed for electron transfer, or to promote the cytochrome P450 - P450 reductase interaction, as only the membrane-binding form of P450 reductase can reduce cytochrome P450 (reviewed in Black, 1992).

1.2.3 Cytochrome b_5 and NADPH dependent cytochrome b_5 reductase.

The precise function of these enzymes in the multi-function oxygenase system is unclear. Indeed it is not even certain whether their presence is a requirement for cytochrome P450-dependent oxidations to occur. Both cytochrome b_5 (M.W. ≈ 18 000) and b_5 reductase (M.W. ≈ 34 000) have been purified from microsomal membranes of rat liver (Strittmater *et al.*, 1978; Schafer and Hultquist, 1980). It has been suggested that they might constitute another pathway for passing reducing equivalents from NADH to cytochrome P450 (Estabrook, 1978), such that electrons would flow:

NADH → b_5 reductase → cytochrome b_5 → cytochrome P450

1.3 Cytochromes P450.

Cytochromes P450 (CYPs) are a superfamily of haemoproteins of molecular weight ≈ 45 000 - 55 000. The haem group is non-covalently bound to the

apoprotein and when it is also complexed to carbon monoxide produces a characteristic Soret peak at 450nm (Omura and Sato, 1964a, Omura and Sato, 1964b). These enzymes receive the reducing equivalents from P450 reductase/cytochrome b_5, bind molecular oxygen and catalyse the metabolism of hydrophobic compounds.

1.3.1 Nomenclature, occurrence and evolution of cytochromes P450.

The extent of the cytochrome P450 superfamily reflects the wide variety of reactions they are involved in. The enzyme system is present in many tissues of a wide variety of organisms, each of which usually contains a multiplicity of cytochrome P450 proteins. There have been 481 cytochrome P450 genes and 22 pseudogenes described to date in 85 eukaryotes (including mammalian, other vertebrate, invertebrate, fungal and plant species) and 20 prokaryotes (Nelson *et al.* 1996). The genes are divided into 74 families, 14 of which are mammalian. Cytochromes P450 are divided into families based on amino acid sequence similarity (≥40% amino acid sequence similarity within a family), and numerous subfamilies (≥46% amino acid similarity) (Nebert *et al.*, 1987). The genes are denoted by "*CYP*" followed by an Arabic numeral designating the family, a capital letter representing the subfamily, and an Arabic numeral to distinguish the individual gene, e.g. *CYP2B2*. The letters used are upper case for all species excepting mouse and Drosophila, where, by convention, the root symbol is "*Cyp*" and a lower case letter denotes the subfamily.

Cytochromes P450 (or CYPs as I shall now call them) occur throughout the phyla, for instance the CYP51 family has been found in mammals, filamentous fungi, yeast and plants, and it is presumed that they were

present in the earliest organisms. Table 1.2 gives an overview of the superfamily. Evolutionary relationships between the genes have been determined using computer based analysis, in conjunction with fossil evidence, to compare primary amino acid sequences of the cytochrome P450 proteins (Nebert, *et al.*, 1987; Nebert *et al.*, 1989; Gonzalez and Nebert, 1990). Many new genes have evolved in the past 400 million years, in particular in the CYP2B subfamily where there have been approximately 30 gene duplications. There are thought to be several contributory factors to this rapid evolution: ~ 1200 million years ago animals and plants diverged and plants began to produce stress metabolites (mainly phytoalexins) to defend themselves from ingestion by animals. Animals had therefore to produce new enzymes to cope with these. Also, when animals moved onto land (~ 400 million years ago), they encountered a host of new plants which gave them new dietary components to be metabolised (Gonzalez and Nebert, 1990). Evolution of the CYP2B subfamily in particular appears to be associated with the development of the distinctive dietary habits of the different species. All these components led to large selective pressures on the superfamily. Gene conversion events and gene duplication, subsequently fixed by natural selection have caused major interspecies differences in drug and carcinogen metabolism. This is obviously of importance when considering the application to humans of pharmaceutical tests of drugs on laboratory animals.

1.3.2 Regulatory mechanisms of cytochromes P450.

Regulation of CYP expression is complex: it is subject to developmental control, CYP expression is tissue, strain and sex specific, and responsive to

Family	Occurence and general characteristics
CYP1	Vertebrates: dioxin-inducible, metabolism of polycyclic hydrocarbons, halogenated and heterocyclic hydrocarbons, and aromatic amines
CYP2	Vertebrates and invertebrates: metabolism of drugs and environmental chemicals
CYP3	Vertebrates: metabolism of drugs and environmental chemicals
CYP4	Vertebrates: fatty acid hydroxylases; invertebrates: unknown functions
CYP5	Vertebrates: thromboxane synthase
CYP6	Insects: metabolism of plant products and pesticides
CYP7A	Vertebrates: cholesterol 7α-hydroxylase
CYP7B	Vertebrates: unknown function
CYP8	Vertebrates: prostacyclin synthase
CYP9	Insects
CYP10	Molluscs: mitochondrial enzyme
CYP11	Vertebrates: cholesterol side-chain cleavage, steroid 11β-hydroxylase, and aldosterone synthase (mitochondrial enzyme)
CYP12	Insects
CYP13	Nematodes
CYP14	Nematodes
CYP15	Insects
CYP16	Nematodes
CYP17	Vertebrates: steroid 17α-hydroxylase
CYP18	Insects
CYP19	Vertebrates: aromatisation of androgens
CYP21	Vertebrates: steroid 21-hydroxylase
CYP24	Vertebrates: steroid 24-hydroxylase (mitochondrial enzyme)
CYP27	Vertebrates: steroid 27-hydroxylase (mitochondrial enzyme)
CYP51	Animals, filamentous fungi, yeast and plants
CYP52	Yeast: alkane hydroxylases
CYP53 to *CYP62*	Fungi
CYP71 to *CYP92*	Plants
CYP101 to *CYP118*	Bacteria

Table 1.2 Overview of the cytochrome P450 superfamily (reproduced from Nelson *et al.*, 1996)

physiological stress and to many drugs and chemicals (reviewed Gonzalez 1989, Porter and Coon, 1991).

1.3.2a Induction by specific chemical agents.

The amount of certain CYPs is significantly increased by exposure to a number of xenobiotics which suggests that they form part of an adaptive response to chemical stress. This phenomenon has wide reaching pharmacological implications, hence the mechanisms of induction are of great interest. There have been several classes of inducers defined:

1) Phenobarbital (PB) and phenobarbital-like inducers.

Phenobarbital (an anti-convulsant drug) and 'PB-like' inducers (including isosafrole, chlordane, allylisopropylacetamide and *trans*-stilbene oxide, figure 1.4) are a large group of structurally unrelated compounds that can increase the expression of members of the CYP2A, CYP2B, CYP2C, and CYP3A subfamilies, amongst others, both in laboratory animals and in humans (reviewed in Waxman and Azaroff, 1992). It would appear, therefore, that regulatory elements involved in the PB induction of CYPs are conserved in many of the genes. PB has a varying effect on the induction of different CYP genes. It causes a 2-fold elevation in the expression of the *CYP2C6* gene compared to a 100-fold increase in the expression of the *CYP2B1* gene (Phillips *et al.*, 1981). There is no correlation between the gene family or subfamily and the responsiveness to PB, for instance *CYP2B3* is not induced by PB whereas *CYP2B1* and *CYP2B2* are (Labbe *et al.*, 1988). The effects of PB are not limited to the induction of cytochrome P450. In rats, PB induces other enzymes including UDP-glucuronosyl transferase, P450 reductase and glutathione-S-transferases. PB

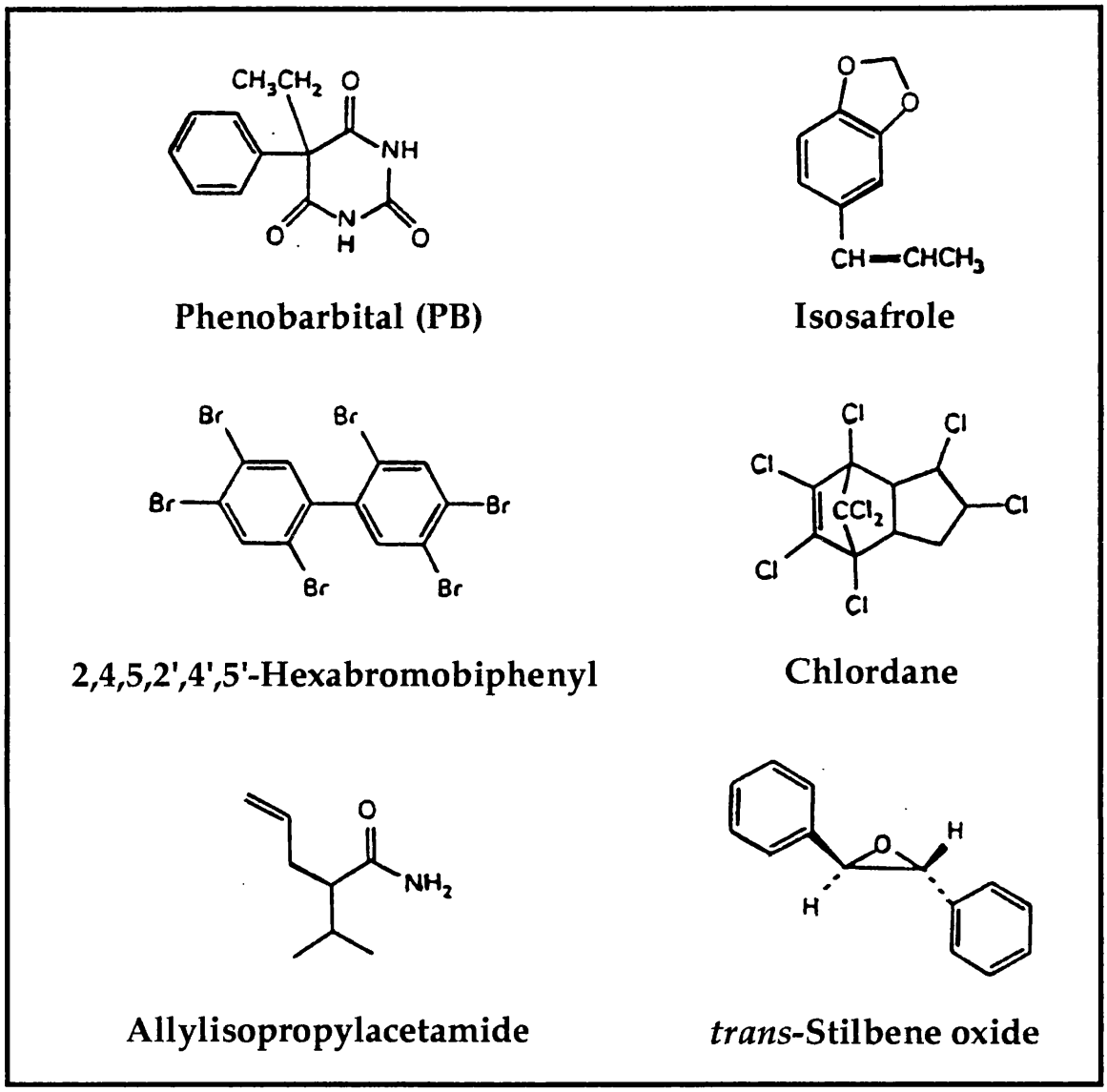


Figure 1.4: Phenobarbital (PB) and 'PB-like' inducers. The diverse structures of phenobarbital and some of the 'phenobarbital-like' inducers of cytochromes P450.

also causes smooth endoplasmic reticulum proliferation (Orrenius *et al.*, 1965) and stabilisation of the endoplasmic reticulum protein in the liver (Omura, 1979), stimulation of weight gain (Conney, 1967), liver tumour promotion (Schulte-Herman, 1974). The induction of the *CYP2B1* and *CYP2B2* genes is the most extensively studied as their response to phenobarbital is extremely pronounced. The regulation of these genes is discussed further in section 1.7.

2) Polycyclic aromatic hydrocarbons (PAH) .

This class of inducers includes 3-methylcholanthrene (3-MC), 2,3,7,8,-tetrachlorodibenzoparadioxin (TCDD), benzo[a]pyrene, benz[a]anthracene and a number of other planar polycyclic aromatic hydrocarbons. These inducers have been extensively studied since the initial observation that xenobiotic molecules induce their own metabolism was associated with 3-MC (Conney *et al.*, 1957). Studies have been mainly based on the mouse liver both in intact animals and in hepatoma cells. The major genes induced by these compounds are the CYP1A subfamily, and the phase II enzymes associated with them: NAD(P)H menadione oxidoreductase (Nmo1), UDP glucuronosyl transferase (UDP-GT), aldehyde dehydrogenase (Aldh1) and glutathione-S-transferase (GST). Collectively, these genes, including the *CYP1A1* and *CYP1A2* are called the Ah (aromatic hydrocarbon) gene battery (Nebert *et al.*, 1990). The promoters of *CYP1A1*, *CYP1A2* and *rGSTA2* genes all contain a xenobiotic response element (XRE) which mediates the response of these genes to PAH (Neuhold *et al.*, 1989; Paulson *et al.*, 1990) (figure 1.5). An Ah receptor that controls this gene battery has been identified. In the absence of inducer, the inactive Ah receptor is normally in the cytosol associated with Hsp 90. The ligand-Ah-receptor complex dissociates from Hsp 90 and binds to the Ah receptor nuclear translocation factor (ARNT). There is some controversy as to

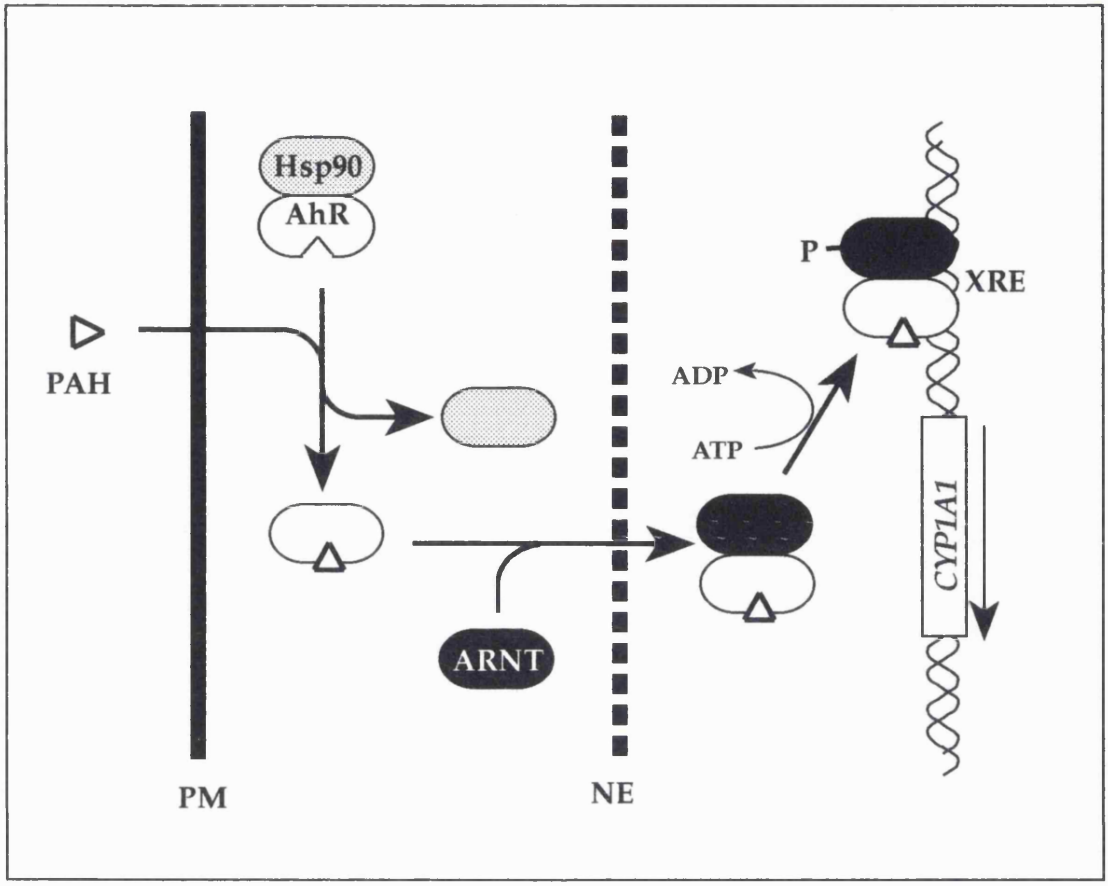


Figure 1.5: Model of the transcriptional regulation of the *CYP1A1* gene by polycyclic aromatic hydrocarbons (PAH). PM is the plasma membrane; NE is the nuclear envelope; AhR is the aromatic hydrocarbon receptor; ARNT is the AhR nuclear translocator; Hsp 90 is Heat shock protein 90 and XRE is the xenobiotic response element. Reproduced from Gonzalez *et al.* (1993).

whether this occurs in or outside the nucleus. However, once in the nucleus the heterodimeric ligand-Ah-receptor-ARNT complex is able to interact with the XRE in the promoters of PAH-regulated genes (Sogawa *et al.*, 1995)

3) Steroids.

CYP3A1 expression in rats and humans is induced by the glucocorticoid dexamethasone (Burger *et al.*, 1990). However, the anti-glucocorticoid pregnenolone-16α-carbonitrile (PCN) also induces *CYP3A1* in rats (Gonzalez *et al.*, 1986) which suggests that the induction of *CYP3A1* is not by the typical glucocorticoid receptor mechanism involved in the dexamethasone induction of other genes such as tyrosine aminotransferase (TAT). Glucocorticoid also stabilises CYP2B1 and CYP2B2 mRNAs in rats (Simmons *et al.*, 1987). Computer analysis of the promoter regions for these genes has indicated a potential glucocorticoid response element (GRE) which does mediate dexamethasone induction of a reporter gene construct in a hepatoma cell line (Jaiswal *et al.*, 1990).

4) Peroxisomal proliferators.

The most studied of these is clofibrate, which induces a battery of enzymes including CYP4A1 in the rat liver and kidney, and CYP4A2 and CYP4A3 in a rat hepatoma cell line (Hardwick *et al.*, 1987). The CYP4A family are involved in the ω-hydroxylation of fatty acids. Induction of these CYPs by peroxisomal proliferators is the result of transcriptional activation. The peroxisomal proliferator activated receptor (PPAR) binds to the peroxisomal proliferator response element (PPRE) in the promoter of the *CYP4A6* gene to mediate the induction of this gene by clofibrate (Muerhoff *et al.*, 1992). The PPAR is a member of the steroid hormone receptor superfamily. It is not certain whether the peroxisomal proliferators bind directly to the PPAR

in a manner analogous to the ligand-Ah-receptor mechanism. It may be that these drugs cause an increase of an endogenous ligand that does bind to the PPAR (Green, 1992).

1.3.2b Regulation of constitutive expression.

1) Hormonal control.

Certain rodent CYPs show sex-specific expression in both the liver and the kidney and their expression is partly dependent on steroid hormones and pituitary factors such as growth hormone (reviewed in Nebert and Gonzalez, 1987). The classic example of such regulation of CYPs is that of *CYP2C11* and *CYP2C12*. These show a high level, sex-dependent, constitutive expression in rat liver. Transcription of *CYP2C11* is detected only in the adult male, and *CYP2C12* only in the adult female. Growth hormone is a major factor in the regulation of these genes. *CYP2C11* is positively regulated by the pulsatile, male specific, growth hormone concentrations, and negatively regulated by the uniform, low amounts of growth hormone that are observed in female rats. The opposite is true of *CYP2C12*. (Sundseth and Waxman, 1992). It is thought that this regulation may be mediated by the STAT family of transcription factors (Ram *et al.*, 1996).

Hormones also regulate the constitutive and phenobarbital inducible levels of CYP2B1 and CYP2B2 (Shapiro *et al.*, 1994). Growth hormone suppresses the expression of *CYP2B1/2*, and continuous, low level secretion repressed the *CYP2B1/2* more than pulsatile secretion. The phenobarbital induction response is consistently lower in the female rat than in the male irrespective of the level of growth hormone, suggesting that another sex-

dependent hormone is involved in the regulation of the induction mechanism.

2) Tissue specific expression.

The majority of cytochromes P450 are expressed in hepatocytes, however some of them have also been found in extrahepatic tissues. For example, those cytochromes P450 involved in steroid biosynthesis have been found in the adrenal glands, the testis and the ovaries. *CYP2B1* is constitutively expressed in the small intestine, but not in the liver (Traber *et al.*, 1990). Tissue specific induction also occurs, for instance TCDD inducible CYP1A1 protein is constitutively expressed in mouse hepatic tissue, but is also found in a variety of tissues after exposure of the animal to TCDD (Kimura *et al.*, 1986).

Even within an organ there may be zonal distribution of the proteins, for example greater induction of *CYP2B1* by PB occurs in the distal region of the small intestine than in the proximal region (Traber *et al.*, 1990). The CYP2B1 and CYP2B2 mRNAs are uniformly distributed in the hepatocytes of the centrilobular and mid-zonal regions of the hepatic lobule, but cells around the periportal tract do not contain them (Traber *et al.*, 1989). The characteristic tissue specificities of the cytochromes P450 are of use in the identification and characterisation of regulatory elements that control the expression of their genes.

3) Developmental regulation.

The constitutive and inducible amounts of CYPs can also be regulated developmentally. Using RT-PCR, Hakkola *et al.* (1994) detected the presence of CYPs 2C8, 2D6, 3A3/4 and 3A7 in the human foetus. More CYPs were detected in the adult liver, where CYPs 1A1, 1A2, 2A6/7, 2B6/7, 2C8, 2C19,

2E1, 3A3/4, and 3A7 were detected. Induction in rat liver of CYP2B1 and CYP2B2 mRNAs can be detected in the foetus at 15 days of gestation. The level of this induction increases post-natally to reach a peak at 3 weeks of age (Tuteja *et al.*, 1985).

4) Genetic polymorphisms.

Cytochromes P450 show a number of genetic polymorphisms. One of the most extensively studied is that of human CYP2D6, because of its clinical significance. This enzyme is responsible for the hydroxylation of debrisoquine to 4-hydroxydebrisoquine. Mahgoub *et al.* (1977) detected debrisoquine, and not the 4-hydroxy derivative, in the urine of certain patients. It was found that amongst Caucasians there is a bimodal distribution of the rate at which debrisoquine is metabolised. This gives two phenotypes - poor metabolisers, about 35% of the population, and extensive metabolisers. CYP2D6 is responsible for the metabolism of a number of drugs, for example the O-dealkylation of codeine to form morphine, its active component (Dayer *et al.*, 1988). Consequently the use of a simple urine test to check the metabolism of debrisoquine can identify patients at risk due to poor metabolism of the drug to be administered.

1.4 Phase II metabolism.

Phase II is considered as the 'true' detoxification step as these reactions alter highly reactive species to more stable, water-soluble metabolites which can then be excreted through the urine or bile. This is achieved by linking the xenobiotic covalently with an endogenous molecule. The drug or its metabolites contain reactive groups (-OH, -COOH, -NH, -SH) which can be conjugated with sugars (e.g. α-D-glucuronic acid, glucose), peptides (e.g.

glutathione), fatty acids (e.g. palmitic acid), acetyl groups or thio groups. The reactions of conjugation usually require the activation of either the drug or the conjugating species and/or the conjugating moiety. The activated form of conjugating agents are usually nucleotides, such as S-adenosylmethionine (SAM) or 3'-phosphoadenosine-5'-phosphosulphate (PAPS) and the reactions are catalysed by enzymes called transferases (reviewed in Mannervick and Danielson, 1988; Bock, 1991; Falany, 1991; Meijer and DePierre, 1988; Cadenas, 1995). Figure 1.6 shows the range of phase II reactions and some examples. Phase II reactions are important clinically as the rate and route of elimination of a drug are determined by the conjugation reactions it undergoes.

1.5 Glutathione-S-transferases (GSTs).

One of the most important groups of phase II drug metabolising enzymes are the glutathione-S-transferases (GSTs). Many drugs are strong electrophiles or are metabolised by phase I reactions to strong electrophiles. These can undergo nucleophilic attack from the sulphur atom of the tripeptide glutathione (Gly-Cys-Glu) to form conjugates. This reaction is catalysed by GSTs. In general:

$$R\text{-}CH_2\text{-}X \xrightarrow[\text{Glutathione-S-Transferase}]{\text{GSH}} R\text{-}CH_2\text{-}SG$$

where $R\text{-}CH_2\text{-}X$ is the electrophilic substrate, $R\text{-}CH_2\text{-}SG$ is the glutathione adduct and GSH is the glutathione tripeptide (reviewed in Armstrong, 1994). The electrophilic functional group may also be nitrogen, or sulphur as well as carbon. The majority of GST substrates are xenobiotics (e.g. aflatoxin B1, benzo[a]pyrene) or products of oxidative stress (e.g. cholesterol α-oxide, fatty acid hydroperoxides). However, GSTs are also involved in the

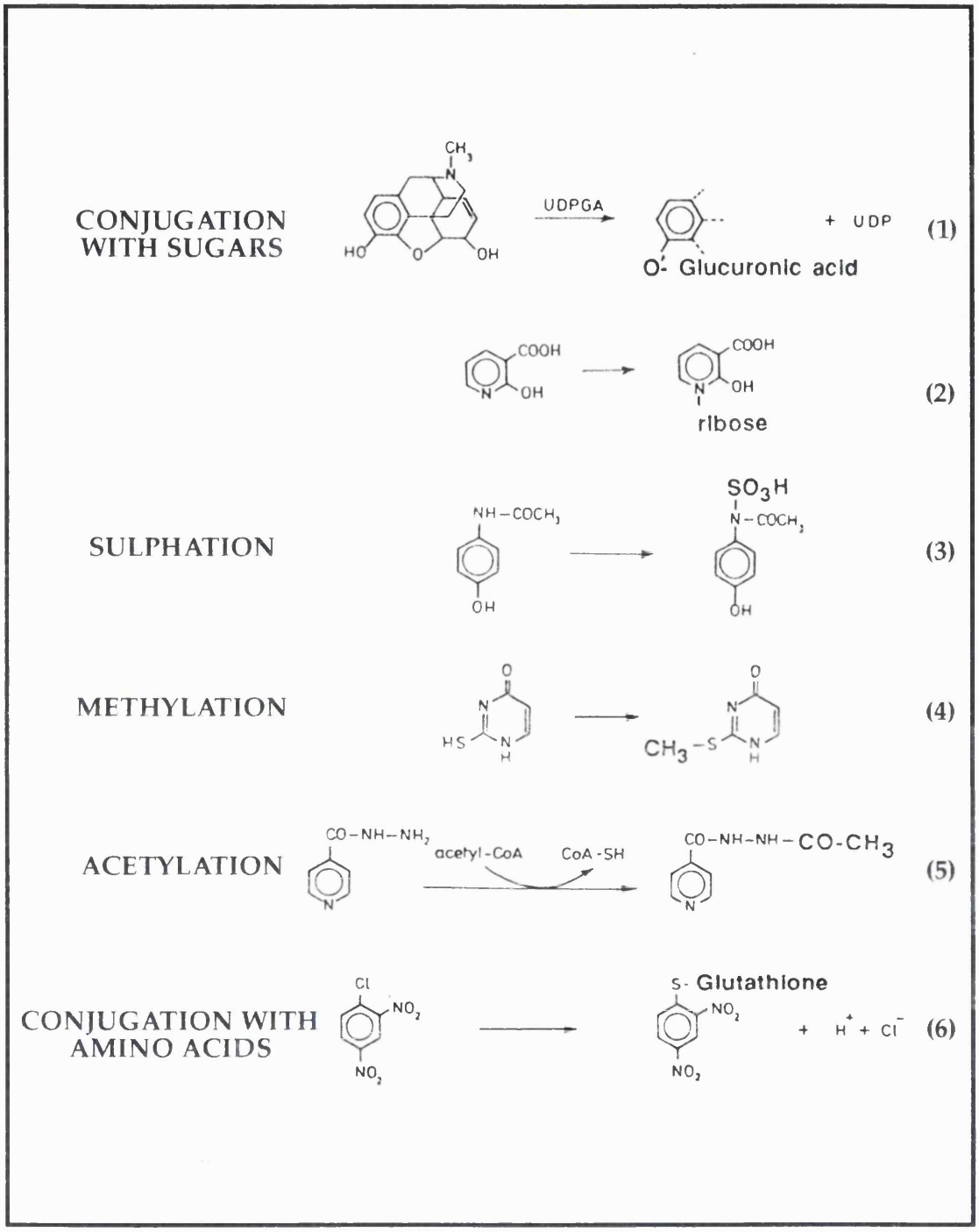


Figure 1.6: Phase II metabolism. Some examples of the diverse conjugative reactions catalysed by Phase II enzymes. (1) The O-glucuronidation of morphine; (2) The N-ribosylation of 2-hydroxynicotinic acid; (3) The sulphate conjugation of paracetamol; (4) The S-methylation of thiouracil; (5) The N-acetylation of isonazid; (6) The glutathione conjugation of 2,4-dinitro-1-chlorobenzene. Compiled from Gibson and Skett (1994).

metabolism of the endogenous compounds leukotriene and prostaglandin. (GST substrates are reviewed in Hayes and Pulford, 1995).

GSTs can be considered as a protective mechanism to prevent cellular macromolecules from being attacked by the sometimes highly reactive electrophilic species. Once the glutathione conjugates are formed, they can be transported from the cell by ATP-dependent glutathione S-conjugate pumps (Ishikawa, 1992). In addition to metabolising xenobiotics, GSTs bind strongly to a number of compounds to sequester these molecules and prevent them from damaging the cellular macromolecules (Ploemen *et al.*, 1994). However, glutathione conjugation does not always result in detoxification; some of the metabolites are more reactive and toxic than the parent compound. This can be exploited in chemotherapy for tumours that over express GST (Hayes and Pulford, 1995).

1.5.1 GST nomenclature.

GSTs are very abundant, and widely distributed in most of the phyla. There are both cytosolic and membrane-bound forms. The cytosolic proteins are divided into five families, classes alpha, mu, sigma, pi and theta. In general, enzymes in the same class share 40% amino acid identity, though the class boundaries are imprecise in some cases. The membrane bound enzymes are called microsomal GST and leukotriene C_4 synthase. My study has concentrated on cytosolic GSTs, so I will restrict my comments to these.

Cytosolic GSTs comprise two subunits and can exist as homodimers or heterodimers. The rat contains as many as 20 cytosolic GST genes from all of the five classes. Through different subunit dimerisations, more than 35

different proteins are formed in the rat. Historically, several different nomenclature systems have been proposed for the GSTs. The current nomenclature is based on the different classes and was suggested by Mannervick *et al.* (1992) for human genes. It is now also being used for rat and mouse GSTs. The species is denoted by a lower case h, r or m, for human, rat or mouse respectively. The class is denoted by an upper case Arabic letter, and the subunit composition by number. Therefore, rGSTA1-1 is a rat GST formed from two Alpha class 1 subunits. Table 1.3 shows the nomenclature for the cytosolic rat genes, possible subunit dimerisation patterns, and previous names.

Whilst the different GST classes show overlapping substrate specificities, for instance the conjugation of GSH with 1-chloro-2,4-dinitrobenze (CDNB) is catalysed by most GSTs (Mannervick and Danielson, 1988), they can also display marked differences in their ability to metabolise certain electrophiles. This can be exploited for identification purposes (reviewed in Hayes and Pulford, 1995), for example rGSTM1-1 has twice the activity of rGSTM1-2 towards 1,2-dichloro-4-nitrobenzene (DCNB). GSTs can also be highly stereoselective: rGSTA5 is highly active towards aflatoxin B_1 exo-8,9-epoxide a highly carcinogenic moiety, but has essentially no activity towards aflatoxin B_1 endo-8,9-epoxide.

1.5.2 Regulation of GSTs.

1) Induction by xenobiotics.

In common with other drug metabolising enzymes, GSTs are induced by many of their substrates, and the response of GSTs to the inducing agent is affected by age, species, strain, sex and organ. Many inducers increase the

Class	Class based subunit nomenclature	"Y" SDS/PAGE subunit nomenclature	Subunit number	Possible dimers
Alpha	rGSTA1	Ya_1	1a	rGSTA1-2
	rGSTA2	Ya_2	1b	rGSTA1-3
	rGSTA3	Yc_1	2	rGSTA1-5
	rGSTA4	Yk	8	rGSTA2-2
	rGSTA5	Yc_2	10	rGSTA2-3
	n.i.	Yl*		rGSTA2-5
				rGSTA3-3
				rGSTA3-5
				rGSTA4-4
Alpha?	n.i.	Ys*		rGSTA6-6
	n.i.			Ya_1Yl
				Yc_1Yl
				YsYs
Mu	rGSTM1	Yb_1	3	rGSTM1-1
	rGSTM2	Yb_2	4	rGSTM1-2
	rGSTM3	Yb_3	6	rGSTM1-3
	rGSTM4*	Yb_4		rGSTM2-2
				rGSTM2-3
				rGSTM3-3
	rGSTM5*	Yn_2	9	rGSTM3-5
	rGSTM6*	Yo	11	rGSTM4-4
				rGSTM6*-6*
Pi	rGSTP1	Yf	7	rGSTP1-1
Sigma	rGSTS1*			rGSTS1*-S1*
Theta	rGSTT1		5	rGSTT1-1
	rGSTT2	Yrs	12	rGSTT2-2
	rGSTT3*		13	rGSTT3-3

Table 1.3: Rat cytosolic GST isoenzymes, subunit nomenclature and dimerisation possibilities. Subunits whose cDNA has not been cloned are shown by *. n.i (not included) indicates that a firm designation cannot be made. Adapted from Hayes and Pulford (1995).

expression of both CYPs and GSTs, for instance phenobarbital and 3-methylcholanthrene. Indeed many of the mechanisms identified in studies on the regulation of CYPs are also involved in GST regulation. The *rGSTA2* gene appears to contain at least four cis-acting elements. One of these is responsible for the induction of the gene by a range of polycyclic aromatic hydrocarbons which is identical to the xenobiotic response element (XRE) in the rat *CYP1A1* gene (Rushmore *et al.*, 1990). There is also an antioxidant-responsive element (ARE) which mediates the response to phenolic antioxidants (Rushmore *et al.*, 1991). This ARE is similar to the 12-*O*-tetradecanoyl phorbol 13-acetate (TPA)-responsive element which binds AP-1 (Lee *et al.*, 1987). The third element in this gene is identical to the glucocorticoid-response element (the GRE, also found in the *CYP2B2* promoter) which may render this gene responsive to dexamethasone (Rushmore *et al.*, 1993). The fourth element in the *rGSTA2* promoter may mediate the phenobarbital induced increase in transcription of this gene. In the murine *GSTA1* gene promoter there is an element, the electrophile response element (EpRE) which is composed of two ARE-like sites in tandem. Binding to this site by AP1 has been shown to increase in response to phenobarbital treatment (Pinkus *et al.*, 1993). An AP1 site has also been identified in *CYP2B2* which is possibly involved in the phenobarbital induction of this gene (Roe *et al.*, 1996). Also, several potential Barbie box elements have been identified (Liang *et al.*, 1995) within the 5' flanking sequence of *rGSTA2* , though these have not been shown to function in the phenobarbital response mechanism.

2) Tissue-specific and developmental regulation of GSTs.

GSTs are regulated in a tissue-specific manner, and the patterns of expression do change with age. Abramovitz and Listowsky (1987), showed that rGSTM3 was localised to the brain and testis. Tee *et al.* (1992) studied

hepatocytes and demonstrated that rGSTP1-1 is expressed in foetal but not in adult hepatocytes, although it has also been observed in hepatomas (Sato *et al.*, 1984).

3) Polymorphic expression of GSTs.

There are several known GST-null phenotypes throughout the phyla. Given the importance of these enzymes in removing harmful species from the body, there is considerable interest as to whether these null phenotypes will have increased susceptibility to disease. In humans there are two known null phenotypes: hGSTM1 and hGSTT1. hGSTM1 has a comparatively high activity towards genotoxic epoxides, and it has been speculated that individuals with the null phenotype would be at increased risk of certain forms of cancer. Indeed hGSTM1 deficient individuals do have an increased susceptibility to DNA adduct formation (Liu *et al.*, 1991), p53 gene mutation (Kawajiri, 1996) and smokers have an increased risk of lung cancer (Kawajiri, 1996). There are very few data on hGSTT1 phenotype, but one study does suggest that the age of onset of colonic cancer is decreased in individuals with this phenotype (Chenevix-Trench *et al.*, 1995).

1.6 Hepatocyte culture.

The regulation of a single cytochrome P450 is difficult to study in the whole organism because of the complexity of the various biotransformation pathways they are involved in. The number of factors that can affect these drug metabolism pathways means that a fully controlled experimental system is very difficult to achieve. It is necessary to have large sample groups of animals to provide meaningful statistical evaluations. This is not

only expensive but time consuming and increasingly unacceptable due to ethical considerations.

One possible alternative to *in vivo* studies is to use a cell culture system. This would provide an experimental environment where a factor could be altered controllably and in isolation from other factors. It also has the advantage that for each experiment all the cells are derived from a single animal, an important consideration in drug metabolism studies where inter-individual differences are so apparent. It should be emphasised, however, that cell culture systems can never fully replace whole animal experiments. They can, however, serve to provide indications of the *in vivo* situation and as a model on which to test hypotheses before *in vivo* experiments are carried out.

Human tissue samples are difficult to obtain, and pose problems due to the variable histories of the patients and lengths of time before the cells can be isolated. Consequently, most investigators use hepatocytes from either mice or rats to culture, and these systems are the best defined. I am going to restrict my comments to rat hepatocyte cultures, unless otherwise indicated, as this is the system that I studied.

Acute toxicity and mechanistic studies are possible using freshly isolated hepatocytes kept in suspension for a few hours (Guillouzo, 1986), but in general longer term studies are necessary for examining the regulation of drug metabolising enzymes. However, most cell lines derived from mammalian liver express little cytochrome P450, either constitutively or after exposure to inducers. Moreover, conventional monolayer primary hepatocyte cultures lose many liver-specific functions leading to foetalisation, or dedifferentiation, of the cells. This includes the rapid loss

of expression of many of the cytochromes P450 and GSTs (Steward *et al.*, 1985; Guillouzo, 1986). However, studies of enzyme activities show that different phase I and II enzymes are affected to different extents by this dedifferentiation process. For instance, Niemann *et al.* (1991) showed that activities associated with phase I and II enzymes belonging to the 3-methylcholanthrene-inducible group (e.g. ethoxyresorufin and 1-napthol UDP-glucuronosyl transferase (UDP-GT)) were maintained better than those belonging to a phenobarbital-inducible group (e.g. pentoxyresorufin UDP-GT).

Dedifferentiation of the hepatocytes seems to be associated with the disruption of cell-cell and cell-substratum interactions during the isolation and plating of the hepatocytes (Bissell and Guzelian, 1980). It has been postulated that the isolation procedure (usually a two-step collagenase perfusion) initiates the repair and growth mechanisms seen after partial hepatectomy and causes the normally quiescent hepatocytes to re-enter the cell cycle. This is supported by the finding that the rate of loss of CYP expression after hepatocyte isolation parallels that after partial hepatectomy and changes in expression of the liver-enriched transcription factors C/EBPα and c-jun are also similar in both cases (Padgham *et al.*, 1993).

Attempts have been made to try to create a primary cell culture system that will support the expression and induction of cytochromes P450 and phase II enzymes. Several factors are known to affect the cellular integrity and biotransformation capacities of hepatocytes *in vitro*: (i) soluble medium factors, (ii) extracellular matrix components and (iii) cell-cell interactions.

1.6.1 The effects of soluble medium factors.

Hepatocytes were conventionally cultured on tissue culture plastic in media containing serum with some other additives. Attempts were made to maintain fully differentiated hepatocytes with additions of hormones (Dich *et al.*, 1988), growth factors (Guzelian *et al.*, 1988), trace elements (Engelman *et al.*, 1985), enzyme inducers (Scheutz *et al.*, 1988), nicotinamide (Steward *et al.*, (1985), dimethylsulphoxide and various other substances (reviewed in Guillouzo, 1986). These approaches only met with limited success. It became clear that it was necessary to use a serum free, hormonally defined medium to maintain levels of CYPs (Enat *et al.*, 1984).

Sidhu *et al.* (1993) compared William's E medium, Chee's medium, Waymouth medium and Ultraculture medium with respect to the maintenance of CYP2B1, CYP2B2 and CYP3A1 mRNAs and their response to phenobarbital. Chee's and William's E media maintained all the mRNAs over a period of 96 hours. Treatment of the hepatocytes with 1mM PB for the final 48 hours resulted in induction of all of these mRNAs. The phenobarbital response was greatest in William's E medium. However, Chee's medium produced an abnormal increase in CYP1A1 expression in response to phenobarbital.

The inclusion of dexamethasone and insulin is important in the maintenance of the hepatocytes differentiated state. Sinclair *et al.* (1990) found that the addition of 0.1μM dexamethasone and 1.7μM insulin separately resulted in an increased response to phenobarbital as measured by the CYP2B1/2-dependent benzyloxyresorufin O-dealkylase (BROD) activity. The inclusion of both led to an additive response to phenobarbital. This finding is supported by the results of Sidhu *et al.* (1995) that if

dexamethasone is omitted from the culture medium, the hepatocytes express rGSTP1-1. This is normally only seen in foetal hepatocytes, and hepatoma cells, and is considered as a mark of dedifferentiation (Tee *et al*, 1992). The removal of 0.1μM dexamethasone from the culture medium also resulted in the deterioration of morphology of the cells. However, concentrations of dexamethasone of 1μM and above led to the repression of phenobarbital induction of CYP2B1/2 mRNAs.

1.6.2 The effects of extracellular matrix components.

The liver extracellular matrix contains collagens, anchorage proteins and proteoglycans (e.g. heparin sulphate). The hepatocytes are attached by anchorage proteins to a collagen scaffolding. Both the anchorage proteins and the proteoglycans are bound to the cell surface via receptors. Within a tissue there can be several types of cell matrix and this can change as the cells differentiate. It has been suggested that the differentiated state of hepatocytes is associated with their polygonal structure, and that this is stabilised by interactions with the extracellular matrix (Reid, 1986).

The addition of proteoglycans and glycosaminoglycans to hepatocytes cultured on plastic was able to restore certain liver specific mRNAs, and to induce gap junction formation (Reid, 1986; Fujita *et al.*, 1987). However, most interest has been in the use of tissue culture plates coated with a substratum derived from extra cellular matrix to attempt to promote the maintenance of the differentiated state, and hence expression of DMEs. Waxman *et al.* (1990) reported that primary rat hepatocytes could be cultured on a collagen-based substratum (Vitrogen, type I collagen) in serum-free modified Chee's medium for 20-30 days. CYP2A1 and CYP2A2

and P450 reductase were maintained in the cultures for 10-20 days and *CYP1A1* and *CYP1A2* were inducible by 3-MC. After the inclusion of 1μM dexamethasone in the culture medium, both *CYP2B1* and *CYP2B2* were induced >50-fold by PB.

Another approach was to use plates coated with a mixture of a crude liver membrane fraction and collagen type I. Saad *et al.* (1993) found that rat hepatocytes cultured on these plates for up to 9 days in a modified Williams' E medium contained 1.5- to 3-fold higher total cytochrome P450 contents than when the cells were cultured on collagen alone. Exposure of the cells to phenobarbital increased CYP 2B1/2 content of the cells by 6-fold.

Schuetz *et al.* (1988) used plates coated with matrigel, an extracellular matrix prepared from Engelbreth-Holm-Swarm sarcoma. Matrigel is rich in laminin and type IV collagen. The cells were cultured in Waymouth MB-725 serum-free culture medium containing insulin (0.1μM). The hepatocytes were rounded and grew in clusters, and they remained attached for weeks. They also supported the expression of several liver specific genes (e.g. albumin) and the induction of CYP2B1/2 by phenobarbital. Sidhu *et al.* (1993) combined culturing rat hepatocytes on collagen with an overlay of matrigel. They found that the overlay prevented the deterioration in morphology of the cells over 2 weeks when they were cultured in Chee's medium. This was also true, though to a lesser extent, when the cells were cultured in William's E medium. Exposure of the overlaid cells to PB (1mM) for 48 hours produced a response resembling the *in vivo* response.

Scheutz *et al.* (1988) compared hepatocytes cultured on vitrogen with those cultured on matrigel. The cells were cultured in serum-free medium based on Waymouth MB-752, containing 0.157μM insulin as the only hormone.

They found that the cell morphology, the expression of albumin and the response of *CYP2B1/2* to phenobarbital were all better maintained using matrigel. However, Trottier *et al.* (1995) also compared hepatocyte cultures grown in Chee's medium (1.7μM insulin, 0.1μM dexamethasone) on collagen coated plates with a matrigel overlay to ones without the overlay. The overlaid cells had only a slightly higher CYP2B1/2 mRNA amount after 24 hours exposure to PB, and similar amounts after 48 or more hours exposure to PB, when compared to the cells without the matrigel overlay.

One problem associated with the use of a matrigel overlay is that it inhibits the transfection of DNA into the hepatocytes (Pasco and Fagan, 1989). Trottier *et al.* (1995) circumvented this by carrying out the transfections before the addition of diluted matrigel in the culture medium.

1.6.3 The effects of cell-cell interactions.

In an attempt to mimic the environment of the hepatocytes in the liver, investigators have cultured hepatocytes with a number of other cell types. The most successful of these has been co-culture with epithelial cells derived from primitive bile ducts. The hepatocytes have a longer viability and better morphology than conventionally cultured hepatocytes. Also between 100% (Bégué *et al.*, 1984) and 25% (Rogiers *et al.*, 1993) of the total CYP content and activity was maintained. Phase II enzymes are also maintained in this system (Rogiers *et al.*, 1990; Vandenberghe *et al.*, 1992). Technically, this system is difficult compared to the simpler cultures on matrigel or vitrogen.

1.7 Cytochromes P450 2B1 and 2B2.

The major PB-inducible forms of cytochrome P450 in the rat liver are CYP2B1 and CYP2B2. The proteins are structurally related: 14 out of 491 amino acids are different (Suwa *et al.*, 1985), and there are similar functional regions including a hydrophobic N-terminal sequence of 20 amino acids that directs insertion of the protein into the membrane and its attachment there (a combined insertion/halt transfer signal peptide) (Monier *et al.*, 1988; Vergeres *et al.*, 1989), a haem binding peptide near the C-terminal end that contains the conserved cysteine region common to all cytochromes P450, a substrate binding site (Edwards *et al.*, 1989), site(s) that interacts with P450 reductase (Nelson and Strobel, 1988) and putative sites of interaction with cytochrome b_5 and phosphorylation by cyclic AMP-dependent protein kinase (Koch and Waxman, 1989, Waxman and Azaroff, 1992).

The CYP2B1 and CYP2B2 proteins are immunochemically crossreactive, though they have similar but distinct substrate specificity profiles, chromatographic and electrophoretic properties (Waxman and Walsh, 1982). The two proteins can have different regioselectivities for the same substrate. For example, CYP2B2 favours 12-methylation of 7,12-dimethylbenzanthracene, whilst CYP2B1 prefers 7-methylation (Wilson *et al.*, 1984). The catalytic activity, *in vitro*, of CYP2B2 is often several fold less than that of CYP2B1; for example CYP2B1 is 5-fold more active than CYP2B2 in metabolising testosterone, and 3-fold more active when metabolising benzo[a]pyrene (Wilson *et al.*, 1984; Waxman and Walsh, 1982).

The two genes are closely linked on rat chromosome 1 (Rampersaud and Walz, 1983) and on human chromosome 19 (Phillips *et al.*, 1985). Both

genes have 9 exons and 8 introns. Several rat allelic variants have been discovered using 2D gel analysis (Rampersaud and Walz, 1983). *CYP2B1* has at least 6 alleles, while *CYP2B2* has two or more. There are 40 coding pair differences between the two genes giving the 14 amino acid differences in their proteins. The changes are grouped in exons 6-9 and are probably due to one or more gene conversion events (Atchison and Adesnik, 1986).

The promoter region for both genes is fairly similar: The transcription initiation sites are 30 base pairs (bp) upstream from the translation initiation sites. There is a modified TATA box (CATAAAA) situated at around -20bp with respect to the transcription initiation site (+1bp). At -81bp in the *CYP2B2* gene there is the sequence GCCAAA, which is a putative binding site for hepatic nuclear factor 1 (HNF 1). At -255bp there is an alternating purine pyrimidine sequence which may form a regulatory Z-helical structure (Suwa *et al.*, 1985). In *CYP2B1* this sequence is $(CA)_5$, and in *CYP2B2* it is $(CA)_{19}$. A region beginning at -1343bp is thought to be a glucocorticoid response unit containing a consensus glucocorticoid response element (-1343 to -1357, GRE) and a CCAAT unit which is involved with dexamethasone inducibility when attached to an heterologous reporter gene construct (Jaiswal *et al.*, 1987). Dexamethasone does act in synergy with phenobarbital to induce CYP2B1/2 mRNA in the adult rat liver (Rao *et al.*, 1990) and increases CYP2B1/2 protein and activity in cultured hepatocytes (Waxman *et al.*, 1990; Sidhu *et al.*, 1995). Phenobarbital leads to a 2-fold increase in the affinity of dexamethasone for the glucocorticoid receptor and a 30% increase in the number of glucocorticoid receptor molecules in a rat hepatoma cell line (Chasserot-Golaz *et al.*, 1990). However, the physiological significance of this GRE is not apparent as glucocorticoid does not induce transcription of these genes, but stabilises their mRNAs (Simmons *et al.*, 1987).

The genes are both constitutively expressed at low levels in the rat liver, though *CYP2B1* has a basal level that is virtually undetectable. Induction of the genes by exposure to phenobarbital (PB) and PB-like inducers increases the amounts of the CYP2B1 protein by more than 100-fold and the CYP2B2 protein by greater than 20-fold. Induced amounts of 2B1 and its mRNA being 2-3 times greater than those of 2B2. The mechanisms by which the diverse PB-like inducers and treatment regimens act to increase liver CYP2B1 and CYP2B2 are not clear. However, the response is inducer-specific, as other lipophilic chemicals, such as dioxin which induces the expression of *CYP1A1,* do not increase the expression of the *CYP2B1* and *CYP2B2* genes (Waxman and Azaroff, 1992).

CYP2B1 and *CYP2B2* are differentially expressed in extrahepatic tissues. Using synthetic oligonucleotide probes to the CYP2B1 and CYP2B2 mRNAs in the rat, it has been observed that there is CYP2B1 present in the brain, lung and testis, and CYP2B2 is present in the brain but not in the lung and testis (Omiecinski, 1986). CYP2B1 is induced in the small intestine by PB (where CYP2B2 is not) and in the adrenal gland (Christou *et al.*, 1987; Traber *et al.*, 1990; A. Elia, Ph.D. thesis, 1996). *In situ* hybridisation studies showed PB-inducible CYP2B1 and CYP2B2 mRNAs are present uniformly across the centrilobular and midzonal regions of the hepatic lobule. However, the cells immediately around the periportal tract do not show an increased expression of CYP2B1 or CYP2B2 in response to PB (Traber *et al.*, 1989; Chinale *et al.*, 1986)

CYP2B1 and *CYP2B2* are also induced by a range of chemicals and natural products, though the natural products are usually less effective than PB as inducers. The anti-epileptic drug sodium valproate is a potent inducer of

CYP2B1 and *CYP2B2* in primary rat hepatocytes (Akrawi *et al.*, 1993; Rogiers *et al.*, 1996). Diallyl sulphide is a natural product derived from garlic and which inhibits tumourogenesis in several animal model systems. It and some other organosulphur compounds lead to transcriptional activation of the *CYP2B1* and *CYP2B2* genes in rat primary hepatocytes. This induction is 22-fold after 36 hours and is suppressed by growth hormone (Pan *et al.*, 1993). Picrotoxin is a toxic natural product that stimulates the central nervous system by blocking chloride ion channels in the GABA receptors, producing convulsions. It is an excellent inducer of the *CYP2B1* and *CYP2B2* genes (Yamada *et al.*, 1993). It has been shown that many terpinoids, including camphor and pinene, are inducers of the CYP2B subfamily (Austin *et al.*, 1988).

1.7.1 Phenobarbital (PB) induction of *CYP2B1* and *CYP2B2* gene expression and the scope of this project.

Phenobarbital-induced expression of the *CYP2B1* and *CYP2B2* genes is mediated by a 20-fold increase in the amount of CYP2B1 and CYP2B2 mRNAs in the cytoplasm, due primarily to increased transcription of the genes (Hardwick *et al.*, 1983; Pike *et al.*, 1985). Hybridisation of *in vitro* nuclear transcripts to an excess of filter bound CYP2B cDNA shows a 16-fold greater concentration of nascent CYP2B2 transcripts in nuclei isolated from animals previously treated with PB than from nuclei isolated from untreated animals (Pike *et al.*, 1985). This, in conjunction with time course studies on the incorporation of radioactive NTP into these transcripts indicate that the mechanism of PB induction involves an increase in transcription.

The protein synthesis inhibitor cycloheximide blocks the accumulation of CYP2B1 and CYP2B2 mRNAs in rat liver treated with PB, and may interfere with basal CYP2B expression (Burger *et al.*, 1990; Chinale *et al.*, 1988). PB-enhanced transcription of a *CYP2B1/2* minigene *in vitro* and increased binding to a DNA element in the *CYP2B1/2* promoter were both blocked by cycloheximide (Rangarajan and Padmanaban, 1989). This suggests a necessity for protein synthesis during PB induction of *CYP2B1* and *CYP2B2* genes. It is not clear whether this requirement is for ongoing or *de novo* synthesis. This indicates that either PB is involved in the induction of a protein factor which positively regulates the *CYP2B* genes, or there is a labile transcription factor which is enhanced by PB, the synthesis of which is blocked by cycloheximide.

Nuclear haem pools could possibly regulate the induction of CYP2B1 and CYP2B2 by PB, as this induction is blocked by haem biosynthesis inhibitors (e.g. $CoCl_2$) (Dwarki *et al.*, 1987). *In vitro* treatment of nuclei isolated from PB-treated rats with $CoCl_2$ blocks the PB-induced increased transcription of a *CYP2B1/2* minigene (Rangarajan and Padmanaban, 1989). Succinylacetone, a more specific haem biosynthesis inhibitor than $CoCl_2$, did not block phenobarbital induction of CYP2B protein or mRNA in primary hepatocytes (Sinclair *et al.*, 1990) or whole animal studies (Srivasta *et al.*, 1989).

It is possible that a receptor mediates the phenobarbital induction in a manner analogous to the Ah receptor in the induction of *CYP1A1* by PAH, or PPAR receptor in the induction of *CYP4A* by clofibrate. A receptor based mechanism is suggested by the facts that PB and PB-like inducers are tissue specific and the regulation shows a saturable dose-response curve both *in vivo* (Lubet *et al.*, 1985) and in primary hepatocytes (Kockarek *et al.*, 1990).

Also genetic differences have been observed in the response to the inducer. Hashimoto *et al.* (1988) observed that CYP2B2 mRNA was present in very small amounts in the liver microsomes of rats from the Qsj:SD strain, even after treatment of the animals with PB. Also the obese fa/fa Zucker rat shows a low level of induction of *CYP2B1* and *CYP2B2* with PB and PB-like inducers (Zannikos *et al.*, 1993; Blouin *et al.*, 1993).

While there does not seem to be any structural or chemical relationship between the PB-like inducers, this does not exclude a receptor-based mechanism as the PPAR receptor interacts with a number of structurally diverse chemicals during the induction of the *CYP4A* genes (Isseman and Green, 1990) but as yet no receptor for phenobarbital in the liver has been isolated. The variety of ligands for a PB receptor would be consistent with a 'sloppy fit' or 'elastic' receptor (Waxman and Azaroff, 1992) and perhaps explains why no PB receptor has been detected by experiments based on a ligand-receptor interaction (e.g. Tierney and Bresnick, 1981).

Alternatively, PB receptor-independent mechanisms have also been postulated (reviewed Waxman and Azaroff, 1992). Phenobarbital could bind with and sequester cytochrome P450 so that an endogenous substrate is not metabolised. The concentration of this substrate would then increase beyond a certain threshold causing transcriptional activation. This is similar to a mechanism which contributes to the basal level of CYP1A1 in liver (RayChaudhuri *et al.*, 1990). A variation of this model postulates the presence of an endogenous inactive repressor. The repressor could be metabolised by CYP to an active repressor, leading to down regulation of the PB inducible CYPs. If PB sequestered the CYP responsible for this activation, the repressor would remain inactive, and the transcription of the PB-inducible CYPs would be de-repressed.

Whether or not a PB receptor is involved in the induction mechanism, the outcome of PB treatment is the activation of transcription of the *CYP2B1* and *CYP2B2* genes. The aim of my study has been to elucidate the events leading up to the transcriptional activation of the *CYP2B2* gene by phenobarbital. Due to the lack of a cell line or culture system that was capable of maintaining the expression and induction of this gene, the response had, initially, to be studied using cell-free systems. I used gel retardation analysis to examine the 5' flanking sequence of this gene in an attempt to identify possible *cis*-acting DNA elements and *trans*-acting factors involved. I then looked at the expression and induction responses of several drug metabolising enzymes in a primary hepatocyte culture system that had been set up in Dr. Shephard's laboratory, to assess whether it could be used to study the phenobarbital response mechanism. I used the primary hepatocytes to examine the effect on the expression of *CYP2B2* of cyclic AMP, a PKA activator and a protein phosphatase inhibitor in conjunction with phenobarbital treatment. Finally, I transfected the primary hepatocytes with constructs containing fragments of the *CYP2B2* 5' flanking sequence attached to a luciferase reporter gene to assess the importance of each of these in the PB response mechanism.

Chapter Two

Methods

2.1 Bacterial cultures.

Solutions:

Ampicillin: A stock solution of the sodium salt of ampicillin in water was prepared at a concentration of 50mg/ml. This was sterilised by using a 0.22μm nitrocellulose filter and stored in aliquots at -20°C. The stock solution was added to sterile media immediately before use to give a final concentration of 50μg/ml for liquid media and 100μg/ml for agar.

Chloramphenicol: A stock solution of 34 mg/ml in 100% v/v ethanol was prepared and stored at -20°C until use. The stock solution was added to sterile media immediately before use to give a final concentration of 170μg/ml.

Luria-Bertani (LB) bacterial growth medium (pH 7.5): Capsules of LB medium were placed in distilled water to give a solution of 10g bacto-tryptone, 5g bacto-yeast extract and 10g NaCl per litre. The solution was autoclaved and cooled to at least 55°C before addition of selective antibiotics, or stored at room temperature until required.

Luria-Bertani (LB) Agar: Capsules of LB agar were placed in distilled water to give a solution of 10g bacto-tryptone, 5g bacto-yeast extract, 10g NaCl and 15g bacto-agar per litre. The solution was autoclaved and stored at room temperature until required.

SOB bacterial medium (pH 7.5): Capsules of SOB medium were placed in distilled water to give a solution containing 20g bacto-tryptone, 5g bacto-yeast extract, 0.58g NaCl and 20mM $MgSO_4$ per litre. The solution was autoclaved and cooled to at least 55°C before addition of selective antibiotics, or stored at room temperature until required.

2.1.1 Preparation of glycerol stocks.

10ml LB medium containing the relevant antibiotic, if necessary, were inoculated with a fresh bacterial colony picked from an agar plate using a sterilised platinum wire loop, and incubated overnight at 37°C with vigorous shaking.

Aliquots of 1ml glycerol in 5ml glass bottles were autoclaved. 4ml of the overnight culture were added to the glycerol and swirled to mix, giving 20%v/v glycerol finally. Alternatively, a Freeze Broth of 30% glycerol in LB medium was prepared and autoclaved. This was used to dilute the overnight culture 1:1 to give 15% glycerol. The stock was frozen on dry ice or in liquid nitrogen and stored at -70°C.

2.1.2 Preparation of agar plates.

The agar was heated by microwave until completely dissolved. It was then cooled to at least 55°C before addition of selective antibiotics. The liquid was poured into sterile 8cm petri dishes and covered until set. The plates were dried at 37°C before use.

2.1.3 Inoculation of cultures and chloramphenicol amplification of plasmid DNA.

Liquid media were inoculated by picking a single colony from an agar plate using a sterile pipette tip and placing it in 10ml of medium, or by scraping a heat sterilised platinum loop across the surface of a glycerol stock and

swirling it in the medium. The culture was grown over night at 37°C with vigorous shaking.

For larger cultures, a 10ml culture was inoculated approximately 8 hours before being transferred to the required volume of selective medium and grown overnight.

For plasmids with a low copy number, particularly those containing the ColE1 origin of replication, a chloramphenicol amplification was necessary. A 10ml culture was inoculated and grown until the optical density at 550nm was approximately 0.6-0.8 OD units with respect to LB medium. This was then transferred to a culture of the required volume and grown for a further 3 hours before addition of chloramphenicol to a final concentration of 170μg/ml.

2.2 Subcloning of DNA into a plasmid vector.

Solutions:

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

Tris EDTA (TE) buffer (pH 8.0): 10mM tris.Cl (pH 8.0), 1mM diaminoethanetetraacetic acid disodium salt (EDTA) (pH 8.0). The solution was aliquotted, autoclaved and stored at room temperature until use.

2.2.1 Ligation of cohesive ends.

The plasmid vector DNA was digested with enzymes to produce ends compatible with those on the insert. The volume of the digest was kept to a minimum. A small aliquot of the digest was run on an agarose gel (section 2.7.1) to check that digestion was complete. The remainder was heated at 65°C for 10 minutes to inactivate the enzyme.

The foreign DNA to be cloned was digested with the appropriate enzymes and purified from a low melting point agarose gel (section 2.7.2). The concentration was measured by the optical density method (section 2.5.1).

The ligation reaction contained approximately equimolar amounts of the vector and the insert, 1mM ATP, 1x One-Phor-All buffer, 2-5 units of T4 DNA ligase per μg of DNA and sterile water to make up the reaction volume to 10-20μl. The reaction mixture was left at room temperature overnight. In addition a control reaction was carried out that contained plasmid only and no insert.

2.2.2 Ligation of blunt ends.

The chosen vector was linearised using a restriction endonuclease, which cut in the multiple cloning site and produced blunt ends (usually Sma I). (section 2.6). The volume of the digest was kept to a minimum. A small aliquot of the digest was run on an agarose gel (section 2.7.1) to check that it was completed. The remainder was heated at 65°C for 10 minutes to inactivate the enzyme.

The fragment of DNA to be cloned was purified from a low melting point agarose gel (section 2.7.2) and the concentration measured by the optical density method (section 2.5.1). Enzymes were chosen that produced blunt ends or 5' overhangs. Overhanging ends were filled in using the Klenow fragment of *E.coli* DNA polymerase I. The filling reaction contained the DNA to be filled, 2-10 units of Klenow enzyme per μg of DNA, 1x One-Phor-All buffer, 1mM dATP, 1mM dGTP, 1mM dTTP, 1mM dCTP and sterile distilled water to make the volume of the mixture up so that the glycerol content from the enzyme was less than 10% v/v of the reaction mix. The reaction mixture was incubated at 37°C for 60 minutes and then at 65°C for 10 minutes to inactivate the enzyme.

The ligation reaction was carried out as for cohesive termini, except the molar ratio of insert to vector was increased to 3:1 and the concentration of ATP was decreased to 0.5mM.

2.3 Transformation of plasmid DNA into E. coli bacteria.

Solutions:

Transformation buffer: 10mM potassium acetate, 45mM $MnCl_2.4H_2O$, 10mM $CaCl_2.2H_2O$, 100mM KCl, 3mM hexaminecobalt chloride and 10% glycerol. The solution was filter sterilised using a 0.45μm nitrocellulose filter, and stored at 4°C until use.

Bacterial strains:

E.coli **strain JM101:** mcr A^+, sup E thi 1, Δ(lac-pro AB), F'[tra D36, pro AB^+, lac lq Z ΔM15], λ^-. (D.Hanahan, 1983).

E.coli **strain DH5α:** deo R, end A1, gyr A96, hsd R17[$r_k^-m_k^+$], rec A1, sup E44, thi 1, Δ(lac ZYA-arg FV169), Φ 80 δ lac Z ΔM15, F-, λ-. (D.Hanahan, 1983)

Protocol:

The method used was a variation of that described in Sambrook *et al.* (1989). 10ml of LB medium were inoculated with the relevant *E.coli* strain. The culture was incubated overnight at 37°C with vigorous shaking. The culture was diluted with LB medium (about a 1 in 5 dilution) and grown at 37°C until the optical density at 550nm was approximately 0.6-0.8 OD units with respect to LB medium. The culture was cooled on ice for 10 minutes, transferred to a sterile plastic universal tube and centrifuged at 700g for 10 minutes. The supernatant was decanted and the cell pellet was resuspended in 8ml ice cold transformation buffer. The suspension was incubated on ice for 10 minutes and centrifuged at 700g for a further 10 minutes. The pellet was resuspended in 2ml ice cold transformation buffer. Working on ice, 200μl of bacterial suspension were transferred to a pre-chilled eppendorf tube and 100ng-1μg of the DNA to be transformed were added. The mixture was incubated on ice for 30 minutes then heat shocked at 42°C for 90 seconds. The mixture was transferred to sterile 50ml polypropylene Falcon tubes and 1ml SOB medium was added to each tube. The culture was incubated at 37°C for 1 hour with vigorous shaking and plated onto LB agar containing the relevant antibiotic for selection.

For each transformation, two plates were made: one spread with 100μl of the final transformation mixture, and one with 800μl of the mixture. In addition, two control plates were made for each transformation experiment using just the competent cells with no addition of DNA. One of these was on the selective medium and one was LB agar without antibiotic. The uptake of DNA was checked using the basic plasmid, without insert, in a

transformation reaction. Colonies containing the transformed cells were used in small scale plasmid preparation (section 2.4.1) and restriction digests (section 2.6) to verify that they contained the correct plasmid and then made into glycerol stocks (section 2.1.1).

2.4 Preparation of plasmid DNA.

Solutions:

Solution 1: 50mM glucose, 10mM EDTA (pH 8.0), 25mM tris.Cl (pH 8.0). The solution was sterilised by autoclaving and stored at 4°C until use.

Solution 2: 0.2M NaOH, 1% w/v SDS. This solution was freshly prepared before each use.

Solution 3: 3M potassium, 5M acetate. This was prepared by mixing 60ml 5M potassium acetate solution, 11.5ml glacial acetic acid and 28.5ml sterile water. The solution was autoclaved and stored at 4°C until use.

Tris EDTA (TE) buffer (pH 8.0): 10mM tris.Cl (pH 8.0), 1mM EDTA (pH 8.0). The solution was aliquotted, autoclaved and stored at room temperature until use.

2.4.1 Small scale preparation of plasmid DNA.

This procedure is based on that described in Sambrook *et al.* (1989) using alkaline lysis of the bacteria. 10ml LB medium containing the relevant antibiotic was inoculated either with a single colony picked from an agar plate using a sterile platinum wire loop or by using a sterile platinum wire loop to scrape the surface of a frozen glycerol stock before swirling it in the medium. The culture was incubated at 37°C overnight with vigorous

shaking. 1.5ml aliquots of the culture were pipetted into sterile eppendorf tubes and centrifuged in an eppendorf centrifuge for 10 minutes. The supernatant was decanted leaving the pellet as dry as possible. The pellet was resuspended in 100µl ice cold solution 1 and incubated at room temperature for 5 minutes. 200µl solution 2 were added, mixed by shaking, and the tube was stored on ice for 5 minutes. 150µl of ice cold solution 3 were added, the tube was gently vortexed and incubated on ice for 5 minutes. The mixture was centrifuged for 5 minutes in an eppendorf centrifuge and the supernatant was decanted into a fresh tube. An equal volume of 1 : 1 phenol and chloroform (pH 8.0) was added and the tube vortexed. The mixture was centrifuged for 2 minutes in an eppendorf centrifuge. The aqueous layer was transferred to a fresh tube. Two volumes of 100% ethanol were added and mixed by inversion. The tube was kept at room temperature for 5 minutes and then centrifuged in an eppendorf centrifuge for 10 minutes. The supernatant was removed by aspiration. The pellet was washed with 70% ethanol, vortexed briefly and recentrifuged. The ethanol was drawn off as fully as possible and the pellet was dried under vacuum. The pellet was resuspended in 10µl TE buffer and stored at -20 °C until use. The DNA present was analysed by restriction digest (section 2.6).

2.4.2 Medium scale plasmid preparation.

This procedure is based on that described in Sambrook *et al.* (1989) using alkaline lysis of the bacteria. LB medium (100ml) containing the appropriate antibiotic was inoculated with the bacteria to be cultured. The culture was incubated at 37°C overnight with vigorous shaking. The culture was poured into sterile plastic centrifuge bottles and centrifuged at 6000g, 4°C, for 10 minutes in a Sorval fixed angle rotor. The supernatant was discarded and

each pellet was fully resuspended in 4ml ice cold solution 1 by pipetting. The suspension was transferred to sterile 30ml plastic centrifuge tubes kept at room temperature for 5 minutes. 8ml of solution 2 were added and the mixture shaken well, then left on ice for 10 minutes. 4ml of ice cold solution 3 were added, the tube shaken and incubated on ice for 10 minutes. The tubes were centrifuged at 30 000g, 4°C, for 30 minutes. The supernatant was decanted through gauze into 30ml corex tubes and 0.6 volumes of isopropanol were added. The solution was mixed by inversion and left at room temperature for 15 minutes. The corex tubes were fitted with rubber adapters and centrifuged at 15 000g, room temperature, for 30 minutes. The supernatant was discarded and the pellet washed briefly with 70% ethanol. The ethanol was removed as fully as possible by aspiration. The pellet was dried under vacuum, resuspended in 1.25ml TE buffer and transferred to a sterile eppendorf tube. 39µl 5M NaCl and 25µl boiled RNase A (10mg/ml) were added and the tubes incubated at 37°C for 60 minutes. The reaction was split between two tubes and an equivalent volume of phenol (pH 8.0) was added and the mixture vortexed thoroughly. The samples were centrifuged for 5 minutes in an eppendorf centrifuge and the aqueous layer was transferred to a fresh tube. The extraction was repeated with 1 : 1 phenol and chloroform (pH 8.0) and then with chloroform alone. The aqueous phase was mixed with 2 volumes of 100% ethanol and 0.1 volumes of 3M NaOAc and stored at -20°C for at least an hour and preferably overnight. The samples were centrifuged for 10 minutes in an eppendorf centrifuge and the supernatant discarded. The pellet was washed with 70% ethanol and then dried under vacuum for 5 minutes. The pellet was resuspended in 10µl TE buffer. The solution was heated at 65°C for 3 minutes and stored at -20°C until use.

2.4.3 Medium scale plasmid DNA preparation using Qiagen columns.

Solutions:

P1 solution: 100µg/ml RNase A, 50mM tris.Cl (pH 8.0), 10mM EDTA (pH 8.0). The solution was stored at 4°C until use.

P2 buffer: 200mM NaOH, 1% SDS. The solution was stored at room temperature until use.

P3 buffer: 3M potassium acetate (pH 5.5). The solution was stored at 4°C until use.

QBT buffer: 750mM NaCl, 50mM MOPS, 15% v/v ethanol, 0.15% Triton X-100, pH 7.0. The solution was stored at room temperature until use.

QC buffer: 1M NaCl, 50mM MOPS, 15% v/v ethanol, pH 7.0. The solution was stored at room temperature until required.

QF buffer: 1.25M NaCl, 50mM tris.Cl, 15% v/v ethanol, pH 8.5. The solution was stored at room temperature until use.

Protocol:

LB medium (100ml culture per Qiagen Tip 500) containing the appropriate antibiotic was inoculated with the bacteria to be grown (section 2.1.3). The culture was incubated at 37°C overnight, with vigorous shaking. The culture was poured into sterile plastic centrifuge bottles and centrifuged at 6000g, 4°C, for 10-15 minutes. The supernatant was discarded and 10ml P1 solution were used to fully resuspend the bacterial pellet. The mixture was transferred to 50ml plastic centrifuge tubes. 10ml P2 buffer were added, mixed by several inversions and kept at room temperature for 5 minutes. 10ml ice cold P3 buffer were added, mixed by inversion and incubated on ice for 20 minutes. The tubes were centrifuged at 30 000g, 4°C, for 30 minutes. In the meantime, the Qiagen Tip 500 was equilibrated with 10ml QBT buffer. After centrifugation, the supernatant was passed through sterile gauze into a

sterile 50ml plastic tube and poured onto the Qiagen Tip. The Qiagen Tip was washed twice with 30ml of QC buffer. A sterile glass corex tube was placed under the Qiagen Tip and 15ml QF buffer were added to the column. 10.5ml (0.7 volumes) of isopropanol were added to the corex tube. The tube was placed in a rubber adapter and centrifuged at 15 000g, 4°C, for 30 minutes. The supernatant was discarded and 15ml 70% ethanol used to wash the pellet. The tube was centrifuged at 15 000g for 15 minutes. The supernatant was discarded and the tube inverted on tissue to allow the remaining ethanol to drain away. When dry, the pellet was resuspended in 500µl TE buffer and stored at -20°C until use.

2.5 Measurement of DNA and RNA concentration.

2.5.1 The optical density method.

The absorbance of an aqueous solution of DNA or RNA at λ=260nm is proportional to the amount of DNA or RNA present (Sambrook *et al.*, 1989). The optical density at λ=260nm (A_{260}) of the DNA or RNA solution diluted in distilled water was measured using a Phillips PU 8720 UV/Vis scanning spectrometer. The solution was contained in 1ml quartz cuvettes and the spectrophotometer was zeroed using a distilled water blank.

For double stranded DNA:

$$\text{concentration } (\mu g/ml) = 50 \times A_{260} \times \text{dilution factor}$$

For single stranded DNA:

$$\text{concentration } (\mu g/ml) = 37 \times A_{260} \times \text{dilution factor}$$

For RNA:

$$\text{concentration } (\mu g/ml) = 40 \times A_{260} \times \text{dilution factor}$$

2.5.2 Using DNA DipStick™ (Invitrogen).

This method was used to measure accurately low concentrations of DNA or RNA (0.1 to 10ng/μl). The kit was used according to the manufacturer's instructions.

2.6 Restriction digests.

Solutions:

10x One-Phor-All buffer (Pharmacia): 100mM tris.acetate (pH 7.5), 100mM magnesium acetate and 500mM potassium acetate. This was used with Pharmacia enzymes according to the manufacturers instructions.

NE 2 Buffer (New England Biolabs): 10mM tris.HCl (pH 7.9), 10mM $MgCl_2$, 50mM NaCl, 1mM DTT. This was used with New England Biolabs' enzymes according to the manufacturer's instructions.

Tris EDTA (TE) buffer (pH 8.0): 10mM tris.Cl (pH 8.0), 1mM EDTA (pH 8.0). The solution was aliquotted, autoclaved and stored at room temperature until use.

Protocol:

Restriction digest mixtures contained 0.5-20μg of DNA and 2-10 units of enzyme per μg of DNA. The volume of the digest was selected so that the glycerol from the enzyme storage buffer formed no more than 10% v/v of the mixture. The relevant restriction digest buffer was added at 1x concentration of and the volume was made up with sterile distilled water. If the DNA used had been made by the small scale plasmid preparation (section 2.4.1), 1μl of RNase A (10mg/ml) per 5μg DNA was added. The digest was incubated at 37°C for 1-16 hours for a full digest. For a partial

digest the incubation time and temperature were reduced until the conditions gave the required result. The digest was stopped, if necessary, by addition of EDTA (pH 8.0) to give a concentration of 10nM. The digest was either loaded directly onto an agarose gel (section 2.7.1) or precipitated by addition of two volumes of 100% ethanol and 0.1 volumes 3M NaOAc and storage at -20°C overnight. After precipitation, the digest was centrifuged in an eppendorf centrifuge for 10 minutes. The pellet was washed with 70% ethanol, dried under vacuum and resuspended in TE buffer before an aliquot was used for agarose gel electrophoresis (section 2.7.1), and the remainder was stored at -20°C until required.

2.7 Agarose gel electrophoresis of DNA.

Horizontal gels were used to separate, identify and purify DNA. The percentage of agarose to be used in the gel was determined by the size of DNA to be separated: the smaller the DNA fragment to be separated, the larger the percentage of agarose. Amounts used varied between 0.4% and 3.0% w/v agarose.

2.7.1 Separation of DNA on an agarose gel.

Solutions:

10x TBE buffer: 0.89M tris.borate, 0.89M boric acid, 0.02M EDTA. 55g boric acid, 9.3g EDTA and 108g trizma base were made up to 1 litre with distilled water. The solution was autoclaved and kept at room temperature until required.

Running Buffer: 10x TBE was diluted by 1:10 before use to make 1x TBE.

Loading buffer: 0.25% w/v bromophenol blue, 30% v/v glycerol in distilled water. The solution was filter sterilised using a 0.45µm nitrocellulose filter and stored in aliquots at -20°C.

Tris EDTA (TE) buffer (pH 8.0): 10mM tris.Cl (pH 8.0), 1mM EDTA (pH 8.0). The solution was aliquotted, autoclaved and stored at room temperature until use.

Protocol:

The amount of agarose to be used was dissolved in the required volume of 1x TBE by heating in a microwave. The agarose mixture was allowed to cool to 50°C with constant stirring by magnetic stirrer. Ethidium bromide solution was added to a final concentration of 0.5µg/ml and stirred in. The edges of the gel mould were sealed with tape, if necessary, and the warm agarose mixture poured in. Care was taken not to create any bubbles. If they did form, they were moved to one side of the mould with a sterile pipette tip. The comb used to form the wells was positioned near one end of the mould with the teeth not quite touching the base, and the gel was left to set. When the gel had completely set it was possible to store it overnight at 4°C wrapped in Saran wrap. To use the gel, the comb was removed and the gel mounted in the electrophoresis tank. 1x TBE containing 5µg/ml ethidium bromide was poured into the tank until the gel was just covered and the wells filled.

DNA samples were made up to the required volume with sterile distilled water and 1.1µl loading buffer were added for every 10µl of sample. The samples were mixed and then pipetted into the wells in the gel. In addition a molecular weight standard (1KB marker) was loaded into one of the wells. The electric circuit was completed and the current was adjusted to 70-100mA. The gel was left to run until the dye front was approximately 1-3cm

from the end, depending on the separation required. The DNA in the gel was visualised using UV light and the sizes of the fragments seen were estimated using the molecular weight standard. The gel was photographed using a Seikostra VP-1500 video imaging system or Polaroid 55 positive negative 4x5 instant sheet film for a more permanent image.

2.7.2 Purification of DNA from a low melting point agarose gel.

Solutions:

50x TAE buffer: 40mM tris.acetate, 2mM EDTA (pH8.0). This was made by dissolving 242g trizma base in distilled water and adding 100ml 0.5M EDTA (pH 8.0) and 57.1ml glacial acetic acid. The volume was made up to 1 litre, the solution autoclaved and stored at room temperature until use.

Running Buffer: 50x TAE was diluted 1: 50 to make 1x TAE.

Loading buffer: 0.25% w/v bromophenol blue, 30% v/v glycerol in distilled water. The solution was filter sterilised using a 0.45µm nitrocellulose filter and stored in aliquots at -20°C.

Protocol:

A gel containing low melting point agarose was poured as described in section 2.7.1. The buffer used to make it was 1x TAE rather than 1x TBE. If a large volume of DNA was to be loaded, several of the teeth of the comb were joined together using masking tape so that one large well was formed when it was positioned in the gel. The gel was set and loaded as in section 2.7.1. The running buffer used was 1x TAE containing 5µg/ml of ethidium bromide. The current applied to the gel was kept at 50-70mA to ensure the gel did not melt. The band to be purified was identified under UV light and excised using a sterile scalpel blade. Exposure of the DNA to UV light was

kept to a minimum. The band of agarose was transferred to a sterile Spin-X centrifuge filtration unit within a sealable tube. The tube was dipped in liquid nitrogen for 1 minute and centrifuged in an eppendorf centrifuge for 10 minutes. This procedure was repeated until almost all of the agarose gel had gone from the upper unit. 100µl TE buffer were added to the unit and centrifuged again to rinse any remaining DNA down. If the percentage of agarose was very high (2-3% w/v) the liquid nitrogen and spin step were alternated with immersion of the tube in a water bath at 65°C for 10 minutes before centrifugation in an eppendorf centrifuge for 10 minutes. The DNA was precipitated by addition of two volumes of 100% ethanol and 0.1 volumes of 3M NaOAc and storage at -20°C for at least 1 hour and preferably overnight. The tube was centrifuged for 10 minutes, the pellet washed with 70% ethanol, dried under vacuum and resuspended in TE buffer. The DNA was stored at -20°C until use.

2.8 Polymerase Chain Reaction.

Solutions:

10x Reaction buffer: 160mM $(NH_4)_2SO_4$, 670mM tris.Cl (pH 8.8), 0.1% v/v Tween-20. This buffer was provided with the Biotaq DNA polymerase and used according to the manufacturer's instructions.

dNTP stock: 10mM dATP, 10mM dTTP, 10mM dGTP, 10mM dCTP. This solution was made using sterile water and stored in 10µl aliquots at -20°C.

Protocol:

Two single stranded primers of approximately 20 base pairs each, were designed and synthesised. These were complementary to the ends of the DNA fragment to be amplified. Each primer was designed to complement

the opposite strand at the opposite end of the DNA fragment to the other. The annealing temperature for the primers to the template was calculated using the formulae below:

i) for each primer:

$$T_m = 69.3 + 0.41(\%G+C) - 650/L$$

where T_m is the melting temperature (°C) for the base paired duplex, %G+C is the percentage of cytosine and guanine residues in the primer, and L is the length of the primer (Dyson, 1991).

ii) annealing temperature (°C) for PCR reaction = $(T_{m1}+T_{m2})/2 - 12$
where T_{m1} is the melting temperature for primer 1 and T_{m2} is the melting temperature for primer 2.

The protocol was carried out using the most sterile conditions possible. All solutions (excepting the primers and template) were filter sterilised using a 0.22μm nitrocellulose filter. Pipette tips, eppendorf tubes, tube racks, pipettes, and solutions (excepting the primers, template and paraffin) were irradiated using a Stratagene UV stratalinker for 5 minutes to break up any contaminating DNA. Gloves were worn and changed frequently, and care was taken not to create aerosols when opening eppendorf tubes, particularly those containing template DNA.

The reaction mixture contained 1x reaction buffer, 1.5mM $MgCl_2$, 10-100ng of the template DNA, 20pmol of each of the primers, 200nM dNTPs, 5 units of BioTaq DNA polymerase and sterile water to make up the volume to 50μl. The reaction buffer, $MgCl_2$, water, primers and template were added to a small eppendorf tube. The solutions were mixed by flicking the side of the tube and collected at the bottom of the tube by a brief centrifugation. The

tube was heated at 95°C for 2 minutes in a Hybaid thermal cycler and immediately cooled on ice for 2 minutes. The rest of the reactants were added to the tube, mixed and collected at the bottom of the tube. 50µl of sterile paraffin were added to prevent evaporation of the reactants during the PCR reaction. Thirty-five cycles of dissociation, annealing and extension were carried out using the thermal cycler. Each cycle consisted of 1 minute at 95°C, 1 minute at the calculated annealing temperature for the reaction and 1 minute at the extension temperature for the DNA polymerase (72°C for BioTaq).

For each experiment a negative control was carried out to identify whether any of the solutions had been contaminated. This was treated exactly the same as the other reactions and contained all the same reactants, excepting the template DNA.

When the reaction was finished, the aqueous phase was removed. An aliquot was checked by agarose gel electrophoresis and the remainder was stored at -20°C until use.

2.9 Preparation of nuclear protein extracts from rat liver tissue.

2.9.1 Treatment of the animals.

Male Sprague-Dawley rats of body weight 180-200g were supplied by U.C.L. Biological Services. They were fed on Harlan Teklad TRM9607 standard rat and mouse pellets. Control (untreated) animals were starved overnight before use. Animals treated with phenobarbital were given freshly-made sodium phenobarbital water (1g/L) each day for 4 days. On the fifth day the

animal was injected with 40mg sodium phenobarbital per kg of body weight in 0.9% NaCl. The animal was starved overnight and given tap water before use 16 hours after the injection. Animals treated with picrotoxin were given picrotoxin water (2g/L) for 3 days (changed daily) and then starved overnight with tap water before use. The animal was sacrificed by cervical dislocation. The liver was removed, weighed and kept in a beaker on ice until use.

2.9.2 Isolation of nuclei.

Solutions:

Homogenization buffer: 10mM N-2-Hydroxymethylpiperazine-N'-2-ethanesulphonic acid (HEPES) (pH 7.6), 15mM KCl, 0.15mM spermine, 0.5mM spermidine, 1mM EDTA, 2M sucrose, 10% v/v glycerol (final concentrations after addition of protease inhibitors). The solution was made using autoclaved or filter sterilised stock solutions and water, and using sterile apparatus. The solution was stored at -20°C. Approximately 1 hour before use, the solution was thawed and dithiothreitol (DTT) and phenylmethylsulfonyl fluoride (PMSF) were added to a final concentration of 0.5mM each. Also added were 0.1µg/ml antipain, 0.1µg/ml pepstatin A, 0.1µg/ml leupeptin and 14µg/ml aprotinin were added. The solution was kept as cold as possible (preferably -10°C) until use.

Nuclear Storage Buffer: 20mM tris.Cl (pH 7.9), 75mM NaCl, 0.5mM EDTA, 50% v/v glycerol (final concentrations after addition of protease inhibitors). The solution was filter sterilised and kept at -20°C. Immediately before use, the solution was thawed to 4°C and DTT and PMSF were added to give concentrations of 0.85mM and 0.125mM respectively.

0.25M PMSF: PMSF was dissolved in 100% ethanol to give a final concentration of 0.25M. The solution was aliquotted and stored at -20°C until use. It was often necessary to heat the mixture to 37°C in a water bath to fully dissolve the solid in the ethanol.

Protocol:

The protocol is based on that described by Sierra (1990). It was carried out entirely at 4°C. The tissue was homogenized as quickly as possible after removal from the animal. If necessary accuracy was sacrificed in favour of speed. Approximately 10ml of homogenization buffer was measured for each gram of tissue. The maximum volume used was calculated from the capacity of the centrifuge tubes, but the tissue never constituted more than 15% of the total volume. The tissue was minced as finely as possible with sterile dissecting scissors and about one third of the measured homogenization buffer was added to it. The tissue was homogenized by 3-4 strokes with a teflon tipped pestle and a Citenco electric overhead homogenizer (7200rpm). The homogenate was mixed with the remainder of the measured homogenization buffer. A cushion of 10ml homogenization buffer was poured into pre-chilled Beckman 39ml polyallomer tubes and the homogenate was poured on top to form 2 layers. The tubes were centrifuged at 104 000g, at 4°C, for 60 minutes in a Beckman L8 centrifuge using the SW28 rotor. The solid disc floating at the top of the tube was removed using a sterile spatula, and the supernatant was poured off. The sides of the tube were wiped clean with a tissue, and then the tube was slightly inverted on ice so that excess buffer ran down the sides away from the nuclear pellet. Care was taken to keep the part of the tube with the pellet in covered by ice. The sides of the tube were washed using distilled water and a large syringe. Care was taken not to wet the nuclear pellet with the water. The nuclei were frozen directly on dry ice (for methods of nuclear protein extraction by Sierra

(1990) and Jose-Estanyol *et al.* (1989)) or resuspended in nuclear storage buffer (for method of nuclear protein extraction by Lavery and Schibler (1993)).

2.9.3 Method of nuclear protein extraction by Sierra (1990).

Solutions:

Nuclear Lysis Buffer: 10mM HEPES (pH 7.6), 100mM KCl, 0.1mM EDTA, 10% v/v glycerol, 3mM $MgCl_2$ (final concentrations after addition of protease inhibitors). The solution was filter sterilised and stored at -20°C until use. Immediately before use 1mM DTT, 0.1mM PMSF, 14µg/ml aprotinin, 0.1µg/ml leupeptin, 0.1µg/ml pepstatin A and 0.1µg/ml antipain were added.

Dialysis Buffer: 25mM HEPES (pH 7.6), 0.1mM EDTA, 40mM KCl, 10% v/v glycerol. The solution was autoclaved and stored at 4°C until use. Just before use, DTT was added to a final concentration of 1mM.

Dialysis Tubing: Strips of 1cm-wide dialysis tubing were cut to approximately 10cm long. The strips were boiled twice for 5 minutes each time in a solution of 2% w/v $NaHCO_3$ and 1mM EDTA, changing the solution between boiling. The tubing was rinsed by boiling several times in distilled water, autoclaved in fresh distilled water and stored at 4°C until use.

Protocol:

The entire protocol was carried out at 4°C. This stage could be carried out immediately after preparation of the nuclear pellet without freezing it. The nuclei were thawed on ice and resuspended in 5ml nuclear lysis buffer per rat liver using a plastic pipette. The suspension was transferred to a sterile

hand-held Dounce homogenizer and the nuclei homogenized by 8 strokes of pestle A. The mixture was transferred to pre-chilled 39ml Beckman polyallomer tubes.

The optical density at λ=260nm of the homogenate was measured. To do this a 1:50 dilution in 0.5% SDS was prepared by adding the sample dropwise to the SDS whilst vortexing vigorously. The solution was vortexed for a further minute before measuring its optical density against a 0.5% SDS blank. The concentration of DNA was calculated as in section 2.5.1.

The amount of lysis buffer needed to dilute the homogenate to a DNA concentration of 0.5mg/ml was calculated and measured out. This was mixed with one tenth of the total volume (lysis buffer + homogenate) of 4M $(NH_4)_2SO_4$. The mixture was added to the homogenate and this was then rolled on a Denly spiramix for 30 minutes. The solution was centrifuged at 126 000g, 4°C, for 60 minutes. The supernatant was pipetted off as quickly as possible after the centrifuge stopped and transferred to fresh tubes. 0.3g of solid $(NH_4)_2SO_4$ was added per ml of supernatant and the tubes rolled for a further 30 minutes. The tubes were centrifuged for 20 minutes, at 126 000g, 4°C. The supernatant was pipetted off and discarded.

The pellet was resuspended in dialysis buffer by gentle pipetting. The amount of dialysis buffer was calculated to give a concentration of approximately 10mg/ml based on the DNA measurement done previously. The solution was transferred to dialysis tubing and dialysed for 2x2hours against 100 volumes of dialysis buffer. The solution was transferred to eppendorf tubes and centrifuged in an eppendorf centrifuge at 4°C for 5 minutes. The supernatant was aliquotted, frozen on dry ice and stored at -70°C before use.

2.9.4 Method of nuclear protein extraction by Lavery & Schibler (1993).

Solutions:

1.1x NUN: 0.3M NaCl, 1% v/v Nonidet P40, 25mM HEPES (pH 7.6), 10% v/v glycerol (final concentrations after addition of urea and protease inhibitors). The solution was filter sterilised and urea (ultra pure, deionised) was added to a final concentration of 1M before storage at -20°C. Immediately before use, the solution was thawed to 4°C and made to the following concentrations of 1mM DTT, 14μg/ml aprotinin, 0.7μg/ml leupeptin and 0.7μg/ml pepstatin A.

Dialysis Buffer: 25mM HEPES (pH7.6), 0.1mM EDTA, 40mM KCl, 10% v/v glycerol. The solution was autoclaved and stored at 4°C until use. Just before use, DTT was added to a concentration of 1mM.

Dialysis Tubing: Strips of 1cm-wide dialysis tubing were cut to approximately 10cm long. The strips were boiled twice for 5 minutes each time in a solution of 2% w/v $NaHCO_3$ and 1mM EDTA, changing the solution between boiling. The tubing was rinsed by boiling several times in distilled water, autoclaved in fresh distilled water and stored at 4°C until use.

Protocol:

The method was carried out entirely at 4°C. The nuclei in nuclear storage buffer were thawed on ice. To estimate the DNA content, the optical density at λ=260nm of the nuclei was measured. To do this a 1:100 dilution in 0.5% w/v SDS was prepared by adding the sample dropwise to the SDS whilst vortexing vigorously. The solution was vortexed for a further minute before measuring its optical density against a 0.5% w/v SDS blank. The concentration of DNA was calculated as in section 2.5.1.

The nuclei were diluted to 25mg/ml DNA content. The nuclei were gently vortexed while nine volumes of 1.1x NUN were added. The tubes were covered with nescofilm, mixed by inversion and left on ice for 15 minutes to precipitate the chromatin. The tubes were centrifuged at 10 000g, 4°C, for 10 minutes. The supernatant was quickly pipetted off, transferred to dialysis tubing and dialysed for 2x2hours against 100 volumes of dialysis buffer. The solution was aliquotted, frozen on dry ice and stored at -70°C.

2.9.5 Method of nuclear protein extraction by Jose-Estanyol *et al.* (1989)

Solutions:

Buffer A: 5mM Na.EDTA (pH 8.0), 15mM β-mercaptoethanol, 10mM tris.HCl (pH 7.4), 0.35M NaCl. The solution was filter sterilised and stored at -20°C. Immediately before use the solution was thawed to 4°C and 14μg/ml aprotinin, 0.1μg/ml leupeptin, 0.1μg/ml pepstatin A, 0.1μg/ml antipain and PMSF to a concentration of 1mM were added.

Protocol:

The protocol was carried out at 4°C. The nuclear pellet was thawed on ice and resuspended in buffer A to a concentration of 1mg/ml of DNA. This was calculated by optical density at λ=260nm as in section 2.5.1. The suspension was transferred to pre-chilled 15ml Beckman polyallomer tubes and rolled on a Denly spiramix for 30 minutes. The tubes were centrifuged for 20 minutes, at 40 000g, 4°C. The supernatant was transferred to a fresh tube and 0.2 volumes of glycerol were added and mixed by several inversions. The solution was aliquotted, frozen on dry ice and stored at -70°C until use.

2.9.6 One hour minipreparation of DNA-binding protein extracts (Deryckere & Gannon, 1994).

Solutions:

Solution A: 0.6% v/v Nonidet P40, 150mM NaCl, 10mM HEPES (pH 7.9), 1mM EDTA. The solution was filter sterilised and stored at -20°C. Immediately before use, the solution was thawed to 4°C and PMSF was added to give a final concentration of 0.5mM.

Solution B: 25% v/v glycerol, 20mM HEPES (pH 7.9), 420mM NaCl, 1.2mM $MgCl_2$, 0.2mM EDTA (final concentrations after addition of protease inhibitors). The solution was filter sterilised and stored at -20°C. Immediately before use, the solution was thawed to 4°C and made to the following concentrations of 0.5mM DTT, 0.5mM PMSF, 14µg/ml aprotinin, 5µg/ml pepstatin A, 5µg/ml leupeptin and 5µg/ml antipain.

Protocol:

The entire method was carried out at 4°C. 100-500mg of rat liver was frozen in liquid nitrogen. It was then broken with a hammer between layers of aluminium foil, transferred to a mortar and ground to a powder. The powder was transferred to a sterile hand-held Dounce homogenizer. 5ml of solution A was added and the tissue homogenized by 5 strokes of pestle B. The homogenate was transferred to a 25ml sterilin tube and centrifuged for 30 seconds, at 3 000g, 4°C. The supernatant was transferred to a fresh sterilin tube and incubated on ice for 5 minutes before centrifuging for a further 5 minutes at 19 600g, 4°C. The pelleted nuclei were resuspended in 100µl solution B per 100mg tissue and incubated on ice for 20 minutes. The lysate was transferred to an eppendorf tube and centrifuged at 2000rpm, 4°C, for 15 seconds. The supernatant was removed as quickly as possible, aliquotted, frozen on dry ice and stored at -70°C before use.

2.9.7 Measurement of protein concentration by the Lowry method (1951).

Solutions:

Solution A: One volume of 2% w/v potassium sodium tartrate solution was mixed with one volume of 1% w/v copper sulphate solution. 100 volumes of a freshly-made 2% w/v sodium carbonate solution in 0.1M sodium hydroxide were added. The solution was prepared immediately before use.

Solution B: Folin and Ciocalteau's reagent was diluted 1:1.5 with water. The solution was freshly prepared before each use.

Protocol:

This method was used for 1-100μg protein per sample. 1ml solution A was added to 0.2ml sample and the mixture was kept at room temperature for 20 minutes. 0.1ml solution B was added and the tube vortexed to mix. The mixture was left at room temperature for 45 minutes and the absorbance at λ=700nm was read using a Phillips 8720 UV/Vis scanning spectrophotometer. In addition to the samples, standard protein dilutions were prepared from a stock solution of bovine serum albumin (B.S.A.) The dilutions were made using dialysis buffer and always contained the same buffer components that were present in the sample. A blank was also prepared of the dialysis buffer alone. Care was taken with the timing to ensure that the optical density of each sample was measured at the same time from the start of its reaction. Detergents interfered with this assay and care was taken not to use buffers containing them.

2.10 The gel retardation assay.

2.10.1 Radioactive end-labelling of DNA.

Solutions:

STE buffer: 20mM tris.Cl (pH 7.5), 100mM NaCl, 10mM EDTA. The solution was autoclaved and stored at room temperature until use.

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

2.10.1a 5' overhang labelling (filling).

This method used the 3' to 5' polymerase activity of the Klenow fragment of *E.coli* DNA polymerase I. The DNA used was either a restriction fragment purified from a low melting point agarose gel (section 2.5.2) or two synthetic oligonucleotides were annealed together by heating to 65°C for 10 minutes and then left at room temperature until cool. The labelling reaction contained 100-500ng of the DNA fragment to be labelled, 2-10 units of Klenow fragment of DNA polymerase I per μg DNA, 1x One-Phor-All buffer, 1mM dATP, 1mM dGTP, 1mM dTTP, 3mM [α^{32}P]dCTP (10μCi/μl, 3000Ci/mmol) and sterile distilled water to make the volume of the mixture such that the glycerol content from the enzyme was less than 10% v/v of the reaction mixture. The reaction mixture was kept at 37°C for 30 minutes. The unincorporated nucleotides were removed on a Nuctrap push column, used according to the manufacturer's instructions.

Using radiolabelled [α^{32}P]dCTP in a filling reaction was not always appropriate. Sometimes the 5' overhang did not have a corresponding guanine residue. Using dCTP to fill opposite the two outermost bases of a four base overhang was not very efficient, for example filling a Hind III restriction fragment (A$\downarrow$AGCTT). In these cases another radionucleotide was chosen, for instance [α^{32}P] dATP was used to fill the Hind III fragment.

2.10.1b Blunt end labelling.

This was achieved using bacteriophage T4 Polynucleotide Kinase to catalyse the transfer of a radiolabelled γ-phosphate group of ATP to the 5' terminus end of a DNA fragment. The DNA used was a PCR product. A 'Ready-to-Go' kit was purchased from Pharmacia and used according to the manufacturer's instructions with [γ^{32}P]ATP (10μCi/μl, 3000Ci/mmol). The unincorporated [γ^{32}P]ATP was removed on a Nuctrap push column.

2.10.1c Measurement of radioactive counts.

Incorporation of radioactivity into the probe was counted by liquid scintillation. 1μl of the labelling reaction was dotted onto two separate pieces of DE81 paper before removal of the unincorporated nucleotides on either column. After the reaction mixture was passed through the column 1μl was again dotted onto two separate pieces of DE81 paper. The paper was allowed to dry completely. One piece from before the column and one from after the column were transferred into 5ml scintillation fluid in a plastic scintillation vial. The remaining papers were washed 6 times for 2 minutes each time in 0.5M Na_2HPO_4 (pH 7.0). The papers were then washed twice for 1 minute

each in distilled water and twice for 1 minute each in 95% v/v EtOH. They were then dried fully and transferred to a plastic scintillation vial containing 5ml scintillation fluid. The counts per minute were measureded using a liquid scintillation counter programmed to count ^{32}P.

2.10.2 The gel retardation.

Solutions:

10x TBE buffer: 0.89M Tris.borate, 0.89M boric acid, 0.02M EDTA. 55g boric acid, 9.3g EDTA and 108g trizma base were made up to 1 litre with distilled water. The solution was autoclaved and kept at room temperature until required.

Running buffer: 10x TBE was diluted by 1/40 before use to make 0.25x TBE.

5x Binding Buffer: 60mM HEPES (pH 7.9), 20mM tris.Cl (pH7.9), 300mM KCl, 150mM NaCl, 25mM $MgCl_2$, 25mM DTT, 0.5mM EDTA, 62.5% glycerol. The solution was filter sterilised using a 0.22µm filter and kept at -20°C until use.

Poly dI.dC DNA copolymer: Made up according to the manufacturer's instructions to 5µg/µl in sterile 5mM NaCl. The solution was kept in aliquots at -20°C until use.

6% nondenaturing polyacrylamide gel: 625µl 10x TBE, 5ml Protogel (30% acrylamide, 0.8% bisacrylamide, gas stabilised), 19.1ml water, 25µl N, N, N', N'-tetramethyl ethyl diamine (TEMED), 250µl 10% w/v ammonium persulphate (freshly prepared). The protogel, TBE and water were mixed together. Immediately before pouring, the ammonium persulphate was added and mixed in followed by the TEMED.

4% nondenaturing polyacrylamide gel: 625µl 10x TBE, 3.33ml Protogel (30% acrylamide, 0.8% bisacrylamide, gas stabilised), 20.77ml water, 25µl TEMED,

250µl 10% w/v ammonium persulphate (freshly prepared). The protogel, TBE and water were mixed together. Immediately before pouring, the ammonium persulphate was added and mixed in followed by the TEMED.
10x Loading Buffer: 30% glycerol, 0.25% bromophenol blue. The solution was filter sterilised using a 0.22µm filter and kept in aliquots at -20°C until use.

Protocol:

This method is based on that described by Rosette and Karin (1995). The polyacrylamide gel was poured on the morning of the experiment using a Bio-Rad gel system (16 x 14cm, 1.5mm spacers, 10 wells). The top plate had been siliconised. Once the gel was set, the wells were marked, the comb removed and the wells carefully rinsed with running buffer using a syringe. The gel was pre-run at 150V for 1 hour before loading. The running buffer in the top well was changed manually every 30 minutes throughout the pre-run and after loading.

Each gel retardation reaction contained 1-2ng radiolabelled DNA ($1x10^4$-$1x10^5$cpm/µl), 2-10µg nuclear protein diluted to the correct concentration with dialysis buffer (see above), 1x binding buffer, 2-10µg poly dI.dC and sterile water to make up the volume to 20µl. All the reactants except the probe were added to the reaction tube and pre-incubated on ice for 10-20 minutes. The probe was added and mixed in by flicking the side of the tube. The reaction mixture was collected at the bottom of the tube by a pulse spin in an eppendorf centrifuge and the tube was incubated on ice for 30 minutes. 2µl of 10x loading buffer was added and the reaction loaded onto the gel using gel loading tips. Two controls were included with each experiment: one was a probe alone reaction, which contained all the reactants except the protein, and the other was a proteinase K digest reaction. For this, all the

reactants were added and incubated as described above. Then 5µg proteinase K was added and the tube incubated at 37°C for 15 minutes before addition of loading buffer.

The gel was run at 150V until the free probe was at the end of the gel. The time depended on the size of the probe and the percentage of the gel. For a 60bp fragment on a 6% gel, the bromophenol blue was just run off the gel. For larger fragments, 0.002% xylene cyanol was added to the loading dye and some of this was loaded into a spare well to track the DNA. For a 200bp fragment on a 4% gel, the xylene cyanol band was 4cm from the end of the gel when electrophoresis was stopped. The gel was removed from the tank and the top (siliconised) plate carefully lifted off. The gel was transferred to 3MM Whatman paper, covered with Saran wrap and dried under vacuum at 80°C until completely dry. An autoradiograph of the dried gel was taken in an aluminium film cartridge using Fuji X-ray film and amplified with Kodak screens. The film was left overnight at -70°C before developing. If sharper bands were required, the gel was autoradiographed for several days at room temperature, without intensifying screens.

2.10.3 Competitive gel retardation.

Competition experiments were carried out using unlabelled DNA fragments to compete for the nuclear proteins. Each of these reactions contained the reactants described in section 2.10.2 and a 100 molar excess of unlabelled DNA compared to the labelled probe. The water, binding buffer, poly dI.dC and unlabelled DNA were added to the tube and pre-incubated on ice for 10-20 minutes. The labelled probe was added, mixed and spun down. Then, the nuclear protein was added, mixed and spun down. The reaction was then

incubated on ice for 30 minutes, loading dye added and the reaction was loaded on to the gel.

2.10.4 Supershift gel retardation.

Supershift retardation reactions contained all the reactants above and included 1μg of antibody to a transcription factor. This was added at the same time as the nuclear protein. The binding incubation was increased from 30 minutes to 1 hour before loading onto the gel.

2.11 Preparation of RNA.

2.11.1 Preparation of total RNA from cell pellets using Ultraspec™ RNA solution.

Protocol:

The procedure was carried out on ice and as much as possible under sterile conditions. The pellets (containing approximately 10^7 cells) were thawed on ice until the liquid above the pellet had melted. The Ultraspec™ RNA solution was thawed at room temperature. 1ml of Ultraspec™ RNA was added to each pellet in a 50ml sterilin tube and a sterile glass homogenizer pestle was used to break up the pellet. The mixture was pipetted up and down to resuspend the cells fully. The suspension was transferred to sterile 2ml eppendorf tubes and incubated on ice for 5 minutes. 0.2ml of chloroform was added to each tube and mixed by vigorous shaking. The tubes were incubated on ice for 5 minutes, then centrifuged at 12 000g, 4°C, for 15 minutes. The aqueous layer was transferred to a fresh eppendorf tube

and an equal volume of isopropanol was added. The tubes were stored on ice for 10 minutes, then centrifuged at 12 000g, 4°C, for 10 minutes. The supernatant was discarded and the pellet washed with 1ml 75% ethanol. The pellet was resuspended in filter sterilised 50µl diethylpyrocarbonate (DEPC)-treated water. The concentration of the RNA was measured using the optical density method (section 2.5). The concentration and integrity of the RNA was checked by agarose gel electrophoresis (section 2.12).

2.11.2 Preparation of total RNA from cell pellets using guanidine thiocyanate.

Solutions:

Lysis buffer: 5M guanidine thiocyanate, 10mM EDTA, 50mM tris.HCl (pH 7.5), 8% v/v β-mercaptoethanol. All the stock solutions, deionised water and the apparatus used in making the solution were autoclaved. The guanidine thiocyanate was dissolved in sterile ultrapure water in a 50°C water bath. The tris.HCl and EDTA were added, and the solution made up to the final volume less the amount of the β-mercaptoethanol. The β-mercaptoethanol was added to the buffer just before use.

RNA solubilisation buffer: 0.1% w/v SDS, 1mM EDTA, 10mM tris.HCl (pH 7.5). All the stock solutions, ultrapure water and the apparatus used in making the solution were autoclaved. Aliquots of the solution could be kept at room temperature for several months before use.

3M LiCl: The solution was made using ultrapure water, autoclaved and stored at room temperature before use.

4M LiCl: The solution was made using ultrapure water, autoclaved and stored at room temperature before use.

Saturated ammonium acetate: The solution was filter sterilised using a 0.45µm nitrocellulose filter and stored at room temperature until use.

Phenol buffering solution: 1mM EDTA, 50mM tris-HCl (pH 7.5). The solution was autoclaved and stored at room temperature before use.

Phenol (pH 6.0): Phenol (pH 8.0) was thawed and poured into a sterile bottle. An equal volume of phenol buffering solution was added. The bottle was vortexed and left until two phases formed. The aqueous layer was removed and more buffering solution was added. The process was repeated 3-5 times until the phenol was pH 6.0, measured with pH paper. The buffered phenol was aliquotted into sterile tubes. These were covered in foil and stored at -20°C until use.

Protocol:

The method is taken from Cathala *et al.* (1983). The cell pellets were thawed on ice covered by 7ml lysis buffer per 1ml of packed cell volume. The cell pellet was resuspended by pipetting. The solution was transferred to sterile corex tubes. The RNA was precipitated with seven volumes of 4M LiCl and incubation at 4°C overnight. The sample was centrifuged at 10 000g, 4°C, for 90 minutes, and the supernatant was discarded. The pellet was resuspended in 3M LiCl by adding 100µl at a time until the RNA went into solution. The solution was centrifuged at 4°C, 11 000g, for 1 hour. The pellet was resuspended in 2ml solubilising buffer by successive rounds of freezing the tube on dry ice and then vortexing. This usually took about 30 minutes. The RNA was extracted with two volumes of 1:1 phenol (pH 6.0) and chloroform and left at -20°C overnight for the interphase to pack. The tube was centrifuged at 400g for 8 minutes. The aqueous phase was transferred into a sterile Falcon tube and re-extracted once with phenol:chloroform and once with chloroform only. The remainder of the protocol was carried out on ice and care was taken to maintain sterility. The final aqueous phase was

transferred into a pre-chilled sterile corex tube. 0.05 volumes of saturated ammonium acetate and two volumes pre-chilled 100% ethanol were added. The tube was vortexed and incubated either for 1 hour at -70°C or on dry ice, or overnight at -20°C to precipitate the RNA. The RNA was pelleted by centrifugation at 11 000g, -10°C, for 70 minutes. The pellet was washed with 1ml cold 100% ethanol and centrifuged again at 11 000g, -10°C, for 60 minutes. The ethanol was carefully pipetted off the pellet. The top of the tube was covered with nescofilm, which was pierced several times with a sterile syringe needle, and the pellet was dried under vacuum. The pellet was resuspended in 100µl sterile DEPC-treated water by incubation at 4°C for 45 minutes. The purity and concentration of the RNA were measured by reading the optical density at λ=260nm and λ=280nm of a 1/500 dilution in sterile water (section 2.5.1). A check gel was run to confirm the concentrations and integrity of the RNA (section 2.12)

2.12 Agarose gel electrophoresis of RNA.

Solutions:

10x MOPS/EDTA buffer (pH 7.0): 0.2M MOPS, 80mM sodium acetate, 10mM EDTA. The solution was adjusted to pH 7.0 using 10M NaOH. The solution was autoclaved and kept at room temperature until use. It could be diluted with distilled water to make 1x MOPS/EDTA buffer that was then reautoclaved and cooled before use as the running buffer.

1.3% agarose RNA gel: 3.9g agarose, 30ml 10x MOPS/EDTA buffer and 270ml water. The mixture was autoclaved and kept sealed at room temperature until use.

RNA loading buffer: 57% v/v deionised formamide, 11.3% v/v 10x MOPS/EDTA, 18.3% v/v formaldehyde, 7.5% v/v glycerol, 0.025% w/v

bromophenol blue, 0.02μg/μl ethidium bromide. The solution was made using filter sterilised stock solutions where possible and stored in aliquots at -20°C.

Protocol:

The gel mixture was microwaved until liquid and cooled to at least 50°C. 15ml formaldehyde per 300ml gel was added and mixed in. The gel mould and comb were thoroughly cleaned and wiped with ethanol. The agarose solution was poured in and left to set in the flow hood. The gel tank was cleaned, washed with ethanol, filled with sterile 1x MOPS and covered until use. The RNA sample to be loaded was made up to a volume of 5μl with sterile DEPC-treated water. 25μl RNA loading buffer were added, the tube vortexed briefly and then spun down. The sample was heated at 65°C for 15 minutes and cooled on ice for 5 minutes before loading immediately onto the gel. The gel was electrophoresed at 50-100V until the dye front was approximately 1-3cm from the end. The RNA bands were visualised with UV light and photographed using Polaroid 55 positive/negative film.

2.13 Slot blot analysis of RNA.

2.13.1 Preparation of the slot blot.

Solutions:

20x SSPE: 3M NaCl, 0.2M sodium phosphate (pH 7.4), 0.02M EDTA. The solution was adjusted to pH 7.4 using 10M NaOH, autoclaved and stored at room temperature until use.

Loading buffer: 0.25% w/v bromophenol blue, 30% v/v glycerol in distilled water. The solution was filter sterilised using a 0.45µm nitrocellulose filter and stored in aliquots at -20°C.

0.2M Sodium phosphate buffer (pH 7.4): 0.2M Na_2HPO_4, was added to 0.2M Na H_2PO_4 until the pH was 7.4. The solution was autoclaved and kept at room temperature until use.

Tris EDTA (TE) buffer (pH 8.0): 10mM Tris.Cl (pH 8.0), 1mM EDTA (pH 8.0). The solution was aliquotted, autoclaved and stored at room temperature until use.

<u>Protocol:</u>

Optimised nylon membrane was cut to fit the slot blot manifold. It was wetted by floating it on top of deionised water and allowing it to wet gradually. The membrane and a piece of 3MM Whatman paper of the same size were soaked in 20x SSPE. The upper block of the manifold was placed on the bench, face down. The wet membrane was placed on top of the block and any air bubbles were smoothed out. The Whatman paper was placed on top of the membrane and the bubbles smoothed out. The middle block of the manifold was placed on top of the filter pad and the lower block on top of that. The manifold was clamped together and the vacuum pump was attached.

Each of the samples to be loaded was suspended in 50µl TE buffer (maximum 10µg per well). 35µl 20x SSPE and 20µl 37% formaldehyde were added to each of the samples. The samples were incubated at 65°C for 15 minutes and stored on ice for 5 minutes before loading onto the blot.

The wells of the manifold were washed out with 500µl 20x SSPE by applying a low vacuum. Too high a vacuum caused the salt to precipitate out and

incorrect loading of the samples. Those wells that did not empty properly were noted and were not used further. 20µl of DNA electrophoresis loading buffer were added to several wells spaced across the blot under vacuum to demonstrate that the blot was tightly and evenly clamped together. The samples were applied under vacuum. Each well was again rinsed with 500µl 20x SSPE. The membrane was placed in a U.V. Stratalinker and the autocrosslink function was used to bind the RNA to the membrane. The membrane was baked at 65-80°C for 10-30 minutes until dry. The blot could be stored at this stage wrapped in Saran wrap in a dessicator at 4°C.

2.13.2 Preparation of the probe by the random priming method.

Solutions:

10 Random Priming (RP) buffer: 900mM HEPES (pH 6.6), 100mM $MgCl_2$. The solution was filter sterilised and stored in aliquots at -20°C until use.

RP dNTP mix: 5mM dATP, 5mM dGTP, 5mM dTTP. The mixture was prepared from sterile stocks and using filter sterilised water.

Protocol:

The method used was based on that described in Sambrook *et al.* (1989). Two reaction mixtures were prepared in separate 0.5ml eppendorf tubes. Tube 1 contained 1µl DTT (20mM), 1µl 10x RP buffer, 1µl RP dNTP mix and 3µl [α^{32}P]dCTP (3000Ci/mmol, 10µCi/µl). Tube 2 contained 1µl random primers (75ng/µl), 1µl plasmid template DNA (250ng/µl) and 1µl water. The reactants were mixed together by flicking the sides of the tubes and collected at the bottom of the tubes by a brief pulse in an eppendorf centrifuge. Tube 1 was boiled for 5 minutes, immediately incubated on ice for 5 minutes and the reactants were spun down. The entire contents of tube 2 were added to

tube 1. 5 units of Klenow DNA polymerase (1µl) was added. The reactants were mixed, collected at the bottom of the tube and incubated at 37°C for 3 hours or overnight. Unincorporated dNTP was removed as in section 2.10.1. The radioactive counts were measured (section 2.10.1c) and the percentage incorporation and cpm/µg were calculated as follows:

% incorporation = (cpm on washed DE81 / cpm on unwashed DE81) x 100

$$\text{cpm}/\mu\text{g} = \frac{\text{cpm on washed DE81 x volume of eluate from column}}{\mu\text{g of template DNA added}}$$

Probes that had an incorporation of > 20% and a cpm/µg of > 1 x 10^8 were used for hybridization reactions.

2.13.3 Hybridisation of the slot blot.

Solutions:

20x SSPE: 3M NaCl, 0.2M sodium phosphate (pH 7.4), 0.02M EDTA. The solution was adjusted to pH 7.4 using 10M NaOH, autoclaved and stored at room temperature until use.

50x Denhardt's reagent: 1% w/v Ficoll, 1% w/v polyvinylpyrrolidone, 1% w/v B.S.A. (pentex fraction V). The solution was made in deionised water, filter sterilised using a 0.45µm nitrocellulose filter and stored in aliquots at -20°C until use.

Salmon sperm DNA, denatured and sheared: Sterile water was added to the DNA in a sterile Falcon® tube to make a final concentration of 10mg/ml. The tube was rotated on a roller-shaker at room temperature overnight to dissolve the DNA. The DNA was sheared by drawing it through a sterile

syringe needle and expelling it again. This was repeated 20 times. Depending on the purity of the DNA, it was sometimes necessary to carry out a phenol and chloroform extraction at this stage. An equal volume of 1:1 phenol (pH 8.0) and chloroform was added to the DNA solution. The mixture was vortexed for 20 seconds and centrifuged at 700g, room temperature, for 10 minutes. The aqueous phase was transferred to a fresh tube and aliquotted. The DNA concentration was measured by optical density (section 2.5.1) and the DNA was stored at -20°C before use. Immediately prior to use, the aliquot was boiled for 5 minutes to denature the DNA and the tube was placed on ice for 5 minutes.

Hybridisation solution: 5x SSPE, 1% SDS, 100µg/µl denatured, fragmented salmon sperm DNA, 5x Denhardt's reagent, 10% w/v dextran sulphate. The solution was made with deionised water and filter sterilised using a 0.45µm nitrocellulose filter before use.

Wash solution 1: 5x SSPE, 0.5% SDS. The solution was made with deionised water and filter sterilised using a 0.45µm nitrocellulose filter before use.

Wash solution 2: 0.1x SSPE, 1% SDS. The solution was made with deionised water and filter sterilised using a 0.45µm nitrocellulose filter before use.

Stripping buffer: 5x SSPE, 1% SDS. The solution was made with deionised water and filter sterilised using a 0.45µm nitrocellulose filter before use.

<u>Protocol:</u>

The slot blot was placed on top of a slightly larger piece of sterile gauze. The blot and gauze were placed in a hybridisation bottle so that they were flat against the side. 15ml of hybridisation solution was added. The bottle was placed in a hybridization oven at 65°C for 3 hours with rolling. The probe was denatured by boiling it for 5 minutes, then placing it on ice for 5 minutes before use. The probe was added to 8ml hybridization solution to give a final DNA concentration of 10-20ng/ml. This solution was used to

replace the hybridization solution in the bottle. Hybridization was carried out overnight at 65°C with rolling. The membrane was removed from the bottle and washed twice in 0.5ml wash solution 1 per cm^2 of blot for 15 minutes each at room temperature. The membrane was washed three times in the same volume of wash solution 2 for 15 minutes each at 65°C. After each wash the blot was monitored with a Geiger counter to check whether all the background radiation had been washed off. When this had been achieved, the washes were stopped. The excess moisture was drained off the blot, but it was not allowed to dry out fully. The blot was wrapped in Saran wrap and autoradiographed at -70°C using a Kodak intensifying screen after which it could be stripped by washing with stripping buffer at 65°C until no more counts could be detected using a Geiger counter. To check that the blot had been fully stripped it was autoradiographed overnight. Once stripped, the blot could be stored while it was still damp wrapped in Saran wrap at 4°C.

2.14 Preparation of whole cell homogenates from cell pellets.

Solutions:

0.25M PMSF: PMSF was dissolved in 100% ethanol to give a final concentration of 0.25M. The solution was aliquotted and stored at -20°C until use. It was often necessary to heat the mixture to 37°C in a water bath to fully dissolve the solid in the ethanol.

0.2M Sodium phosphate buffer (pH 7.25): 0.2M Na_2HPO_4, was added to 0.2M Na H_2PO_4 until the pH was 7.25. The solution was autoclaved and kept at room temperature until use.

Homogenization buffer: 10mM sodium phosphate buffer (pH 7.25), 1mM EDTA (pH 8.0), 20% v/v glycerol. The solution was autoclaved and stored at

room temperature. Immediately before use, PMSF was added to give a final concentration of 4mM.

Protocol:

The whole procedure was carried out on ice. The cell pellet (approximately 10^7 cells) was thawed on ice. Any excess liquid present on top of the pellet was removed by pipetting off as much as possible without disturbing the pellet. 100µl homogenization buffer were added per 100µl volume of the pellet (estimated by eye). The pellet was resuspended by pipetting up and down. The suspension was transferred to a pre-chilled, sterile, hand-held homogenizer and homogenized by 10 strokes of the pestle. The liquid was poured into a pre-chilled sterile eppendorf tube. The homogenate was aliquotted into pre-chilled eppendorf tubes and snap frozen on dry ice. Care was taken before and during aliquotting to pipette the homogenate up and down to ensure full mixing and equal concentration in all the aliquots. The concentration of each aliquot was measured using the Lowry method (section 2.9.7).

2.15 Western blot analysis of proteins.

2.15.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE).

Solutions:

0.25M PMSF: PMSF was dissolved in 100% ethanol to give a final concentration of 0.25M. The solution was aliquotted and stored at -20°C until use. It was often necessary to heat the mixture to 37°C in a water bath to fully dissolve the solid in the ethanol.

0.2M Sodium phosphate buffer: (pH 7.25) 0.2M Na_2HPO_4, was added to 0.2M Na H_2PO_4 until the pH was 7.25. The solution was autoclaved and kept at room temperature until use.

pH 8.8 buffer: 1.5M tris.HCl (pH 8.8), 0.4% w/v SDS. The pH of the solution was adjusted to pH 8.8 with HCl and NaOH. The solution was autoclaved and stored at room temperature until use.

pH 6.8 buffer: 0.5M tris.HCl pH 6.8, 0.4% w/v SDS. The pH of the solution was adjusted to pH 6.8 with HCl and NaOH. The solution was autoclaved and stored at room temperature until use.

2x Protein loading buffer: *1%* w/v SDS, 10mM EDTA, 10mM sodium phosphate buffer (pH 7.0), 1% v/v β-mercaptoethanol, 15% v/v glycerol, 0.01%w/v bromophenol blue. The solution was sterilised using a 0.45μm nitrocellulose filter. PMSF was added to give a final concentration of 4mM and the solution was stored at -20°C until use.

Running buffer: 0.025M Tris, 0.192M glycine, 0.1% w/v SDS. The solution was freshly-made before each use.

3% acrylamide stacking gel: 2ml protogel (30% acrylamide), 5ml pH 6.8 buffer, 12.78ml sterile water, 20μl TEMED, 200μl freshly-made 10% w/v ammonium persulphate. The protogel, buffer and water were mixed together and then the TEMED and ammonium persulphate added and mixed in immediately before pouring.

7% acrylamide separating gel: 23.33ml protogel (30% acrylamide), 25ml pH 8.8 buffer, 50.57ml sterile water, 100μl TEMED, 1ml freshly-made 10% w/v ammonium persulphate, mixed together in the same way as for the 3% acrylamide gel.

10% acrylamide separating gel: 16.67ml protogel (30% acrylamide), 12.5ml pH 8.8 buffer, 20.28ml sterile water, 50μl TEMED, 0.5ml freshly-made 10% w/v ammonium persulphate, mixed together in the same way as for the 3% acrylamide gel.

13% acrylamide separating gel: 13ml protogel (30% acrylamide), 7.5ml pH 8.8 buffer, 9.17ml sterile water, 30µl TEMED, 30µl freshly-made 10% w/v ammonium persulphate, mixed together in the same way as for the 3% acrylamide gel.

Protocol:

The percentage of acrylamide used in the separating gel depended on the size of the proteins and the separation required. Less acrylamide provided a gel that gave a better separation of high molecular weight proteins. More acrylamide gave sharper bands and better resolution of low molecular weight proteins. The separating gel was set, the night before the experiment. A Hoefer Mighty Small long gel system (8.2 x 9.4cm, 0.75mm thick, vertical gel) or a Bio-Rad gel system (14 x 12cm, 1.5mm thick, vertical gel) was used. The plates were set up as in section 2.10.2. The combs were placed between the plates and a mark made 1.5cm below the base of the comb. The separating gel was poured up to this mark. Immediately afterward an overlay of 0.1% SDS solution was gently pipetted on top of the gel to a depth of approximately 0.5cm. The gel was left to set, covered in Saran wrap and left overnight. The SDS was poured off and the stacking gel was poured on top of the separating gel. The combs were pushed in and the gel was left to set for at least an hour. The whole cell extracts were thawed on ice and diluted to a convenient concentration with homogenization buffer. The sample was diluted 1:1 with 2x protein loading buffer. The sample could now be stored at -20°C. An aliquot containing the amount of sample to be run on the gel was pipetted into a fresh tube. An aliquot of mid-range protein molecular weight marker positive controls for each experiment were also prepared. The sample tubes were boiled for three minutes before addition of 1µl β-mercaptoethanol per 20µl of sample. The wells in the gel were rinsed out with running buffer and the samples loaded. The gel was

run at 8mA per gel (Hoefer gel system) or 15mA per gel (Bio-Rad gel system). Once the tracking dye had formed a thin line at the boundary of the two gels and run slightly into the separating gel, the current was doubled. The gel was run until the dye was approximately 1cm from the end.

2.15.2 Staining of SDS-PAGE gels with Coomassie Brilliant Blue.

Solutions:

Coomassie Brilliant Blue Stain: 0.2% w/v brilliant blue R, 10%v/v glacial acetic acid, 45% v/v methanol, 45% v/v water. The dye was dissolved in the methanol, then the water and the acid were added in that order. The solution was kept at room temperature, and could be reused several times.

Coomassie Destain: 10%v/v glacial acetic acid, 45% v/v methanol, 45% v/v water. The solution was kept at room temperature until use.

Protocol:

The gel apparatus was dismantled and the top plate of the gel carefully removed. The gel was transferred to a sealable Tupperware container. The Coomassie stain was poured on top and the gel was left to stain for up to 16 hours. The stain was poured off and could be retained for future use. Destain solution was poured on to the gel and the container was sealed and shaken using an orbital shaker. The destain solution was changed as it became saturated with colour. This was continued until the bands of protein were clearly seen and the rest of the gel became clear. The gel was then photographed. Stained gels were not used for blotting.

2.15.3 Transfer of the proteins to a nitrocellulose filter.

Solutions:

Transfer buffer: 192M glycine, 25mM Tris, 20% v/v methanol, 0.1% w/v SDS. This solution was made up freshly for each use.

Protocol:

Hybond C-extra nitrocellulose filter was cut to the size of the gel, labelled with pencil, wet in deionised, high purity water and soaked in transfer buffer for 10 minutes. Four pieces of 3MM Whatman paper were cut just larger than the filter and soaked in pairs in the transfer buffer. Four blotting pads were also soaked in the transfer buffer. The gel apparatus was dismantled and the top plate of the gel carefully removed. The filter was laid on top of the gel, any bubbles were smoothed out and the filter peeled back, removing the gel with it. The transfer cassette (Bio-Rad Trans Blot Cell System) was opened, and two blotting pads laid on top of one side. Then two sheets of the soaked Whatman paper were placed on top of the pads and overlaid with the filter and gel, with the gel uppermost. Two more sheets of Whatman paper were placed on the gel and any bubbles smoothed out. The remaining blotting pads were laid on top. The cassette was closed and positioned in the transfer tank so that the nitrocellulose filter was closer to the anode than the gel. The tank was filled with transfer buffer and the current set at 100mA overnight. The current was turned up to 200mA for 1 hour.

2.15.4 Probing the Western blot.

Solutions:

10x TBS (pH 7.5): 200mM Tris, 5M NaCl. The pH of the solution was adjusted to pH 7.5. The solution was autoclaved and kept at room temperature until use. It was diluted by 1/10 with distilled water to make 1x TBS.

1x TTBS: 20mM Tris, 500mM NaCl, 0.05% Tween-20, pH 7.5. 200µl of Tween-20 was added per 400ml 1x TBS. This solution was made up freshly before each use.

Blocking solution: 3% w/v gelatin (blotting grade) in 1x TTBS. 3g of gelatin were melted in 100ml 1x TTBS and cooled to less than 50°C with stirring before use. This solution was made up freshly before each use.

Antibody buffer: 1% gelatin (blotting grade) in 1x TTBS. The gelatin was melted in the buffer and the solution stored at 4°C until use.

Primary antibody solution: The rabbit serum antibodies to the relevant protein were diluted to the appropriate titre using the antibody buffer. The dilution was stored at -20°C until use. The antibody could be reused several times.

Secondary antibody solution: 60µl of the goat-anti-rabbit IgG conjugated to horseradish peroxidase (GAR-HRP) was mixed into 60ml 1x TBS immediately before use.

Protocol:

The nitrocellulose filter was placed in a sealable Tupperware container, rinsed with distilled water and the blocking solution was poured on. The container was covered and placed on an orbital shaker set at a slow rate for 1 hour. The blocking solution was tipped away and the filter washed in 1x TTBS for 5 minutes. The primary antibody was added and the container

shaken for 2 hours. The primary antibody was decanted off and reserved for future use. The filter was washed twice for 10 minutes each with 1x TTBS, then incubated with the secondary antibody for 1 hour. The antibody was discarded and the filter washed twice for 10 minutes each with 1x TTBS and once for 5 minutes with 1x TBS.

2.15.5 Visualisation of the antibodies using the Bio-Rad Immun-Blot Assay Kit and the National Diagnostics HRPL kit.

Both of these kits were used according to the manufacturers' instructions. The Immun-Blot assay colour development solution was poured onto the blot in a Tupperware container and the reaction was stopped by washing the blot in double distilled water. The HRPL assay development solution was pipetted onto the blot and dispersed across the blot by tipping and rotating it. The excess solution was poured off after a minute. Tissue was used to remove the last of the solution. The blot was covered with polythene, bubbles were smoothed out and the blot was exposed to Fuji X-ray film for up to 4 minutes.

2.15.6 Staining of a nitrocellulose filter using Amido Black.

Solutions:

Amido Black stain: 0.1% w/v amido black 10-B, 45% v/v methanol, 10% v/v glacial acetic acid. The solution was kept at room temperature until required.

Amido Black destain: 90% v/v methanol, 2% v/v glacial acetic acid, 8% v/v water. The solution was kept at room temperature until use.

The filter was placed in a Tupperware container and the staining solution poured on top. After 2 minutes the stain was poured off and amido black destain was poured on. The container was shaken at room temperature using an orbital shaker. The destain solution was changed as it became saturated with colour. This was continued until the protein bands were clearly seen and the background filter had almost returned to its original colour.

2.16 Culture of primary rat hepatocytes.

2.16.1 Isolation of primary hepatocytes:

Solutions:

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

10x EBSS: Earle's Balanced Salt Solution without calcium and magnesium. The solution was diluted 1:10 with sterile water before use.

EGTA stock: 25mM EGTA in 0.1M NaOH. The solution was autoclaved and kept at 4°C until use within one week.

Perfusion Buffer 1: 1x EBSS, 0.2% w/v $NaHCO_3$, 0.5mM EGTA, 0.002M NaOH, 100µg/ml streptomycin, 100U/ml penicillin, pH 7.5. 700ml of perfusion buffer 2 were poured into a sterile 1L Duran bottle. 16ml of EGTA stock solution were added and the pH adjusted if required to 7.4-7.5 using sterile 1M HCl or sterile 1M NaOH and judging by colour. The solution was made up to 800ml with sterile deionized water and kept at room temperature until use on the same day.

Perfusion buffer 2: 1x EBSS, 0.2% w/v $NaHCO_3$, 100U/ml penicillin, 100µg/ml streptomycin, pH 7.5. 60ml of filter sterilised 7.5% $NaHCO_3$

solution were added to 200ml of 10x EBSS in a sterile 2L Duran bottle. Deionized autoclaved water was added to make the volume to almost 2L. The pH of the solution was corrected to 7.4-7.5 using sterile 1M NaOH or sterile 1M HCl, and judging the pH by colour. The solution was made up to 2L with sterile deionized water and kept at room temperature until use on the same day.

Perfusion buffer 3: 1x EBSS, 0.2% w/v $NaHCO_3$, 100U/ml penicillin, 100µg/ml streptomycin, 5mM $CaCl_2$, 0.08 U/ml Collagenase H, 0.001% w/v soya bean trypsin inhibitor, pH 7.5. 450ml of perfusion buffer 2 were poured into a sterile 500ml Duran bottle. 2.5ml of sterile 1M $CaCl_2$ solution were added and the pH adjusted to 7.4-7.5 if necessary using sterile 1M NaOH or sterile 1M HCl, and judging the pH by colour. The solution was made up to 500ml with sterile deionized water and kept at room temperature until use on the same day. Immediately before use 0.08 units/ml of collagenase H and 0.001% trypsin inhibitor were added.

Dispersal buffer: 10mM HEPES, 142mM NaCl, 7mM KCl, 2.5% w/v B.S.A. The solution without the B.S.A. was prepared, autoclaved and kept at 4°C until use within one week. On the day of the experiment the B.S.A. was added and mixed in.

Heparin solution: A stock of 5000U/ml of heparin in sterile 0.9% NaCl was prepared and filter sterilised. 100µl of this stock were added to 2400µl of sterile 0.9% NaCl and 1ml of this solution was used for injection.

<u>Protocol:</u>

This method is a modification of the two-step collagenase perfusion of Seglen (1976). On the day of the experiment, before the operation was started, the perfusion apparatus (figure 2.1) was set up in a sterile class 2 biological hood and was washed out once with 70% ethanol and six times with sterile deionized water. The platform height was adjusted so that it was

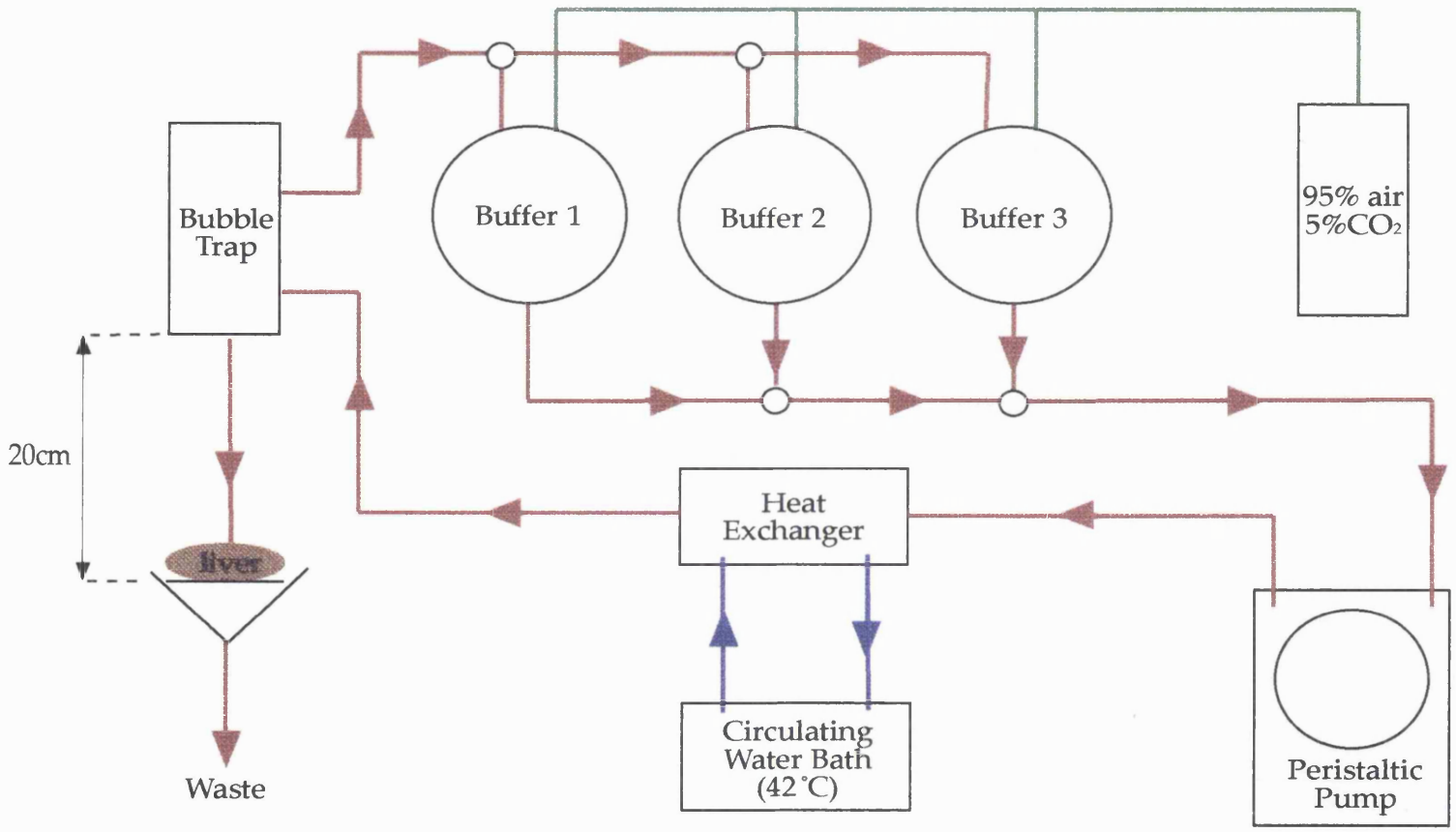


Figure 2.1: Schematic diagram of perfusion apparatus. Buffer 1 contains perfusate + EGTA, buffer 2 contains perfusate and buffer 3 contains perfusate + calcium chloride + collagenase. The red arrows show the direction of flow of the perfusion buffers, the blue arrows indicate circulating water, and the green lines indicate the flow of gas (95% air, 5% carbon dioxide). Open circles denote three-way taps.

20cm immediately below the bubble trap. The tube to be connected to the cannula was checked to ensure that it contained no air bubbles. The perfusion buffers were warmed to 37°C.

The hepatocytes were isolated from 230-250g male Sprague Dawley rats obtained from U.C.L. Biological Services. The animal was anaesthetised by an intraperitoneal injection of a sterile 60mg/ml solution of sodium pentobarbital in 0.9% w/v NaCl (100µl per 100g body weight). A sterile glass cannula was filled with perfusion buffer 1 using a sterile syringe. It was then capped at the wide end with a sterile plastic cap, also filled with perfusion buffer 1. Care was taken to ensure that no air bubbles were caught in the cannula or cap.

The pedal reflex was tested to ascertain when the animal was fully unconscious. The abdomen was cleaned with 70% v/v ethanol. The abdomen was cut open using blunt-ended scissors and the sternum was clamped away from the abdominal cavity. The intestines were pushed to the right and the liver lobes pushed upwards to reveal the *vena porta*. Two ligatures were tied loosely around the *vena porta* using a Deschamps ligature needle and Mersilk Black 3 ligature thread. These were placed approximately 7.5-10mm apart. A third ligature was tied loosely around the *vena suprarenalis*. Any membrane connecting the small liver lobe and the left kidney was cut. The animal was given an intravenous injection of 1ml of a sterile solution of heparin (200U/ml). After 20-30 seconds, the lower ligature on the *vena porta* was tightened and knotted. A flat cut was made to slit the *vena porta* below the upper ligature without severing it and the cannula was inserted into the cut. The cannula was secured by tightening the upper ligature as firmly as possible and knotting it. The lower ligature on the *vena porta* was also knotted around the cannula to keep it in

position. The ligature around the *vena suprarenalis* was also tightened and knotted. The liver was moved forwards. The diaphragm and ribcage were cut upwards to the forelimbs. The liver was gently removed using blunt-ended, curved Mayo scissors. The connections underneath the diaphragm were severed first. The liver was pushed back. The stomach was gripped in the left hand and pulled away as the rest of the connections were cut with the right hand. Care was taken to ensure the cannula remained in position in the vein and the Glisson's capsule on the liver was not damaged.

The liver was placed on the platform of the perfusion apparatus. The cap was removed from the cannula and the cannula was connected to the perfusion apparatus using Tygon 50 dialysis tubing (4mm diameter). The liver was perfused for 2 minutes with perfusion buffer 1, 10 minutes with perfusion buffer 2 and 20-25 minutes with perfusion buffer 3. During the perfusion, the buffers were gently bubbled with 95% air/5% CO_2 to correct their pH and heated so their temperature was 37°C as they reached the liver. A peristaltic pump set at a flow rate of >50ml/min was used to pass the buffers continuously through the heat exchanger. If the perfusion was proceeding correctly, the liver lost its red colour almost immediately. Lobes that did not do this could be massaged gently with a finger to encourage the perfusion buffer to enter them. When the perfusion was complete, the liver had swelled and the lobes looked pinky-white and spongy. It was immediately transferred to a petri dish containing 30ml of Leibovitz L-15 medium supplemented with 2.5% w/v B.S.A. (fraction V). The Glisson's capsule was gently removed with sterile forceps and the cells released into the medium by gently squeezing them out. The cell suspension was filtered through a sterile 62µm nylon mesh to remove large debris. The mesh was washed through with 20ml Leibovitz L-15 and squeezed gently against the filter funnel to maximise the cell yield. The cells were left to settle out for 10

minutes. The supernatant was gently pipetted off and the cells were resuspended in 50 ml dispersal buffer. The suspension was centrifuged for 2 minutes, at 50g, 4°C. The cell pellets were resuspended in complete culture medium (2ml per plate) supplemented with 5% v/v dialysed foetal calf serum.

Viability of the cells was determined using a trypan blue exclusion test (Jauregui *et al.*, 1981). Those cells that excluded trypan blue, but did not have a well defined outline or a shiny appearance, were counted as not viable (Seglen, 1976).

2.16.2 Culture of hepatocytes.

Solutions:

Complete culture medium: William's E medium without phenol red or L-glutamine was supplemented with 1.7 μM insulin, 0.1μM dexamethasone, 100μg/ml streptomycin, 100U/ml penicillin, 2.5μg/ml amphotericin B, 200μg/ml neomycin and 4mM L-glutamine.

Plate coating solution: 90-100μg/ml Vitrogen 100 (type I collagen) and 130μg/ml CDI (1-cyclohexyl-3(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulphonate). The solution was made freshly with sterile water before each use.

Saline solution: 0.9% w/v NaCl. The solution was autoclaved and kept in aliquots at room temperature until use.

Protocol:

Before the experiment, vitrogen coated plates were prepared as described by Waxman *et al.* (1990). Permanox plates were coated with 3ml of the coating

solution and incubated overnight at 37°C. The unbound collagen was removed by aspiration and the plates washed twice with sterile saline solution. The plates were stored for up to one week before use at 4°C and were washed once more with saline solution immediately before use.

The isolated hepatocytes were plated onto the vitrogen coated plates (4-5x 10^6 per plate) in a volume of 2ml and left to attach for 3 hours at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The culture medium was removed by aspiration and replaced with 7ml of fresh complete culture medium. Cells were then incubated at 37°C in a humidified atmosphere of 95% and 5% CO_2. Culture medium was changed at 24 hour intervals.

2.16.3 Transfection of the hepatocytes.

This protocol is a variation of the one used by Gaunitz et al. (1996).

2.16.3a Preparation of DNA for transfection.

DNA for the transfection experiment was prepared using Qiagen columns (section 2.4.3). To produce as much supercoiled plasmid as possible, a second 70% ethanol wash was added at the end of the procedure and the DNA pellet was not over-dried. This reduced the amount of pipetting that was needed to dissolve the final pellet. The DNA was desalted and concentrated in Centricon-30 columns (Amicon) according to the maufacturer's instructions. The optical density of the DNA was measured at λ=260nm and λ=280nm. The ratio of the two (A_{260}/A_{280}) was between 1.6 and 1.8, otherwise the DNA was not used for transfection. The DNA was diluted to 1μg/μl before addition to the $CaCl_2$ solution.

2.16.3b Preparation of $CaPO_4$/DNA precipitate for transfection.

Solutions:

250mM $CaCl_2$: This was freshly-made with sterile ultrapure water and $CaCl_2.4H_2O$ Suprapur®.

2x HeBS: 0.28M NaCl, 10mM KCl, 1.5mM Na_2HPO_4, 42mM HEPES, 2% w/v glucose. Sterile stock solutions and utensils were used. The solution was made to just less than the final volume and the pH was carefully adjusted to pH 7.10 using sterile 1M HCl and 10M NaOH solutions. The solution was kept at room temperature for up to a week before use.

Protocol:

500µl of a solution of 12µg DNA in 250mM $CaCl_2$ were added dropwise using a glass Pasteur pipette to 500µl of 2x HeBS in a polystyrene conical falcon tube (Falcon® 2095, 17x 120mm). This addition took at least 1 minute and was carried out while the mixture was bubbled with air from the bottom of the tube using a mechanical pipettor and a glass Pasteur pipette. Immediately after this addition the mixture was vortexed at maximum speed for 2-3s. It was then incubated at room temperature for 25-30 minutes before addition to the cells. The mixture was checked for even precipitation under a microscope. These amounts were altered according to the final amount needed. For a 35 mm plate, a total of 2.4µg of DNA were used. This could be a mixture of different vectors.

2.16.3c Transfection of the hepatocytes.

Solutions:

Glycerol shock solution: 15% sterile glycerol was freshly-made in 1x HeBS solution.

Hank's solution: 137mM NaCl, 5.4 mM KCl, 0.4mM $MgSO_4.7H_2O$, 0.5mM $MgCl_2.6H_2O$, 0.35mM $Na_2HPO_4.2H_2O$, 0.44mM KH_2PO_4, 2mM HEPES. The solution was made using sterile stock solutions and utensils to just less than the final volume. The pH was adjusted to pH 7.4 using sterile 10μ NaOH. The solution was freshly-made before use.

Complete culture medium + FCS: William's E medium without phenol red or L-glutamine was supplemented with 5% v/v dialysed foetal calf serum (FCS), 1.7 μM insulin, 0.1μM dexamethasone, 100μg/ml streptomycin, 100U/ml penicillin, 2.5μg/ml amphotericin B, 200μg/ml neomycin and 4mM L-glutamine.

Protocol:

The volumes given are for 60mm culture dishes. The hepatocytes were plated as described previously (section 2.16.2) and allowed to attach for 3 hours. The cells were washed with sterile saline and cultured in complete culture medium + FCS overnight (37°C, 95% air, 5% CO_2). 20 hours post-plating, the medium was removed and 2.6ml fresh medium + FCS were added per plate. The CaPO4 solution was made up and mixed by pipetting with a 1000μl pipette. The mixture (343μl) was added to the cells using the same pipette tip while the culture dish was gently swirled. The culture dishes were not allowed to remain at room temperature for more than 6 minutes during addition of the DNA. The cells were returned to the incubator for 8 hours. The cells were washed twice with approximately 3ml Hank's solution, and 860μl of glycerol shock solution was added to each

plate at room temperature. After exactly two minutes the glycerol solution was removed and the cells were washed again, once in Hank's solution and once in fresh complete culture medium without FCS. 2.5 ml fresh culture medium without FCS were added to the cells and they were incubated at 37°C in 5% CO_2, 95% air. The culture medium was changed at 48 hours post-plating and the cells were lysed at 72 hours post-plating. Treatment with phenobarbital and other chemicals began at 48 hours post-plating.

2.17 Densitometry.

Densitometric readings were taken using a BioRad GS-670 imaging densitometer, and analysed using Molecular Analyst software. For western blots, the band on the blot was selected and local background was subtracted automatically from the reading. For gel retardations, a profile across the whole track was selected. The probe alone track was used as a baseline and subtracted from each of the other reactions. Each analysis was done in triplicate and the mean was taken as the final reading.

Chapter Three

Results and Discussion

Section I: Analysis of the 5' flanking sequence of the CYP2B2 gene.

3.1 Optimisation of gel retardation assay conditions.

The gel retardation assay was initially designed by Fried and Crothers (1981) to study the kinetics of DNA-protein interactions. It has since been widely used to identify and characterise DNA-binding factors and to analyse their target sequences.

The literature provides many and varying protocols for this assay so several of its parameters were studied in an attempt to optimise it. A fragment of the *CYP2B2* 5' flanking sequence (-34 to -86bp) was chosen for these experiments. It had previously been shown to bind nuclear proteins in a gel retardation assay (Shephard *et al.*, 1994).

3.1.1 Generation of the -34 to -86bp fragment of the CYP2B2 gene

This fragment was generated using a PCR strategy. Oligonucleotides were synthesised corresponding to the -67 to -86 residues on the positive strand of the *CYP2B2* gene and the -34 to -53 residues on the negative strand (figure 3.1a). A PCR reaction was carried out using the λP4502B2 clone as a template to synthesise the 52bp fragment from -34 to -86bp. Figure 3.1b shows preliminary PCR reactions using different amounts of the DNA template to optimise the conditions of the reaction. The 52bp fragment is clearly seen in all the reactions which included the template. Once synthesised, the fragment was purified from a low melting point agarose gel and labelled with $\gamma^{32}P$ using the blunt end labelling protocol before use in gel retardation assays.

A

-86bp

5' GCT AAA GCA GGA GGC GTG AAC ATC TGA
3' CGA TTT CGT CCT CCG CAC TTG TAG ACT

AGT TGC ATA ACT GAG TGT AGG GGC AG 3'
TCA ACG TAT TGA CTC ACA TCC CCG TC 5'

-34bp

B

Sample	M	1	2	3	4
Track	1	2	3	4	5

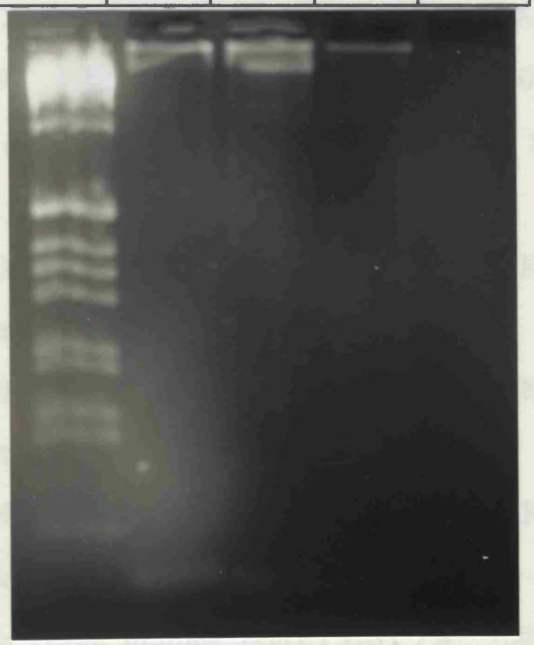


Figure 3.1: P.C.R. amplification of the -34 to -86bp fragment of the *CYP2B2* promoter sequence. (A) The sequence from -34 to -86bp of the *CYP2B2* gene promoter. The synthetic oligonucleotides used as primers in the P.C.R. reaction are shown in red. (B) A 2% agarose gel showing the P.C.R. reactions. The reactions were carried out as described in section 2.8 and contained 10ng (sample 1), 100ng (sample 2) or 250ng (sample 3) of template DNA. Sample 4 contained no template λP4502B2 DNA. The size of the P.C.R. product is indicated on the left of the photograph. The sizes of all of the bands in the 1KB molecular weight marker (M) are shown in figure A.1.

3.1.2 The effect of different binding buffers

It is essential that the composition of the binding buffer is correct in order to achieve optimal protein-DNA binding. Buffers may be Tris- or HEPES-based; they may have varying concentrations of salt (typically NaCl or KCl); they may have varying concentrations of divalent cations (usually Mg^{2+} or Ca^{2+}); and they may include non-ionic detergents. To assess the effects of different binding buffers on the gel retardation experiment a range of buffers were made:

10x Buffer 1 (Hassanain, 1993): 100mM HEPES-KOH (pH 7.9), 500mM KCl, 50% v/v glycerol, 1mM EDTA, 1mM PMSF, 2.5mM DTT, 10mM $MgCl_2$.

10x Buffer 2 (Hassanain, 1993): 100mM HEPES-KOH (pH 7.9), 500mM KCl, 50% v/v glycerol, 1mM EDTA, 1mM PMSF, 2.5mM DTT, 10mM $MgCl_2$, 5% v/v Nonidet P-40.

10x Buffer 3 (Scott *et al.*, 1994): 100mM Tris-HCl (pH 7.6), 500mM KCl, 10mM EDTA, 50% v/v glycerol, 10mM DTT.

10x Buffer 4 (Scott *et al.*, 1994): 100mM Tris-HCl (pH 7.6), 750mM KCl, 10mM EDTA, 50% v/v glycerol, 10mM DTT.

10x Buffer 5 (Scott *et al.*, 1994): 100mM Tris-HCl (pH 7.6), 1M KCl, 10mM EDTA, 50% v/v glycerol, 10mM DTT.

10x Buffer 6 (Shephard *et al.*, 1994): 100mM Tris.Cl (pH 7.4), 700mM NaCl, 10mM EDTA (pH 8.0), 100mM β-mercaptoethanol, 30mM MgCl2, 40% v/v glycerol.

5x Buffer 7 (Rosette and Karin, 1995): 60mM HEPES (pH 7.9), 20mM Tris.Cl (pH 7.9), 300mM KCl, 150mM NaCl, 25mM $MgCl_2$, 25mM DTT, 0.5mM EDTA, 62.5% v/v glycerol.

DNA-protein complexes formed with each of the buffers used, though to different amounts (figure 3.2). The HEPES-based buffers (tracks 1 and 2) showed the greatest binding compared to the Tris-based buffers (tacks 3 to 6). The addition of 5% nonidet-P40 (buffer 2, track 2) increased this binding further. As the final concentration of KCl was increased from 50mM (buffer 3, track 3) to 75mM (buffer 4, track 4) and again to 100mM (buffer 5, track 5) the amount of binding was seen to diminish. As each DNA-protein binding interaction is different, it was necessary to carry out a similar experiment to this one for each new DNA fragment used as a gel retardation probe in order to produce optimal binding conditions. I have not presented these results, but the conditions used in the binding assay for each fragment are given in the relevant figure legend.

3.1.3 Binding temperature

Initially, the binding incubations were carried out at room temperature, but several papers (e.g. Rosette and Karin, 1995) suggested that these incubations should be at 4°C as the protein-DNA interaction is labile and decreasing the temperature will cause it to be more stable. Figure 3.3 shows an experiment using a range of different binding buffers and carrying out the binding incubation on ice. The number of complexes seen have dramatically increased as compared to the experiment carried out at room temperature (figure 3.2).

In addition, the buffers that produced the most abundant complexes with the least smearing down the lane were buffers 5 (Scott *et al.*, 1994, track 5) and 7 (Rosette and Karin, 1995, track 7). Buffer 7 was never tested at room temperature as these experiments were carried out before the paper by

Probe: -34 to -86bp *CYP2B2*							
Buffer	1	2	3	4	5	6	6
Nuclear protein	+	+	+	+	+	+	–
Track	1	2	3	4	5	6	7

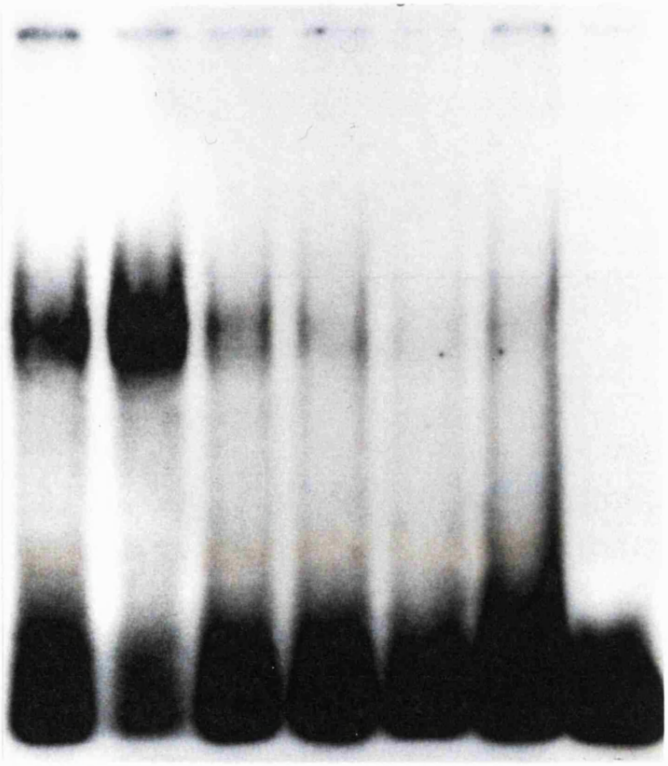


Figure 3.2: The effect of different binding buffers at room temperature. The -34 to -86bp fragment of *CYP2B2* was incubated in the presence (+) or absence (-) of 5µg nuclear protein extract made using the method of Sierra (1990) from the livers of rats treated with phenobarbital. The incubations were carried out at room temperature and included 2µg poly dI.dC and a variety of binding buffers (1-6, see text for buffer formulae).

Probe: -34 to -86bp *CYP2B2*									
Protein	+	+	+	+	+	+	+	+	−
Buffer	1	2	3	4	5	6	7	6	6
Track	1	2	3	4	5	6	7	8	9

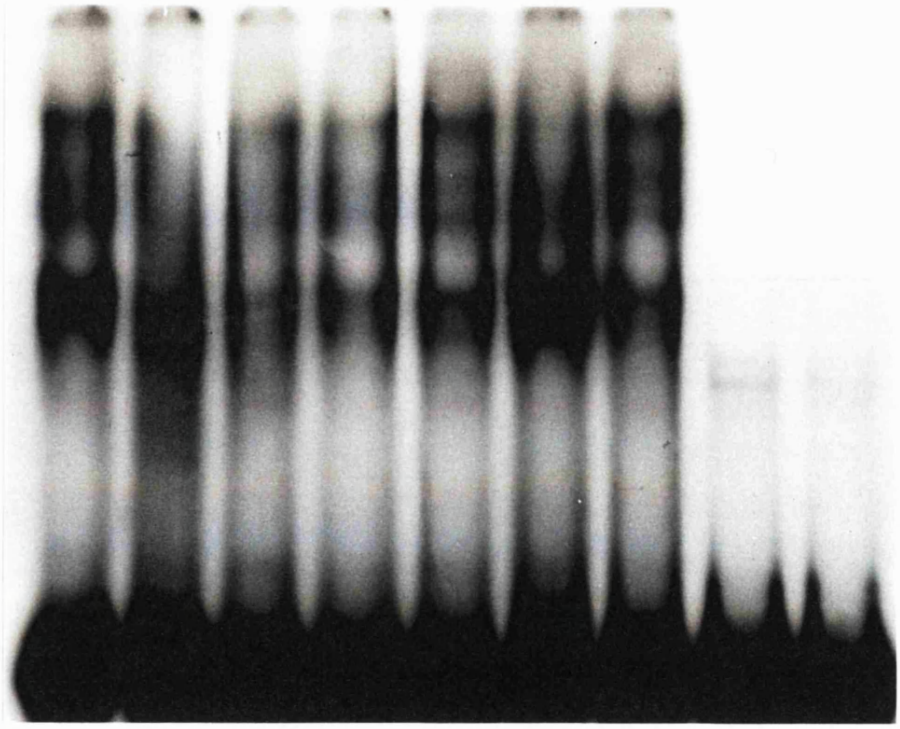


Figure 3.3: The effect of binding buffers on the gel retardation of -34 to -86bp *CYP2B2* at 4˚C. The -34 to -86bp fragment of the 5' flanking sequence of the *CYP2B2* gene was incubated in the presence (+) or absence (-) of 5µg of nuclear protein extract made using the method of Sierra (1990) from the livers of rats treated with phenobarbital. The binding incubations were carried out at 4˚C and contained 2µg of poly dI.dC. A variety of binding buffers were used (1-7; see text for buffer formulae). The reaction in track 8 was digested using 5µg of proteinase K before loading on to the gel.

Rosette and Karin (1995) was published. Buffer 7 was chosen to use in further experiments with this fragment.

3.1.4 Nuclear protein extraction

Figure 3.4 shows a comparison of four different protocols for protein extraction (section 2.9) in a gel retardation assay using the -34 to -86bp fragment. The protocol of Sierra (1990) was more exacting than the others, but it gave a high yield of an extract rich in DNA-binding proteins. The mini-preparation protocol (Deryckere and Gannon, 1994) gave a low yield of a crude extract, and as much as 20μg of protein were needed for any binding to be seen (results not shown). The complexes which formed with the extracts made by the other two methods (Lavery and Schibler, 1993 and Jose-Estanyol *et al.*, 1989) were similar to those seen with the extracts prepared using the method of Sierra (1990), but they were much less abundant in general. Extracts prepared according to the protocol of Lavery and Schibler (1993) gave two extra DNA-protein complexes in comparison with the extracts made using the protocol of Sierra (1990). One of these extra complexes was very abundant. From these results I decided to use the protocol of Sierra (1990) to produce nuclear protein extracts.

Each new batch of nuclear protein extracts was checked for integrity on an SDS-PAGE gel (e.g. figure 3.5), and compared with a previously made extract in a gel retardation experiment. Figure 3.6 shows two such experiments. In this case, preparation 2 (tracks 3 and 4, figure 3.6a) was discarded as the PB extract appeared slightly degraded. A variation in the abundances of some of the complexes was observed between the different preparations (figure 3.6b).

Probe: -34 to -86bp *CYP2B2*									
Protocol	1		2		3		4		–
Treatment	U	PB	U	PB	U	PB	U	PB	–
Track	1	2	3	4	5	6	7	8	9

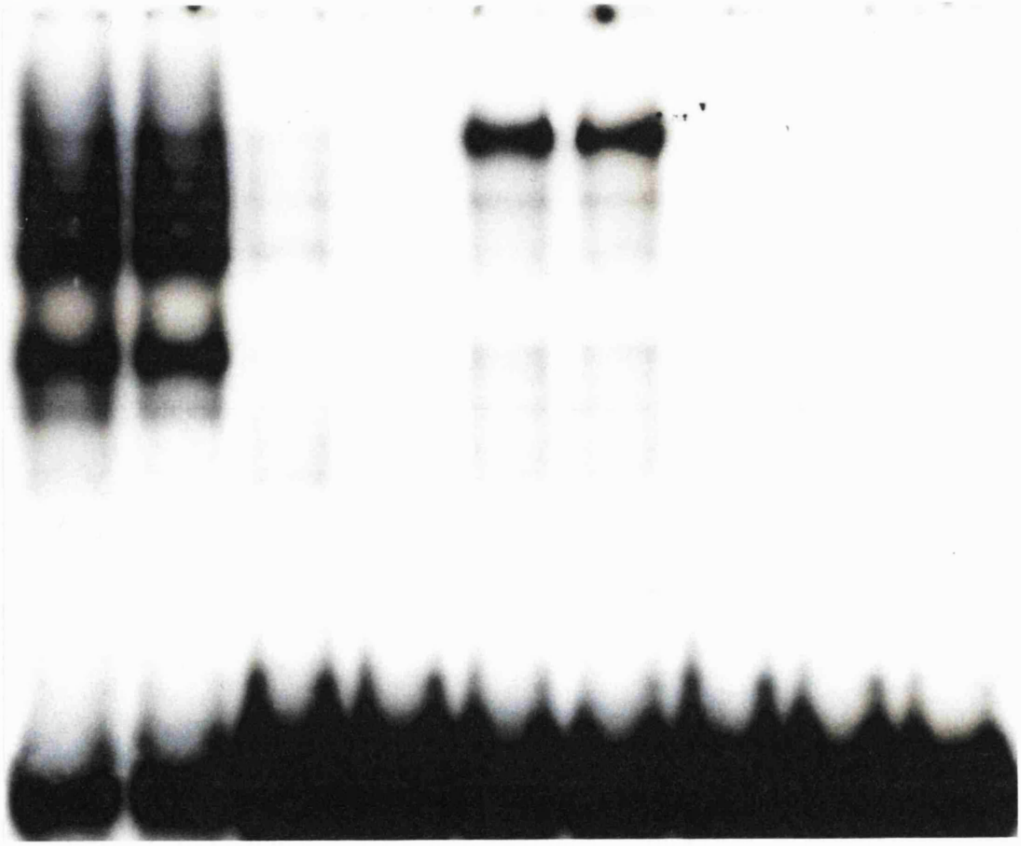


Figure 3.4: A comparison of different methods for the extraction of nuclear proteins. The -34 to -86bp fragment of the *CYP2B2* gene was incubated with 5µg of nuclear protein extract made using the method of Sierra (1990) (protocol 1, tracks 1 and 2), the method of Jose-Estanyol (1989) (protocol 2, tracks 3 and 4), the method of Lavery and Schibler (1993) (protocol 3, tracks 5 and 6) and the one hour mini-preparation method (Deryckere and Gannon, 1994) (protocol 4, tracks 7 and 8). In each case, protein extracts were made from the livers of untreated rats (U) or rats treated with phenobarbital (PB). The binding reactions were carried out at 4°C and contained 2µg of poly dI.dC. The reaction in track 9 (-) contained no nuclear protein.

Protocol	—	Sierra		Mini-prep		Lavery		Jose-Estanyol	
Treatment	—	U	PB	U	PB	U	PB	U	PB
Track	M	1	2	3	4	5	6	7	8

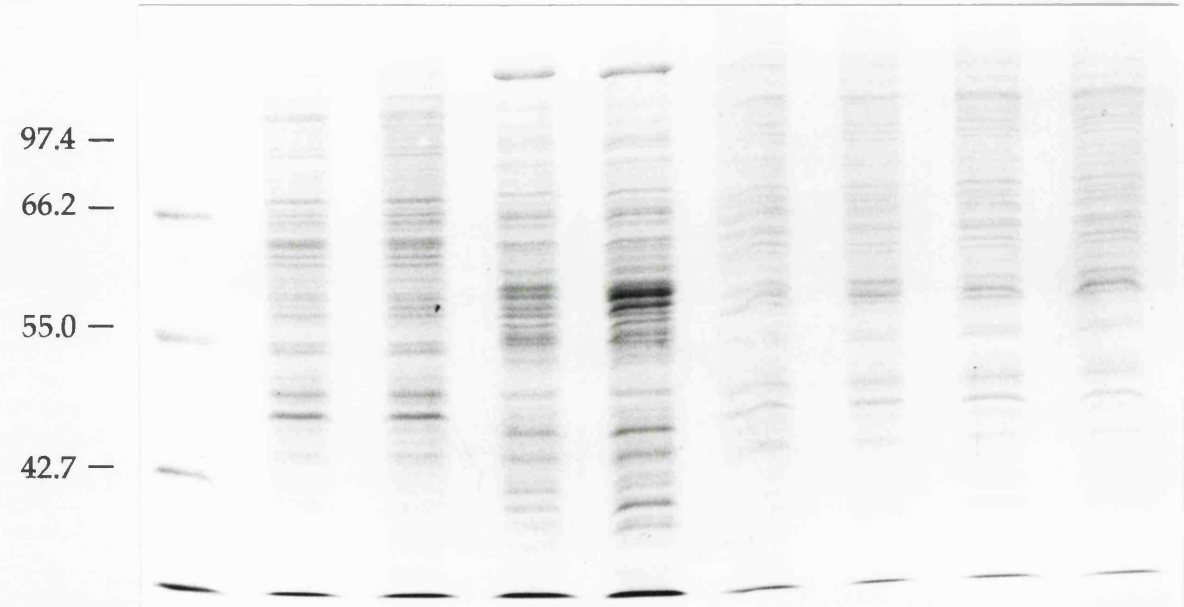


Figure 3.5: SDS-PAGE of nuclear protein extracts. 10µg of nuclear protein extracts made from the livers of untreated (U) or phenobarbital-treated (PB) rats were separated on a 10% polyacrylamide gel. The proteins were made using the method of Sierra (1990) (tracks 1 and 2), the mini-preparation method (Deryckere and Gannon, 1994) (tracks 3 and 4), the method of Lavery and Schibler (1993) (tracks 5 and 6) and the method of Jose-Estanyol (1989) (tracks 7 and 8). The apparent molecular weights ($x10^3$) of the mid-range moleular weight standard (M) are shown on the left of the photograph.

Probe: -34 to -86bp *CYP2B2*					
Preparation	1		2		–
Treatment	U	PB	U	PB	–
Track	1	2	3	4	5

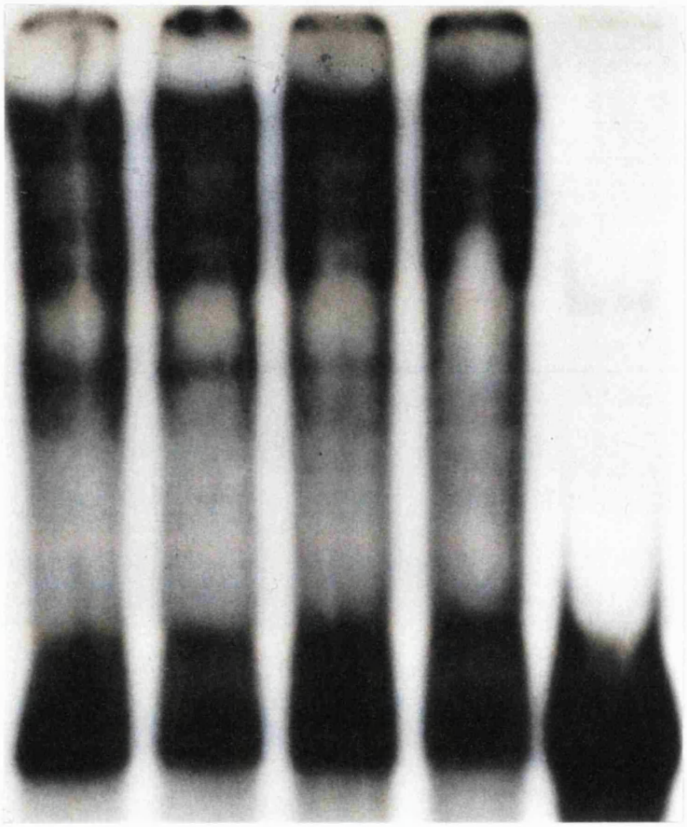


Figure 3.6a: Comparison of two different preparations of nuclear protein extracts. The -34 to -86bp fragment of the 5' flanking sequence of the *CYP2B2* gene was incubated without (-) or with 5µg of nuclear protein extract made from the livers of untreated rats (U) or rats treated with phenobarbital (PB) using the method of Sierra (1990). Tracks 1 and 2 contain protein from extracts made in January 1996 (preparation 1) and tracks 3 and 4 contain protein from extracts made in July 1996 (preparation 2). The binding reactions were carried out on ice and included 2µg of poly dI.dC.

Probe: -34 to -86bp *CYP2B2*				
Preparation	1		3	
Treatment	U	PB	U	PB
Track	1	2	3	4

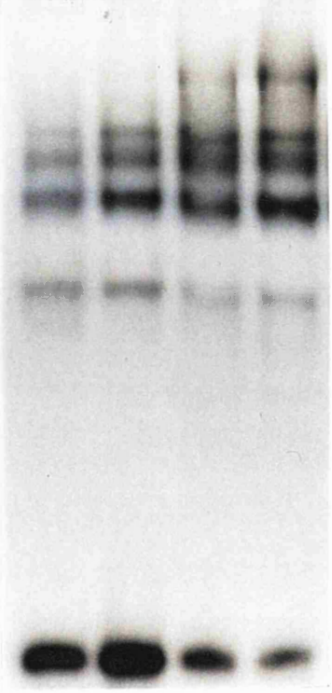


Figure 3.6b: Comparison of two different preparations of nuclear protein extracts. The -34 to -86bp fragment of the 5' flanking sequence of the *CYP2B2* gene was incubated with 5µg of nuclear protein extract made from the livers of untreated rats (U) or rats treated with phenobarbital (PB) using the method of Sierra (1990). Tracks 1 and 2 contain nuclear protein from extracts made in January 1996 (preparation 1) and tracks 3 and 4 contain nuclear protein from extracts made in August 1996 (preparation 3). The binding reactions were carried out on ice and included 2µg of poly dI.dC.

3.1.5 Poly dI.dC concentration

Poly dI.dC acts as an heterologous competitor to reduce non-specific interactions. It is therefore important to get the concentration correct, as too little will allow non-specific interactions to be seen, and too much will begin to compete out even specific interactions. Figure 3.7 shows a gel retardation experiment using increasing amounts of poly dI.dC whilst keeping the nuclear protein concentration the same. As the poly dI.dC concentration increases, the abundance of the complexes seen decreases. At the same time, the amount of complex caught in the wells of the gel also decreases. This titration of poly dI.dC was carried out for each DNA fragment used as a probe, as those fragments that formed more complexes required more poly dI.dC to avoid them sticking in the wells (results not shown). Poly dI.dC was therefore added in proportion to the amount of protein used in the gel retardation reaction.

Probe: -34 to -86 bp *CYP2B2*									
Poly dI.dC (μg)	1		2		5		10		2
Treatment	U	PB	U	PB	U	PB	U	PB	–
Track	1	2	3	4	5	6	7	8	9

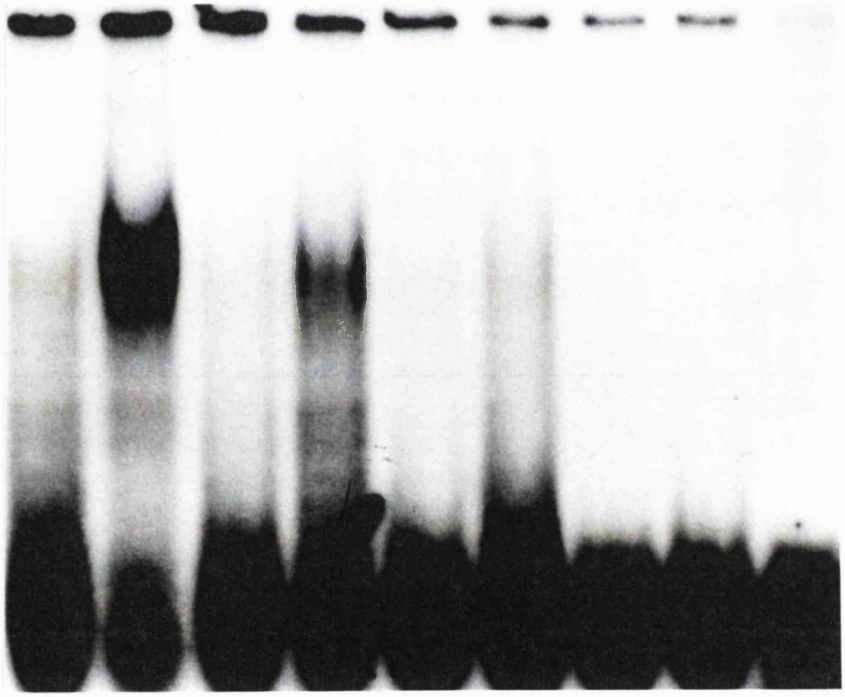


Figure 3.7: The effect of altering the concentration of poly dI.dC. The -34 to -86bp fragment of the *CYP2B2* gene was incubated without (-) or with 5μg of nuclear protein extracted from the livers of untreated (U) or phenobarbital-treated (PB) rats using the method of Sierra (1990). The binding reactions were carried out at room temperature and included varying amounts of poly dI.dC.

3.2 Analysis of the 5' flanking sequence of the *CYP2B2* gene.

3.2.1 Gel retardation assay with -44 to -67bp of the *CYP2B2* 5' flanking sequence.

The region between -86 and -34bp of the *CYP2B2* 5' flanking sequence has been identified by gel retardation and DNase footprinting assays as binding more nuclear protein from extracts from the livers of phenobarbital-treated rats as compared to extracts from the livers of untreated rats (Shephard *et al.*, 1994). For simplicity, in this discussion I shall refer to extracts from phenobarbital-treated animals as PB extracts and to extracts from untreated animals as U extracts. Computer analysis of this region suggested that it might contain a CCAAT/Enhancer Binding Protein (C/EBP) binding site. To determine whether this putative site is important in the phenobarbital induction response, oligonucleotides were synthesised corresponding to the *CYP2B2* sequence from -44 to -67bp which included the site (figure 3.8a). The oligonucleotides were designed with 5' overhangs to facilitate radiolabelling. The ability of this shorter fragment to bind nuclear proteins was analysed by gel retardation.

Several distinct DNA-protein complexes were formed with nuclear extracts isolated from the livers of both phenobarbital-treated (PB) and untreated (U) rats (figure 3.9a). The binding was shown to be specific, as both competition with 100-fold molar excess of the unlabelled -44 to -67 bp fragment and proteinase K digestion eliminated the formation of all DNA-protein complexes. Competition with 100-fold molar excess of an unlabelled sequence corresponding to the consensus C/EBP binding site (figure 3.8b) also abolished all DNA-protein complexes. However, analysis of the autoradiographs by image densitometry showed no significant difference in

5' TCGA ACA TCT GAA GTT GCA TAA CTG AGT 3'

3' TGT AGA CTT CAA CGT ATT GAC TCA AGCT 5'

-67bp -44bp

Figure 3.8a: Sequence from -44 to -67bp of the *CYP2B2* 5' flanking sequence showing putative C/EBP binding site. The putative C/EBP binding site is shown in colour. Red bases match the consensus C/EBP binding site exactly. Green bases do not match the consensus sequence. The small print indicates the 5' overhangs added to the oligonucleotides to facilitate radiolabelling.

5' TGCAGA TTGCGCAA TCTGCA 3'

3' ACGTCT AACGCGTT AGACGT 5'

Figure 3.8b: Consensus C/EBP binding sequence (Mahoney *et al.*, 1992). The consensus binding site is shown in red.

Probe: -44 to-67bp *CYP2B2*							
Protein (µg)	2.5		5		7.5		–
Treatment	U	PB	U	PB	U	PB	–
Track	1	2	3	4	5	6	7

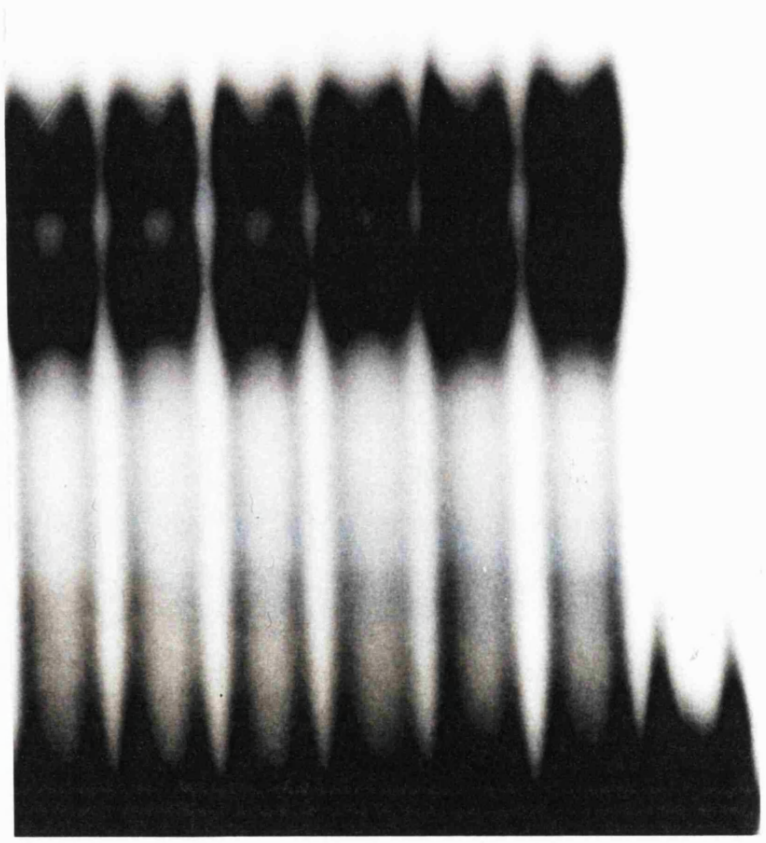


Figure 3.9a: Gel retardation with the -44 to-67bp fragment of the *CYP2B2* 5'flanking sequence. The -44 to -67bp fragment of the *CYP2B2* promoter was radiolabelled and incubated without protein (-), or with 2.5-7.5µg of nuclear protein extract. The proteins were extracted from the livers of phenobarbital-treated (PB) or untreated (U) rats. The amount of poly dI.dC included in each reaction was 40% of the amount of nuclear protein, or 2µg for the reaction without nuclear protein.

the relative abundance of complexes formed with PB extracts as compared to U extracts.

The gel retardation assays show several discrete DNA-protein complexes, suggesting that the -44 to -67bp region is capable of binding various proteins of different molecular weights. This binding is specific and is competed out with the consensus C/EBP binding site, suggesting that it is C/EBP that is binding (figure 3.9b).

The name 'CCAAT enhancer-binding protein' refers to a family of transcription factors. The C/EBP and C/EBP related proteins (CRP) are capable of forming homo- and heterodimers and they bind to similar DNA sequences (Williams *et al.*, 1991). All the known members of the family contain a region which is rich in basic amino acids and a flanking leucine zipper domain. These regions are necessary for DNA-binding and dimer formation with other members of the family, and there is a high level of amino acid sequence similarity within these regions between the members of the family (Cao *et al.*, 1991). To date there have been 6 of these proteins identified: C/EBPα, C/EBPβ (LAP, NF-IL6, IL-6DBP, CRP-2, AGP/EBP), C/EBPγ (Ig/EBP), C/EBPδ (CRP-3), CHOP and C/EBP related protein 1(CRP-1). In addition, there are 2 differently spliced forms of C/EBPβ, LIP and LAP.

To confirm that the -44 to -67bp region of the *CYP2B2* gene promoter was binding one or more forms of C/EBP, it was analysed using a supershift assay with antibodies to C/EBPα, C/EBPβ and C/EBPδ (figure 3.10). The antibodies were obtained from Santa Cruz Biotechnology. They are polyclonal antibodies raised against the carboxy terminal epitope of each of the proteins and are not cross-reactive with the other family members.

Probe: -44 to -67bp *CYP2B2*					
Treatment	U	U	U	U	–
Competitor	–	C/EBP consensus	-44 to -67bp *CYP2B2*	–	–
Proteinase K	–	–	–	+	–
Track	1	2	3	4	5

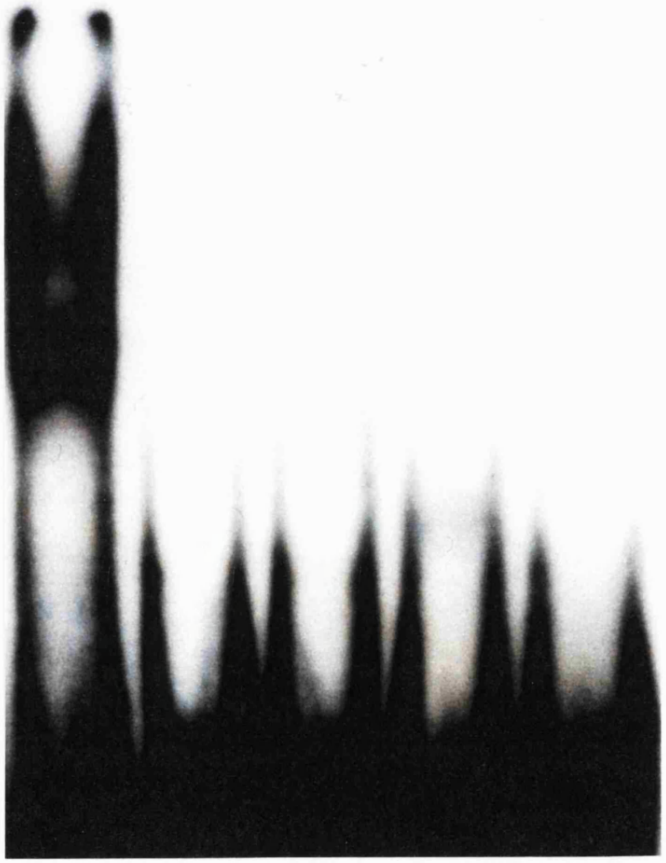


Figure 3.9b: Competitive gel retardation of the -44 to -67bp fragment of the *CYP2B2* promoter. The -44 to -67bp fragment of the *CYP2B2* 5'flanking sequence was radiolabelled and incubated with (+) or without (-) 5µg of nuclear protein extract from the livers of untreated rats in the presence of 2µg of poly dI.dC. Unlabelled competitor DNA was added at 100-fold molar excess over the labelled probe to the reactions in tracks 2 and 3. Competitors used were the -44 to -67bp fragment of the *CYP2B2* promoter and an oligonucleotide corresponding to the consensus binding sequence of C/EBP. The reaction in track 4 was incubated with 5µg of proteinase K.

Probe: -44 to -67bp *CYP2B2*								
Treatment	U	U	U	U	PB	PB	PB	PB
Anti-C/EBPα	−	+	−	−	−	+	−	−
Anti-C/EBPβ	−	−	+	−	−	−	+	−
Anti-C/EBPδ	−	−	−	+	−	−	−	+
Track	1	2	3	4	5	6	7	8

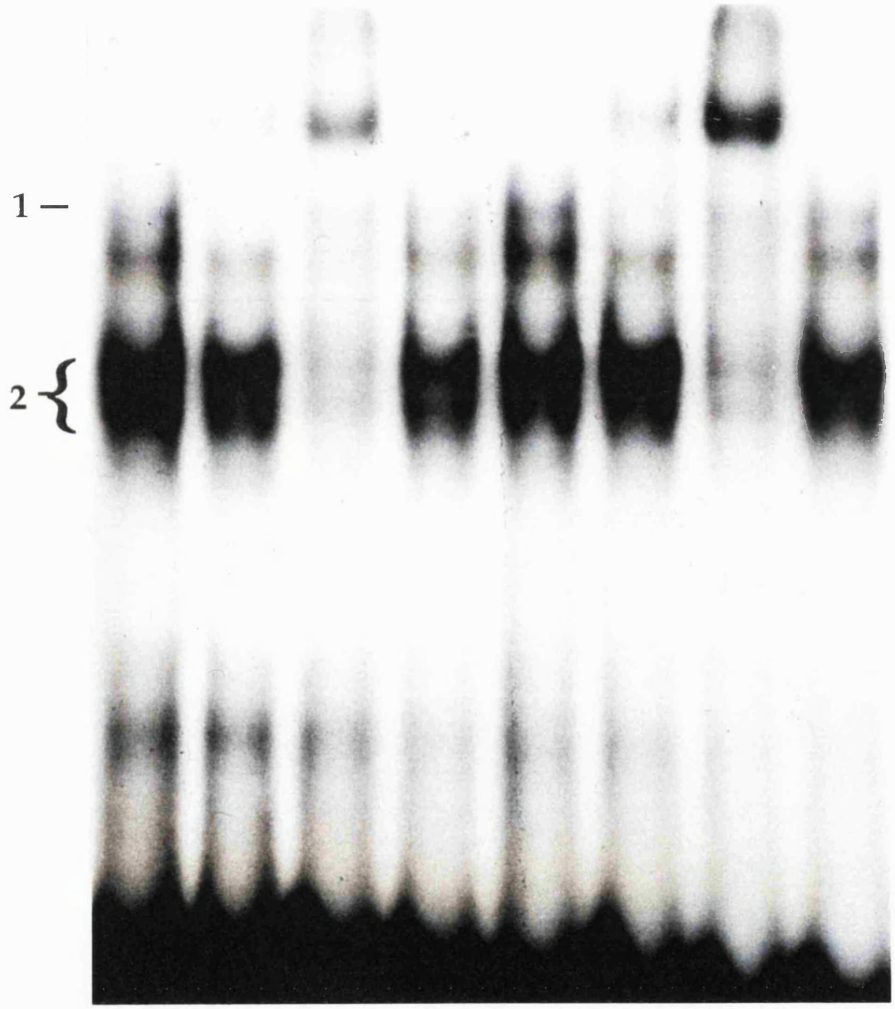


Figure 3.10: Supershift gel retardation with the -44 to -67bp fragment of the *CYP2B2* promoter and anti-bodies to C/EBP. The -44 to -67bp fragment of the *CYP2B2* promoter was radiolabelled and incubated with 2.5μg of nuclear protein extracted from the livers of phenobarbital-treated (PB) or untreated (U) rats. The reactions included 2.5μg of poly dI.dC and occured in the presence (+) or absence (-) of 1μg of anti-C/EBPα, anti-C/EBPβ or anti-C/EBPδ. The numbers on the left of the photograph indicate complexes that have been supershifted by C/EBPα (1) and C/EBPβ (2).

Supershifts were seen for both C/EBPα and C/EBPβ. The incubation with C/EBPδ did not show any change. In the case of C/EBPα a specific complex has been shifted (band 1, figure 3.10), so it can be identified as a homo- or heterodimer of C/EBPα. It is difficult to discern a particular band that has been shifted in the C/EBPβ incubation, however the lower complexes are significantly reduced (region 2, figure 3.10). This suggests that C/EBPβ forms several complexes either as a homodimer, or as a heterodimer with other unidentified transcription factors. As yet the group of transcription factors that can dimerise with the different forms of C/EBP has not been fully elucidated. However, it is known that all combinations between the different family members are possible (Williams *et al.*, 1991).

3.2.2 Gel retardation with -6 to -38bp fragment of the *CYP2B2* gene promoter.

DNase I footprinting analysis of the *CYP2B2* promoter region identified a proximal footprint at -6 to -38bp that was less prominent with PB extracts than with U extracts (Shephard *et al.*, 1994). This region (-6 to -38bp) was used in a gel retardation experiment to study further its involvement in the phenobarbital response. Oligonucleotides were synthesised corresponding to -38 to -13bp of the positive strand, and -30 to -6bp of the negative strand of *CYP2B2*. The two oligonucleotides were annealed together and radiolabelled by filling in the ends as described in section 2.10.1a.

Figure 3.11 shows the gel retardation experiment with this fragment. No complexes were formed with this fragment at all. This suggests that any binding of proteins to this region relies on stabilisation due to interactions with either another region of the promoter, or proteins bound to the promoter elsewhere.

Probe: -6 to -38bp *CYP2B2*					
Treatment	U	PB	PB	PB	–
SP1 competitor	–	–	+	–	–
Proteinase K	–	–	–	+	–
Track	1	2	3	4	5

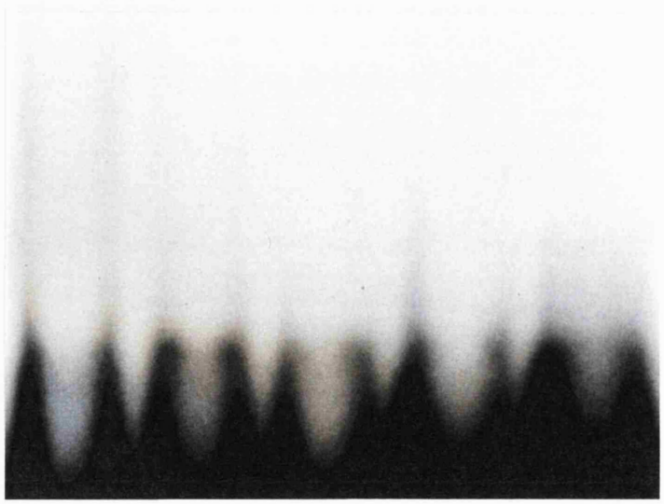


Figure 3.11: Gel retardation with the -6 to -38bp fragment of the *CYP2B2* promoter. The -6 to -38bp fragment was radiolabelled and incubated without protein (-), or with 5µg of nuclear protein extract made from the livers of untreated rats (U) or rats treated with phenobarbital (PB). Each reaction contained 2µg of poly dI.dC. The reaction in track 3 included a 100-fold molar excess of the unlabelled -6 to-38bp fragment and the reaction in track 4 was incubated with 5µg proteinase K.

3.2.3 Gel retardation with the -34 to -86bp region of *CYP2B2* gene promoter.

Although the presence of a C/EBP binding site in the -44 to -67bp region of *CYP2B2* had been demonstrated, this region did not show any differences in the abundance of complexes formed with PB extracts as compared to U extracts, so I was no further on in trying to clarify the PB induction mechanism of this gene.

The next approach was to return to the original result (Shephard *et al.*, 1994), where the region -34 to -86bp did show a difference in the abundances of complexes formed with PB extracts as compared to U extracts. Scott *et al.* (1994) observed that a shorter oligonucleotide (16bp) had a lower affinity for its binding protein than the corresponding 30bp oligonucleotide. It is possible that this is the case for the short -44 to -67bp oligonucleotide (23bp), so that the full effect is not seen. It was also possible that the changes I had made in extracting the proteins and carrying out the gel retardation experiment had altered this result. The -34 to -86bp fragment was generated by P.C.R. as in section 3.1.1, radiolabelled and used in a series of gel retardation experiments.

Several complexes were formed with both U and PB extracts (figure 3.12). Complexes in groups A and B all appear to be increased in abundance in response to phenobarbital-treatment of the rats, particularly complex 3. Over the series of experiments with this fragment there was a variation in the relative abundances of these complexes in group A and B, such that there did not appear to be a difference between the U and the PB extracts in some cases. This variation could be due to the different preparations of nuclear

Probe: -34 to-86bp *CYP2B2*								
Protein (µg)	2.5		5		7.5		5	–
Treatment	U	PB	U	PB	U	PB	PB	–
Proteinase K	–	–	–	–	–	–	+	–
Track	1	2	3	4	5	6	7	8

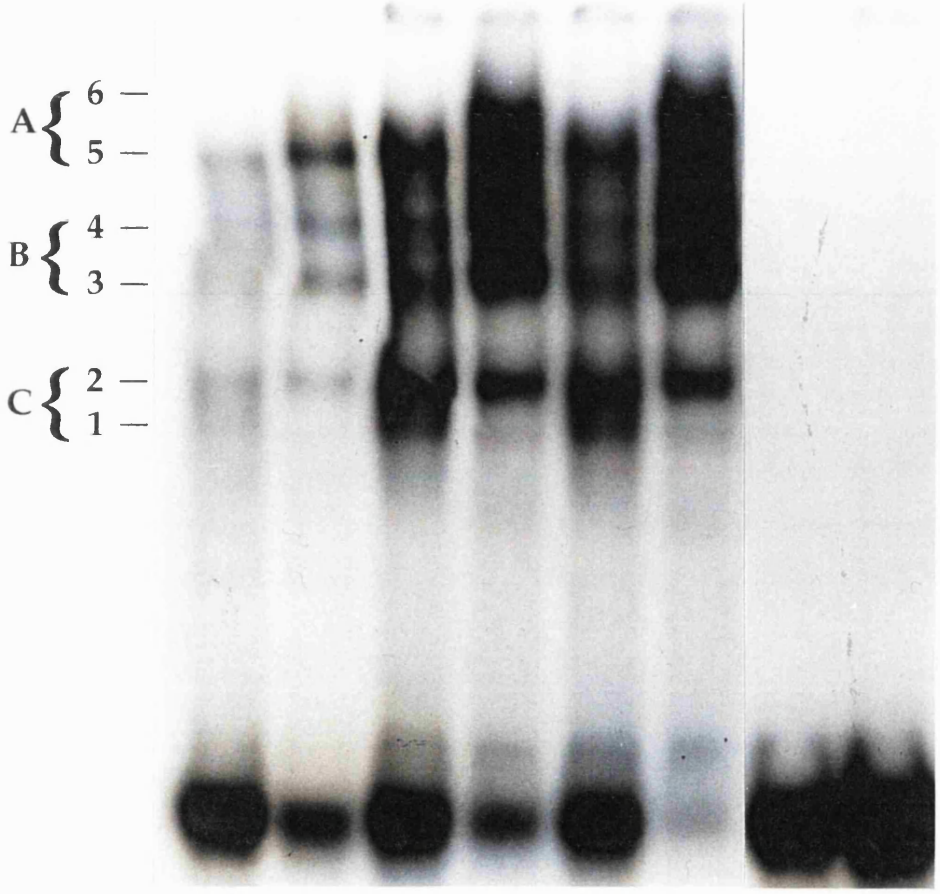


Figure 3.12: Gel retardation with the -34 to -86bp fragment of the *CYP2B2* gene promoter. The -34 to -86bp region of the *CYP2B2* gene was incubated without nuclear proteins (-), or with varying amounts of nuclear protein extracts made from the livers of untreated rats (U) or rats treated with phenobarbital (PB). The amount of poly dI.dC included in the reactions was 0.4x the amount of nuclear protein, or 2µg in the absence of nuclear protein. The reaction in track 7 was incubated with 5µg of proteinase K. The different protein-DNA complexes are numbered and grouped by letter to the left of the photograph.

proteins and different preparations of purified P.C.R. product used during the course of these experiments. It is also probable that the ratio of unlabelled to labelled -34 to -86bp DNA fragment would vary with each labelling reaction. The unlabelled DNA would act as a competitor, possibly masking effects seen when there was less unlabelled DNA present in the reactions. However, in every case complex 3 was 1.5- to 4.3-fold more abundant with the PB extract than with the U extract. It was also observed that complex 6 was only present in reactions carried out in the presence of PB nuclear protein extract.

There were two complexes formed in group C, the lowest molecular weight one is more abundant with the U extract than the PB extract. Again there was a variation between 1.5- to 5-fold more of complex 1 with the U extract than with the PB extract over the series of experiments. It should be noted that not all of the relative abundances of the complexes varied in this way. The larger of the two complexes in group C was between 1.25- to 1.5-fold more of the complex with the U extract than with the PB extract throughout the series of experiments.

The difference in abundance of complexes formed with 2.5µg of nuclear protein extract as compared to 5µg of nuclear protein extract was much greater than the difference between 7.5µg and 5µg of nuclear protein extract. In the case of the PB extract, this could be due to the probe becoming limiting with 7.5µg nuclear protein (there is very little free probe), so not all of the possible complexes are seen. However, with the U extract where the same large increase in abundance of complexes between 5µg and 7.5µg was seen, the probe is still in excess with 7.5µg nuclear protein. Also, the complex with the slowest mobility was not seen until 5µg of nuclear protein extract was added to the reaction. These results suggest some extra

* once the gel retardation had been optimised,

stabilisation of the protein-DNA complexes at a higher protein concentration.

This gel retardation (figure 3.12) was compared to one using the -44 to -67bp fragment of the *CYP2B2* gene promoter (figure 3.9). The complexes formed with the -44 to -67bp fragment were the same as those formed in groups B and C with the untreated extracts. So increasing the length of the gel retardation probe from -44/-67bp to -34/-86bp resulted in the formation of several extra higher molecular weight complexes (group A), and a difference between the abundances of complexes formed with the U and PB extracts.

A supershift gel retardation assay was carried out using the -34 to -86bp probe and anti-sera to C/EBPα, C/EBPβ and C/EBPδ to determine the involvement of these forms in the complexes (figure 3.13). The complexes are numbered to the left of the photograph. Complex 1 forms in greatest abundance with U extracts and complex 3 is the complex predominantly increased in response to phenobarbital treatment of the animal. In this experiment complexes 4 and 5 also increase with the PB extracts.

Complex 5 is supershifted when anti-C/EBPα is included in the gel retardation reaction. Complexes 1 through 5 are supershifted when anti-C/EBPβ is included. Anti-C/EBPδ does not shift any of the complexes discernibly. It seems, therefore, that complex 5 is a heterodimer between C/EBPα and C/EBPβ, and that complexes 1 through 4 also contain C/EBPβ either as a homodimer, or as a heterodimer with transcription factors that are unidentified as yet. It is difficult to discern whether the C/EBPα or C/EBPβ anti-sera had any effect on complex 6.

Probe: -34 to -86bp *CYP2B2*								
Treatment	U				PB			
Anti-C/EBPα	−	+	−	−	−	+	−	−
Anti-C/EBPβ	−	−	+	−	−	−	+	−
Anti-C/EBPδ	−	−	−	+	−	−	−	+
Track	1	2	3	4	5	6	7	8

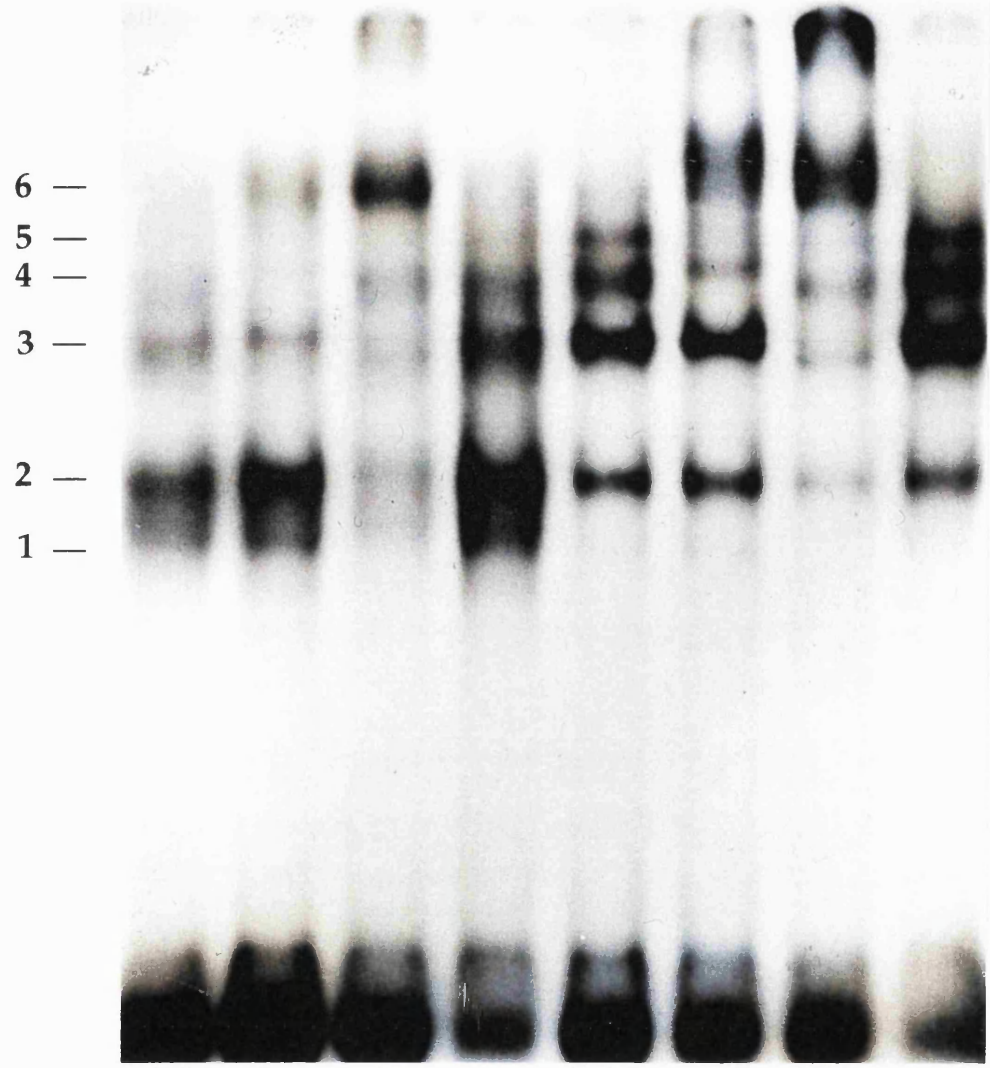


Figure 3.13: Supershift gel retardation with the -34 to -86bp fragment of *CYP2B2* and anti-bodies to C/EBP. The -34 to -86bp fragment of the *CYP2B2* gene promoterwas radiolabelled and incubated with 2.5µg of nuclear protein extracted from the livers of phenobarbital-treated (PB) or untreated (U) rats. The reactions included 2.5µg of poly dI.dC and occured in the presence (+) or absence (-) of 1µg of anti-C/EBPα, anti-C/EBPβ or anti-C/EBPδ. The different complexes are labelled on the left of the photograph.

Competition analysis confirmed that all the complexes seen with the -34 to -86bp fragment were specific as self competition abolished all binding (tracks 3 and 4, figure 3.14).

When 100-fold molar excess of the unlabelled -44 to -67bp fragment was included in the reactions (tracks 5 and 6, figure 3.14), all the lower molecular weight complexes (complexes 1 to 5) were competed away. The highest molecular weight complex (complex 6) increased in abundance once all the other complexes were eliminated. This is perhaps due to increased access to the binding site of this protein once the other proteins had been removed.

The fragment -6 to -38bp was also used as a competitor (tracks 7 and 8, figure 3.14) . This did not appear to compete away any complexes, however the abundance of all the complexes formed with the U extract increased so that they appeared very similar to the complexes formed with the PB extracts. This effect was also seen in the repeat experiment, so it was not the result of a mistakenly large amount of protein being added. It is possible that a protein in the U extract binds to this -6 to -38bp region that slightly destabilises, or interferes with the formation of the complexes seen with the -34 to -86bp fragment.

The last competitor used in this experiment was a consensus oligonucleotide for the binding site of Sp1 (Stratagene, figure a) (tracks 9 and 10, figure 3.14). Sp1 is a transcription factor involved in the regulation of many vertebrate genes, including a number of the housekeeping genes. It binds to a GC-rich consensus sequence known as the GC box. However this GC box has also been shown to bind proteins that are unrelated to Sp1. There are possible Sp1 binding sites at bases -45 to -36 and -79 to -70 of the *CYP2B2* promoter (figure 3.15b). Also Imataka *et al.* (1992) postulated a basic

Probe: -34 to -86bp *CYP2B2*											
Competitor	–		1		2		3		4		–
Treatment	U	PB	U	PB	U	PB	U	PB	U	PB	–
Track	1	2	3	4	5	6	7	8	9	10	11

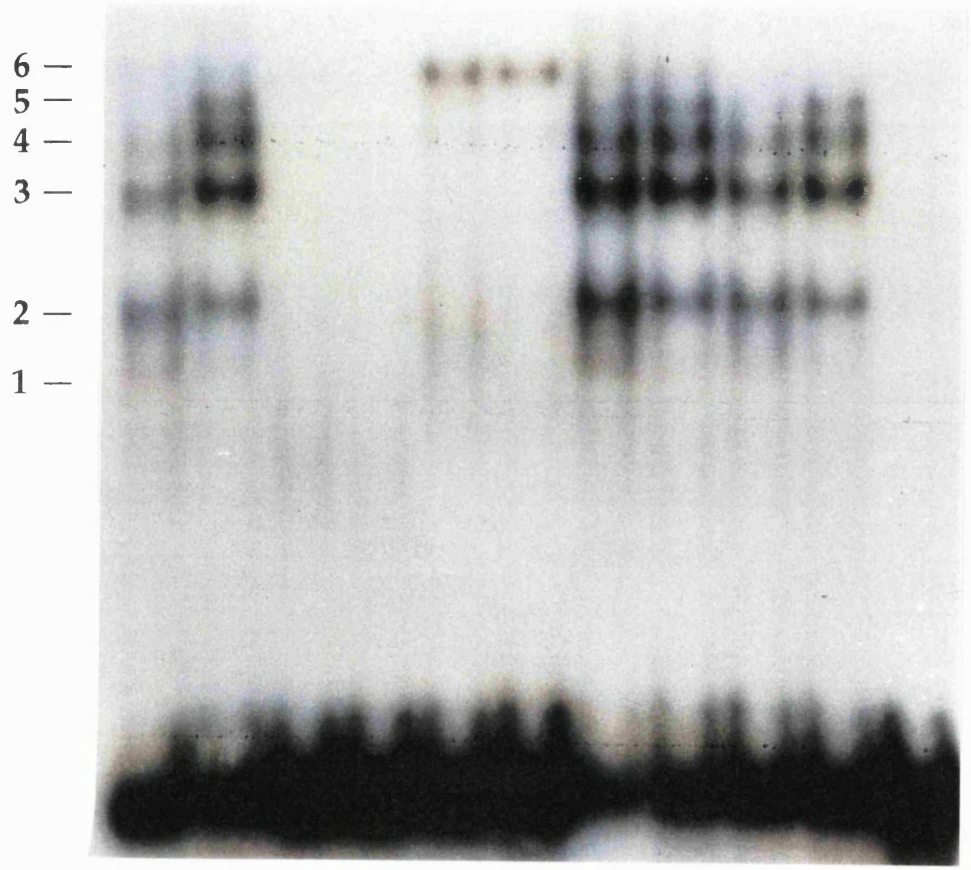


Figure 3.14: Analysis of the -34 to -86bp fragment of the *CYP2B2* promoter by competitive gel retardation. The -34 to -86bp fragment of the*CYP2B2* promoter was incubated with 5µg nuclear protein extract made from the livers of untreated (U) or phenobarbital-treated (PB) rats, or without nuclear protein (-). The incubations included 2µg poly dI.dC, and were carried out in absence (-) or presence (tracks 3-10) of 100-fold molar excess of unlabelled competitor DNA. Competitor 1 (tracks 3 and 4) was the -34 to -86bp fragment of *CYP2B2*, competitor 2 (tracks 5 and 6) was the -44 to -67bp fragment of *CYP2B2*, competitor 3 (tracks 7 and 8) was the -6 to -38bp fragment of *CYP2B2* and competitor 4 (tracks 9 and 10) was an oligonucleotide corresponding to the consensus binding site of Sp1 (Stratagene). The different protein-DNA complexes are numbered to the left of the photograph.

5' GATCGATC GGGGC GGGGC GATC 3'

3' CTAGCTAG CCCCG CCCCG CTAG 5'

Figure 3.15a: Oligonucleotide for the consensus binding sequence of Sp1 (Stratagene). The high affinity Sp1 binding site is shown in green.

-45 to-36bp *CYP2B2*:

5' GTGTAGGGGC 3'

-79 to -70bp *CYP2B2*:

5' CAGGAGGCGT 3'

Consensus Sp1 binding site (Lee *et al.*, 1994):

(G/T)(G/A)GG(C/A)G(G/T)(G/A)(G/A)(C/T)

Figure 3.15b: Comparison of possible Sp1 binding sites in the proximal CYP2B2 promoter region to a consensus sequence. The letters in red indicate the bases that exactly match the consensus sequence shown below. Most strong Sp1 binding sites do not differ from this consensus in more than one position.

5' CGCTTGA TGACTCA GCCGGAA 3'

3' GCGAACT ACTGAGT CGGCCTT 5'

Figure 3.15c: The consensus oligonucleotide for the AP1 binding site (Santa Cruz). The AP1 binding site is highlighted in green.

transcription element (BTE) in the region -103 to -66bp of *CYP2B2* by comparison with other liver specific genes. The BTE has been shown to interact with Sp1 in other CYP genes (Kobayashi *et al.*, 1996). The Sp1 consensus binding site did compete out complex 6, the highest molecular weight complex which did not form with the shorter -44 to -67bp probe. When this consensus Sp1 binding site was used as the probe for a gel retardation experiment, no difference was observed in the relative amounts of the protein complexes formed with the U extract as compared to the PB extract (figure 3.16).

3.2.4 Gel retardation with the -118 to -2bp fragment of the *CYP2B2* gene promoter.

This fragment was chosen to include both of the DNase I footprints observed in the proximal region of the promoter (Shephard *et al.*, 1994), in an attempt to study possible interactions between the proteins binding to this region. The -118 to -2bp region is a Nla III fragment which was cloned into the Sph I site of pUC 19 (section 2.2). For use in the gel retardation assay, it was excised from the vector using a Pst I and Hind III double digest, and purified from a 1% low melting point agarose gel.

Figure 3.17 shows a competitive gel retardation analysis using this fragment. Tracks 1 and 2 are without competitor. The complexes labelled 1 through 6 correspond to the complexes formed with the -34 to -86bp fragment of *CYP2B2* (figure 3.12). Several high molecular weight complexes, larger than complex 6, are formed with the -118 to -2bp fragment that were not observed with the smaller fragment. There are also extra bands seen interspersed between complexes 2 and 5, though these may have been masked with the

Probe: Consensus Sp1 binding site					
Treatment	U	PB	PB	PB	–
Consensus SP1 competitor	–	–	+	–	–
Proteinase K	–	–	–	+	–
Track	1	2	3	4	5

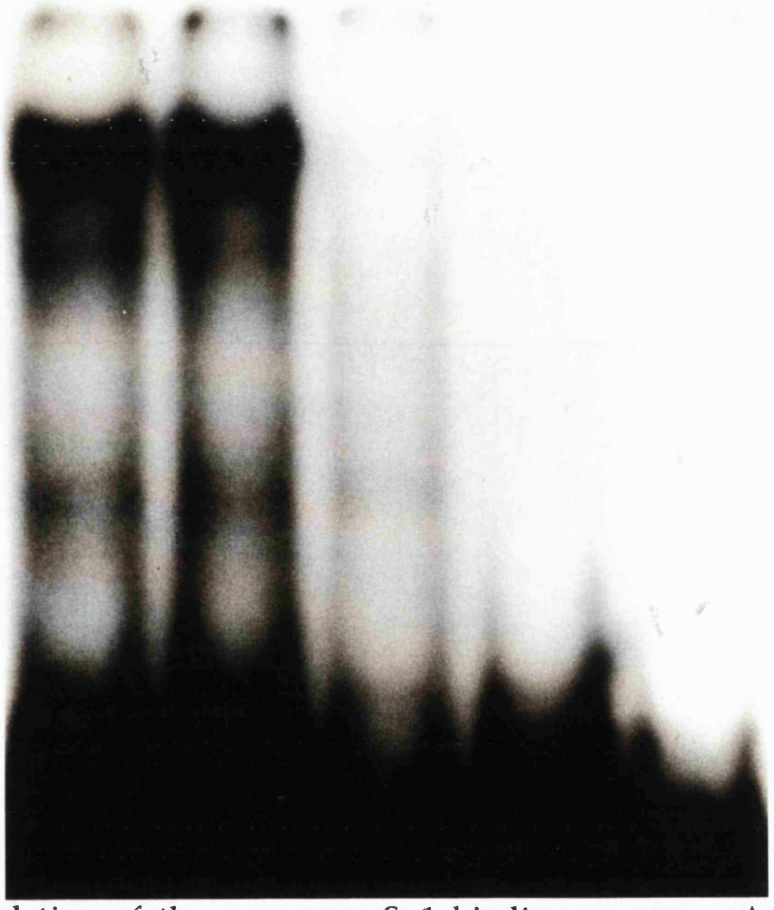
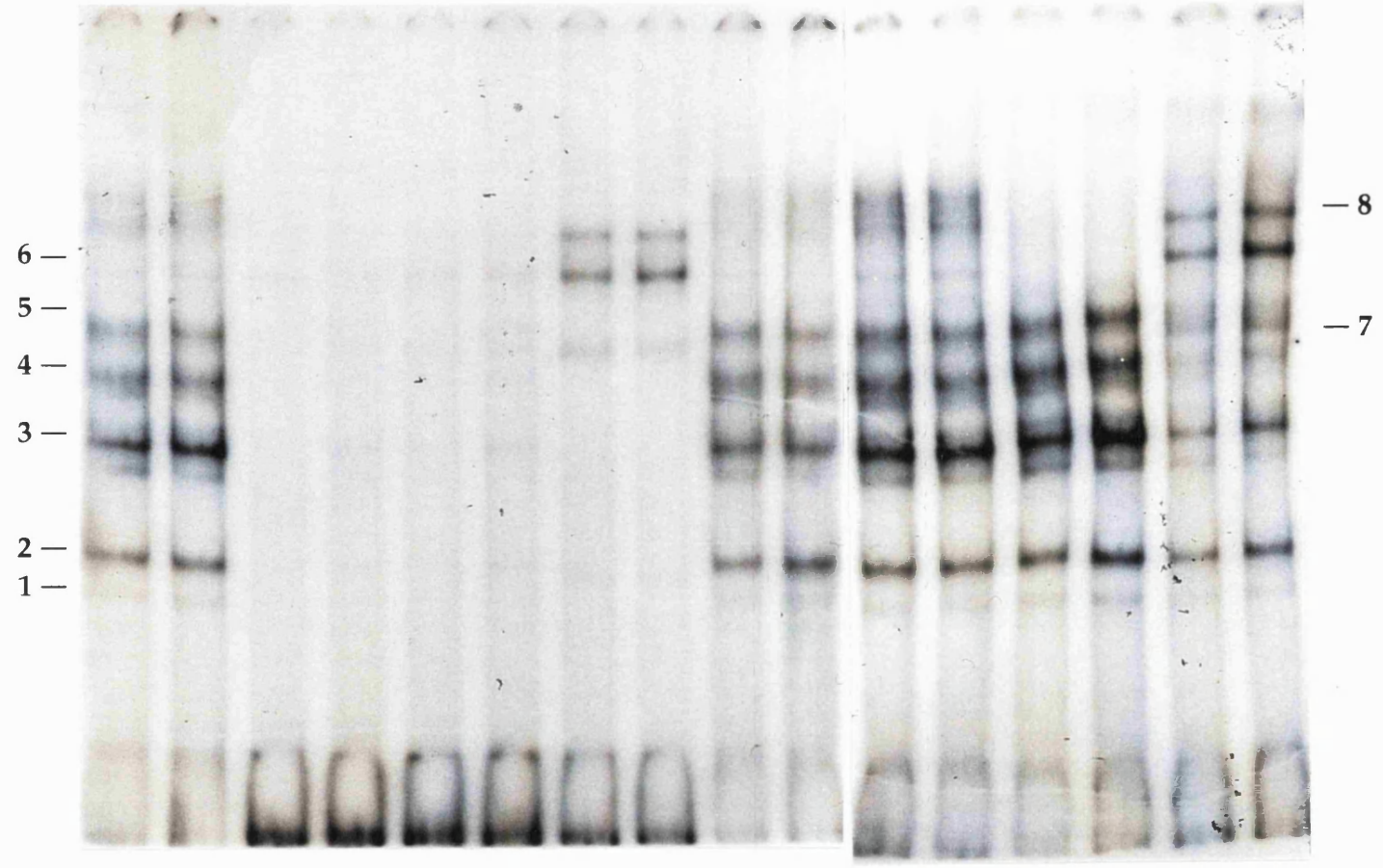


Figure 3.16: Gel retardation of the consensus Sp1 binding sequence. An oligonucleotide corresponding to the Sp1 binding site (Stratagene) was radiolabelled and incubated without (-) nuclear protein, or with nuclear proteins extracted from the livers of untreated (U) or phenobarbital-treated (PB) rats. The binding reactions were carried out in the presence of 2µg of poly dI.dC. The reaction in track 3 also included a 100-fold molar excess of the unlabelled Sp1 consensus oligonucleotide and the reaction in track 4 was incubated with 5µg proteinase K.

Figure 3.17: Analysis of the -118 to -2bp fragment of the *CYP2B2* gene promoter by competitive gel retardation. The -118 to -2bp fragment was incubated with 2.5μg of nuclear protein extracted from the livers of phenobarbital-treated (PB) or untreated (U) rats. The incubations were in the absence (-) or presence (A-G) of 100 fold molar excess of unlabelled competitor DNA. Competitor A (tracks 3 and 4) was -118 to -2bp *CYP2B2*, competitor B (tracks 5 and 6) was -118 to -34bp *CYP2B2*, competitor C (tracks 7 and 8) was -44 to -67bp *CYP2B2*, competitor D (tracks 9 and 10) was -34 to -2bp *CYP2B2*, competitor E (tracks 11 and 12) was -38 to -6bp *CYP2B2*, competitor F (tracks 13 and 14) was the consensus sequence for the Sp1 binding site (Stratagene) and competitor G (tracks 15 and 16) was the consensus sequence for the AP1 binding site (Santa Cruz). Each reaction contained 2.5μg of poly dI.dC. The numbers at the sides of the photograph identify particular protein-DNA complexes discussed in the text.

Probe: -118 to -2bp *CYP2B2*																
Competitor	–		A		B		C		D		E		F		G	
Treatment	U	PB	U	PB	U	PB	U	PB	U	PB	U	PB	U	PB	U	PB
Track	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16



-34 to -86bp fragment, as the bands in this experiment are a lot sharper and clearer than previously. Complex 1 is much less abundant relative to the other complexes formed with this longer fragment as compared to the -34 to -86bp fragment. Densitometric analysis of this and two other gel retardation experiments with the same fragment (not shown) showed that none of the complexes formed with the PB extracts are significantly more abundant than the complexes formed with the U extracts.

Self competition (tracks 3 and 4, figure 3.17) removed all the complexes, as did competition with the -118 to -34bp fragment (tracks 5 and 6). When oligonucleotides corresponding to -34 to -2bp or -6 to -38bp (tracks 9 and 10; and tracks 11 and 12 respectively, figure 3.17) were included as competitors, none of the complexes was competed away. However, the -338 to +33bp region of *CYP2B2* showed a footprint at -6 to -38bp (Shephard *et al.*, 1994). This competition analysis would suggest that any complex that formed in the -6 to -38bp region must require some stabilisation from a DNA element, or proteins binding to a DNA element that is between -2 and +33bp, or -338 and -118bp of the *CYP2B2* promoter.

As with the -34 to -86bp competition analysis, when the -118 to -2bp fragment was competed with the -67 to -44bp fragment from *CYP2B2* (tracks 7 and 8, figure 3.17), complexes 1 through 5 were abolished, and complex 6 was more abundant than without competition. In addition, one of the higher molecular weight complexes (complex 8) is increased in abundance, and there is the formation of a complex (complex 7) that has not previously been observed, following competition with this fragment. These results indicate that the formation of complexes 6, 7 and 8 is inhibited, to varying extents, by the proteins binding to the -44 to -67bp region.

The oligonucleotide for the consensus sequence of the Sp1 binding site was included as a competitor in the reactions in tracks 13 and 14 (figure 3.17). All of the complexes with slower mobilities than complex 5 are competed away showing that these include a GC box binding protein.

Several phase II enzymes contain a sequence called the EpRE (or electrophile response element) in their promoters. This sequence contains 2 AP1-like binding sites and has been shown to mediate the phenobarbital induction of the GST Ya and quinone reductase genes in rats (Pinkus *et al.*, 1993; Friling *et al.*, 1990). To determine whether AP1 might be important in the PB responsiveness of the *CYP2B2* gene, I included an oligonucleotide corresponding to the AP1 consensus binding site (Santa Cruz Biotechnology, figure 3.15b) as a competitor to the -118/-2 fragment.

This AP1 consensus oligonucleotide had previously been used in a gel retardation (figure 3.18) where it formed several protein-DNA complexes. Complex a was shown to be non-specific as self competition failed to eliminate it, though the oligonucleotide did compete out complexes b and c. The abundance of complex b remained unchanged and of complex c increased 4- and 5-fold (2.5µg and 5µg respectively) in response to phenobarbital treatment of the animal (densitometric analysis of three separate experiments).

When this oligonucleotide was used as a competitor to the -118 to -2bp fragment (tracks 15 and 16, figure 3.17), a similar pattern of binding was seen as that for -44 to -67bp competition (tracks 7 and 8, figure 3.17), in that the abundances of complexes 3, 4 and 5 were reduced, complexes 6 and 8 were increased and the formation of complex 7 was again observed. Complexes 1 and 2 were not competed away as they had been with the -44 to -67bp region.

Probe: Consensus AP1 binding site							
Protein (µg)	2.5		5				–
Treatment	U	PB	U	PB	PB	PB	–
Competitor DNA	–	–	–	–	+	–	–
Proteinase K	–	–	–	–	–	+	–
Track	1	2	3	4	5	6	7

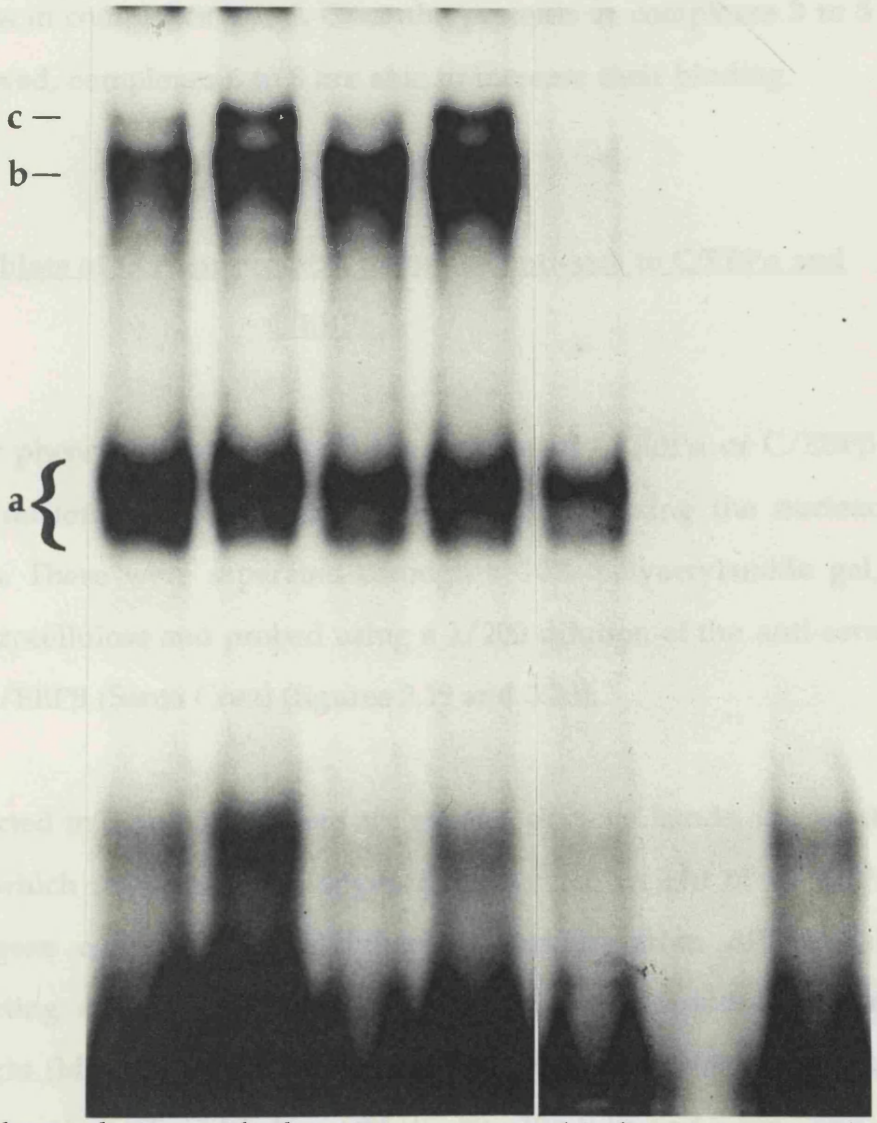


Figure 3.18: Gel retardation with the AP1 consensus binding sequence. An oligonucleotide corresponding to the consensus binding sequence for AP1 (Santa Cruz) was radiolabelled and incubated with 2.5µg or 5µg of nuclear protein extract, or without protein (-). The extracts were made from the livers of untreated rats (U) or rats treated with phenobarbital (PB). The amount of poly dI.dC added to the reactions was 40% of the amount of nuclear protein. The reaction in track 5 included unlabelled AP1 consensus sequence DNA at 100-fold molar excess over the labelled DNA. The reaction in track 6 was incubated with 5µg proteinase K. The letters on the left of the photograph label complexes discussed in the text.

Also the AP1 consensus oligonucleotide was not as efficient as a competitor for complexes 3, 4 and 5 as the -44 to -67bp *CYP2B2* promoter fragment.

This result suggested either that the proteins involved in the complexes 3, 4 and 5 can also bind weakly to the AP1 consensus sequence oligonucleotide, or that a protein(s) binding to the AP1 consensus sequence also interacts with the proteins in complexes 3 to 5. Once the proteins in complexes 3 to 5 have been removed, complexes 6 to 8 are able to increase their binding.

3.2.5 Western blots of nuclear proteins using the anti-sera to C/EBPα and C/EBPβ.

To see whether phenobarbital increased the amount of C/EBPα or C/EBPβ present in the nucleus, western blots were carried out using the nuclear protein extracts. These were separated through a 10% polyacrylamide gel, blotted onto nitrocellulose and probed using a 1/200 dilution of the anti-sera to C/EBPα or C/EBPβ (Santa Cruz) (figures 3.19 and 3.20).

C/EBPα is detected in figure 3.19. There are several obvious bands, the most prominent of which migrates at an apparent molecular weight of 42 $x10^3$. The C/EBPα gene codes for two polypeptides arising from differential translation starting at two separate sites. The full length product has a molecular weight (M.W.) of 42 $x10^3$. In addition there is a shorter transcript, the translation product of which has a M.W.=30 $x10^3$ (Lin and Lane, 1994). One of the other bands seen on the blot migrates at this apparent weight. The amounts of the bands identified as C/EBPα are not affected by phenobarbital treatment of the animal.

Probe: Anti-C/EBPα					
Sample	M	Ex U	Ex PB	Nu U	Nu PB
Track	1	2	3	4	5

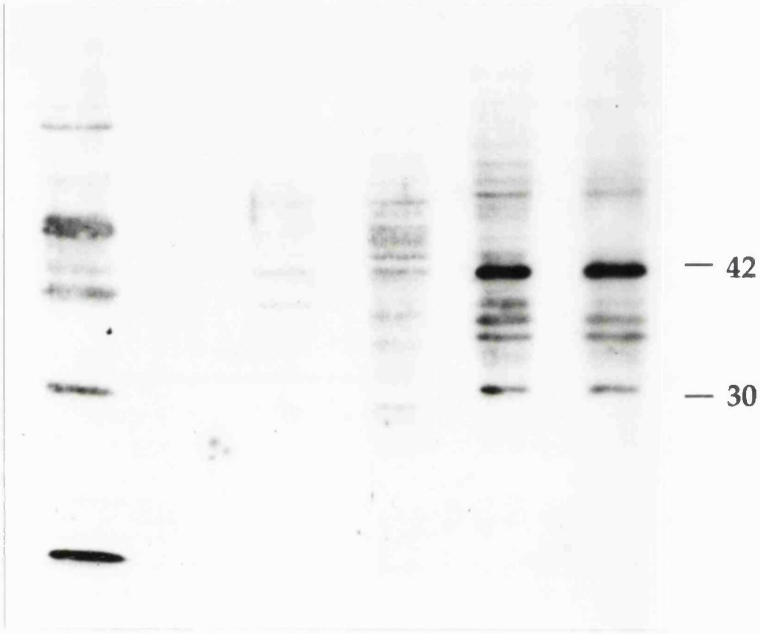


Figure 3.19: Western blot to detect C/EBPα. Nuclear proteins (Nu, tracks 4 and 5) and extra-nuclear fractions (Ex, tracks 2 and 3) were prepared from the livers of phenobarbital-treated (PB, tracks 3 and 5) or untreated (U, tracks 2 and 4) rats. 10μg of each sample was separated through a 10% polyacrylamide gel. The proteins were transferred to a nitrocellulose filter which was probed with 0.1μg/ml C/EBPα anti-serum. A mid-range protein molecular weight standard (M) was also loaded onto the gel. The apparent molecular weights ($x10^3$) of certain bands are indicated to the right of the photograph. The sizes of the bands in the standard are shown in figure A.1.

The C/EBPβ blot is shown in figure 3.20. Again several bands are seen. Descombes and Schibler (1991) showed that the rat C/EBPβ gene has three translation products. These are full-length LAP (FL-LAP)(M.W.=38 $x10^3$), LAP (M.W.=34 $x10^3$) and LIP (M.W.=20 $x10^3$). The designation of the bands seen on the blot is not as clear as for the C/EBPα blot; possible identifications appear on the figure. There are two bands that are larger than FL-LAP. It is possible that they are one of the other C/EBP family members, although according to the manufacturer, the antibody is not immunologically cross-reactive with other C/EBP proteins. They do not correspond to the size of C/EBPα. None of the bands seen changes in abundance in response to phenobarbital treatment of the animal.

3.2.6 Phosphatase treatment of the nuclear protein extracts.

To determine whether the phosphorylation status of the proteins binding to the proximal CYP2B2 promoter was important, the nuclear protein extracts were treated with alkaline phosphatase before addition to a gel retardation reaction. 5μg of nuclear protein extract was digested with 4 units of calf intestinal alkaline phosphatase (Pharmacia) in a volume of 30μl for 1 hour at 37°C. The digestion was stopped by addition of EDTA to a final concentration of 0.1mM. The digest was added to the gel retardation reaction tube. The amounts of the other reactants were adjusted to take account of the increase in total volume from 20μl to 45μl, otherwise the reactions were carried out as previously.

Figure 3.21 shows a gel retardation carried out using the -118 to -2bp *CYP2B2* probe. The nuclear protein extracts in tracks 3 and 4 have been treated with alkaline phosphatase, whilst those in tracks 1 and 2 have not. All of the

Probe: Anti-C/EBPβ					
Sample	M	Ex U	Ex PB	Nu U	Nu PB
Track	1	2	3	4	5

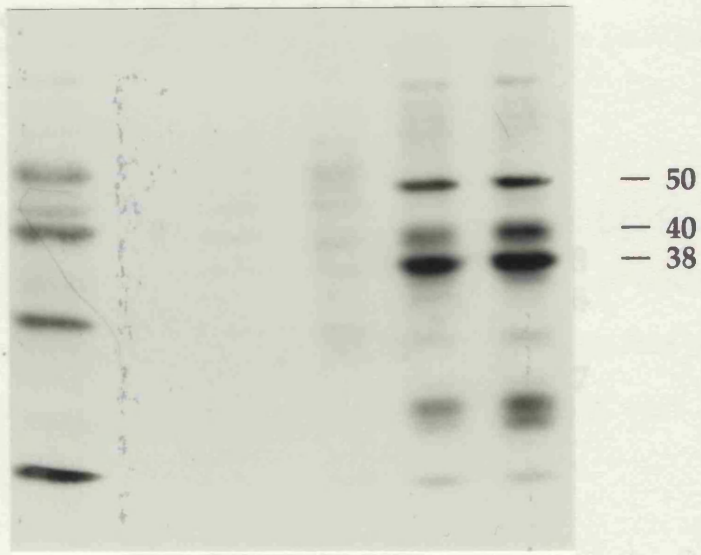


Figure 3.20: Western blot to detect C/EBPβ. Nuclear proteins (Nu, tracks 4 and 5) and extra-nuclear fractions (Ex, tracks 2 and 3) were prepared from the livers of phenobarbital-treated (PB, tracks 3 and 5) or untreated (U, tracks 2 and 4) rats. 10μg of each sample was separated through a 10% polyacrylamide gel. A mid-range protein molecular weight standard (M) was also loaded onto the gel. The proteins were transferred to a nitrocellulose filter which was probed with 0.1μg/ml C/EBPβ anti-serum. The apparent molecular weights ($x10^3$) of certain bands are indicated to the right of the photograph. The sizes of the bands in the standard are shown in figure A.1.

Probe: -118 to -2bp *CYP2B2*					
Phosphatase	−		+		−
Treatment	U	PB	U	PB	−
Track	1	2	3	4	5

Figure 3.21: The effect of phosphatase treatment on the binding of nuclear proteins to the -118 to -2 bp fragment of the *CYP2B2* promoter. The -118 to -2bp region of the *CYP2B2* promoter was incubated with 5µg nuclear protein extracted from the livers of phenobarbital-treated (PB) or untreated (U) rats. Calf intestinal alkaline phosphatase was used to treat some of the nuclear protein extract (+) as described in the text. The reactions included 5µg of poly dI.dC. The reaction in track 5 was carried out in the absence of nuclear protein. Numbers to the right of the photograph refer to DNA-protien complexes discribed in the text

complexes with the phosphatase-treated extracts have a slightly slower mobility in the gel compared to those from extracts which were not treated with phosphatase. This is possibly due to the larger volume of reaction loaded on to the gel. Complexes 3 to 5 and the complexes in region 9 have been significantly reduced by the phosphatase treatment. Complex 7 forms only with the phosphatase-treated extracts. The DNA-binding ability of the proteins in complexes 1, 2, 6 and 8 is unchanged by the phosphatase treatment. There is not any difference between the extracts from untreated rats as compared to phenobarbital treated rats.

So the proteins in complexes 3 to 5 need to be phosphorylated for DNA-binding to be achieved.* It is interesting to note that the pattern of binding observed after phosphatase treatment of the extracts strongly resembles that when binding to the -118 to -2bp *CYP2B2* fragment was competed with the oligonucleotide for the consensus AP1 binding sequence.

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* It is also possible that the addition of EDTA to the nuclear proteins affected their binding to the DNA, or that the presence of alkaline phosphatase alone somehow affected the binding. It would be possible to control for these possibilities by i) adding the same amount of EDTA to undigested nuclear protein before the binding reaction and ii) adding the EDTA before the alkaline phophatase and carrying the incubation out as usual before adding the nuclear proteins to the gel retardation binding reaction.

3.3 Section I: Discussion

3.3.1 Optimisation of the gel retardation assay.

Gel retardation experiments are useful in identifying potential regulatory sequences in the promoter regions of genes. However it is important to remember that this assay is not an *in vivo* system. The DNA fragments involved are comparatively short, so potential interactions between transcription factors with widely spaced binding sites may not be detected. Changes in the DNA-binding ability of transcription factors in response to a certain treatment can be observed, but changes in their trans-activating ability cannot. There will also be a whole cascade of events leading up to the binding or activation of transcription factors that cannot be examined by gel retardation (reviewed Calkhoven and Ab, 1996). In addition, the assay uses naked DNA and takes no account of the influence of chromatin structure on the regulation transcription (reviewed Travers, 1994; Becker, 1994).

The results showed how varying several of the parameters affected the gel retardation assay. The optimised protocol that I used in subsequent experiments is set out in the methods section. However, adjustments in the amount of poly dI.dC, and the percentage of the gel, were required for each new DNA probe. The amount of poly dI.dC is discussed in each figure legend. The percentage of the gel used was 6% for probes > 60bp and 4% for larger probes.

The variation between batches of nuclear protein extracts observed in the experiments described in section 3.1.4 is not unique. Ram *et al.* (1996) studying the binding of STAT proteins to an element in the β-casein gene

show a comparison of 3 different nuclear protein batches which do vary slightly from each other, for instance one batch shows one less complex than the other two under the same set of conditions. It would, perhaps, have been useful to prepare several different batches of extracts using each of the protocols discussed, and use each of them in a gel retardation assay with each new DNA fragment studied so as to identify as many complexes as possible. As time was running short, however, this was not attempted.

These results were a reminder that while the gel retardation assay can give some useful information with regard to potential transcription factor binding sites, it is an *in vitro* assay and as such is limited by the conditions provided by the experimenter.

3.3.2 Analysis of the 5' flanking sequence of the CYP2B2 gene.

The putative regulatory elements in the first hundred base pairs of the *CYP2B2* promoter are summarised in figure 3.22.

3.3.2a Binding at -44 to -67bp of the *CYP2B2* gene promoter.

The results demonstrate that there is a C/EBP binding site at -44 to -67bp of the *CYP2B2* 5' flaking sequence, to which both C/EBPα and C/EBPβ can bind. This finding is in agreement with reports from Luc *et al.* (1996) and Park and Kemper (1996). Both groups studied the 5' flanking regions of the *CYP2B1* and *CYP2B2* genes (the promoters of these two genes are very similar for the first 200 base pairs) by DNase footprinting and identified a footprint at -45 to -64bp in both promoters. This footprint was competed

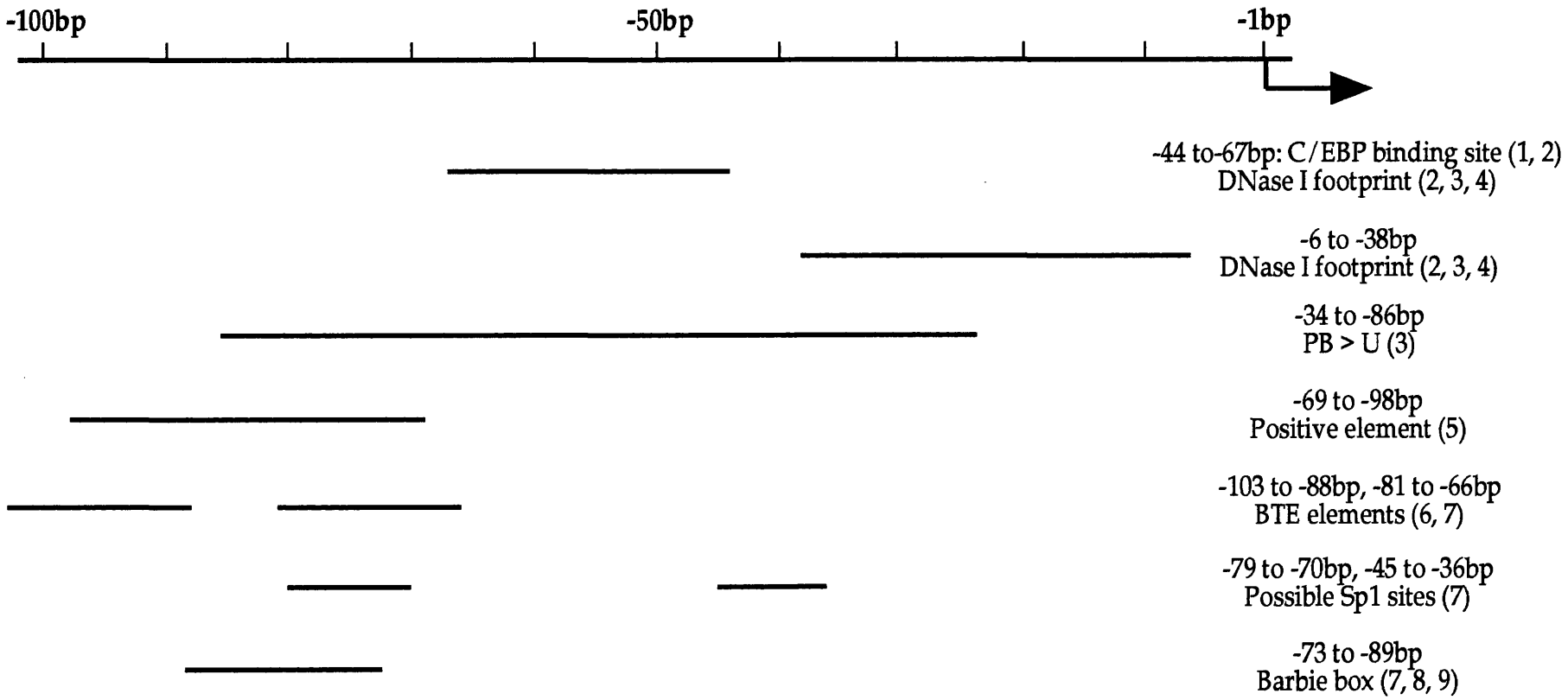


Figure 3.22: Summary of putative regulatory elements in the proximal *CYP2B2* gene 5' flanking sequence. The positions of various DNA elements possibly involved in the phenobarbital-mediated regulation of the *CYP2B2* gene are respresented. (1) Park and Kemper (1996), (2) Luc *et al.* (1996), (3) Shephard *et al.* (1994), (4) Sommer *et al.* (1996), (5) Upadhya *et al.* (1992), (6) Imataka *et al.* (1992), (7) Hoffman *et al.* (1992), (8) Liang and Fulco, (1995), (9) He and Fulco (1991).

away by an oligonucleotide containing a known C/EBP binding sequence, but not by an oligonucleotide containing a mutated C/EBP binding site. A DNase I footprinting experiment using a recombinant C/EBPα C-terminal polypeptide created an identical footprint to that obtained with rat liver nuclear extracts (Luc *et al.*, 1996).

Park and Kemper (1996) identified several protein-DNA complexes when they used the -110 to +1bp fragment of the *CYP2B1* promoter in a gel retardation experiment. The number and pattern of the DNA-protein complexes are the same as the ones observed in the gel retardation carried out with the -118 to -2bp region of the *CYP2B2* promoter (figure 3.17), with the exception of the complex that is labelled complex 1 and the complexes which migrated more slowly than complex 6, which are all missing in Park and Kemper's experiment. In agreement with my results, this 110bp fragment did not display any difference in the abundance of complexes formed with nuclear proteins extracted from the livers of phenobarbital-treated as compared to those from untreated rats. The -110 to +1bp *CYP2B1* fragment was also used by Park and Kemper (1996) in a supershift gel retardation with anti-sera to C/EBPα and C/EBPβ. A comparison of their results with those shown in figure 3.13 identified the equivalents of complexes 4 and 5 as containing C/EBPα. My supershift results also showed complexes 4 and 5 involve C/EBPα. Complexes 1 to 5 were identified as C/EBPβ in the supershift gel retardation. However the results of the western blots with the C/EBP anti-sera show that the C/EBPβ does cross-react with at least one other unknown species, so it is possible that some of the complexes that I have identified as C/EBPβ are different proteins. Park and Kemper (1996) confirmed that complexes 3 and 4 contain C/EBPβ.

Luc *et al.* (1996) transfected Hep G2 cells with the first 1.4kb of the *CYP2B2* promoter driving a luciferase reporter gene. When this vector was co-transfected with C/EBPα or C/EBPβ expression vectors, the expression of the luciferase gene was enhanced 15- and 5- fold respectively. When they carried out the same experiment using the same region of the *CYP2B1* promoter, Park and Kemper found the activation to be slightly lower at 7- to 9-fold with C/EBPα and 5- to 6- fold with C/EBPβ. However, in both experiments, if the C/EBP site on the *CYP2B1/2* promoter was mutated, there was no activation of luciferase expression. Thus the -44 to -67bp region in both the *CYP2B1* and the *CYP2B2* promoters is a functional binding site for C/EBPα and C/EBPβ.

The results of the phosphatase treatment of the nuclear protein extracts (figure 3.21) showed that the proteins in complexes 3 to 5 need to have a phosphate group for DNA-binding to be achieved. The activity of C/EBPβ is known to be regulated by phosphorylation, however there is very little information as to how C/EBPα is affected by phosphorylation (reviewed in Johnson and Williams, 1994).

3.3.2b Binding to the -118 to -67bp region of the *CYP2B2* promoter.

The analysis of the -118 to -2bp region of the *CYP2B2* 5' flanking sequence by competitive gel retardation showed that there must be proteins binding in the region -86 to -118bp. This is at odds with several DNase I footprinting studies (Shephard *et al.*, 1994; Sommer *et al.*, 1996; Park and Kemper, 1996, Luc *et al.*, 1996) where no footprint was seen in this region. However, Sommer *et al.* (1996) did consistently find multiple DNase I hypersensitive sites at -81 to -91bp, and Shephard *et al.* (1994)comment on a weak DNase I

footprint between -85 and -90bp. Rangarajan and Padmanaban (1989) describe a footprint between -56 and -88bp of a *CYP2B1/2* promoter, but did not see the footprint in the region between -40 and -67bp which is described by all of the reports above.

Upadhya *et al.* (1992) reported that the -69 to -98bp fragment of the *CYP2B1/2* gene promoter bound rat liver nuclear proteins in gel retardations. They designated this a positive element (PE), as the binding of proteins was increased in response to phenobarbital treatment of the animal. Hoffman *et al.* (1992) also reported binding to the region -104 to -71bp.

From the study of the barbiturate-regulated induction of the bacterial *CYP102* gene He and Fulco (1991) identified a 17bp sequence (designated the Barbie box, consensus sequence 5' ATCAAAAGCTGGAGG 3') as the binding site for a barbiturate-regulated factor. Based on sequence similarities and protein binding studies it is suggested that this region is involved in the regulation of *CYP2B2* and other mammalian PB-inducible genes. There is a region in the *CYP2B2* promoter that is similar to this box (-89 to -73bp) (Liang and Fulco, 1995; Hoffman *et al.*, 1992). However when this 17-mer was used in a gel retardation no binding was seen (Shephard *et al.*, 1994) and it did not compete away binding to the -31 to -85bp fragment of the *CYP2B2* promoter (Shephard *et al.*, 1994). Also no DNase I footprint has been observed in the region (Sommer *et al.*, 1996; Park and Kemper, 1996; Luc *et al.*, 1996).

Recent interest has focussed on the BTE (basal transcription element, Yanagida *et al.*, (1990)) identified by Hoffman *et al.* (1992) at -66 to -103bp. This includes two half sites at -82 to -67bp and -87 to -102bp. The BTE has

been shown to interact with Sp1 in other genes (Imataka *et al.*, 1992; Kobayashi *et al.*, 1996). Park and Kemper carried out a DNase I footprint experiment using the -211 to +1bp fragment of the *CYP2B2* promoter and competed it with the -82 to -67bp oligonucleotide. This did not compete away binding to the -64 to -45bp footprint. This agrees with my result (figure 3.17) that competition of binding to the -118 to -2bp fragment with the Sp1 consensus oligonucleotide did not compete away complexes identified as including C/EBP and binding to the -44 to -67bp region.

Sp1 initially binds to DNA as a monomer. As the amount of Sp1 is increased, and the free DNA becomes limiting, higher order complexes form "stacked" on top of the original Sp1 unit. The proteins form the dimers and tetramers through protein-protein interactions only (Pascal and Tjian, 1991). Hoffman *et al.* (1992) noted that in the gel retardation with the -104 to -71bp fragment, as they increased the amount of protein (either crude nuclear protein, or purified protein), and the free DNA became limiting, there was the formation of extra, slower mobility complexes. My results showed the same was true for complex 6 in the gel retardation using the -34 to -86bp *CYP2B2* probe which was not seen until 5µg of protein extract were added to the gel retardation reaction. This would be consistent with Sp1 binding at a site within the -34 to -86bp region of the *CYP2B2* promoter.

Park and Kemper (1996) transfected Hep G2 cells with the first 1.4kb of the *CYP2B1* 5' flanking sequence attached to a luciferase reporter gene. They reported that the deletion of various sequences to -110bp had only small effects on the transcriptional activity of the vectors, but a further deletion to -57bp caused a drop of transcriptional activity of 90%. They also carried out experiments in which the C/EBP binding sequence, or the BTE sequence

between -82 and -67bp, or both, were mutated. All of these possibilities reduced transcriptional activity by 70-80%.

The results of the competitive gel retardation studies with the -118 to -2bp fragment (figure 3.17) showed that there were proteins that bound in the absence of the proteins involved in the C/EBP complexes, that these proteins did not bind to the -6 to -38bp region, and that their binding was competed by a oligonucleotide for the consensus Sp1 binding site. In conjunction with the results described above, this would suggest a weak site for a GC-box binding protein (possibly Sp1) in the BTE region. Stronger binding to this site is seen in the absence of the proteins binding to the -44 to -67bp region (this was also observed by Park and Kemper, 1996) which would explain why when the -98 to -69bp or -104 to -71bp fragments are used in gel retardations binding is seen (Upadhya *et al.*, 1992 and Hoffman *et al.*, 1992 respectively) even though several investigators did not observe a footprint in this region.

Park and Kemper's transfection studies showed that there must be binding to both the BTE region and the C/EBP region for transcriptional activity above a low basal level. Given the close proximity of the two elements it is tempting to postulate an interaction between the proteins binding to them, however there is no direct evidence for this at present. While protein binding to the BTE has not been fully identified as Sp1, Sp1 does bind to the BTE sequence in other genes and interacts with other proteins bound to separate elements to regulate these genes. The AhR-ARNT complex interacts with Sp1 in the regulation of *CYP1A1*. When either the AhR-ARNT complex is bound to the xenobiotic response element (XRE) or Sp1 is bound to the basal transcription element (BTE), the binding of the second factor is facilitated (Kobayashi *et al.*, 1996). In the regulatory region of the

CYP2D5 gene there is a poor C/EBP binding site adjacent to an Sp1 binding site. Lee *et al.* (1994) demonstrated that C/EBPβ alone was unable to bind to the *CYP2D5* promoter, but it could form a complex when recombinant Sp1 was added.

3.3.2c Binding of proteins at more distal regions of the *CYP2B2* gene 5' flanking sequence.

Competing binding to the -118 to -2bp fragment of the CYP2B2 promoter with an oligonucleotide for the consensus AP1 binding sequence showed either that the proteins involved in the complexes 3, 4 and 5 can also bind weakly to the AP1 consensus sequence, or that a protein(s) binding to the AP1 consensus sequence also interacts with the proteins in complexes 3 to 5. Roe *et al.* (1996) have identified an AP1 binding site at -1441bp of the *CYP2B2* gene 5' flanking sequence which binds 5- to 7-fold more protein with extracts from the livers of phenobarbital-treated rats as compared to those from untreated rats. They also found that binding was competed away by self competition but not by a consensus Sp1 oligonucleotide.

Hsu *et al.* (1994) found that C/EBPβ anti serum can co-immunoprecipitate c-jun and c-fos from mixtures of bacterially expressed proteins and that this required the presence of the C/EBPβ leucine zipper domain. Also they showed that over expression of Fos and Jun inhibited C/EBPβ-mediated transactivation of a promoter containing C/EBP binding sites. This would suggest that dimerisation of AP1 and C/EBPβ does occur and that it alters the trans-activation capability of C/EBPβ. Whether AP1 is contained in any of the complexes seen with the proximal *CYP2B2* promoter, or whether it interacts with any of them has not been studied.

3.3.2d Phenobarbital inducibility of the *CYP2B2* gene.

My studies have concentrated on putative regulatory elements in the proximal promoter of the *CYP2B2* gene (figure 3.22). When a gel retardation was carried out with the -34 to -86bp fragment of the *CYP2B2* promoter, a number of the complexes formed showed changes in abundance in response to treatment of the rat with phenobarbital. However when a longer fragment (-118 to -2bp) was used there was no difference between the two extracts. Park and Kemper (1996) also found that phenobarbital had no effect on the binding of rat liver nuclear proteins to the -110 to +1bp sequence of the *CYP2B1* gene, which is extremely similar in sequence to the *CYP2B2* gene in this region. If the difference in binding to the -34 to -86bp sequence (PB compared to U extracts) were the result of an extra interaction with a protein that does not bind to this region (i.e. through a direct protein-protein interaction), as the results of the AP1 competition in figure 3.18 suggest it might be, the larger complex may not be very stable in this *in vitro* assay. The longer fragment of DNA might be enough to destabilise this protein-protein interaction. Alternatively, it might interfere directly with the binding of proteins to the DNA.

I did not find any binding to the -38 to -6bp region, nor did it compete binding to the longer 116bp fragment. However, Shephard *et al.* (1994) reported that the fragment from -6 to -33bp bound more nuclear protein from extracts from untreated animals as compared to phenobarbital-treated animals. Both Sommer *et al.* (1996) and Luc *et al.* (1996) also found DNase I footprints in this region, but their reactions showed no difference between extracts from PB-treated or untreated animals.

Rangarajan and Padmanaban (1989) showed that a *CYP2B1/2* mini-gene containing the first 179bp of the promoter and part of the first exon of the gene was transcribed in freeze-thawed rat liver nuclei and that phenobarbital treatment of the animal increased this transcription. They proposed that this -179 to +1bp was a minimal PB-responsive promoter. Trottier *et al.* (1995) found that regions between -2015bp and the transcription start site did not have an effect on the responsiveness of reporter gene activity to phenobarbital. Park *et al.* (1996) also found that the transcription of a reporter gene construct attached to the first 1.4kb of the *CYP2B1* promoter transiently transfected *in situ* into rat liver was not inducible by phenobarbital.

Other studies argue for the role of distal elements in the phenobarbital induction of the *CYP2B2* gene. A transgenic mouse line carrying up to -800bp of the *CYP2B2* 5' flanking sequence expressed CYP2B2 mRNA only constitutively. PB-dependent induction was observed when additional sequences were present (Ramsden *et al.*, 1993). Also the Qsj:SD rat strain in which the *CYP2B2* gene has sequences up to -800bp the same as the parent strain and an intact Barbie box does not express more CYP2B2 in response to phenobarbital, although its *CYP2B1* gene is induced by PB (Hashimoto *et al.*, 1988).

Trottier *et al.* (1995) transfected primary hepatocytes with vectors containing various sections of the *CYP2B2* 5' flanking sequence attached to a CAT reporter gene. They identified an element between -2155 and -2318bp of the *CYP2B2* gene (the phenobarbital responsive element, or PBRE) that conferred PB responsiveness to a heterologous promoter and was active in both orientations. Constructs with promoter fragments from -2015bp or

-1680bp to +1bp (containing the proposed AP1 site) were not responsive to phenobarbital. The PBRE bound sequence-specific proteins in a gel retardation. The binding of the proteins was increased in response to treatment of the rat with phenobarbital. As yet, the proteins have not been identified.

Park *et al.* (1996) carried out transient *in situ* transfections of rat liver with vectors containing 1-3 copies of the PBRE attached to a minimal *CYP2B1* promoter (110bp). They reported that reporter gene activity with these constructs was PB inducible. They transfected similar vectors with 3 copies of the PBRE and 110bp of the *CYP2B1* promoter, but in which either the BTE, the C/EBP binding site, or the Barbie box sequence had been mutated. Mutation of the BTE or the C/EBP binding sites reduced expression of the reporter gene, but a 3- to 4-fold induction was maintained in response to phenobarbital. However, mutation of the Barbie box sequence had no effect on either the expression or induction levels of the reporter genes. The low induction of the reporter gene in response to phenobarbital compared to the *in vivo* response suggests that either the spacing of the PBRE in relation to the start of the reporter gene is not optimal, or there are other, as yet unidentified, elements involved in the PB mechanism as well.

In conclusion, there are at least two elements, the BTE and the C/EBP-binding region, in the proximal *CYP2B2* promoter that interact with nuclear proteins. Whether they have a role in the phenobarbital-mediated induction of this gene, as well as regulating basal transcription, is unclear. More distal elements (particularly the PBRE) are also implicated in the phenobarbital mechanism.

Section II: Use of a primary hepatocyte culture system to study the phenobarbital induction of the CYP2B2 gene.

3.4 Characterisation of the primary hepatocyte culture system.

Whilst gel retardation experiments produce useful information, they are artificial environments and their results need to be confirmed using a system that provides an environment closer to the whole animal. In the absence of a cell line that can maintain expression and induction of CYP2B1 and CYP2B2, it was decided to use a primary rat hepatocyte system to study the phenobarbital induction of these genes further. Dr. G. Ciaramella had previously examined various methods of isolating hepatocytes and several combinations of culture plates and media (Ciaramella, Ph.D. Thesis, 1995). The protocol used in this study (section 2.16.3) was the one that gave the best results in terms of the morphology of the cells and expression and induction of CYP2B1/2 mRNAs.

For an hepatocyte culture system to be of use in the study of drug metabolism and drug metabolising enzymes, it is necessary for the cells not only to support the expression and induction of phase I enzymes, but also for the products of these enzymes to be further detoxified by phase II enzymes, as they would be in the whole liver. To examine whether any phase II enzymes were expressed in our hepatocyte cultures, immunoblots were carried out using whole cell homogenates. The GST family of drug metabolising enzymes was chosen for analysis as it is the largest group of phase II drug metabolising enzymes. Also the different GST isoforms have distinct and well documented expression patterns which change dramatically during conventional hepatocyte culture.

GSTπ is not expressed in the adult rat liver, but it is found in the hyperplastic nodules of chemically induced hepatomas, well developed hepatomas and also in foetal hepatocytes (Sato *et al.*, 1984; Tee *et al.*, 1992).

However, when hepatocytes are cultured conventionally, there is a marked expression of this gene (Abramovitz *et al.*, 1989). It is consequently a widely used marker for the dedifferentiation, or foetalisation, of hepatocytes.

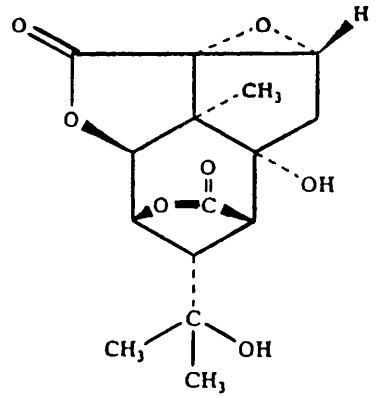
The levels of GSTα and GSTμ are also altered during primary hepatocyte culture. In general α class GSTs decrease and μ class GSTs are either maintained or increase (Abramovitz *et al.*, 1989; Vandenberghe *et al.*, 1992; Dwivedi *et al.*, 1993). While these changes are not as striking as those seen in the expression of GSTπ, they are also indicative of dedifferentiation of the hepatocytes.

In addition to the GST isoforms, the expression of NADPH-dependent cytochrome P450 reductase (P450 reductase) was examined. P450 reductase is required for the catalytic activity of cytochromes P450. If the hepatocytes are capable of carrying out biotransformation reactions, this enzyme must be present and its expression should be maintained during culture. Finally, the expression and induction of CYP2B1 and CYP2B2 were observed, as these enzymes were the focus of my thesis.

The effects of treating the hepatocytes with phenobarbital and with picrotoxin on the amounts of the enzymes discussed above were also observed. Picrotoxin is a toxic natural product used as an antidote to barbiturates and is a stimulator of the central nervous system. It is a molecular compound containing equal parts of picrotin (which is non-toxic) and picrotoxinin (the toxic component) (figure 3.23). Yamada *et al.* (1993) showed that picrotoxin induces the expression of CYP2B1 and CYP2B2 proteins and increases the activity of GST towards 1-chloro-2,4-dinitrobenzene (CDNB). They also noted that picrotoxin inhibits the binding of [^{3}H]PB to rat liver which would suggest that PB and picrotoxin

PICROTIN

M.W. 310.29



PICROTOXININ

M.W.292.28

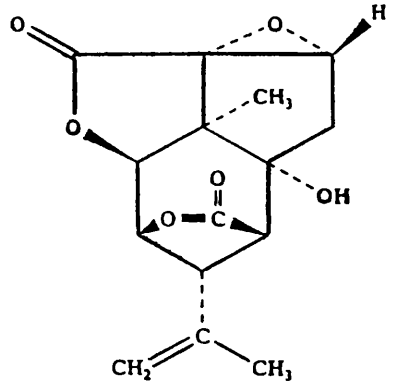


Figure 3.23: The structures of picrotin and picrotoxinin. Picrotoxin is a molecular compound containing equimolar amounts of picrotin and picrotoxinin.

act at the same site on the liver (Hamaguchi, T., Oguri, K., and Yashimura, H., unpublished observation). Treatment of the hepatocytes was also carried out to determine whether they were also responsive to another PB-like inducer.

3.4.1 Expression of Glutathione-S-Transferases (GSTs) in primary hepatocyte culture.

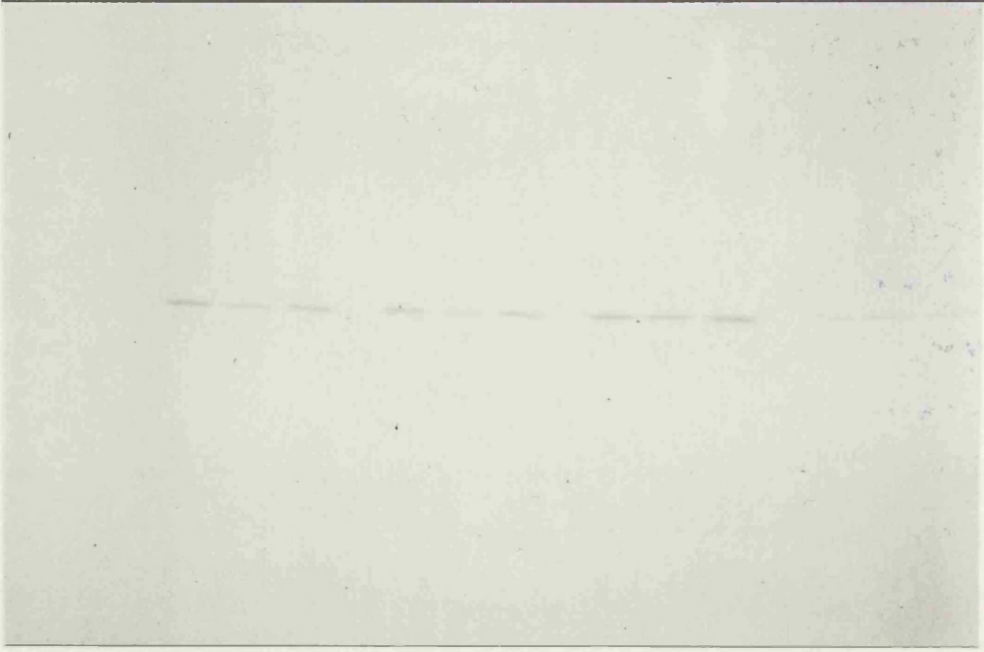
The results shown are from two separate culture experiments. I have counted the isolation and culture of hepatocytes from one animal as one culture. In describing these results, the GST 'subunit number' nomenclature is used (see table 1.3), as this is how the purchased antibodies were described. The expression of GST 2-2/1-1 (α class), GST 3-3/4-4 (μ class) and GST 7-7 (π class) was detected on the western blots using rabbit anti-rat GST 2-2, 3-3 and 7-7 sera purchased from Biotrin International. The anti-GST 2-2 serum cross reacts with GST 1-1 and the anti-GST 3-3 serum cross reacts with GST 4-4 (Biotrin technical bulletin) so that, for instance, immunoblots probed with the anti-GST 2-2 serum therefore detected both GST 1-1 and GST 2-2 expression. The effects of phenobarbital and of picrotoxin on the levels of these GST isoforms were observed.

3.4.1a Expression of GST 2-2/1-1 in primary rat hepatocytes.

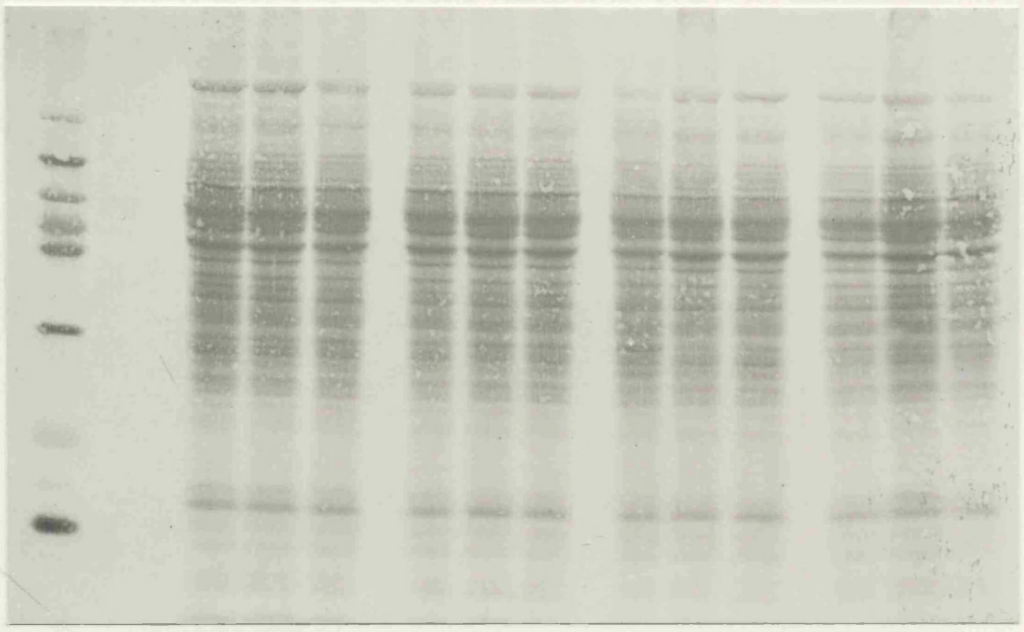
GST subunits 1 and 2 were detected after 72 hours and 96 hours of culture in both of the culture experiments. The length of culture time affected the separate cultures slightly differently. The hepatocytes in the first culture (half of which was treated with phenobarbital, figure 3.24) expressed GST2-

Anti-GST 2-2/1-1												
M	U 72			PB 72			U 96			PB 96		
1	2	3	4	5	6	7	8	9	10	11	12	13

A



B



C

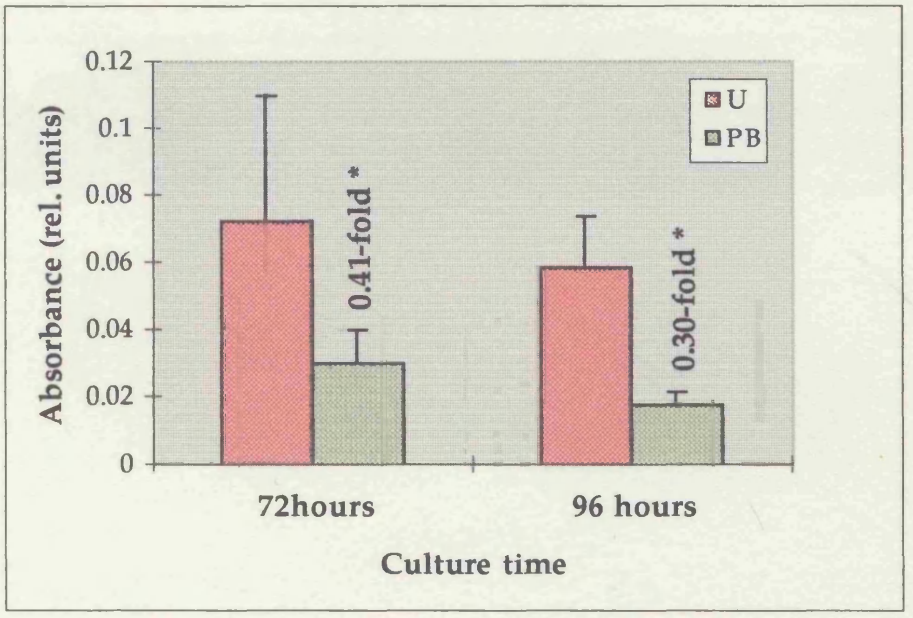
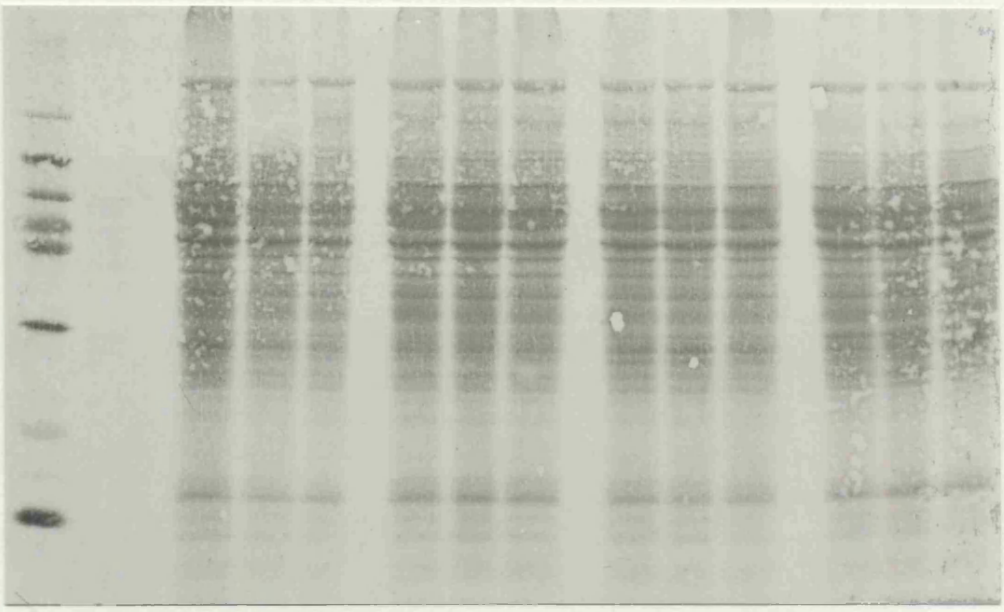


Figure 3.24: Western blot of whole cell homogenate from cultured primary hepatocytes probed with anti-GST 2-2/1-1. Primary hepatocytes from one rat liver were harvested after a total of 72 hours (tracks 1-6) or 96 hours (tracks 7-12) of culture. Cells were either untreated (U, tracks 1-3 and 7-9) or exposed to 0.75mM phenobarbital 48 hours after seeding until harvesting (PB, tracks 4-6 and 10-12). A minimum of 3 culture plates was pooled to make one cell pellet. (A) Each track contains 50µg of whole cell homogenate from one cell pellet. The proteins were separated through a 13% SDS polyacrylamide gel before blotting onto a nitrocellulose membrane. In addition a mid-range protein molecular weight marker was run on the gel (M). (B) The membrane was probed using a 1/500 dilution of the GST 2-2/1-1 antibody. The membrane was then stained with amido black to show the total protein loaded in each track. The blot was scanned using an imaging densitometer and the results for each sample were normalised against the amount of protein loaded. Graph C shows the average of the normalised results for each treatment condition. The error bars show the standard deviation. The numbers printed next to bars on the graph are fold differences (PB/U) for each culture period. A significance of $p < 0.05$ calculated by a two-tailed, paired, Student's T-test is denoted by *.

Anti-GST 2-2 / 1-1												
M	U 72			Px 72			U 96			Px 96		
1	2	3	4	5	6	7	8	9	10	11	12	13

A

B



C

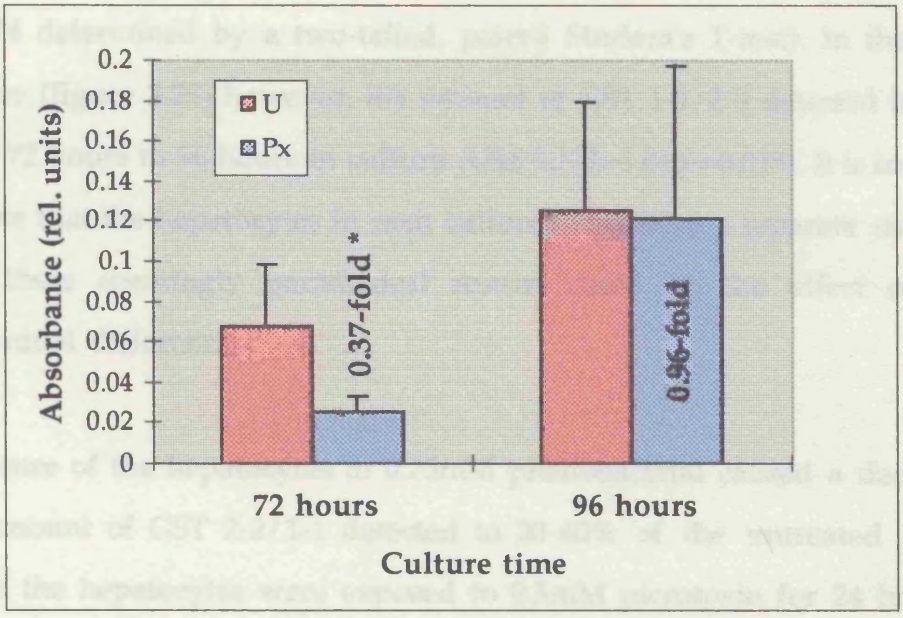


Figure 3.25: Western blot of whole cell homogenate from cultured primary hepatocytes probed with anti-GST 2-2/1-1. Primary hepatocytes were harvested after a total of 72 hours (tracks 1-6) or 96 hours (tracks 7-12) of culture. Cells were either untreated (U, tracks 1-3 and 7-9) or exposed to 0.5mM picrotoxin from 48 hours post-plating until harvesting (Px, tracks 4-6 and 10-12). A minimum of 3 culture plates was pooled to make one cell pellet. (A) Each track contains 50µg of whole cell homogenate from one cell pellet. The proteins were separated through a 13% SDS polyacrylamide gel before blotting onto a nitrocellulose membrane. In addition a mid-range protein molecular weight marker was run on the gel (M). The membrane was probed using a 1/500 dilution of the GST 2-2/1-1 antibody. (B) The membrane was then stained with amido black to show the total protein loaded in each track. The blot was scanned using an imaging densitometer and the results for each sample were normalised against the amount of protein loaded. Graph C shows the average of the normalised results for each treatment condition. The error bars show the standard deviation. The numbers printed in or next to bars on the graph are fold differences (Px/U) for each culture period. A significance of $p < 0.05$ calculated by a two-tailed, paired Student's T-test is denoted by *.

2/1-1 at approximately the same level at both time points (U96/U72=0.8; p=0.74 determined by a two-tailed, paired Student's T-test). In the second culture (figure 3.25) however, the amount of GST 1-1/2-2 detected increased from 72 hours to 96 hours in culture (U96/U72=1.84;p=0.018). It is important to note that the hepatocytes in each culture came from a separate animal, so that these seemingly paradoxical results could be the effect of inter-individual differences.

Exposure of the hepatocytes to 0.75mM phenobarbital caused a decrease in the amount of GST 2-2/1-1 detected to 30-40% of the untreated amount. When the hepatocytes were exposed to 0.5mM picrotoxin for 24 hours (Px 72) there was a decrease in the amount of GST 2-2/1-1 to 37% of the untreated amount. However, after 48 hours exposure to picrotoxin (Px 96), the amount of GST 2-2/1-1 had returned to the untreated level.

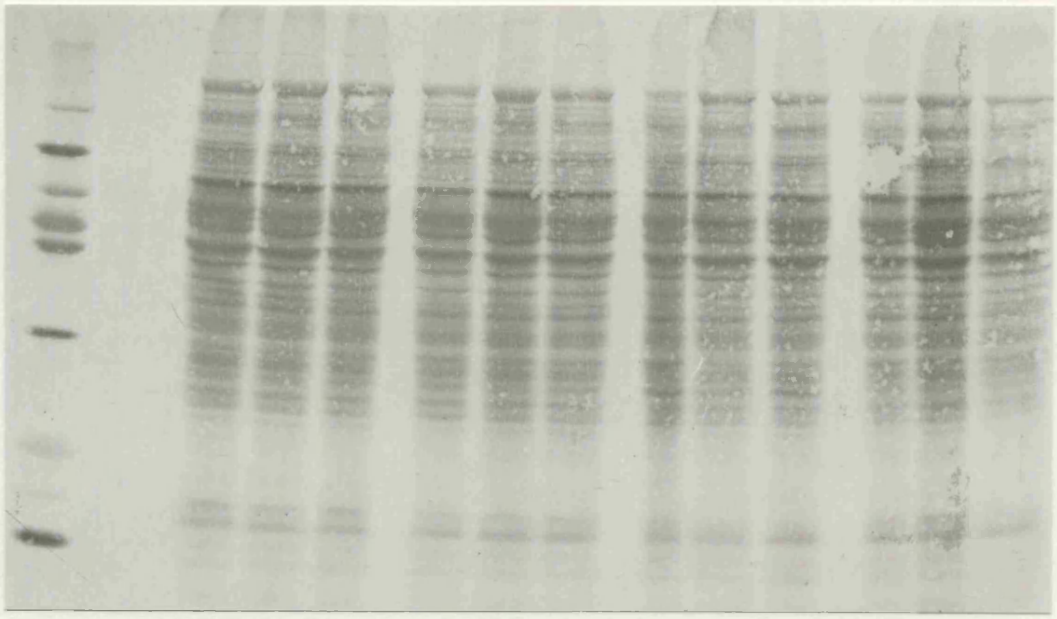
3.4.1b Expression of GST 3-3/4-4 in primary rat hepatocytes.

The amount of GST 3-3/4-4 detected increases by 4.25-fold (p=0.09, culture 1, figure 3.26) and 4.65-fold (p=0.07, culture 2, figure 3.27) between 72 and 96 hours. When the hepatocytes were exposed to phenobarbital for 24 hours (PB 72), there was a slight increase (1.18-fold) in the amount of GST 3-3/4-4. After 48 hours treatment with phenobarbital, however, the amount of GST 3-3/4-4 was only 40% of the untreated level. Exposure to picrotoxin had a similar effect on the amount of GST 3-3/4-4 as on GST 2-2/1-1, in that, after 24 hours of treatment, there was a significant decrease to 55% of the untreated level, but after 48 hours treatment, the amount was the same as the untreated level.

Anti-GST 3-3 / 4-4													
M	+	U 72			PB 72			U 96			PB 96		
1	2	3	4	5	6	7	8	9	10	11	12	13	14

A

B



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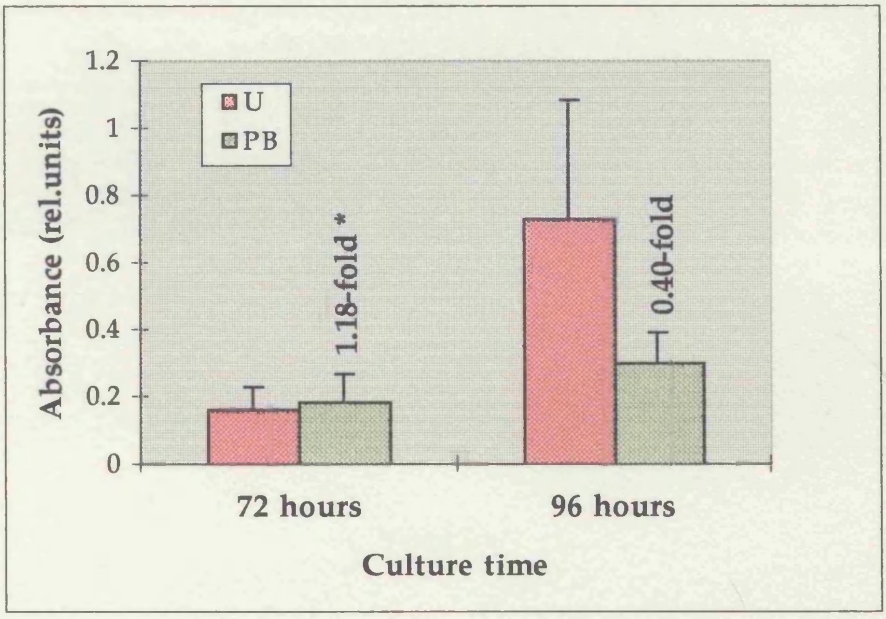
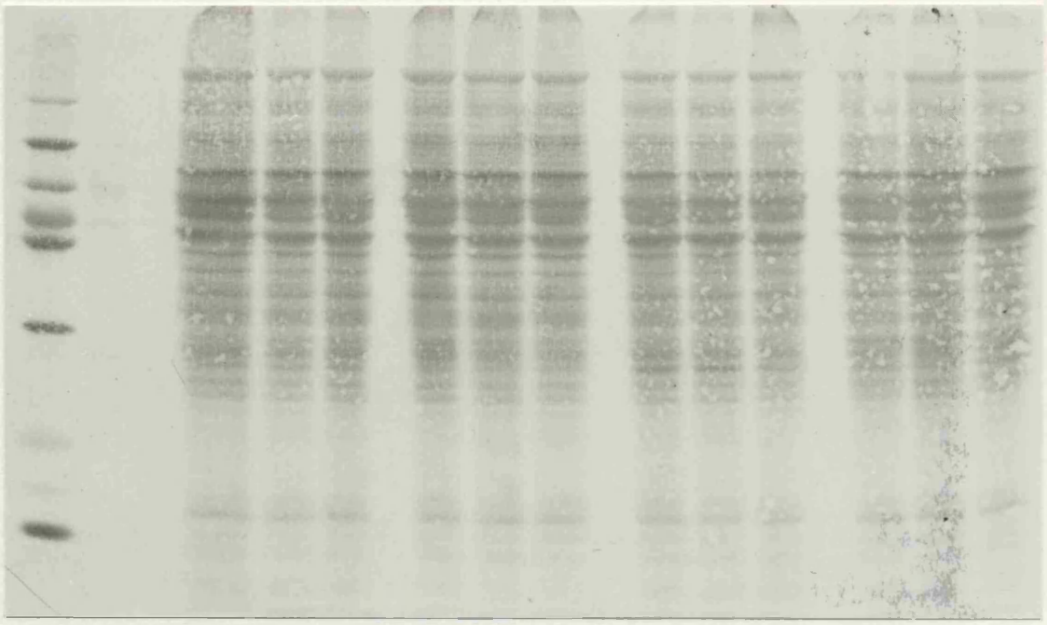


Figure 3.26: Western blot of whole cell homogenate from cultured primary hepatocytes probed with anti-GST 3-3/4-4. Primary hepatocytes were harvested after a total of 72 hours (tracks 1-6) or 96 hours (tracks 7-12) of culture. Cells were either untreated (U, tracks 1-3 and 7-9) or exposed to 0.75mM phenobarbital from 48 hours post-plating until harvesting (PB, tracks 4-6 and 10-12). A minimum of 3 culture plates was pooled to make one cell pellet. (A) Each track contains 50µg of whole cell homogenate from one cell pellet. The proteins were separated through a 13% SDS polyacrylamide gel before blotting onto a nitrocellulose membrane. In addition a mid-range protein molecular weight marker (M) and 3µg of an extra-nuclear fraction from the liver of a rat treated with phenobarbital (+) were run on the gel. The membrane was probed using a 1/500 dilution of the GST 3-3/4-4 antibody. (B) The membrane was then stained with amido black. The blot was scanned using an imaging densitometer and the results for each sample were normalised against the amount of protein loaded. Graph C shows the average of the normalised results for each treatment condition. The error bars show the standard deviation for each treatment condition. The numbers printed next to the bars on the graph are fold differences (PB/U) for each culture period. A significance of $p < 0.05$ calculated by a two-tailed, paired, Student's T-test is denoted by *.

Anti-GST 3-3 / 4-4													
M	+	U 72			Px 72			U 96			Px 96		
1	2	3	4	5	6	7	8	9	10	11	12	13	14

A

B



C

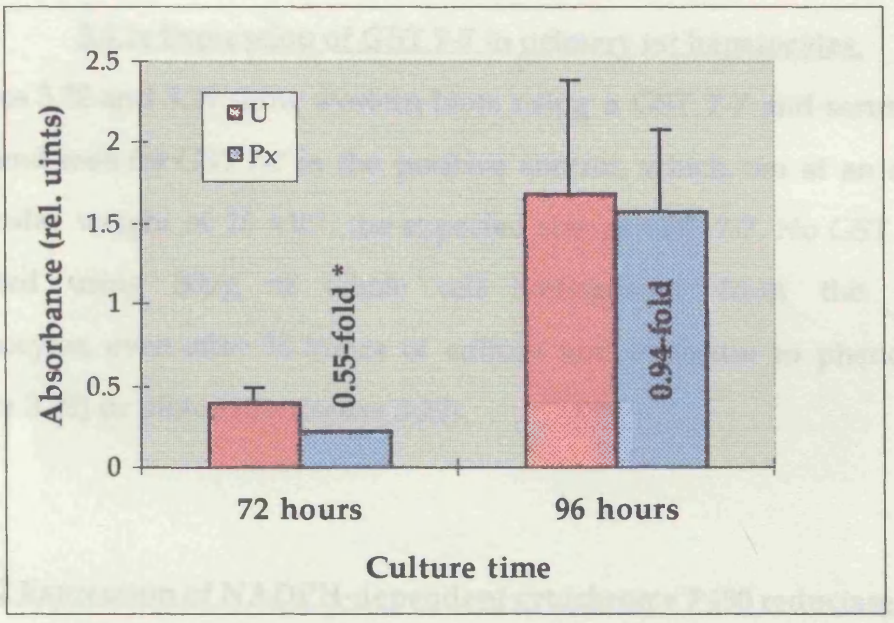


Figure 3.27: Western blot of whole cell homogenate from cultured primary hepatocytes probed with anti-GST 3-3/4-4. Primary hepatocytes were harvested after a total of 72 hours (tracks 1-6) or 96 hours (tracks 7-12) of culture. Cells were either untreated (U, tracks 1-3 and 7-9) or exposed to 0.5mM picrotoxin from 48 hours post-plating until harvesting (Px, tracks 4-6 and 10-12). A minimum of 3 culture plates was pooled to make one cell pellet. (A) Each track contains 50µg of whole cell homogenate from one cell pellet. The proteins were separated through a 13% SDS polyacrylamide gel before blotting onto a nitrocellulose membrane. In addition a mid-range protein molecular weight marker was run on the gel (M). The membrane was probed using a 1/500 dilution of the GST 3-3/4-4 antibody. The membrane was then stained with amido black (B). The blot was scanned using an imaging densitometer and the results for each sample were normalised against the amount of protein loaded. Graph C shows the average of the normalised results for each treatment condition. The error bars show the standard deviation for each treatment condition. The numbers printed in or next to bars on the graph are fold differences (Px/U) for each culture period. A significance of $p < 0.05$ calculated by a two-tailed, paired, Student's T-test is denoted by *.

3.4.1c Expression of GST 7-7 in primary rat hepatocytes.

Figures 3.28 and 3.29 show western blots using a GST 7-7 anti-serum. There is a band seen for GST 7-7 in the positive control, which ran at an apparent molecular weight of 25 $x10^3$, the expected size of GST 7-7. No GST 7-7 was detected using 50µg of whole cell homogenate from the primary hepatocytes, even after 96 hours of culture and exposure to phenobarbital (figure 3.28) or picrotoxin (figure 3.29).

3.4.2 Expression of NADPH-dependent cytochrome P450 reductase (P450 reductase) in primary rat hepatocytes.

The expression and induction of this enzyme in the primary hepatocytes was detected using immunoblots probed with a rabbit anti-rat P450 reductase serum previously prepared by Dr E.A. Shephard.

P450 reductase was detected as a band at the expected apparent molecular weight of 78 $x10^3$. The amount of the enzyme remained the same between 72 and 96 hours of culture. The fold-difference between the amounts of P450 reductase in untreated hepatocytes for the two culture times is 0.94 for culture 1 (p=0.31, figure 3.30) and 1.04 for culture 2 (p=0.002, figure 3.31). Exposure to phenobarbital for 24 hours caused a slight decrease in the amount to 83% of the untreated level, but after 48 hours of phenobarbital treatment, there was an induction of 1.48-fold. Picrotoxin had little or no effect after 24 hours of treatment, but 48 hours of exposure resulted in a 1.28-fold increase in the detected amount of P450 reductase.

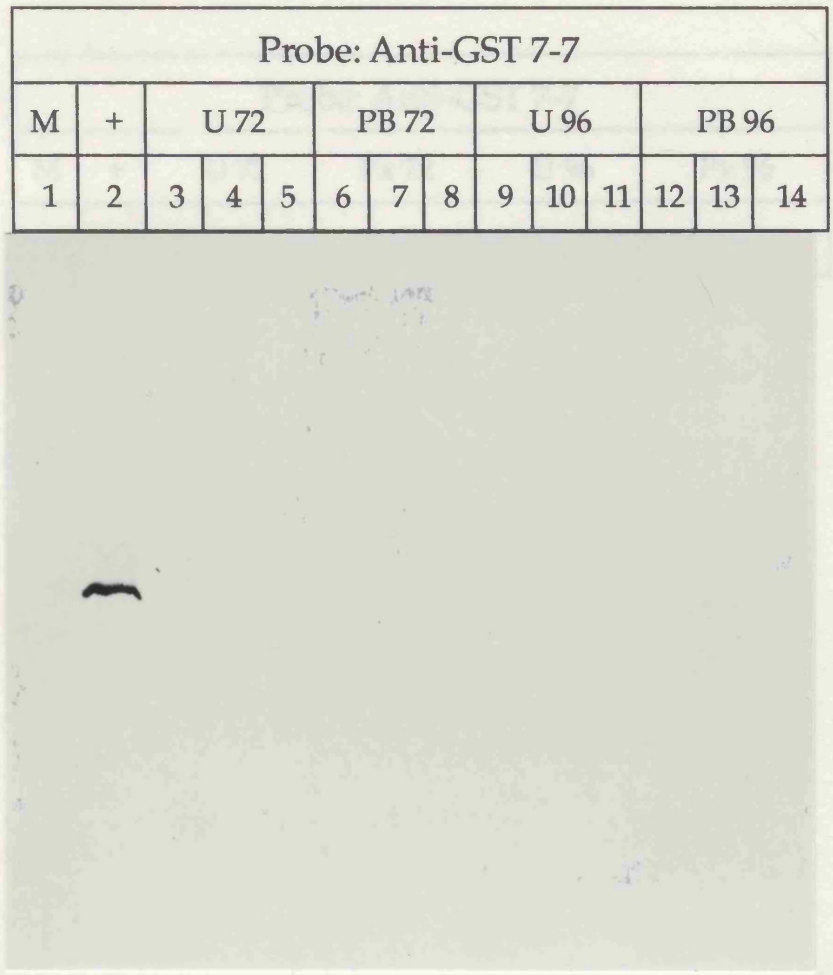


Figure 3.28: Western blot of whole cell homogenates from primary hepatocytes probed with anti-GST 7-7. The Western blot was prepared as in figure 3.26, except that the positive control (+) was 40µg of whole cell extract from HaCaT immortalised human keratinocytes. The blot was probed with a 1/200 dilution of the GST 7-7 antibody.

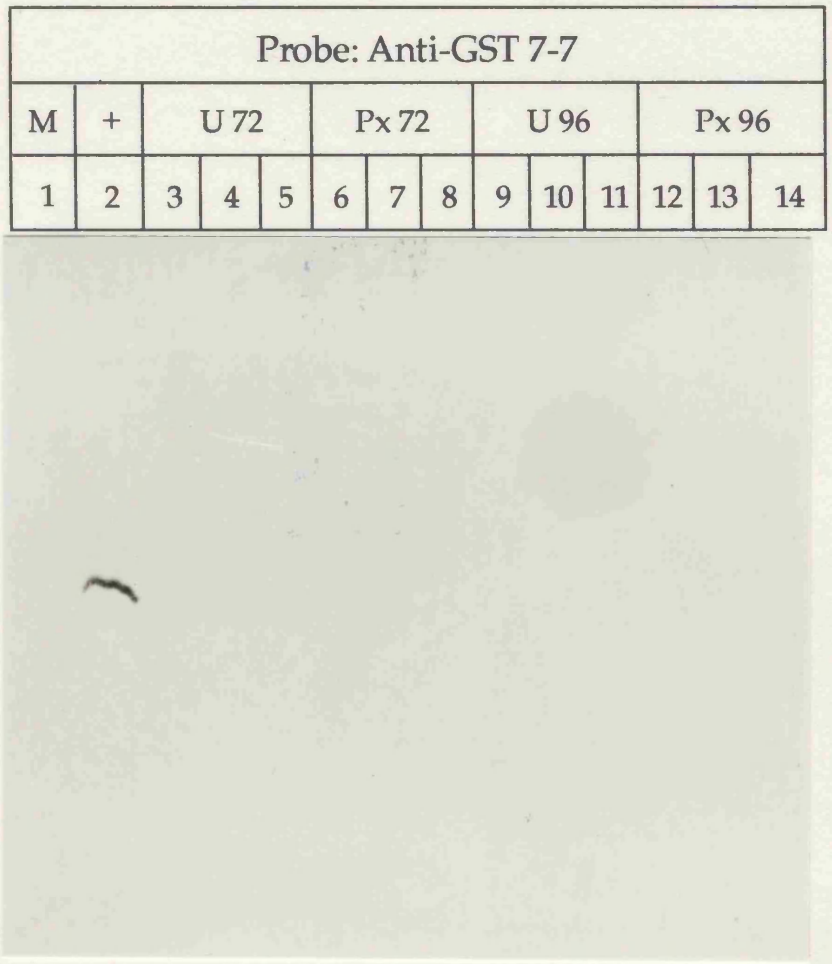
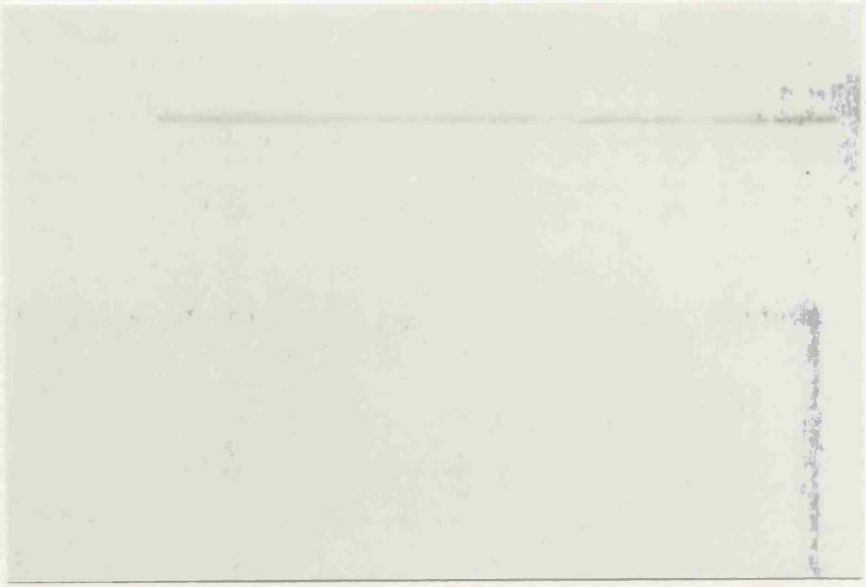


Figure 3.29: Western blot of whole cell homogenates from primary hepatocytes probed with anti-GST 7-7. The Western blot was prepared as in figure 3.27, except that the positive control (+) was 40μg of whole cell extract from HaCaT immortalised human keratinocytes. The blot was probed with a 1/200 dilution of the GST 7-7 antibody.

Anti-P450 Reductase													
M	+	U 72			PB 72			U 96			PB 96		
1	2	3	4	5	6	7	8	9	10	11	12	13	14

A



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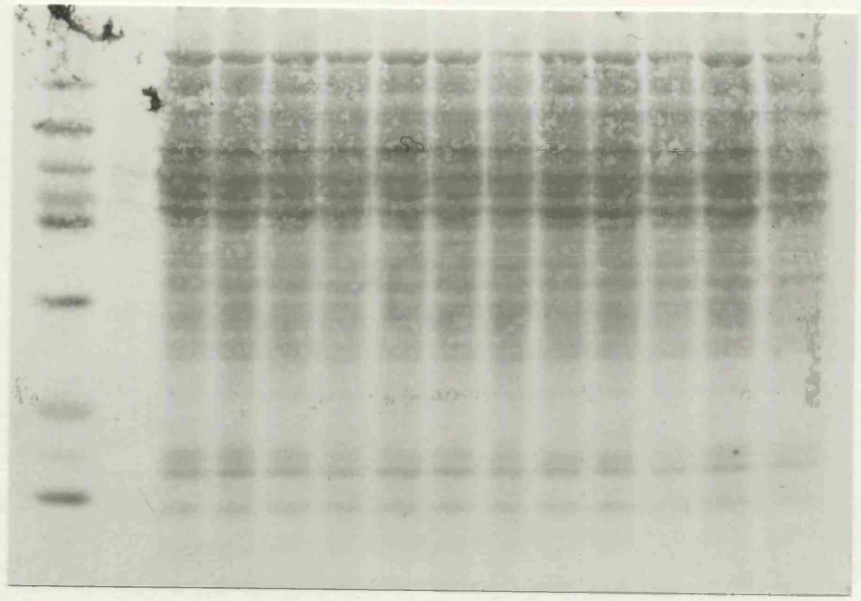


C

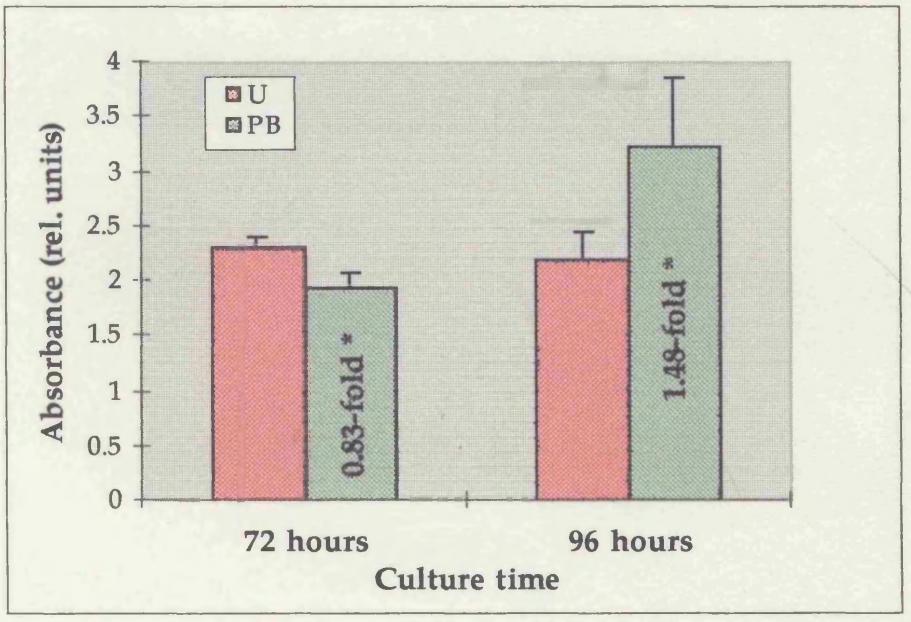
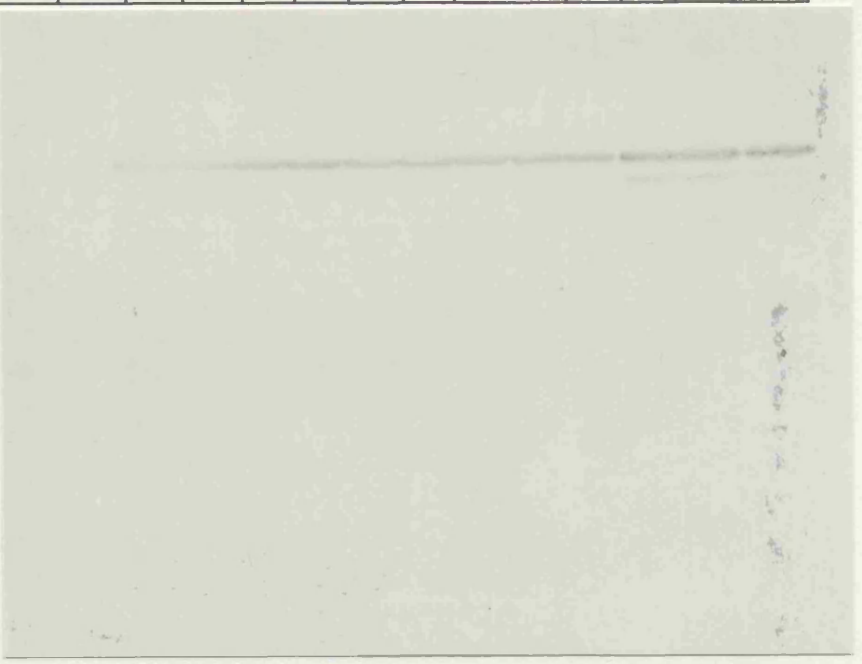


Figure 3.30: Western blot of whole cell homogenate from cultured primary hepatocytes probed with anti-P450 reductase. The blot was prepared as previously (figure 3.26). The positive control (+, track 2) is 1µg of the extra-nuclear fraction from untreated rat liver. The membrane was probed using a 1/500 dilution of the anti-P450 reductase serum(A). The membrane was then stained with amido black (B) to show the total protein loaded in each track. Graph C shows the average of the normalised results for each treatment condition. The error bars show the standard deviation. The numbers printed in the bars on the graph are fold differences (PB/U) for each culture period. A significance of $p < 0.05$ calculated by a two-tailed, paired, Student's T-test is denoted by *.

Anti-P450 Reductase													
M	+	U 72			Px 72			U 96			Px 96		
1	2	3	4	5	6	7	8	9	10	11	12	13	14

A



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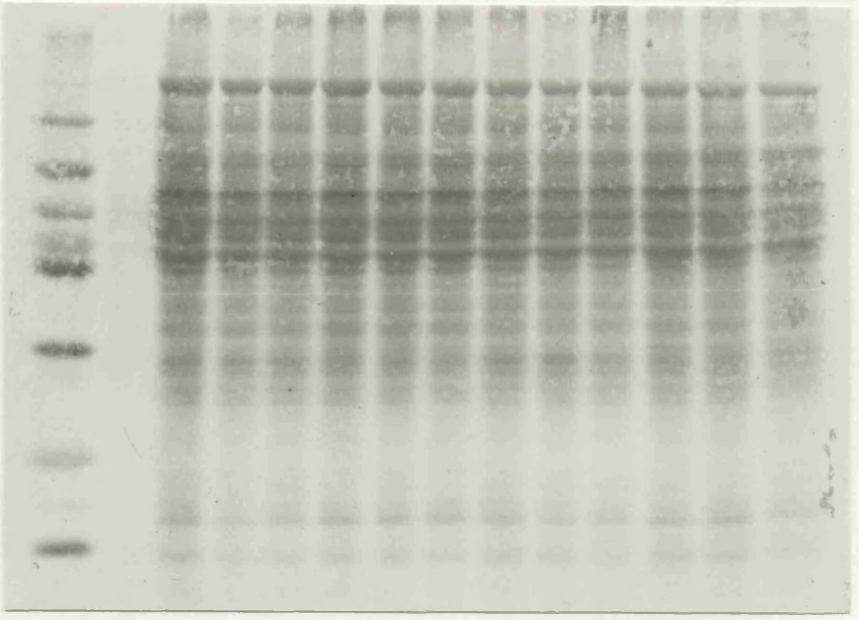

C

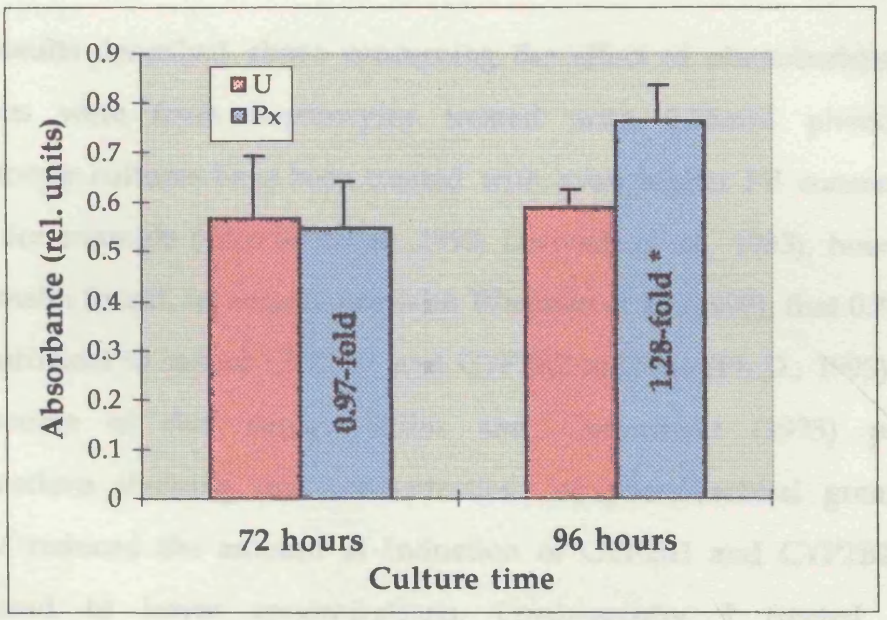

Figure 3.31: Western blot of whole cell homogenate from cultured primary hepatocytes probed with anti-P450 reductase. The blot was prepared as previously (figure 3.27). The positive control (+, track 2) is 1µg of the extra-nuclear fraction from untreated rat liver. The membrane was probed using a 1/500 dilution of the anti-P450 reductase serum(A). The membrane was then stained with amido black (B) to show the total protein loaded in each track. Graph C shows the average of the normalised results for each treatment condition. The error bars show the standard deviation. The numbers printed in the bars on the graph are fold differences (Px/U) for each culture period. A significance of $p < 0.05$ calculated by a two-tailed, paired, Student's T-test is denoted by *.

3.4.3 Expression of CYP2B1 and CYP2B2 in primary hepatocyte culture.

The results described above concerning the effect of phenobarbital on the cultures were from hepatocytes treated with 0.75mM phenobarbital. Hepatocyte cultures have been treated with even higher PB concentrations, 2mM for example (Akrawi *et al.*, 1993; Dwivedi *et al.*, 1993), however Dr. Ciaramella found, in accordance with Waxman *et al.* (1990), that 0.75mM PB was sufficient to induce CYP2B1 and CYP2B2 mRNAs (Ph.D., 1995). During the course of this study, Sidhu and Omiecinski (1995) published observations showing that concentrations of phenobarbital greater than 0.5mM reduced the amount of induction of CYP2B1 and CYP2B2 mRNA compared to lower concentrations. Consequently, I treated primary hepatocytes cultured using our system with various concentrations of phenobarbital and observed the effect using western blot and slot blot analysis.

The slot blot (figure 3.32) was prepared using RNA made with the guanidine thiocyanate method (section 2.11.2). Figure 3.35a shows the agarose gel run to check the integrity and concentration of the RNA. The 28S, 18S and 5.8S bands are sharp and there is no evidence of degradation or protein or DNA contamination of the samples. 15µg total RNA from each treatment were loaded onto a slot blot in triplicate. The blot was hybridized with a probe of ^{32}P radiolabelled DNA made using the random priming method with the plasmid 4G12 as a template. This plasmid contains a 1400bp fragment of the CYP2B2 cDNA* in the pAT153 vector. The probe is capable of hybridization to both CYP2B1 and CYP2B2 mRNA (Phillips *et al.*, 1983).

* (from amino acid 168 to the poly A tail)

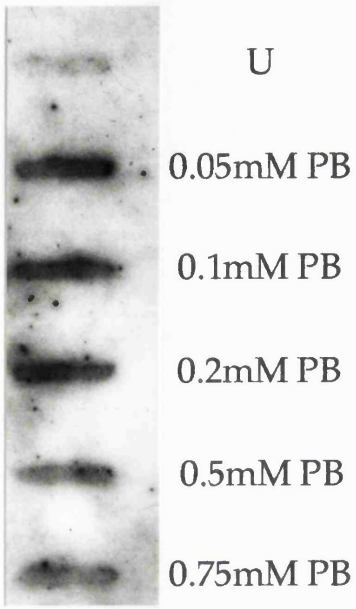

Figure 3.32: Slot blot showing CYP2B1/2 mRNA from primary hepatocytes treated with varying amounts of phenobarbital. Total RNA was extracted from primary hepatocyte cell pellets using the guanidine thiocyanate method. Each cell pellet contained cells from four culture plates. 15µg of RNA from one cell pellet of each condition were loaded onto the slot blot. The hepatocytes were untreated (U), or treated with 0.05mM. 0.1mM, 0.2mM, 0.5mM or 0.75mM phenobarbital (PB) from 48 hours post-plating for 24 hours until the cells were harvested. The blot was probed with a CYP2B1/2 probe made using the random priming method from the p4G12 template.

Whole cell homogenates were made from cell pellets from the same hepatocyte culture. These were used to prepare western blots to determine the relative quantities of CYP2B1/2 protein present in the cells (figure 3.33).

The results of the two blots are presented in figure 3.34. The Western blot did not detect any CYP2B1/2 in the untreated sample. The fold inductions are therefore calculated from the slot blot results only. Phenobarbital treatment increases the amount of the CYP2B1/2 mRNA and of both the CYP2B1 and CYP2B2 proteins. The relative amount of CYP2B1 protein is far greater than that of the CYP2B2 protein. As the two bands were so close together, it was not possible to distinguish between the two during the densitometric analysis, and the results on the graph are a sum of both bands.

A similar trend was observed in relative amounts of both the protein and the mRNA. The greatest response to the phenobarbital treatment was seen at concentrations of 0.05mM to 0.2mM. The maximal induction was 2.74-fold at 0.2mM PB, however the amounts of mRNA were very similar between 0.05mM and 0.2mM. The relative amounts of CYP2B1/2 dropped off after the concentration of PB was raised to 0.5mM and 0.75mM.

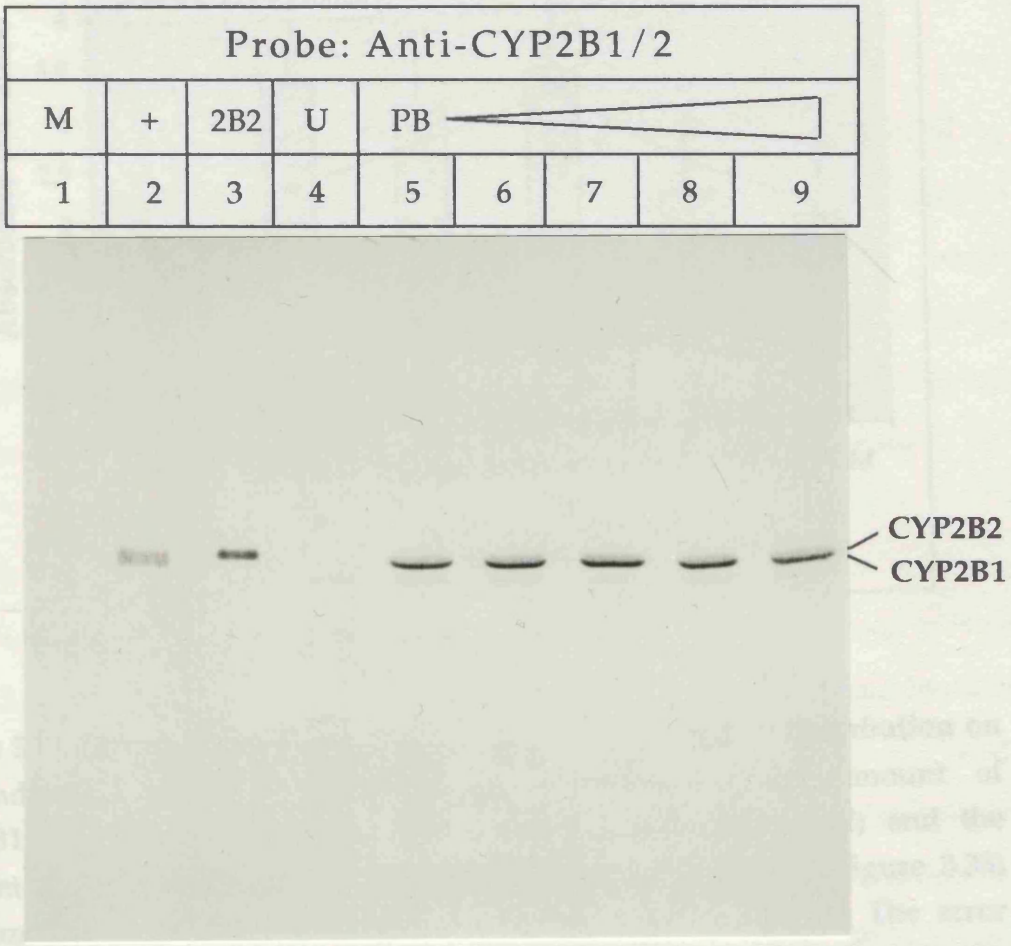

Figure 3.33: Western blot of primary hepatocyte whole cell homogenates using anti-CYP2B1/2 serum. Primary hepatocytes were either untreated (U) or exposed to 0.05mM, 0.1mM, 0.2mM, 0.5mM or 0.75mM phenobarbital (tracks 5 to 9 respectively) from 48 hours of culture until harvesting at 72 hours of culture. 50µg of whole cell homogenate from each cell pellet were separated on a 10% SDS-PAGE gel before blotting onto a nitrocellulose filter. Also loaded onto the gel was a mid-range protein molecular weight marker (M), 3µg of the extra-nuclear fraction from the livers of phenobarbital treated rats (+), and 10ng of purified CYP2B2 (2B2). The two different CYP2B isoforms are identified to the right of the photograph.

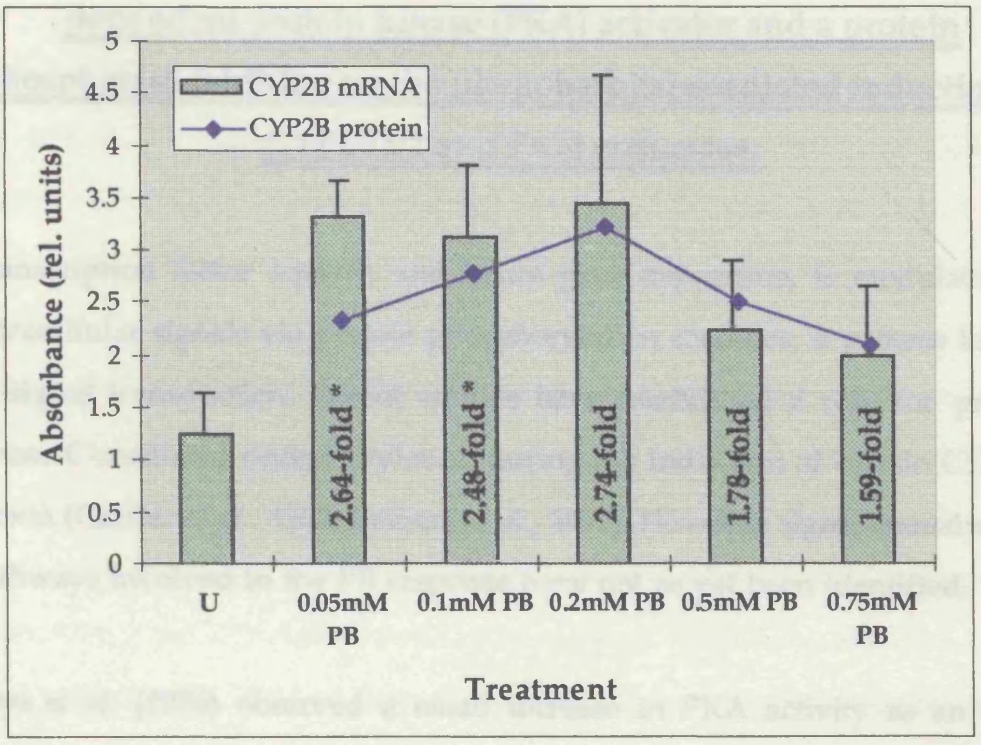

Figure 3.34: Graph of the effect of changing phenobarbital concentration on the induction of CYP2B1/2 in primary hepatocytes. The amount of CYP2B1/2 mRNA observed on the slot blots (e.g. figure 3.32) and the amount of CYP2B1/2 protein seen on the Western blots (e.g. figure 3.33) were measured densitometrically and are shown on the graph. The error bars are the standard deviation. The figures associated with the bars in the histogram are the fold induction (PB/U) of the mRNA for each treatment. A significance of $p < 0.05$ as determined using a two-tailed, paired, Student's T-test is denoted by *.

3.5 The effects of a cyclic AMP (cAMP) analogue, a cAMP-dependent protein kinase (PKA) activator and a protein phosphatase inhibitor on the phenobarbital-mediated induction of CYP2B1/2 and P450 reductase.

Transcription factor activity, and hence gene expression, is modulated by extracellular signals via protein phosphorylation cascades, a process known as signal transduction. Recent studies have established a role for protein kinase C-mediated phosphorylation during the induction of certain CYPs by dioxin (Carrier *et al.*, 1993; Reiners *et al.*, 1993). However signal transduction pathways involved in the PB response have not as yet been identified.

Buys *et al.* (1976) observed a small increase in PKA activity as an early response to phenobarbital treatment *in vivo*. Also, the treatment of rats with cAMP analogues (which activate PKA) inhibited the increase in total CYP content in response to phenobarbital treatment (Hutterer *et al.*, 1975). In accordance with this result, a recent study by Sidhu and Omiecinski (1995) using cultured primary rat hepatocytes showed that cAMP analogues and a specific PKA activator inhibited the accumulation of CYP2B1, CYP2B2 and CYP3A1 mRNAs after phenobarbital treatment.

In contrast to this, Nirodi *et al.* (1996) found that treatment of rats with 2-aminopurine (a general protein kinase inhibitor) also leads to the inhibition of the PB-mediated increase of CYP2B1/2 mRNA. It has also been reported that cAMP potentiates the induction of CYP synthesis by PB in isolated hepatocytes (Canepa, *et al.*, 1985).

My results (figure 3.21) indicated that phosphorylation plays a part in the binding of transcription factor(s) to the proximal *CYP2B2* promoter at a site

possibly involved in the phenobarbital response. In an attempt to elucidate the role that PKA plays in the PB response, I treated primary hepatocytes with 8-(4-chlorophenylthio)-cAMP [8-CPT, a cAMP analogue (Sandberg *et al.*, 1991)], the S-phosphorothioate stereoisomer of cAMP [(Sp)cAMPS, a potent PKA activator (Rothermel and Botelho, 1988)] and okadaic acid [(Ok), a protein phosphatase inhibitor (reviewed Cohen *et al.*, 1990)].

Cell pellets from each treatment were used to prepare RNA using Ultraspec™ solution (section 2.11.2). Previously a sample of COS 7 cells had been used to prepare RNA in the same way, to test out the procedure. Figure 3.35b shows part of the agarose gel run to check the integrity of the RNA. Using the Ultraspec™ solution to prepare RNA from the COS 7 cells gave a reasonable yield of very clean RNA. There was no smearing on the gel (figure 3.35b, track 5), and no evidence of protein in the sample or degradation of the RNA. However when RNA was prepared from the hepatocyte cell pellets using the Ultraspec™ solution, the yield was very low, and the RNA contained contaminants. The gel shows smearing in all of the tracks (1-4, figure 3.35b), evidence of protein contamination, although the 28S and 5.8S bands can be seen on the gel which suggested that there was some RNA present. This difference in the quality of RNA produced from the different cell types is possibly due to the fact the hepatocytes were cultured on collagen, whilst the COS 7 cells were not. Either a collagenase digest should be carried out before the cells are pelleted or a lot more Ultraspec™ solution would need to be added to remove the contaminants. As the yield of RNA was so low, extraction with phenol:chloroform was not practical. A slot blot was prepared using this RNA, however it would not hybridize to any probe and it was abandoned.

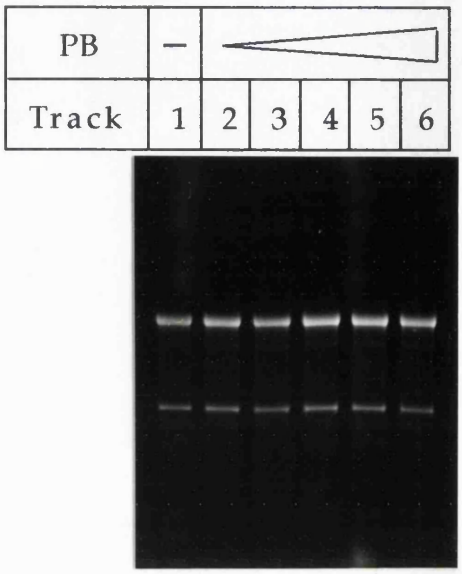

Figure 3.35a: Preparation of total RNA from primary hepatocytes using the guanidine thiocyanate method. Primary hepatocytes were untreated (-) or exposed to 0.05mM, 0.1mM, 0.2mM, 0.5mM or 0.75mM phenobarbital (tracks 2-5 respectively) from 48 hours post-plating until harvesting at 72 hours of culture. Total RNA was prepared from the hepatocyte cell pellets using the guanidine thiocyanate method. 5µg was run on a denaturing agarose gel to check integrity.

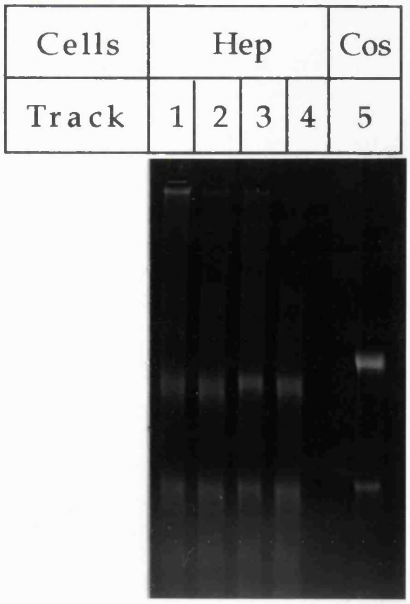

Figure 3.35b: Preparation of total RNA from primary hepatocytes and Cos 7 cells using Ultraspec solution. Primary hepatocytes (Hep) were either untreated (tracks 1 and 3) or treated with 0.75mM phenobarbital from 48 hours of culture (tracks 2 and 4) until harvesting at 72 hours of culture. A pellet of untreated Cos 7 cells (Cos) was also prepared. Total RNA was extracted from the cells using Ultraspec solution and run on a denaturing agarose gel to check its integrity.

A second culture was carried out and RNA was prepared from one cell pellet of each condition using the guanidine thiocyanate method. This produced a large yield of uncontaminated, intact RNA (results not presented). 15μg of each sample was loaded on to a slot blot in triplicate and the blot was hybridized to a CYP2B1/2 probe as previously (section 3.4.3) (figure 3.36). This hybridization was carried out together with the blot in figure 3.32.

The results of this blot are presented in figure 3.37, however the slot blot did not detect the amount of phenobarbital-mediated induction of CYP2B2 mRNA that would be expected from the amounts of CYP2B1/2 protein detected and from previous studies on this culture system (Ciaramella, PhD. thesis; discussed further in section 3.7.1). It seems that either the hybridization conditions are at fault, or that the blot had been stripped too many times. It is possible that the probe is detecting some other RNA species as well as the CYP2B1/2. It would have been preferable to have carried out a northern blot or RNase protection assay to ensure that only CYP2B1/2 mRNA was detected. However, at this point time was limited, and these were not attempted. As the results are not reliable, they have been excluded from further discussion.

Whole cell homogenates were made from a second cell pellet of each condition from the same culture that the RNA was made from. Western blots were prepared and were probed with anti-CYP2B1/2 (figure 3.38) and then reprobed with anti-P450 reductase (figure 3.38). Excepting one, none of the samples showed any CYP2B1/2 protein, so it was not possible to analyse them densitometrically. The P450 reductase results were analysed densitometrically and are presented in figure 3.39. The only sample in which CYP2B1 and CYP2B2 were detected was from the cells which had

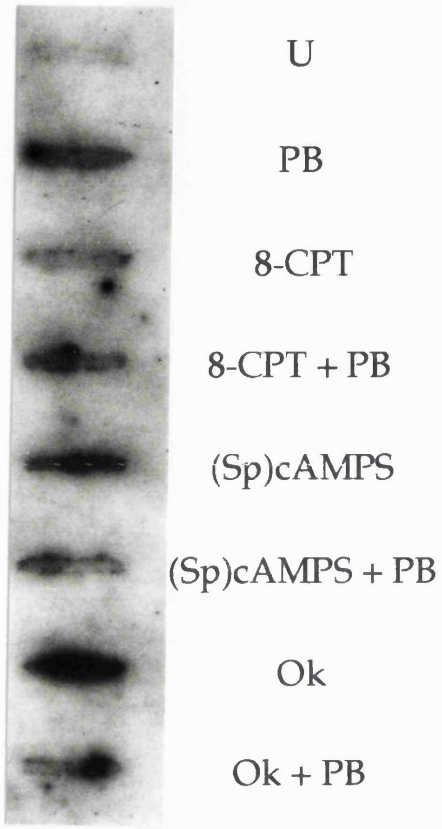

Figure 3.36: Slot blot showing CYP2B1/2 mRNA from primary hepatocytes treated with 8-CPT, (Sp)cAMPS or okadaic acid. Primary hepatocytes were untreated (U), treated with 0.1mM phenobarbital (PB), 10μM 8-(4-chlorophenythio)-cAMP (8-CPT), 10μM (Sp)cAMPS or 100nM okadaic acid (Ok). The cells were harvested 72 hours post-plating. The cells were exposed to 8-CPT, (Sp)cAMPS or okadaic acid at 48 hours post-plating for one hour. The medium was removed and fresh medium was added which contained the same chemical wit,h or withou,t 0.1mM phenobarbital. The untreated cells, and cells treated with phenobarbital only, underwent the same pattern of medium changes. Total RNA was extracted from primary hepatocyte cell pellets using the guanidine thiocyanate method. Each cell pellet contained cells from four culture plates. 15μg of RNA from one cell pellet of each condition were loaded onto the slot blot. The blot was probed with a CYP2B1/2 specific probe made using the random priming method from the p4G12 template.

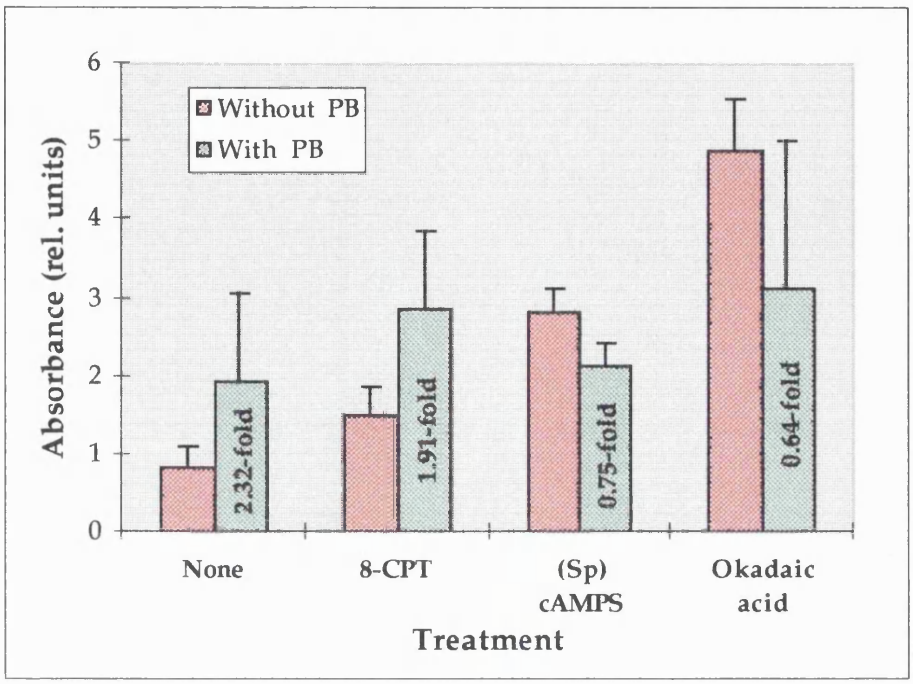

Figure 3.37: Graph of the effect of 8-CPT, (Sp)cAMPS and okadaic acid on the amount of CYP2B1/2 mRNA. The relative amounts of CYP2B1/2 mRNA observed on the slot blot shown in figure 3.36 and on two other blots prepared the same way were determined densitometrically. The numbers printed in the bars on the graph are fold differences (With PB/ without U) for each treatment. A two-tailed, paired, Student's T-test was carried out, but none of the differences were significant.

Probes: Anti-CYP2B1/2 and Anti-P450 Reductase										
Sample	M	+	1	2	3	4	5	6	7	8
Track	1	2	3	4	5	6	7	8	9	10

Figure 3.38: Western blot to show the effect of 8-CPT, (Sp)cAMPS, and okadaic acid on the amounts of CYP2B1/2 and P450 reductase in primary heptocytes. Whole cell homogenates were prepared from one cell pellet containing primary hepatocytes from 3 culture plates each. The hepatocytes were treated with 8-CPT (samples 3 and 4), (Sp)cAMPS (samples 5 and 6) and okadaic acid (samples 7 and 8). In addition samples 2, 4, 6 and 8 were treated with phenobarbital. Sample 1 remained untreated. The treatment regimen was carried out as described in figure 3.36. 50µg of each whole cell homogenate was separated on a 10% polyacrylamide gel and blotted onto a nitrocellulose filter. Also loaded onto the gel were 1µg of mid-range protein molecular weight marker (M) and 3µg of the extra-nuclear fraction prepared from the livers of phenobarbital treated rats (+). The filter was probed with a 1/100 dilution of anti-CYP2B1/2 serum and then re-probed with a 1/500 dilution of anti-P450 reductase serum. The different proteins detected are labelled on the left of the photograph.

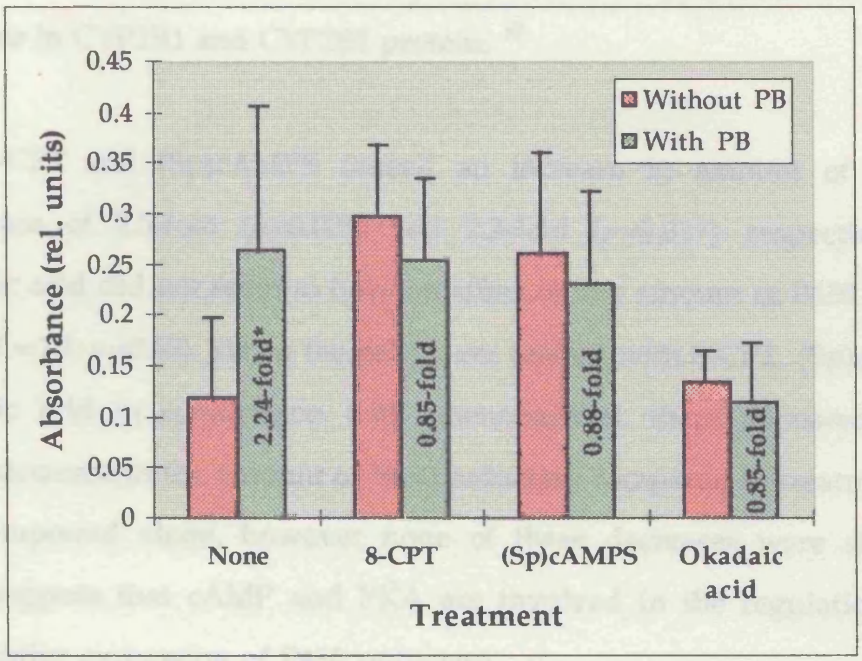

Figure 3.39: Graph of the effect of 8-CPT, (Sp)cAMPS and okadaic acid on the amount of P450 reductase. The relative amounts of P450 reductase observed on the western blot shown in figure 3.38 and on two other blots prepared the same way were determined densitometrically. The results were normalised against the amount of protein loaded in each track (the amido black stained filter is not presented) and plotted on a graph. The numbers printed in the bars on the graph are fold differences (With PB/ without U) for each treatment. A significance of $p < 0.05$ calculated by a two-tailed, paired, Student's T-test is denoted by *.

been treated with phenobarbital alone. The treatments with 8-CPT, (Sp)cAMPS and okadaic acid all inhibited the phenobarbital induced increase in CYP2B1 and CYP2B2 protein. *

The 8-CPT and (Sp)cAMPS caused an increase in amount of the P450 reductase of 2.5-fold (p=0.025) and 2.2-fold (p=0.017) respectively. The okadaic acid did not seem to have an effect on the amount of P450 reductase (Ok/U = 1.1; p=0.68). When the cells were treated with 8-CPT, (Sp)cAMPS or okadaic acid in conjunction with phenobarbital, there appeared to be a slight decrease in the amount of P450 reductase compared to treatment with the compound alone, however none of these decreases were significant. This suggests that cAMP and PKA are involved in the regulation of the constitutive expression of P450 reductase.

Treatment with 0.1mM PB caused a larger induction of P450 reductase than treatment with 0.75mM PB (figure 3.30). There was a 2.24-fold increase with 0.1mM PB after 72 hours as compared to a 1.48-fold increase with 0.75mM PB after 96 hours. However these are results from separate cultures and should be interpreted with caution, as this difference could be due to interindividual differences between the animals and the viability of the cells obtained from them.

* It may be that the 8-CPT, (Sp)cAMPS and okadaic acid are cytotoxic to the cells at the concentration used. This would seem unlikely as the expression of P450 reductase is maintained, however it is possible.

3.6 Analysis of the 5' flanking sequence of the *CYP2B2* gene using the dual luciferase reporter gene assay.

3.6.1 Cloning for the luciferase assay.

My aim was to create a series of constructs containing the firefly luciferase reporter gene regulated by various sections of the *CYP2B2* 5' flanking region. Several constructs had been used in a luciferase assay in the laboratory previously, but no expression of the luciferase gene by these constructs could be detected (Ciaramella, Ph.D. Thesis, 1995). It was thought that a possible reason for this was that the constructs did not include the translation start site for the gene. Trottier *et al.*, 1995 reported an PB-mediated induction of 6.6-fold using a -2318 to +31bp fragment of the *CYP2B2* gene promoter attached to a chloramphenicol acetyl transferase reporter gene and transfected into primary rat hepatocytes.

The clone λP4502B2 containing the full length *CYP2B2* gene (6.4kb) and 5.6kb of the 5' flanking sequence of the gene, cloned into the Eco RI site of pBluescript had previously been constructed by the laboratory (Shephard *et al.*, 1994). The pGL3 enhancer luciferase reporter vector was purchased from Promega (pGL3 (En), figure 3.40). This vector contains an SV40 enhancer downstream of the luciferase *luc+* gene, which causes higher levels of transcription of the gene making its detection easier.

a: -5.6kb to +29bp *CYP2B2*

The λP4502B2 clone had been sequenced between -2915bp and +165bp (-1401 to +165bp: Shephard *et al.*, 1994; -2915bp to -1401: Shephard *et al.*, unpublished). Computer analysis of this sequence detected Nco I restriction sites at -2255bp, -983bp and +29bp. The Nco I site at +29bp, is exactly at the

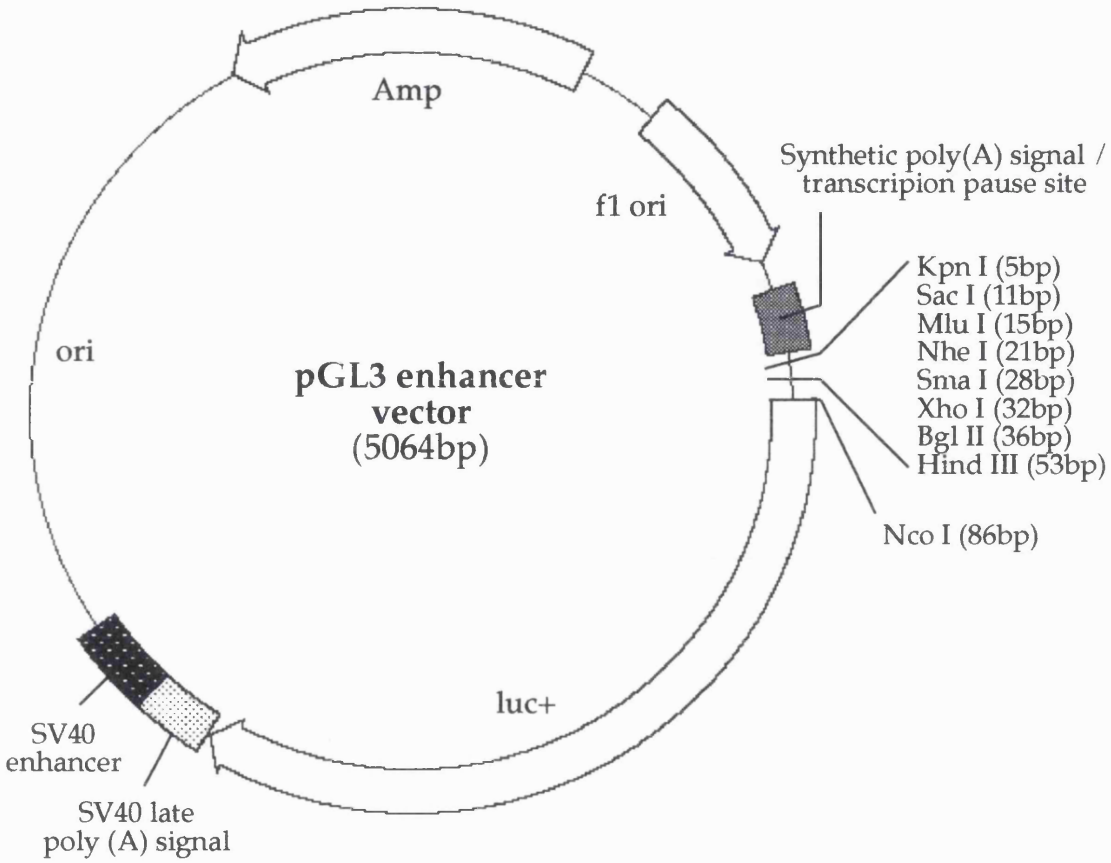

Figure 3.40: The pGL3 enhancer vector map. luc+ is the cDNA encoding the modified firefly luciferase; Amp is the gene for ampicillin resistance in *E. coli*; f1 ori is the origin of replication derived from filamentous phage; ori is the origin of plasmid replication in *E. coli*. Arrows on the luc+ and Amp genes indicate the direction of transcription; the arrow in f1 ori indicates the direction of ss DNA strand synthesis. Reproduced from the Promega technical manual for the pGL3 luciferase reporter vectors.

translation start site for the gene. Initially I attempted to subclone the full promoter sequence from -5.6kb to the Nco I site at +29bp into pGL3 enhancer vector. Once this had been achieved I hoped it would be comparatively easy to delete further fragments from this clone to create a series of clones containing various lengths of the promoter sequence.

Partial restriction digests of λP4502B2 with Nco I and Eco RI were carried out (figure 3.41). The fragments -5600bp to +29bp; -2255bp to +29 bp and -983bp to +29bp were purified from a low melting point agarose gel. The 5' overhangs of each fragment were filled, and a blunt ended ligation into the Sma I site of the pGL3 enhancer vector was attempted. Once the ligation had been transformed, mini preparations of the plasmid DNA from 12 of the colonies produced were carried out. Restriction digest analysis of this DNA showed that all of the colonies picked contained the pGL3 vector that had religated to itself without any insert. A further group of plasmid DNA mini preparations showed the same result. The ligation was carried out several more times and increasing the amount of insert each time, but they were all unsuccessful in producing clones.

b: -983bp to +29bp *CYP2B2*

This Nco I fragment was cloned into the unique Nco I site of the pGL3 enhancer vector (86bp). The fragment was again purified from a low melting point agarose gel and a ligation was carried out with Nco I cut pGL3 enhancer DNA under conditions suitable for ligating cohesive ends. Once the transformed colonies had been grown and the plasmid DNA extracted, one clone was found with the correct insert (figure 3.43a, track 4). Having created this clone (pGL -983), further subclones were possible. Figure 3.42 shows the restriction sites used in the cloning strategy, and the series of constructs created.

Reaction	M	A				B				M
Time (min)	–	15	30	45	60	15	30	45	60	–
Track	1	2	3	4	5	6	7	8	9	10

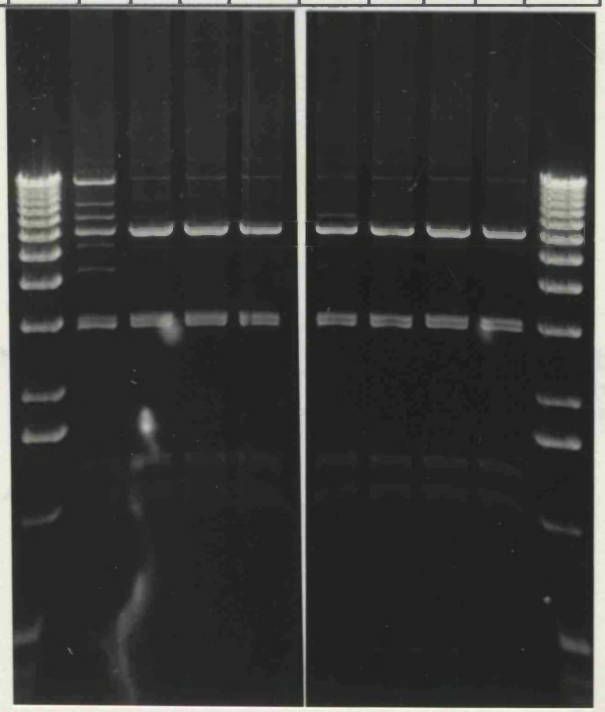

Figure 3.41: Partial digests of λP4502B2 by Nco I and Eco RI. λP4502B2 DNA (500ng) was digested for 15, 30, 45 or 60 minutes, at 37˚C, with 2.5 units of each enzyme (reaction A) or 5 units of each enzyme (reaction B). After the specified time the reaction was stopped by addition of EDTA to a final concentration of 100mM. The reactions were then run on a 0.8% agarose gel. Aliquots of 1KB marker were also run on the gel (M). The sizes of the bands in the marker are show in figure A.1.

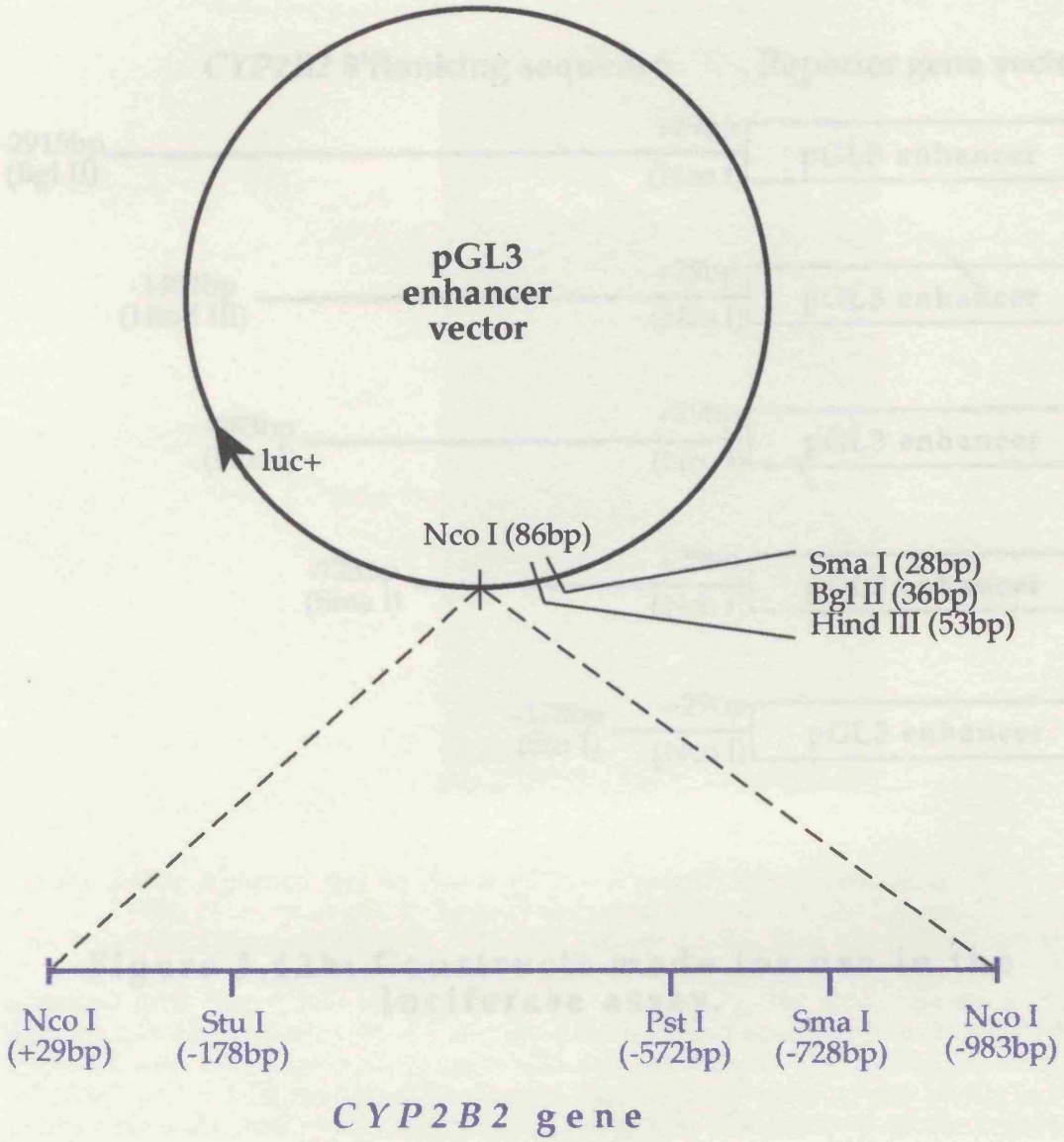

Figure 3.42a: Cloning strategy to create luciferase reporter gene constructs.

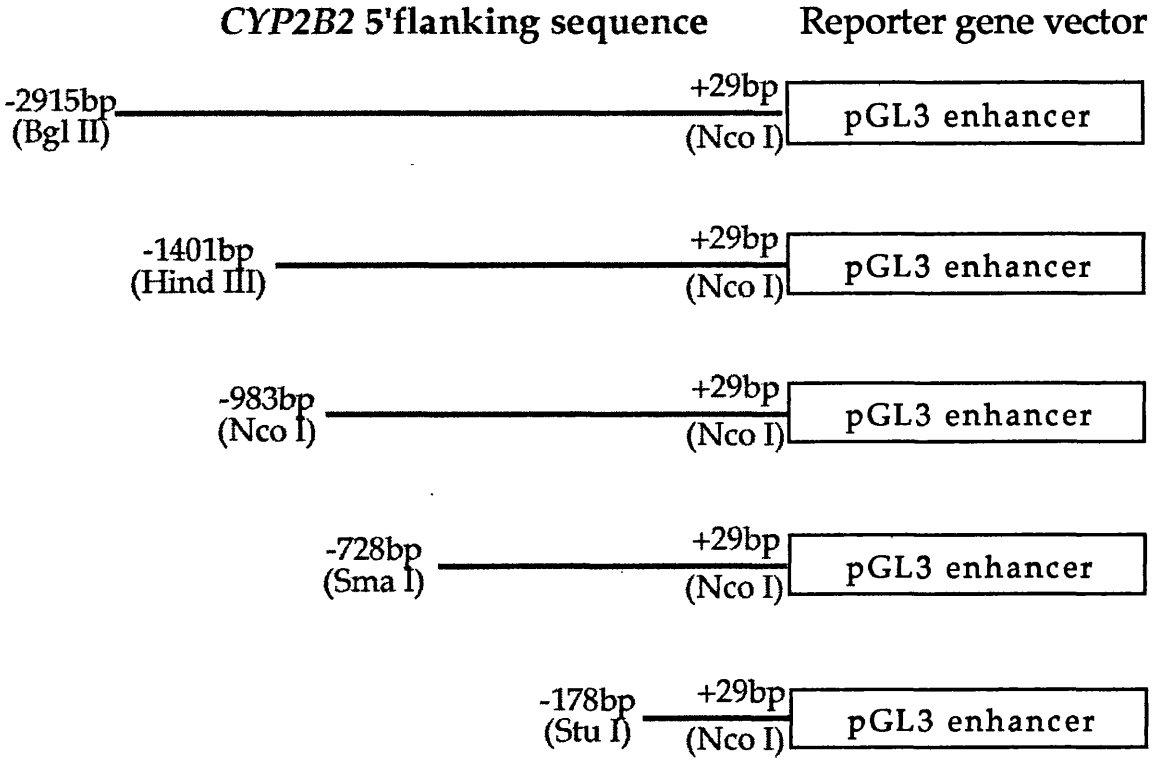

Figure 3.42b: Constructs made for use in the luciferase assay.

Sample	M	1	2	3	4	5	M	6	7	8	9
Track	1	2	3	4	5	6	7	8	9	10	11

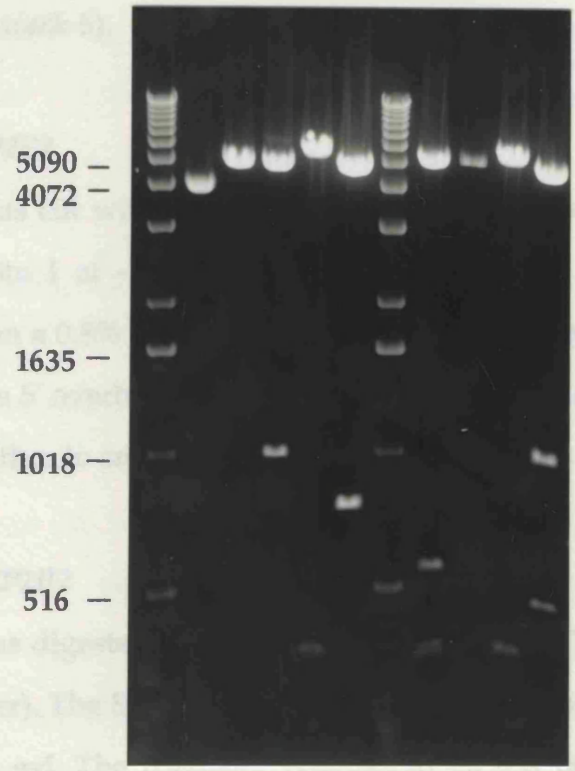

Figure 3.43a: Agarose gel to show pGL - *CYP2B2* constructs. pGL -983 was digested with Nco I (sample 3), Sma I (sample 4), Sma I and Stu I (sample 6) and Sma I and Pst I (sample 8). pGL -728 was digested with Nco I and Sac I (sample 5). pGL -178 was digested with Nco I and Sac I (sample 7). pGL -1401 was digested with Nco I and Sac I (sample 9). All the digests were run on a 0.8% agarose gel. The vectors pRL-TK digested with Eco RI (sample 1) and pGL3 (En) digested with Nco I (sample 2) were also run on the gel. The molecular weight standard (M) is 1KB marker. The sizes in base pairs of some of the bands in the marker are indicated on the left of the photograph. The sizes of all the bands in the marker are shown in figure A.1.

c: -728bp to +29bp *CYP2B2*

The pGL -983 clone was cut with Sma I at -728bp (*CYP2B2*) and 28bp (pGL3 (En)) (figure 3.43a, track 5).The 5.8kb band was purified from a 0.8% low melting point agarose gel. The blunt ends were ligated together to create pGL -728 (figure 3.43a, track 6).

d: -178bp to +29bp *CYP2B2*

The pGL -983 clone was cut with Sma I at -728bp (*CYP2B2*) and 28bp (pGL3 enhancer) and with Stu I at -178bp (*CYP2B2*) (figure 3.43a, track 8). The digest was separated on a 0.8% low melting point agarose gel and the 5.27kb band was purified. The 5' overhang created by Stu I was filled and the blunt ends were ligated together to create pGL-178 (figure 3.43a, track 9)

e: -1401bp to +29bp *CYP2B2*

The pGL -983 clone was digested with Pst I at -572bp (*CYP2B2*) and Hind III at 53bp (pGL3 enhancer). The 5.63kb fragment was purified from a 0.8% low melting point agarose gel. The fragment -1401bp to -572bp of *CYP2B2* was created by a Pst I/Hind III double digest of p(Pst I/Hind III), and it was purified from a 1.0% low melting point agarose gel. The two fragments were ligated together under conditions suitable for ligating cohesive ends to create the clone pGL -1401 (figure 3.43a, track 11).

f: -2915bp to +29bp *CYP2B2*

The restriction analysis of the λP4502B2 clone identified a Bgl II restriction site at -2915bp. This was repeated and confirmed (results not shown). The pGL -983 clone was digested with Pst I at -572bp (*CYP2B2*) and Bgl II at 36bp (pGL3 enhancer). The 5.63kb fragment was purified from a 0.8% low melting point agarose gel. The fragment -2915bp to +29bp of *CYP2B2* was

made by a Bgl II/Pst I double digest of λP4502B2. As more than one fragment of approximately 3kb was formed by this digest, the whole digest was used in a ligation with the purified luciferase fragment. The conditions were chosen for cohesive end ligation. The clone with the correct Bgl II/Pst I fragment (pGL -2915) was identified using a Nco I digest of possible clones, as only the clone containing the -2915bp to +29bp CYP2B2 fragment contains three Nco I sites (figure 3.43b).

3.6.2 Transient transfection of the hepatocytes with the luciferase constructs.

The hepatocytes were transfected with each of the *CYP2B2*-firefly luciferase constructs in conjunction with treatment with phenobarbital and/or okadaic acid. To control for variations in transfection efficiency, the cells were co-transfected with a vector containing the *Renilla* firefly reporter gene under the control of the thymidine kinase promoter to provide low-level constitutive expression of *Renilla* luciferase (pRL-TK, figure 3.43a, track 2). The thymidine kinase promoter is relatively weak in hepatocytes (Ponder *et al.*, 1991), and it was used to reduce the possibility of *trans* effects between the promoters as much as possible. The activity of the Renilla luciferase was not affected by treatment of the hepatocytes with phenobarbital and/or okadaic acid (results not presented). Figure 3.44 shows the firefly luciferase activities normalised against the *Renilla* activity for each transfection.

The pGL3 enhancer vector without any insert (pGL3 (En)) was transfected alongside the *CYP2B2* constructs, as a control. However, there was an appreciable amount of firefly luciferase activity, even though the gene was promoterless (figure 3.40). The pGL -178 construct caused firefly luciferase

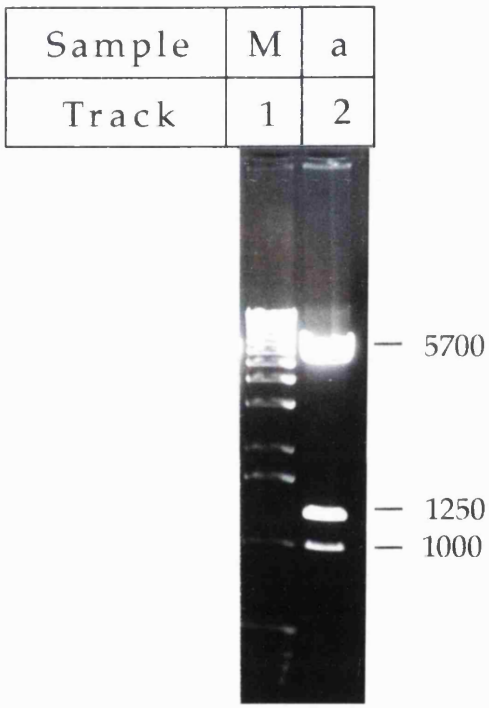

Figure 3.43b: Agarose gel to show the pGL -2915 clone. The pGL -2195 clone was digested with Nco I and run on a 0.8% agarose gel (a, track 2) together with an aliquot of 1KB molecular weight standard (M, track 1). The sizes in base pairs of the bands seen are indicated on the right of the photograph. The sizes of the fragments in the 1KB marker are shown in figure A.1.

Sample	Construct	Restriction Enzyme	Restriction Site Position	Fragment Size (bp)
1	pRL-TK	Eco RI	683 (vector)	4045
2	pGL3 (En)	Nco I	86 (vector)	5064
3	pGL -983	Nco I	86 (vector)	5064 1012
4	pGL -983	Sma I	28 (vector) -728 (*CYP2B2*)	5761 315
5	pGL -728	Nco I Sac I	86 (vector) 11 (vector)	5304 772
6	pGL -983	Sma I Stu I	28 (vector) -728 (*CYP2B2*) -178 (*CYP2B2*)	5213 548 315
7	pGL -178	Nco I Sac I	86 (vector) 11 (vector)	5304 224
8	pGL -983	Sma I Pst I	28 (vector) -728 (*CYP2B2*) -572 (*CYP2B2*)	5607 315 154
9	pGL -1401	Nco I Sac I	86 (vector) -983 (*CYP2B2*) 11 (vector)	5304 1012 149
a	pGL -2915	Nco I	-2255 (*CYP2B2*) -983 (*CYP2B2*) 86 (vector)	5695 1269 1012

Figure 3.43c: Table of the sizes of DNA fragments expected from restriction digests of the pGL-*CYP2B2* constructs. The restriction digests were run on the agarose gels shown in figures 3.43a and 3.43b. The sample numbers in the tables are the same as for figures 3.43a and 3.43b.

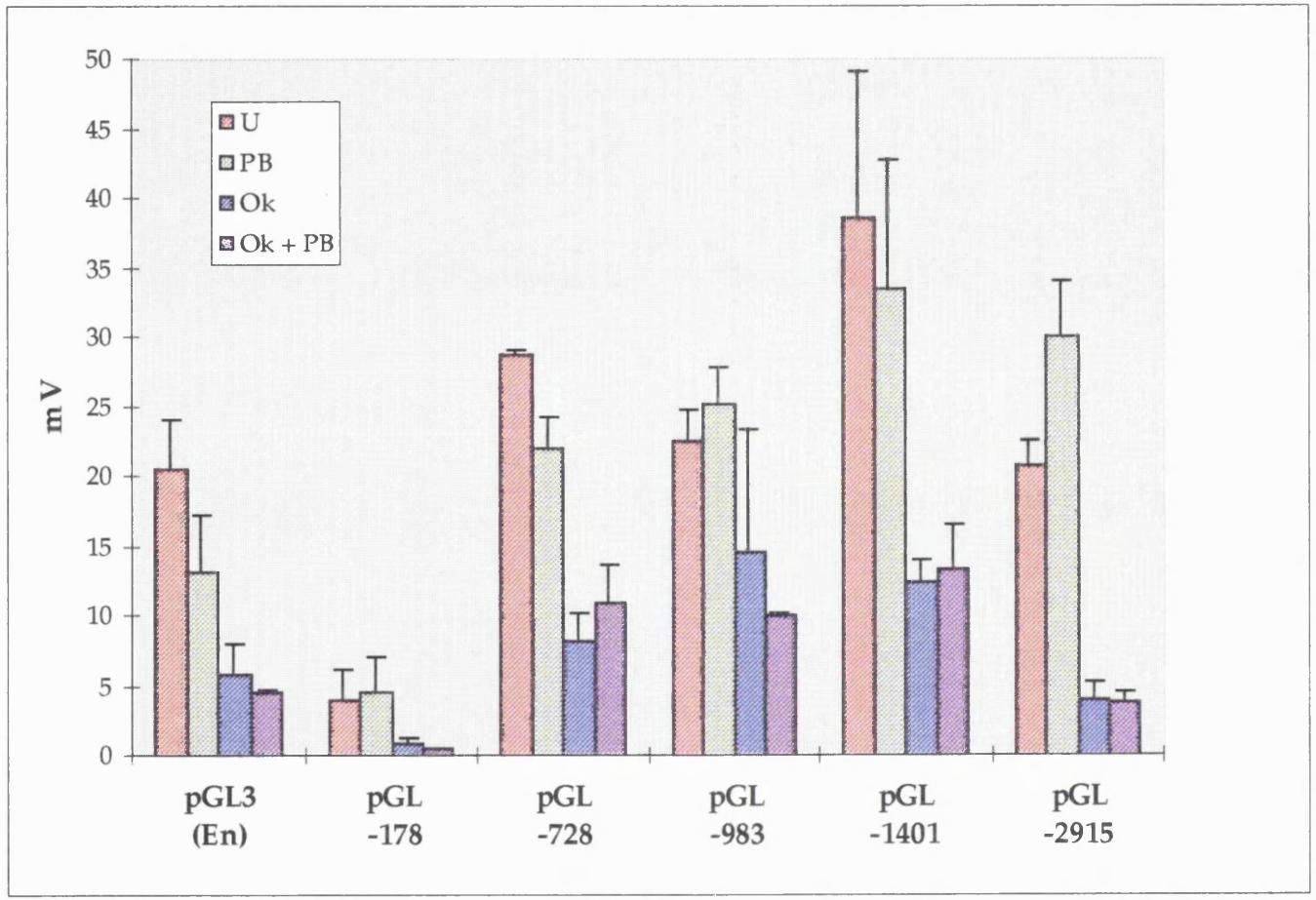
50
45
40
35
30
25
20
15
10
5
0
m V
U
PB
Ok
Ok + PB
pGL3 (En)
pGL -178
pGL -728
pGL -983
pGL -1401
pGL -2915

Figure 3.44: Luciferase activity in primary hepatocytes transfected with *CYP2B2* promoter constructs.

Primary hepatocytes from one rat liver were transfected with a series of constructs containing fragments of the *CYP2B2* promoter and the firefly luciferase reporter gene (see text for details). The ratio of pGL construct to pRL-TK control was 2:1. Twelve plates were transfected with each construct. Of those plates, three were untreated (U) and three were treated with 0.1mM phenobarbital (PB) from 48 hours to 72 hours post-plating. At 48 hours post-plating, the six remaining plates were treated with 100nM okadaic acid for one hour. The medium was then changed and three of the plates were treated with 100nM okadaic acid (Ok) and three were treated with 100nM okadaic acid and 0.1mM phenobarbital (Ok + PB). As a control, the cells were co-transfected with the *Renilla* firefly luciferase gene controlled by the thymidine kinase promoter. All the cells were lysed at 72 hours post-plating and the activities of the firefly luciferase and the *Renilla* luciferase were determined using the Promega Dual-Luciferase Assay. The activities of both luciferases were determined in a sample of untransfected, untreated hepatocytes from the same rat liver, cultured alongside the transfected cells to provide a background measurement. The background signal was subtracted from the results and each result was normalised against the *Renilla* luciferase activity for that plate. The graph shows the average normalised firefly luciferase activity for each treatment and each construct. The error bars show standard deviation.

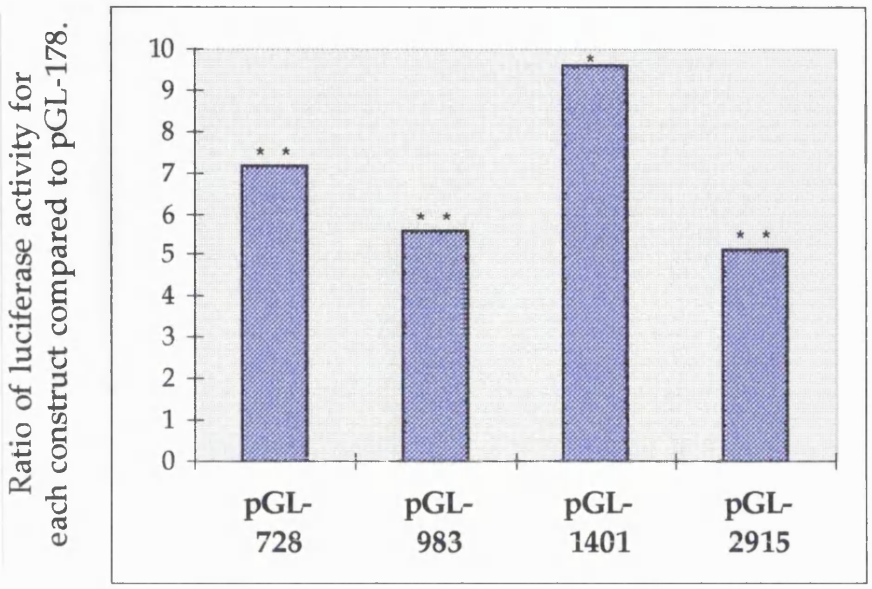

Figure 3.45: Graph of relative activity of the various constructs in transfected hepatocytes. The figures used are from the same experiment as in figure 3.44. The ratio of luciferase activity from each of the constructs to that for pGL-178 in untreated cells is shown on the graph. The * denotes a significance of $p < 0.05$ and ** shows significance of $p < 0.01$ as calculated by a two-tailed, paired, Student's T-test.

activity which was lower than the pGL3 (En) control. These observations could be explained as cryptic transcription of the promoterless luciferase gene. Technical advisors at Promega did report that other workers had observed cryptic transcription from the pGL3 (En) vector in other systems (personal communication). Furthermore, if when a fragment of *CYP2B2* is inserted into the vector it interfered with the cryptic signals, and the -178 to +29bp had a very low promoter activity, lower than the cryptic signal, this would produce the results observed.

The relative amounts of firefly luciferase activity for the different *CYP2B2* constructs compared with that for the pGL -178 construct are shown on figure 3.45. All of the other fragments (-728 to +29bp, -983 to +29bp, -1401 to +29bp, and -2915 to +29bp) cause considerably more transcription than the -178 to +29bp fragment. The greatest firefly luciferase activity was seen with the pGL-1401 construct, although this was not significantly greater than pGL-728, pGL-983 or pGL-2915 (p=0.25, 0.16 and 0.10, respectively).

Phenobarbital treatment of the transfected hepatocytes did not have a great effect (figure 3.46). The expression of firefly luciferase was slightly inhibited by phenobarbital with the pGL -728 construct and the pGL-1401 construct. With the largest *CYP2B2* fragment, there was a significant induction of expression of luciferase activity of approximately 1.5-fold. There was a slight increase in expression of the firefly luciferase with the pGL -178 and pGL -983 constructs in response to phenobarbital treatment, though neither of these was significant.

A considerable inhibition of expression of firefly luciferase was caused by treatment of the transfected hepatocytes with okadaic acid (figure 3.46). This occurred with all the constructs. Treatment of the cells with phenobarbital

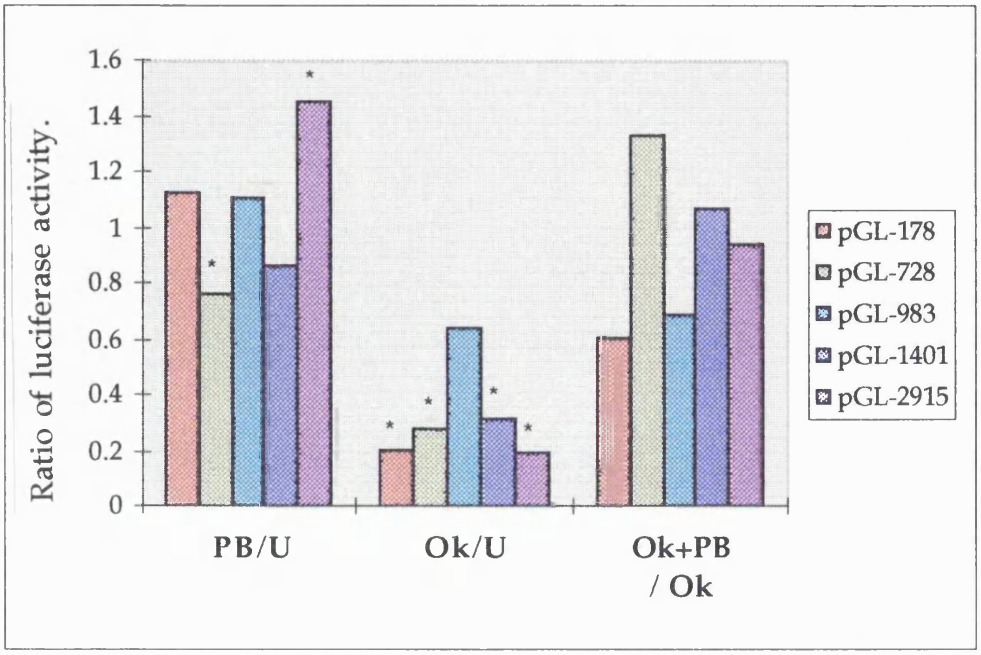

Figure 3.46: Graph of the effects of phenobarbital and/or okadaic acid treatment on the activity of firefly luciferase reporter gene in transfected hepatocytes. The figures used are from the same experiment as in figure 3.44. The ratio of average luciferase activity after treatment with phenobarbital (PB) or okadaic acid (Ok) over average luciferase activity in untreated (U) cells for each construct is shown on the graph. The effect of adding phenobarbital as well as okadaic acid on the average luciferase activity is also presented (Ok + PB/Ok). The * denotes a significance of $p < 0.05$ as calculated by a two-tailed, paired, Student's T-test.

and okadaic acid caused the opposite effect to treatment with phenobarbital alone. Expression from constructs which had been inhibited by phenobarbital alone (pGL -728 and pGL -1401) was induced when the cells were treated with phenobarbital together with okadaic acid, compared to okadaic acid alone. Also phenobarbital induction of expression from pGL -178, pGL -983 and pGL -2915 was inhibited when the cells were treated with okadaic acid in conjunction with phenobarbital. However, none of the differences between the Ok and the Ok + PB populations was significant.

3.7 Section II: Discussion

3.7.1 Characterisation of the hepatocyte culture system.

The expression and induction of various phase I and phase II drug metabolising enzymes were studied in primary hepatocyte to discern whether our cell culture system could be used to study the phenobarbital response mechanism.

Several investigators have analysed the amount and activity of the GST isoenzyme subunits as a function of culture time and conditions. In general, it has been found that dedifferentiation of primary hepatocytes in culture is associated with alpha class GST expression decreasing and mu class GST expression increasing (Abramovtiz *et al.*, 1989; Vandenberghe *et al.*, 1989; Dwivedi *et al.*, 1993). My results with our hepatocyte culture system, showed GST 2-2/1-1 (alpha class) decreased slightly over time in one of the cultures, and increased in the other. The cells for each culture experiment came from different animals, and it is possible that this difference in expression patterns between the two cultures is the result of interindividual differences between the animals, or the differences in the viability of the cells obtained for each experiment. The expression of GST 3-3/4-4 (mu class) does increase with time in the primary hepatocytes.

The most marked change during foetalisation of hepatocytes is that pi class GST begins to be expressed (Abramovtiz *et al.*, 1989; Dwivedi *et al.*, 1993). In the cell culture system used in this study, GST 7-7 (pi class) is not present at all during the culture period.

Treatment of rats with phenobarbital increases the GST associated 1-chloro-2,4-dinitrobenzene (CDNB) activity 1.6- to 2.6-fold (Igarashi, *et al.*, 1987; Yamada *et al.*, 1993). *In vivo* treatment of rats with picrotoxin also increases CDNB activity by approximately 1.6-fold. (Yamada *et al.*, 1993).

Cultured hepatocytes have a similar response to phenobarbital, but to my knowledge, the effects of picrotoxin have not been studied. Dwivedi *et al.*, 1993 observed a 1.5-fold increase in CDNB activity after 48 hours treatment of hepatocytes cultured in Chee's medium on Vitrogen coated plates with 2mM PB. Subunits Ya (alpha class), Yc (alpha class) and Yb (mu class) increase 1.5- to 2-fold after treatment with phenobarbital in cultured hepatocytes (Dwivedi *et al.*, 1993) and 2- to 6-fold in *vivo* (Ding and Pickett, 1985).

In this study, treatment of the cells with 0.75mM phenobarbital or 0.5mM picrotoxin caused a decrease in the amounts of GST 2-2/1-1 and GST 3-3/4-4. To my knowledge this response has not been observed previously in cultured hepatocytes, and it is difficult to account for. It is possible that some component of William's E medium produces this aberrant response, analogous to Chee's medium causing an abnormal response of *CYP1A1* expression in response to phenobarbital (Sidhu *et al.*, 1993). Also the concentration of phenobarbital used to treat the hepatocytes in this study (0.75mM) is lower than that in the reports cited above (2mM), which may affect the response of the GST genes.

P450 reductase was expressed in the hepatocytes and increasing culture time from 72 to 96 hours did not change the amount observed. When treated with 0.75mM phenobarbital or 0.5mM picrotoxin, a slight induction was seen after 48 hours of treatment (PB 96 or Px 96), but not 24 hours (PB 72 or

Px 72). When the concentration of phenobarbital was decreased to 0.1mM, a 2.24-fold induction was seen after 24 hours of treatment (72 hours total culture time). The different concentrations of phenobarbital were used with different cultures, so this could be the result of interindividual differences of the rats. However, it is plausible that the concentration of phenobarbital that most induces CYP2B1/2 expression would also give the greatest change in P450 reductase expression.

These results are consistent with the findings of other investigators. *In vivo* the amount of P450 reductase increases 5- to 6-fold after treatment of rats with phenobarbital (Shephard *et al.*, 1983). *In vitro,* this response is slightly reduced. Clark *et al.* (1996) reported that exposure for 24 hours to 0.75mM phenobarbital caused a 2-fold induction of P450 reductase in hepatocytes cultured in William's E medium on a Matrigel basement. Waxman *et al.* (1990) found that exposure of hepatocytes that had been cultured for 16 days to 0.75mM phenobarbital for a further 96 hours induced P450 reductase activity 3-fold. These hepatocytes were cultured in Chee's medium on Vitrogen coated plates.

The amount of CYP2B1/2 in the primary hepatocytes changed in response to the concentration of phenobarbital used to treat the cells. The fold-inductions of CYP2B1/2 mRNA measured from the slot blot were very low. Using RNase protection assays Ciaramella (Ph.D. thesis, 1995) showed that there was an increase of approximately 60-fold in CYP2B1/2 mRNA after the hepatocytes had been treated for 48 hours with 0.75mM phenobarbital using the same culture system. The change in the amount of CYP2B1/2 protein detected appeared much greater than that of the CYP2B1/2 mRNA detected by the slot blot. However, since there was no CYP2B1/2 protein detected in the untreated cells, this induction could not be calculated. The

seemingly low level of induction of CYP2B1/2 mRNA is probably due to difficulties experienced in the hybridization of the probe to the mRNA. The hybridization was carried out several times under different conditions with different probes, so the blot had been stripped several times. The hybridization conditions still do not seem to be optimal as the fold induction for the CYP2B1/2 mRNA is expected to be a lot larger. However, as time was restricted, the hybridization was not repeated. The slot blot does show same trend as the western blot in the amount of CYP2B1/2 detected in response to different concentrations of phenobarbital. The greatest response was to 0.1mM - 0.2mM phenobarbital. This was in good agreement with the results of Sidhu and Omiecinski (1995). However Clark *et al.* (1996) found that benzyloxyresorufin O-deethylase (BROD) activity (CYP2B1/2 specific) and the amount of CYP2B1/2 detected on western blots were maximal at a concentration of phenobarbital between 0.3 and 1mM in hepatocytes cultured on Matrigel in William's E medium. My subsequent experiments used phenobarbital at a concentration of 0.1mM. Other investigators have reported 15- to 100-fold inductions of CYP2B1/2 mRNA in response to phenobarbital treatment of hepatocytes cultured using a variety of systems (Sinclair *et al.*, 1990; Waxman *et al.*, 1990; Niemann *et al.*, 1991; Williams *et al.*, 1991; Akrawi *et al.*, 1993; Saad *et al.*, 1993; Sidhu *et al.*, 1993; Kocarek *et al.*, 1994).

The hepatocytes cultured using this system had an abnormal response to phenobarbital in terms of amounts of GST 2-2/1-1 and GST 3-3/4-4 detected. However, the expression of GST 7-7 was not detected throughout the culture period and CYP2B1, CYP2B2 and P450 reductase proteins are induced by phenobarbital. So, whilst this system would not be suitable to study the expression and induction of all drug metabolising enzymes, it

could be used to examine the response of *CYP2B1/2* and P450 reductase to phenobarbital in these cells.

3.7.2 The effects of 8-CPT, (Sp) cAMPS and okadaic acid on the expression of CYP2B1/2 and P450 reductase, and on their induction by phenobarbital.

To investigate the involvement of cAMP dependent protein kinase (PKA) and protein phophatases I and IIA in the regulation of *CYP2B2*, the primary hepatocytes were treated with 8-(4-chlorophenylthio)-cAMP (8-CPT), the S phosphorothioate stereoisomer of cAMP ((Sp)cAMPS) and okadaic acid. 8-CPT is a cAMP analogue that is membrane permeable and stable towards cAMP-associated phosphodiesterase hydrolysis (Sandberg *et al.*, 1991). (Sp)cAMPS is a potent and specific PKA activator (Rothermel and Botelho, 1988). Both of these compounds were used at a concentration of 10μM as this was reported to be sufficient to cause an inhibition of the PB-mediated accumulation of CYP2B1 mRNA (Sidhu and Omiecinski, 1995).

Okadaic acid is a potent inhibitor of protein phosphatase type 1 (PP1) and protein phosphatase type 2A (PP2A). Technical staff at Sigma suggest using okadaic acid at a concentration of 1μM for all cell types (personal communication). However, it is a highly toxic chemical and studies with rat hepatocytes showed that it induces apoptosis at concentrations of 1μM (Bøe *et al.*, 1991). Reaven *et al.* (1993) demonstrated that a concentration of 100nM was sufficient to induce hyperphosphorylation of a number of proteins, and that whilst functions of the Golgi body were disrupted, other hepatocyte cell functions such as albumin synthesis and lactate dehydrogenase activity were unchanged at this concentration. *In vitro*, 50%

inhibition of PP1 by okadaic acid (IC_{50}) occurs at 0.1-1.0nM, and that of PP2A occurs at 20-100nM (reviewed Schönthal, 1995). Consequently, the hepatocytes in this study were treated with 100nM okadaic acid.

It should be noted that the results presented here are from one culture experiment only. This should be repeated at least twice to reach any firm conclusion. It would also be extremely useful to carry out a reliable analysis of the amounts of CYP2B1/2 mRNA for each of these experiments.

CYP2B1 and CYP2B2 are phosphorylated by PKA at the serine 128 residue. This phosphorylation causes a shift in the carbon monoxide difference peak from 450nm to 420nm, and a decrease in enzyme activity both in vitro (Pyerin and Taniguchi, 1989) and in vivo (Koch and Waxman, 1989). Eliasson *et al.* (1990) suggest that this signals the start of the degradation of the protein. It is possible, therefore that no protein is detected on the Western blot after treatment with 8-CPT or (Sp) cAMPS (with or without PB) because it has been degraded via this PKA-mediated phosphorylation pathway. There are no reports of the involvement or otherwise of PP1 or PP2A in this degradation process.

However, Sidhu and Omiecinski (1995) report that 8-CPT and (Sp) cAMPS both inhibit the accumulation of CYP2B1 mRNA caused in response to phenobarbital treatment, and conclude that PKA has a negative regulatory role in the phenobarbital induction mechanism. This mechanism would also be the reason why there was no CYP2B1/2 protein detected in my experiments.

Treatment of the hepatocytes with 8-CPT, (Sp)cAMPS or okadaic acid as well as PB prevents the increase of P450 reductase to amounts greater than

treatment with the compound without PB. Assuming that the PB-induction mechanism is the same for both P450 reductase and CYP2B1/2, it is plausible that this is also the case for the CYP2B1/2 proteins. This would also agree with Sidhu and Omiecinski (1995).

These results appear to be contradictory to those of Salonpää *et al.*, (1994), who reported that treatment of primary mouse hepatocytes with dibutyryl cAMP (a cAMP analogue) or forskolin (which stimulates the production of cAMP) potentiated the induction of Cyp2a5 by phenobarbital. This may be a result of the different cell culture system, or a species difference. However, Cyp2a5 is regulated differently to other CYPs during liver injury (Camus-Randon *et al.*, 1996), it is possible that it is also regulated differently by phenobarbital.

Dibutyryl cAMP also potentiates the phenobarbital induction of δ-aminolevulinate synthase mRNA in rat hepatocytes (Varone *et al.*, 1994). This enzyme catalyses the rate limiting step of haem biosynthesis, and it is induced co-ordinately with CYPs to create haem for CYP biosynthesis (May *et al.*, 1995). So these results would also seem to contradict the fact that dibutyryl cAMP and other cAMP analogues inhibit the phenobarbital induction of CYP2B1 mRNA (Sidhu and Omiecinski, 1995).

Nirodi *et al.* (1996) observed that okadaic acid inhibited the phenobarbital-mediated induction of transcription of a *CYP2B1/2* minigene construct in vitro. However in the same system they found that 2-aminopurine (a general protein kinase inhibitor) also inhibited the phenobarbital response. Dogra and May (1996) also found that treatment of foetal chick hepatocytes with 2-aminopurine blocked the phenobarbital-induced activation of *CYP2H1* (the major phenobarbital-inducible gene in the chicken). This effect

was not due to the inhibition of protein kinase C or tyrosine kinase activity. It may be that there are two separate phosphorylation/dephosphorylation steps involved in the phenobarbital response mechanism.

3.7.3 Transfection of the primary hepatocytes with constructs containing the luciferase reporter gene controlled by various fragments of the *CYP2B2* gene promoter.

Transfection of the primary hepatocytes was carried out 20 hours after plating the hepatocytes to give the cells time to stabilise. Padgham *et al.* (1993) demonstrated marked changes in the amounts of several transcription factors (including C/EBPα) in cultured rat hepatocytes during the first 2-3 hours after liver perfusion. Foetal calf serum (FCS) was included in the culture medium during the transfection as it has been shown to maintain the hepatocytes better (Vandenberghe, *et al.*, 1992; Gaunitz, F. personal communication). After the transfection the FCS was removed as it has been shown to be refractile to phenobarbital induction (Waxman *et al.*, 1990).

During this study, several other investigators have carried out similar transfection experiments using sections of the *CYP2B2* promoter to control various reporter genes in primary hepatocytes, in hepatoma cell lines and *in vivo*. The results from the different experiments do not correlate very well with each other. Trottier *et al.*, (1996) studied the activity of a chloramphenicol acetyl transferase (CAT) reporter gene in primary hepatocytes cultured in Chee's medium on Vitrogen coated plates. The cells were transfected 4 hours after plating and once the transfection was

complete, a matrigel overlay was added. Maximal CAT activity was observed with a construct containing the -2015 to +31bp sequence of *CYP2B2*. The activity of a -1681 to +31bp and a -729 to +31bp construct were approximately equal at 60% maximal activity. Park and Kemper (1996) reported greatest activity with the first 725bp of the *CYP2B2* promoter attached to a luciferase reporter gene and transfected into Hep G2 cells. This was compared to activity for a 1400bp construct, a 211bp construct and a 110bp construct.

My results most closely matched those of Honkakoski *et al.* (1996), who transfected CAT constructs containing sequences from the phenobarbital-inducible mouse *Cyp2b10* gene into primary mouse hepatocytes. The first 1405bp of the *Cyp2b10* gene promoter sequence is 83% identical to the *CYP2B2* gene. One of the noteable features of this promoter sequence is that there is a 42bp insert into the middle of the Barbie box sequence identified in the *CYP2B2* gene, yet the gene is phenobarbital inducible. The results of these transfection studies showed maximal CAT activity with a -1401 to +4bp construct as compared to a -775 to +4bp and a -4300 to +4bp construct. My results also showed pGL -2915 < pGL -728 < pGL -1401.

Phenobarbital treatment of the transfected hepatocytes did not have a great effect. Significant phenobarbital-induction of luciferase activity (1.5-fold) was only seen with pGL -2915. There was a slight increase in expression of the firefly luciferase with the pGL -178 and pGL -983 constructs in response to phenobarbital treatment, though neither of these were significant. Also the expression of firefly luciferase was slightly inhibited by phenobarbital with pGL -728 and pGL-1401. None of the transfection studies discussed above have been successful in producing phenobarbital induction of reporter gene expression at levels comparable to the *in vivo* induction of

* The low response to phenobarbital in all of these studies could be because not all of the elements required for the full phenobarbital response have been included in the constructs, or interactions between various elements are not occurring due to different DNA conformations or lack of chromatin structure of the plasmids compared to the DNA *in vivo*. It is also possible that the actual transfection experiment disrupts some of the signalling pathways involved in the phenobarbital response, or that a transcription factor that is required for the full response is not maintained in the cultured hepatocytes.

CYP2B2. Trottier *et al.* (1996) found a maximal induction of 6.6-fold with a -2506 construct, and Honkakoski *et al.* (1996) showed a maximal induction of 3.3-fold using the -1401 to +4bp construct. The -179 to +1bp *CYP2B2* gene attached to growth hormone structural sequences was transfected into rat liver *in vivo* as an asialoglycoprotein-polylysine complex (Prabhu *et al.*, 1995). Treatment of the rat with phenobarbital resulted in a significant increase of the reporter mRNA, although the amount of induction could not be calculated as the untreated levels were undetectable. Neither of the other studies show phenobarbital induction with promoter fragments corresponding to this region. Otherwise the pattern of expression resulting from phenobarbital treatment of the cells was different to my results, and the different studies (Honkakoski *et al.*, 1996, Trottier *et al.*, 1996) did not correlate with each other either. *

Okadaic acid treatment of the transfected hepatocytes caused a considerable inhibition of expression of firefly luciferase (figure 3.46), both with and without phenobarbital. This occurred with all the constructs. Treatment of the cells with phenobarbital and okadaic acid caused the opposite effect to treatment with phenobarbital alone, although the differences involved were slight. None of the studies described above treated the transfected cells with okadaic acid, so a comparison cannot be made. Okadaic acid did inhibit the phenobarbital-mediated induction *in vitro* transcription of a *CYP2B2* minigene construct containing the first 179bp of the *CYP2B1/2* promoter (Padmanaban *et al.*, 1994). However, okadaic acid treatment alone enhanced the transcription of this construct, which would seem to be contradictory to my results.

3.8 General Discussion

The regulation of the amounts of transcription factors, and their DNA-binding and transactivation activities is very complex and can occur at many stages (summarized in figure 3.47). The activity is often controlled by (de)phosphorylation, which can then affect other functions such as nuclear translocation, or dimerisation capabilities. How phenobarbital is involved in the regulation of transcription factors is still a matter for debate. My results suggest that C/EBPα and C/EBPβ are implicated in the phenobarbital response mechanism (complexes 4 and 5 [C/EBPα], and complexes 3, 4 and 5 [C/EBPβ] from the figure 3.13). They also require a phosphate group for binding to the *CYP2B2* gene promoter.

The activity of C/EBPα/β is known to be regulated by several signaling pathways. They are involved in the acute phase inflammatory response mechanism and are regulated by several mediators in this cascade, particularly Interleukin 6 (IL6). C/EBPα is down-regulated slightly and C/EBPβ is up-regulated at the mRNA level by IL6 (Alam *et al.*, 1992). Also the transactivation ability of C/EBPβ, but not C/EBPα, is increased several fold by treatment of Hep 3B cells with IL6 (Poli *et al.*, 1990). The regulation of C/EBPα/b by IL6 is thought to occur through a Ras/MAP kinase signaling pathway (reviewed Poli and Ciliberto, 1994).

Cyclic AMP levels also regulate C/EBP activity. Raising cAMP levels in PC12 cells by treatment with forskolin causes increased phosphorylation of C/EBPβ, and increased its nuclear translocation and enhanced its transactivation activity (Metz and Ziff, 1991).

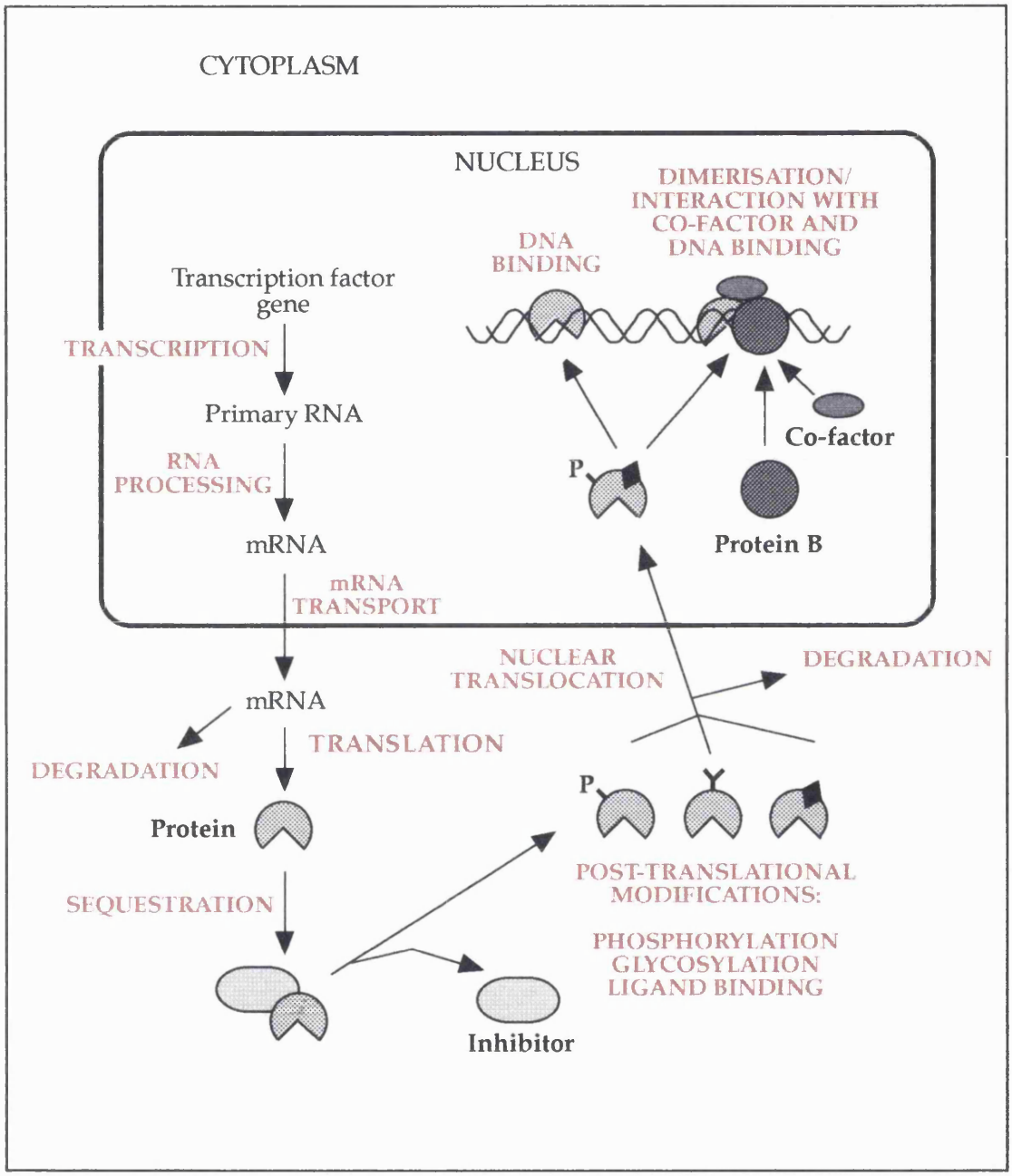

Figure 3.47: Regulation of the expression and activity of DNA-binding proteins. A schematic representation of crucial steps in the synthesis, activation and action of DNA-binding proteins. Potential regulatory points are written in red. Reproduced from Calkhoven and Ab (1996).

Protein phosphatases are implicated in the regulation of C/EBPα activity during the activation of GLUT4 (insulin responsive glucose transporter) by tumour necrosis factor (TNF) in 3T3-L1 adipocytes. Treatment of the adipocytes with okadaic acid co-ordinately repressed both C/EBPα and GLUT4 transcription (Stephens and Pekala, 1992).

It is interesting to note that all of these treatments markedly inhibit the phenobarbital-mediated expression of *CYP2B1/2*. IL6 treatment of primary hepatocytes inhibited the phenobarbital induction of BROD activity (CYP2B-specific) and CYP2B1/2 mRNA (Clark *et al.*, 1996, Abdel-Razzak *et al.*, 1995). The results presented here (figure 3.38) and those of Sidhu and Omiecinski (1995) suggest that raising cAMP levels and stimulating PKA inhibits phenobarbital-mediated accumulation of CYP2B1/2 mRNAs and proteins, and P450 reductase protein. Treatment of primary hepatocytes or whole animals with okadaic acid also inhibited phenobarbital induction of CYP2B protein and in vitro transcription of a *CYP2B1/2* minigene (figure 3.38; Prabhu *et al.*, 1995). It is possible that the change in ratio of C/EBPα:C/EBPβ associated with these treatments could be involved in inhibiting the phenobarbital response. The developmental regulation of the alcohol dehydrogenase gene (ADH) is reliant on an analogous shift from C/EBPα homodimers and HNF1 in the foetal liver to C/EBPα-C/EBPβ heterodimers, C/EBPβ homodimers and DBP in the postnatal liver (reviewed Johnson and Williams, 1994).

Appendix

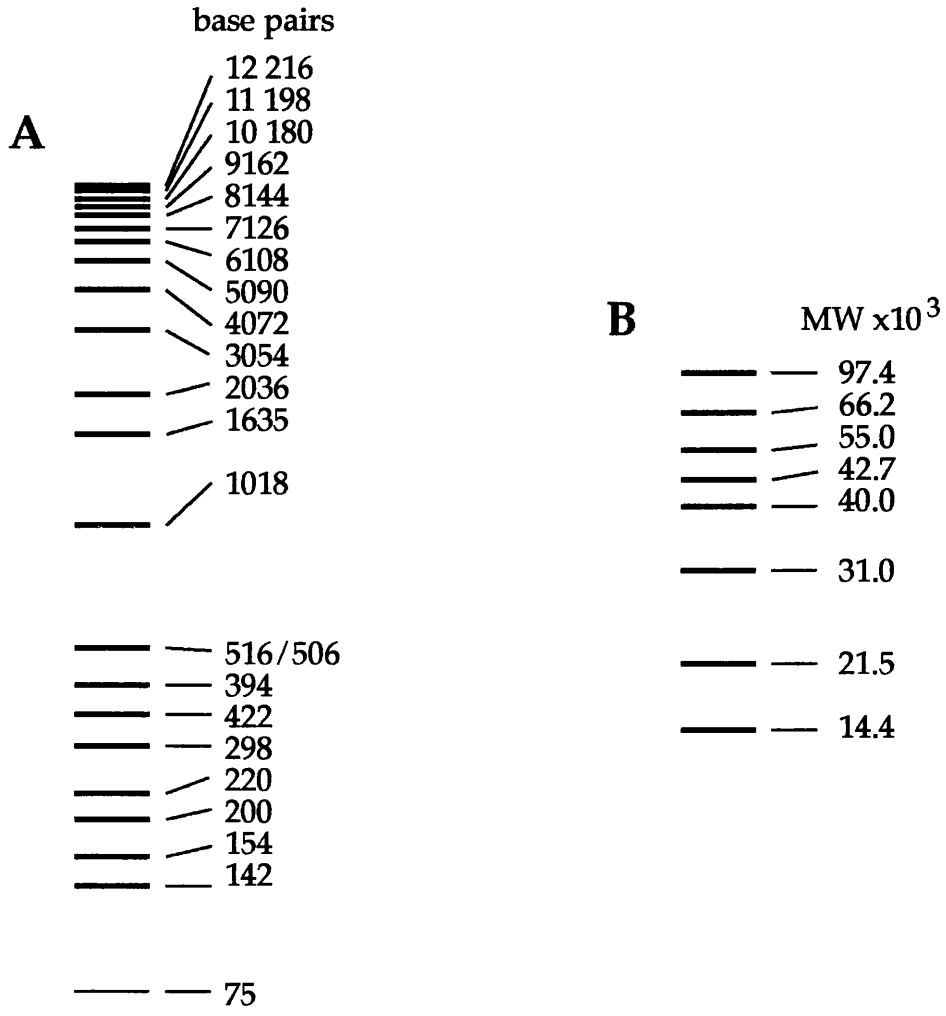

Figure A.1: Molecular weight standards. (A) The sizes in base pairs of bands seen with the 1KB DNA molecular weight marker (Gibco BRL). (B) The apparent molecluar weights (MW) of bands seen with the mid-range protein molecular weight marker (Promega).

Materials

Unless otherwise stated all materials were obtained from Sigma Chemicals Co. Ltd. or BDH Laboratory Supplies and all restriction endonucleases were purchased from Pharmacia Biotech Ltd..

Agarose	Sigma Chemicals Co. Ltd.
Agarose (low melting point)	Gibco BRL
Amido black stain	Sigma Chemicals Co. Ltd.
Ammonium acetate	Fisons Scientific Equipment
Ammonium persulphate	Fisons Scientific Equipment
Amphotericin B	Gibco BRL
Ampicillin, sodium salt, injection BP	BERK Pharmaceuticals Ltd.
Antibodies to C/EBP	Santa Cruz Biotechnology
Antibody to CYP2B1/2	Prepared by Dr.E.A.Shephard
Antibodies to Glutathione-S-transferases	Biotrin International
Antibody to P450 reductase	Prepared by Dr.E.A.Shephard
Antipain	Sigma Chemicals Co. Ltd.
Aprotinin	Sigma Chemicals Co. Ltd.
ATP	Pharmacia Biotech Ltd.
[γ^{32}P]ATP, specific activity 3000 Ci/mmol	New England Nuclear Research Products
BioTaq DNA polymerase	Bioline
Boric acid	Fisons Scientific Equipment
Bovine serum albumin (B.S.A.), 1mg/ml	New England Biolabs
B.S.A. (pentex fraction V)	Sigma Chemicals Co. Ltd.
Bromophenol blue	Bio-Rad Laboratories Ltd.
Centricon-30 unit	Amicon
Chloramphenicol	Sigma Chemicals Co. Ltd.
8-(4-chlorothiophenyl)-cAMP	Sigma Chemiclas Co. Ltd.
Chymostatin	Sigma Chemicals Co. Ltd.
Collagenase H	Boehringer Mannheim Ltd.
Coomassie brilliant blue	Sigma Chemiclas Co. Ltd.

1-Cyclohexyl-3(2-morpholinoethyl)carbo diimidemetho-*p*-toluenesulphonate (CDI)	Aldrich Chemicals
dATP, 100mM	Pharmacia Biotech Ltd.
dCTP, 100mM	Pharmacia Biotech Ltd.
[α^{32}P]dCTP, specific activity 3000 Ci/mmol	New England Nuclear Research Products
DE81 paper	Whatman Scientific Ltd.
Deschamps ligature needle	Lawton Gmb.H. & Co.
DEPC treated, RNase free water	Sigma Chemicals Co. Ltd.
Dexamethasone, (4mg/ml), Injection B.P.	David Bull Laboratories
dGTP, 100mM	Pharmacia Biotech Ltd.
DTT	Sigma Chemicals Co. Ltd.
dTTP, 100mM	Pharmacia Biotech Ltd.
Earle's Balanced Salt Solution	Gibco BRL
E.coli DH5α	Clonetech Laboratories Inc.
E.coli JM101	Pharmacia Biotech Ltd.
EDTA	Fisons Scientific Equipment
EGTA	Sigma Chemicals Co. Ltd.
Ethidium Bromide, 10mg/ml	BDH Laboratory Supplies
Ethidium Bromide, 10mg tablets	BDH Laboratory Supplies
Ficoll	Sigma Chemicals Co. Ltd.
Foetal calf serum (F.C.S.) (dialysed)	Gibco BRL
Folin and Ciocalteu's Reagent	Sigma Chemicals Co. Ltd.
Formaldehye (37%)	Sigma Chemicals Co. Ltd.
Formamide, deionised	Ambion
Gauze, PA-12-112/XX	Nybolt
Gel loading pipette tips	Costar
Glacial acetic acid	Fisons Scientific Equipment
Glucose	Fisons Scientific Equipment
Glycerol	Fisons Scientific Equipment
Guanidine thiocyanate	Sigma Chemicals Co. Ltd.
Heparin, injection B.P.	Fisons Scientific Equipment
HEPES	Sigma Chemiclas Co. Ltd.
Hexamine cobalt chloide	Sigma Chemicals Co. Ltd.

Hybond-C extra, 0.45μm nitrocellulose filter	Amersham International
Insulin	Sigma Chemicals Co. Ltd.
1KB molecular weight standard, 1μg/μl	Gibco BRL
Klenow DNA polymerase, 10u/μl	Pharmacia Biotech Ltd.
LB medium capsules	Bio 101 Inc.
LB agar capsules	Bio 101 Inc.
Leupeptin	Sigma Chemicals Co. Ltd.
Leibovitz L-15 medium	Sigma Chemicals Co. Ltd.
L-glutamine	Sigma Chemicals Co. Ltd.
Mayo scissors (curved)	Lawton Gmb. H. & Co.
β-mercaptoethanol	BDH Laboratory Supplies
Mersilk Black 3 ligature thread	Ethicon Ltd.
3MM paper	Whatman Scientific Ltd.
MOPS	Sigma Chemicals Co. Ltd.
Mse I, 4u/μl	New England Biolabs
NE2 Buffer	New England Biolabs
Neomycin	Gibco BRL
Nitrocellulose filter (supported)	BDH Laboratory Supplies
0.22μm nitrocellulose syringe filter unit	Sartorius
0.45μm nitrocellulose syringe filter unit	Millipore
Nonidet P40	Fisons Scientific equipment
Nuctrap push columns	Stratagene
Nylon membrane (optimised)	BDH Laboratory Supplies
Nylon mesh (62μm)	BDH Laboratory Supplies
Okadaic acid	Sigma Chemiclas Co. Ltd
One-Phor-All Buffer	Pharmacia Biotech Ltd.
Pepstatin A	Sigma Chemicals Co. Ltd.
Penicillin	Sigma Chemicals Co. Ltd.
Permanox® culture plates (60mm)	Nunc Inc.
Phenol, pH8.0	Fisons Scientific Equipment
Picrotoxin	Sigma Chemicals Co. Ltd.
PIPES	Sigma Chemicals Co. Ltd.

PMSF	Sigma Chemicals Co. Ltd.
Poly (dI.dC) DNA copolymer	Pharmacia Biotech Ltd.
Polyvinylpyrrolidone	Sigma Chemicals Co. Ltd.
Potassium chloride	Fisons Scientific Equipment
Potassium dihydrogen orthophosphate	Fisons Scientific Equipment
Proteinase K	Boehringer Mannheim Ltd.
Protein molecular weight marker (mid-range)	Promega
Protogel, 30% acrylamide 0.8% bisacrylamide, gas stabilised.	National Diagnostics
Random primers (75ng/µl)	Gibco BRL
RNase A, 10mg/ml	Sigma Chemicals Co. Ltd.
Salmon sperm DNA	Sigma Chemicals Co. Ltd.
Scintillation fluid, Ecoscint	National Diagnostics
SDS	BDH Laboratory Supplies
SOB medium capsules	Bio 101 Inc.
Sodium acetate	Fisons Scientific Equipment
Sodium pentobarbital	Sigma Chemicals Co. Ltd.
Sodium phenobarbital	Sigma Chemicals Co. Ltd.
Soya bean trypsin inhibitor	Sigma Chemicals Co. Ltd.
(Sp)cAMPS	Sigma Chemicals Co. Ltd.
Spermidine	Sigma Chemicals Co. Ltd.
Spermine	Sigma Chemicals Co. Ltd.
Spin TE-10 column	Clonetech Laboratories Inc.
Spin-X centrifugation filtration units	Costar
Streptomycin	Sigma Chemicals Co. Ltd.
Sucrose	Fisons Scientific Equipment
T4 DNA ligase, 6.7u/µl	Pharmacia Biotech Ltd.
T4 polynucleotide kinase (Ready-to-go)	Pharmacia Biotech Ltd.
TEMED	Bio-Rad Laboratories Ltd.
Trypan blue	Sigma Chemicals Co. Ltd.
Tween-20	Bio-Rad Laboratories Ltd.
Tygon 50 dialysis tubing	Verder Belgium
Ultraspec™ RNA solution	Biotecx

Vitrogen 100®	Imperial Laboratories
William's E medium (without phenol red)	Gibco BRL
X-Ray film	Fuji Photo Film Co. Ltd.
Xylene cyanol	BDH Laboratory Supplies

Kits:

Bio-Rad immun-blot assay kit:
10x TBS, gelatin (blotting grade), tween-20 (blotting grade), GAR-HRP conjugate solution, colour development reagent (4-chloro-1-napthol).

Bio-Rad protein assay:
Dye reagent, lyophilised bovine plasma albumin (BSA)

Promega Dual Luciferase Assay kit:
Luciferase Assay Buffer II, Luciferase Assay Substrate, Stop and Glo Buffer, Stop and Glo Substrate, Stop and Glo Substrate Buffer, Passive Lysis Buffer.

Qiagen plasmid preparation kit:
Qiagen tip 500 (Qiagen column), P1 solution, P2 solution, P3 solution, QBT buffer, QC buffer, RNase A.

National Diagnostics HRPL kit:
Development buffers A and B.

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