# ISOLATION, CHARACTERIZATION AND INDUCTION STUDIES OF HUMAN CYTOCHROMES P450

BY

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# *ABSTRACT*

Animal models, particularly rodents, are used for biotransformation studies. Marmosets may provide a better model as they are more closely evolutionarily linked to man. A human CYP2A6 and three marmoset CYP2A cDNA clones were isolated from their respective liver cDNA libraries. The DNA sequences of the three marmoset CYP2As were 92% identical to the human CYP2A6 DNA sequence whereas the predicted protein sequences were 88% identical.

In experimental animals anti-epileptic drugs, such as phenobarbital and valproic acid, lead to increased expression of CYPs. It has been shown using western blot analysis with polyclonal antibodies to CYP2As, CYP2B6, CYP2Cs, CYP3As and NADPH-cytochrome P450-reductase that both these drugs induce the expression of the above enzymes in cultured human hepatocytes derived from two different individuals. The anti-epileptic agents induced the expression of these enzymes after four and seven days (three and six days of treatment with the drugs, respectively) of culture. The antibodies used in this study recognise all isoforms of each subfamily and were mainly raised to non-human CYPs.

The major aim of this thesis was to produce monoclonal antibodies to two human CYPs, CYP2A6 and CYP3A4, using an adaptation of the recombinant phage antibody system. These CYPs were purified from insect cell microsomes and injected separately into mice. Antibody ScFv cDNA libraries were constructed from mice spleen mRNA using an RT-PCR based cloning strategy. The antibodies were expressed in *E. coli* using a flexible phagemid vector that permitted either phage-displayed or soluble antibody expression. Soluble antibodies that recognised a CYP3A4 antigen preparation were isolated from the CYP3A4 ScFv antibody library by a two-membrane immunodetection screen.

TO MY MOTHER,

MAUREEN LINDEY

(1947-1987)

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# ABBREVIATIONS

mA = Milliamperes
Ah = Aromatic hydrocarbon
AMP = Ampicillin
AP = Alkaline phosphatase
ATP = Adenosine triphosphate
B.D.H. = British Drug Houses
BHT = Butylated hydroxy-toluene
bp = Base pairs
BSA = Bovine serum albumin
cDNA = Complementary DNA
C = Constant
$C_H$ = Constant heavy
$C_L$ = Constant light
CDRs = Complementarity-determining regions
C/EBP = CCAAT/enhancer binding protein
Ci = Curie
μCi = Microcurie
4-CN = 4-chloro-1-napthol
C-terminus = Carboxy-terminus
CTP = Cytidine triphosphate
CYP = Cytochrome P450
CYPs = Cytochromes P450
dNTP = Deoxynucleoside triphosphate
ddNTP = Dideoxynucleoside triphosphate
D = Diversity
DEAE = Diethylaminoethyl

DEPC = Diethylpyrocarbonate

DMSO = Dimethyl sulphoxide

DNA = Deoxynucleic acid

DTT = Dithiothreitol

$e^-$ = Electron

*E. coli* = *Escherichia coli*

EDTA = Diaminoethanetetra-acetic acid

ELISA = Enzyme-linked immunosorbent assay

etc. = Etcetera

F = Forward

Fab = Fragment antibody

FAD = Flavin adenine dinucleotide

$FAD^{\bullet}$ = Flavin adenine dinucleotide radical form

$FADH_2$ = Flavin adenine dinucleotide reduced form

FMN = Flavin mononucleotide

$FMN^{\bullet}$ = Flavin mononucleotide radical form

$FMNH_2$ = Flavin mononucleotide reduced form

Fv = Fragment variable

FW = Framework

g = Gravitational force (where appropriate}

g = Gram

g3p = gene 3-encoded adsorption protein

H = Heavy

kg = Kilogram

mg = Milligram

μg = Microgram

ng = Nanogram

pg = Picogram

GST = Glutathione *S*-transferase

GSTs = Glutathione *S*-transferases
GTP = Guanosine triphosphate
HAT = Hypoxanthine, aminopterin and thymidine
HEPES = N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HRP = Horseradish peroxidase
Ig = Immunoglobulin
IgG = Immunoglobulin G
IPTG = Isopropyl-β-D-thiogalactopyranoside
J = Joining
kb = Kilobases
KCl = Potassium chloride
L = Light
Lac = Lactose
LB = Luria-Bertani
LiCl = Lithium chloride
ml = Millilitres
μl = Microlitres
nm = Nanometres
μm = Micrometres
M = Molar
mM = Millimolar
μM = Micromolar
nM = Nanomolar
min = Minute
mseconds = Milliseconds
M-MLVRT = Moloney-murine leukaemia virus reverse transcriptase
MOPS = 3-[*N*-morpholino] propanesulfonic acid buffer
MW = Molecular weight
N = Normal

NaCl = Sodium chloride

NAD = Nicotinamide adenine dinucleotide

NADH = Nicotinamide adenine dinucleotide reduced

NADP = Nicotinamide adenine dinucleotide phosphate

NADPH = Nicotinamide adenine dinucleotide phosphate reduced

NDEA = *N*-nitrosodiethlyamine

NDMA = *N*-nitrosodimethylamine

NNK = (methylnitrosamino)-1-(3-pyridyl)-1-butanone

no. = Number

N-terminus = Amino-terminus

$O.D._x$ = Optical density at x nanometres

Oligo = Oligonucleotide

$OPA^+$ = One-Phor-All Buffer Plus

PB = Phenobarbital

PBS = Phosphate buffered saline

PCR = Polymerase chain reaction

PEG = Polyethylene glycol

*Pfu* = *Pyrococcus furiosis*

Poly (A)+ = Polyadenylated ribonucleic acid

PMSF = Phenylmethylsulphonylfluoride

P450-reductase = NADPH-cytochrome P450-reductase

q = Long arm

qter = Tip of long arm

r = Radius

R = Reverse

® = Registered

RACE = Rapid amplification of cDNA ends

RCF = Relative centrifugal force

rel = Relative

RH = Drug substrate

RIA = Radioimmunoassay

RNA = Ribonucleic acid

mRNA = Messenger RNA

RNase = Ribonuclease

ROH = Hydroxylated drug metabolite

RPAS = Recombinant phage antibody system

rpm = Revolutions per minute

RS = Restriction site

RT = Reverse transcription

S = Svedberg

ScFv = Single-chain Fv fragment

SDS = Sodium dodecyl sulphate

SDS-PAGE = Sodium dodecyl sulphate polyacrylamide gel electrophoresis

*Sf9* = *Spodoptera fugiperda*

$[\alpha\text{-}^{35}\text{S}]$dATP = Sulphur radiolabelled dATP at the $\alpha$-phosphate

TBE = Tris-borate buffer

TBS = Tris buffered saline

TEMED = N, N, N', N'-Tetramethylethylenediamine

™ = Trade mark

*Tni* = *Trichoplusia ni*

Tris = Tris (hydroxy methyl) aminomethane

TTBS = Tween-20 Tris buffered saline

TTP = Thymidine triphosphate

U.K. = United Kingdom

U.S.A. = United States of America

U.V. = Ultraviolet

V = Variable

$V_H$ = Variable region of heavy chain of an antibody

$V_L$ = Variable region of light chain of an antibody

VP = Valproic acid

w/v = Weight/volume

v/v = Volume/volume

X-gal = 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

+ve = Positive

-ve = Negative

$1^{o}$ = Primary

$2^{o}$ = Secondary

~ = Approximately

# = Clone

# *CHAPTER ONE*

# INTRODUCTION

## 1.1. CYTOCHROMES P450-MEDIATED METABOLISM

Cytochromes P450 are involved in the oxidative, peroxidative and reductive metabolism of numerous endogenous compounds such as steroids, bile acids, fatty acids, prostaglandins, leukotrienes and biogenic amines (Nelson *et al.*, 1993). These microsomal enzymes are also involved in the metabolism of a wide array of foreign chemicals including drugs, environmental pollutants, natural plant products, odorants and alcohols (Nelson *et al.*, 1993). Included in the xenobiotics metabolised by cytochromes P450 are carcinogens. P450 enzymes metabolise carcinogens such as polycyclic aromatic hydrocarbons and nitrosamines (Gonzalez, 1989) or create carcinogenic intermediates such as epoxides during metabolism of procarcinogens. These metabolites attack the cellular biomolecules DNA, RNA and protein resulting in toxicity, cell death, mutations and cell transformation.

In 1958 Klingenberg and Garfinkle separately identified a reduced pigment that had an absorption band with a $\lambda_{max}$ at 450 nm after binding to carbon monoxide (Klingenberg, 1958; Garfinkle, 1958). Omura and Sato further characterised this pigment in 1961 as being a P450 haemoprotein (Omura and Sato, 1961). Four years later Cooper *et al.* (1965) linked the role of cytochromes P450 to the microsomal mixed function monooxygenase system. During the next twenty years it became evident, from protein purification studies, that multiple forms of cytochromes P450 exist in mammals and other species.

The majority of cytochrome P450-mediated metabolism results from the insertion of a single atom of oxygen, derived from molecular oxygen, into the substrate via a wide range of reactions depending on the substrate. These are often referred to as Phase I reactions of xenobiotic metabolism and include: oxidative and reductive dehalogenation; N-hydroxylation and N-oxidation; oxidative deamination; S-, N- and O-dealkylation; and aliphatic and aromatic hydroxylation (Gonzalez, 1989). Examples of the diverse monooxygenase activities of the cytochromes P450 are illustrated in figure 1.1.

A common feature of these enzymes is that a single cytochrome P450 can metabolise many structurally diverse compounds. This leads to a collective capacity to metabolise and detoxify countless substances in the diet and environment (Gonzalez and Nebert, 1990).

Cytochromes P450 are part of the mixed-function oxidase system located in the microsomes of mainly the liver, kidney, lung and intestine (Gibson and Skett, 1994). The key enzymatic components of this system are the flavoprotein NADPH-cytochrome P450-reductase, cytochromes P450 and lipid. Cytochromes P450 are classified as haem-containing enzymes with iron protoporphyrin IX as the prosthetic group. The haem is non-covalently bound to the apoprotein via a cysteine residue situated within a conserved haem-binding domain of fifteen amino acids (Gotoh *et al.*, 1983). NADPH-cytochrome P450 reductase is described in section 1.2. Phosphatidylcholine has been identified as being the lipid component of the mixed-function oxidase system (Lu *et al.*, 1969; Strobel *et al.*, 1970). The exact function of lipid in the monooxygenase system has not been resolved

## 1.2. NADPH-CYTOCHROME P450-REDUCTASE

The flavoprotein NADPH-cytochrome P450-reductase (P450-reductase) contains one mole of flavin adenine dinucleotide (FAD) and one mole of flavin mononucleotide (FMN) per mole of apoprotein (Iyangi and Mason, 1973; Vermilion and Coon 1974). This distinguishes NADPH-cytochrome P450-reductase from other flavoproteins as normally only FAD or FMN is present as the prosthetic group. The enzyme has a monomeric molecular weight of approximately 78,000 and it exists in close association with cytochrome P450 in the endoplasmic reticulum membrane. P450-reductase is

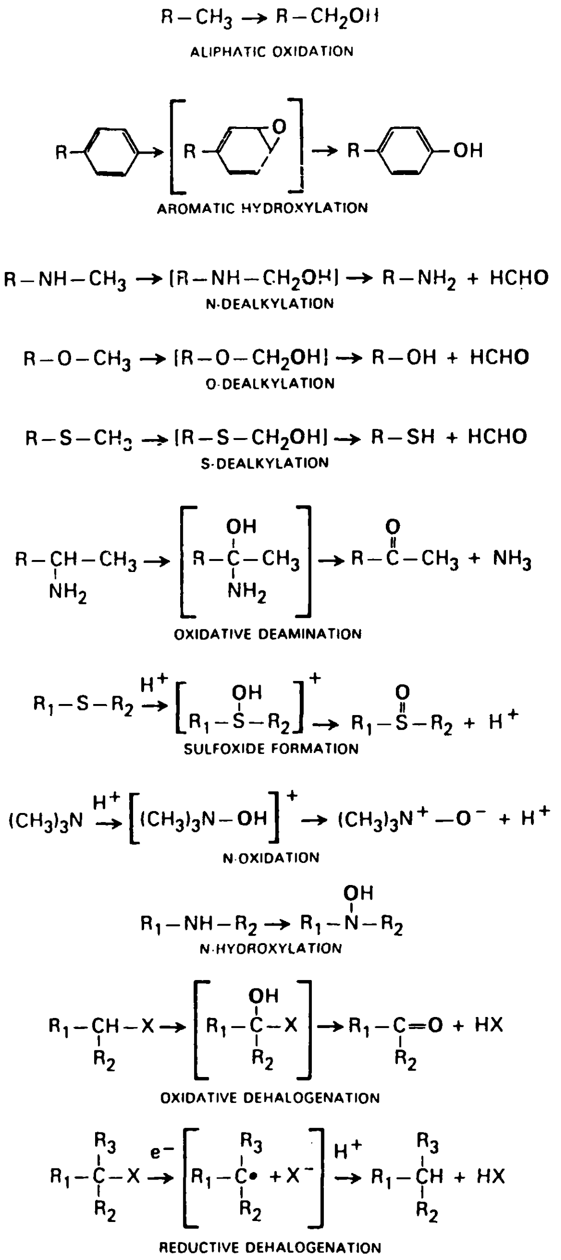

ALIPHATIC OXIDATION
AROMATIC HYDROXYLATION
N-DEALKYLATION
O-DEALKYLATION
S-DEALKYLATION
OXIDATIVE DEAMINATION
SULFOXIDE FORMATION
N-OXIDATION
N-HYDROXYLATION
OXIDATIVE DEHALOGENATION
REDUCTIVE DEHALOGENATION

Figure 1.1: Examples of the many kinds of monooxygenase reactions carried out by CYPs (reproduced from Nebert and Gonzalez et al., 1987).

susceptible to proteolytic cleavage; proteolysis yields a protein of apparent molecular weight of 66,000 (Lu *et al.*, 1969) and a polypeptide of approximate apparent molecular weight of 12,000. The cleaved polypeptide is very hydrophobic and is found at the N-terminus of the parent protein (Black *et al.*, 1979; Black and Coon, 1982). This polypeptide anchors the native enzyme in the membrane (Gum and Strobel, 1979; French *et al.*, 1980).

P450-reductase transfers two electrons separately from NADPH + $H^+$ to cytochrome P450; these electrons are required during steps two and five of the catalytic cycle of cytochrome P450 (section 1.3.). The oxidation/reduction state of P450-reductase during the cytochrome P450 catalytic cycle has not yet been precisely determined.

## 1.3. CATALYTIC CYCLE OF CYTOCHROME P450

During the cytochrome P450 catalytic cycle the haem iron undergoes cyclic oxidation/reduction reactions in conjunction with substrate binding and oxygen activation (reviewed in Porter and Coon, 1991). The nature and degree of oxygenation of the haem iron in certain of the intermediates is unsure. The catalytic cycle of cytochrome P450 is shown in figure 1.2.

The first step in the reaction cycle involves the binding of the substrate RH to the oxidized ferric ($Fe^{3+}$) form of cytochrome P450. In the second step the first electron reduction of the substrate-bound ferric ($Fe^{3+}$) cytochrome P450 to the ferrous ($Fe^{2+}$) form of the haemoprotein takes place. The reducing equivalent necessary for this reduction is originally derived from NADPH + $H^+$ and is transferred by the flavoprotein P450-reductase (figure 1.3A). The flavoproteins FAD/FMN are involved in this electron transfer event. The third step involves the binding of molecular oxygen to the ferrous cytochrome P450-substrate adduct. The oxy-ferrous-substrate complex is unstable and hence this reaction is not well characterised. The fourth, fifth and sixth

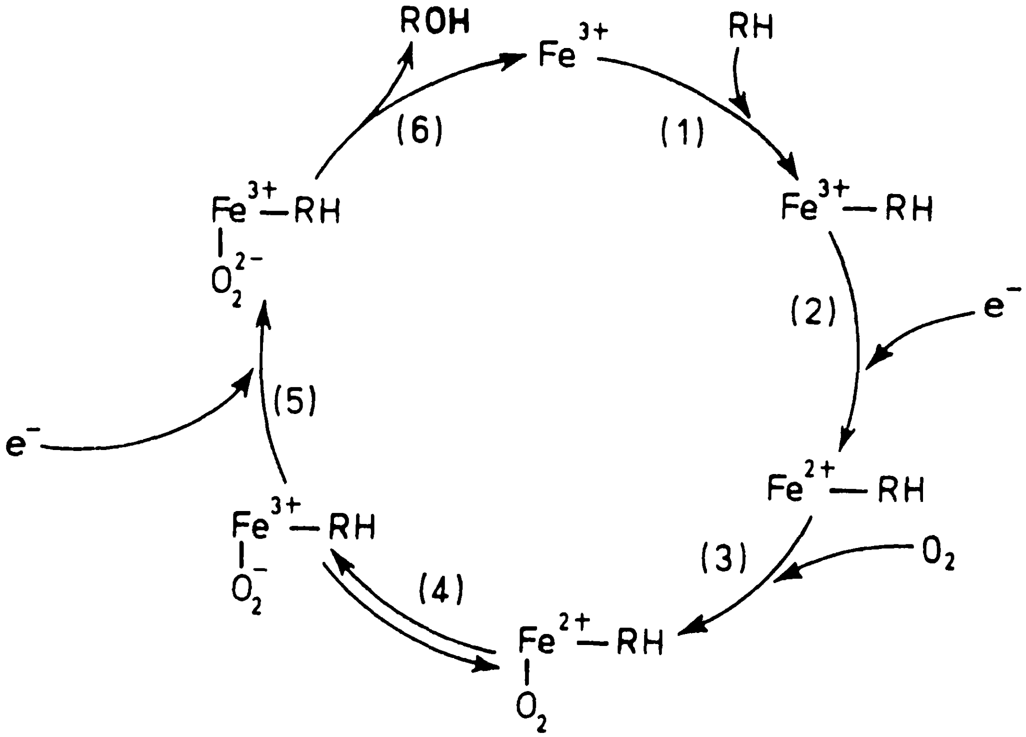

ROH
Fe3+
RH
(6)
(1)
Fe3+—RH
Fe3+—RH
O2 2-
(2)
e-
(5)
e-
Fe2+—RH
Fe3+—RH
O2 -
(3)
O2
(4)
Fe2+—RH
O2

Figure 1.2: Catalytic cycle of cytochrome P450.

RH represents the drug substrate, ROH the corresponding hydroxylated metabolite and e- an electron (adapted from Gibson and Skett, 1994).

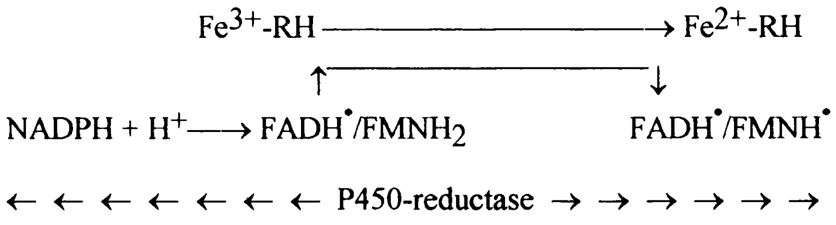

$Fe^{3+}$-RH
$Fe^{2+}$-RH
NADPH + $H^+$
$FADH^•/FMNH_2$
$FADH^•/FMNH^•$
P450-reductase

Figure 1.3A: First electron reduction of cytochrome P450.

The reducing equivalent is transferred from NADPH + $H^+$ to cytochrome P450 by P450-reductase. $Fe^{3+}$ and $Fe^{2+}$ represents the oxidized (ferric) and reduced (ferrous) forms of cytochrome P450 haem iron respectively, bound to a substrate RH; $FADH^•$ and $FMNH^•$ represent the radical forms of FAD and FMN, respectively; $FMNH_2$ represents the reduced form of FMN (adapted from Gibson and Skett, 1994).

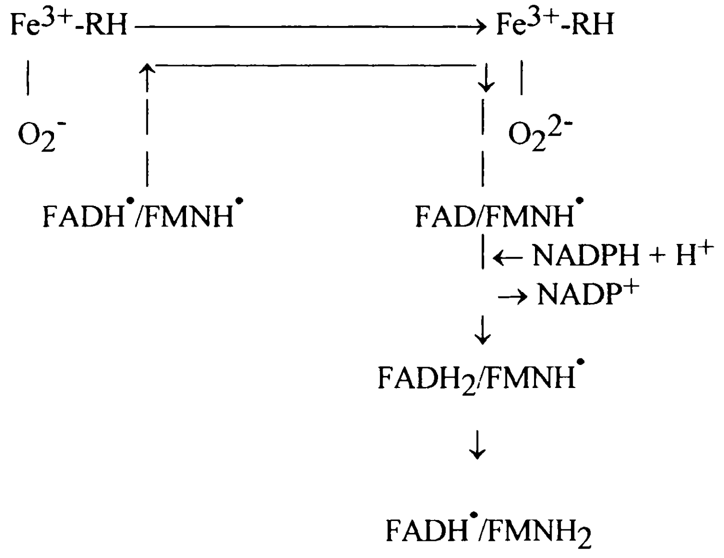

$Fe^{3+}$-RH
$Fe^{3+}$-RH
$O_2^-$
$O_2^{2-}$
$FADH^•/FMNH^•$
$FAD/FMNH^•$
NADPH + $H^+$
$NADP^+$
$FADH_2/FMNH^•$
$FADH^•/FMNH_2$

Figure 1.3B: Second electron reduction of cytochrome P450; $FADH_2$ represents the reduced form of FAD (adapted from Gibson and Skett, 1994).

steps involve putative electron rearrangement, introduction of the second electron and subsequent oxygen insertion and product release. The precise oxidation states of iron and oxygen in these intermediates are not fully known. A second electron enters the cycle in the fifth step, this is usually derived from NADPH + H via P450-reductase (figure 1.3B) and is sometimes thought to be donated via cytochrome $b_5$. Once again, the flavoproteins FAD/FMN are involved in the electron transfer step. Step six has not yet been fully elucidated, it involves the actual chemical mechanism of oxygen insertion into the cytochrome P450 substrate. The subsequent metabolic product is then released.

## 1.4. MEMBRANE TOPOLOGY OF MAMMALIAN CYTOCHROMES P450

The membrane topology of the mammalian P450s has been extensively studied. Recent findings indicate that the microsomal P450s are bound to the endoplasmic reticulum by only one or two transmembrane peptides located at the amino-terminal end, and that the active site is part of a large cytoplasmic domain that may have one or two additional peripheral membrane contacts (Black, 1992). Sequences of the microsomal cytochromes begin with a highly hydrophobic segment of about twenty amino acids which is followed by a short polycationic segment; the remainder of the sequence exhibits alternating hydrophilic and hydrophobic character, but typically of greater polarity than that of the amino-terminal region (Black, 1992).

## 1.5. MITOCHONDRIAL CYTOCHROMES P450

Mitochondrial cytochrome P450s together with some microsomal cytochromes P450s are involved in the anabolic synthesis of steroids in the adrenal glands, ovaries and testes. These proteins include the microsomal enzymes steroid 17α-hydroxylase and 21α-hydroxylase; and the mitochondrial enzymes cholesterol side chain cleavage enzyme and steroid 11β-hydroxylase (reviewed in Gonzalez, 1989). The mitochondrial P450s differ from their microsomal counterparts in that they are deficient in a hydrophobic N-terminus (Black, 1992) and they collect electrons via adrenodoxin and adrenodoxin reductase (Fevold, 1983; Jefcoate, 1986). Mitochondrial P450s are synthesised in the cytosol with a transient targeting sequence of 24-39 amino acids at the amino-terminus; the amphipathic target sequences are rich in leucine, alanine and arginine and are proteolytically removed after movement of the precursor to the inner mitochondrial membrane (Black, 1992). The genetic lack of the mitochondrial cytochromes P450s is associated with severe defects in cortisol biosynthesis (Miller, 1988; Miller and Levine, 1987).

## 1.6. CLASSIFICATION AND NOMENCLATURE OF CYTOCHROMES P450

A vast array of P450 genes exist, 481 genes and 22 putative pseudogenes have been identified in 85 eukaryotes (including vertebrates, invertebrates, fungi and plants) and 20 prokaryote species (Nelson *et al.*, 1996). These genes have been classed into 74 gene families; a general overview of cytochrome P450 families/subfamilies is shown in table 1.1. Within a single family, the P450 protein sequences are > 40% similar to each other. Further classification into subfamilies brings the similarity to > 55% in mammals (Nelson *et al.*, 1993). To date each subfamily appears to represent a cluster of tightly linked genes. Each P450 gene almost always encodes a single protein, a few exceptions exist where functional alternative mRNA splicing may occur (Nelson *et al.*, 1993). Almost all mammalian tissues contain one or more cytochromes P450, predominantly in the endoplasmic reticulum and mitochondria (Coon *et al.*, 1992)

The P450 Nomenclature Committee recommend that for naming a P450 gene the italicised root symbol "*CYP*" ("*Cyp*" for the mouse and Drosophila), denoting Cytochrome P450, an Arabic number designating the P450 family, a letter indicating the subfamily when two or more subfamilies are known to exist within that family, and an Arabic numeral representing the individual gene are included (Nelson *et al.*, 1996). "*P*" ("*ps*" in mouse and Drosophila) after the gene number is used to denote a

| | |
|---|---|
| *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 function(s) |
| *CYP5* | Vertebrates; thromboxane synthase |
| *CYP6* | Insects; metabolism of plant products and pesticides |
| *CYP7A* | Vertebrates; cholesterol 7α-hydroxylase |
| *CYP7B* | Vertebrates; unknown function(s) |
| *CYP8* | Vertebrates; prostacyclin synthase |
| *CYP9* | Insects |
| *CYP10* | Mollusks (mitochondrial enzyme) |
| *CYP11* | Vertebrates; cholesterol side-chain cleavage, steroid 11β-hydroxylase and aldosterone synthase (mitochondrial enzyme) |
| *CYP12* | Insects (mitochondrial enzyme) |
| *CYP13* | Nematodes |
| *CYP14* | Nematodes |
| *CYP15* | Insects |
| *CYP16* | Nematodes |
| *CYP17* | Vertebrates; steroid 17α-hydroxylase |
| *CYP18* | Insects |
| *CYP19* | Vertebrates; aromatization 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; sterol biosynthesis |
| *CYP52* | Yeast; alkaline hydroxylases |
| *CYP53-CYP62* | Fungi |
| *CYP71-CYP92* | Plants |
| *CYP73* | Plants, cinnamic acid hydroxylase |
| *CYP101-CYP118* | Bacteria |

Table 1.1: General overview of cytochrome P450 families/subfamilies and enzyme functions in various species (reproduced from Nelson *et al.*, 1996).

pseudogene (Nelson *et al.*, 1996). If no second subfamily or second gene exists in a family, the subfamily and gene number are not included e.g. *CYP5* or *CYP19*. The above nomenclature applies to the corresponding gene product (mRNA, cDNA or enzyme). For example, italicized "*CYP1A2*" ("*Cyp1A2*" in mouse) for the gene; non-italicized and capital letters "CYP1A2" for the cDNA, mRNA and protein in all species (including mouse and Drosophila; Nelson *et al.*, 1996).

## 1.7. EVOLUTION OF THE CYP GENE SUPERFAMILY

The CYP superfamily is very ancient, it is thought the ancestral gene existed more than 3.5 billion years ago (reviewed in Nelson *et al.*, 1993). The ancestral gene predates the divergence of prokaryotes and eukaryotes, the occurrence of therapeutic drugs, animal-plant interactions and carcinogens arising from the combustion of organic material. Nebert (1991) has proposed that the CYP enzymes play(ed) a vital role in maintaining the steady-state levels of endogenous ligands involved in ligand-modulated transcription of genes affecting homeostasis, growth, differentiation and neuroendocrine functions.

The formation of new CYP genes occurs through DNA turnover events or "molecular drive"; new variant genes can ultimately be spread throughout a population as a consequence of the genomic turnover mechanisms themselves (Dover, 1986). However genetic drift and natural selection might account for a small number of new CYP genes. These processes allow continual adaptation to changing environmental conditions. It seems highly probable that a new CYP gene that has just appeared during the past several hundred million years of evolution will not carry out a critical life function (for example, synthesis or degradation of a ligand responsible for ligand-activated transcriptional activation) such as a housekeeping CYP gene that has existed for more than 500 or 1000 million years (Nebert, 1991). Nebert (1991) has suggested that if a CYP gene is no longer required for survival, mutations are accumulated and the gene is eventually inactivated. The early stages of gene inactivation can lead to genetic polymorphisms where only a small percentage of alleles encoding a CYP gene are defective.

The species specificity in CYP catalytic activities and regulation can be accounted for by many molecular events. These include gene duplication, gene inactivation or terminal mutational events and a variety of other DNA turnover mechanisms such as replication slippage, insertions and gene conversions (Gonzalez and Nebert, 1990).

## 1.8. PHASE II REACTIONS OF XENOBIOTIC METABOLISM

Drug metabolism is divided into two phases, phase I (functionalistaion reactions) and phase II (conjugation reactions). Phase II reactions conjugate products from phase I metabolism to endogenous moieties such as glucuronide, glutathione and sulphate. Phase II enzymes include UDP glucuronosyltransferases; glutathione *S*-transferases; sulfotransferases; quinone reductases; NADPH diaphorase; azo reductases; NAD- and NADP-dependent alcohol, aldehyde and steroid dehydrogenases; aldoketoreductases; acetyltransferases; methyltransferases; transaminases; and a wide variety of esterases and hydrolases (Nebert, 1991). These hydrophilic conjugated compounds are then easily passed from the body via the urine or bile (Gonzalez, 1989). The expression of a human glutathione *S*-transferase, hGSTP1-1, was examined in human hepatocyte cultures in this thesis, therefore a brief description of the GST family of enzymes is given in section 1.8.1.

### 1.8.1. GLUTATHIONE *S*-TRANSFERASES

The glutathione *S*-transferases (GSTs) are a family of detoxification enzymes involved in phase II metabolism. Eukaryotic species possess cytosolic and membrane-bound GST isoenzymes (reviewed in Hayes and Pulford, 1995). In man, there are at least twenty cytosolic GSTs found mainly in the liver, kidney and gut; they conjugate glutathione (Gly-Cys-Glu) to strong electrophiles produced during the phase I metabolism of drugs and to strong electrophilic xenobiotic compounds (Gibson and Skett, 1994). The membrane-bound GSTs are microsomal GST and leukotriene $C_4$ synthetase. The microsomal GST is involved in the metabolism of xenobiotics; leukotriene $C_4$ synthetase conjugates glutathione to leukotriene $C_4$ (reviewed in Hayes and Pulford, 1995). The glutathione *S*-conjugates are actively exported out of cells by an ATP-dependent glutathione *S*-conjugate export pump (reviewed in Ishikawa, 1992).

The cytosolic GSTs are encoded by at least five gene families, class alpha, mu, pi, sigma and theta GST; enzymes in the same family share >40% amino acid identity. The GSTs are composed of two subunits; the enzymes are either homodimers or heterodimers. The enzymes are named using the nomenclature system proposed by Mannervick *et al.*, (1992); the species is denoted by a lower case letter, the class is denoted by an upper case letter and the subunit composition by two Arabic numbers. Therefore hGSTP1-1 is a human GST formed from two pi class 1 subunits. The membrane-bound enzymes, microsomal GST and leukotriene $C_4$ synthetase are encoded by single genes.

## 1.9. SPECIES DIFFERENCES

Animal models are used to further our understanding of genetic and biochemical pathways such as gene regulation, signal transduction and metabolic pathways. Most information on the enzymology, structure and regulation of CYPs has been found through the study of rodent systems. Since rodent and human lineages diverged over 60 million years ago, caution must be applied when comparing rodent CYPs with human CYPs due to the evolutionary differences (Gonzalez *et al.*, 1988). There have been several reports identifying the existence of a particular CYP gene (and corresponding enzyme activity) in the laboratory animal but not in man and vice versa (Nebert and Gonzalez, 1987; Gonzalez, 1989).

Species differences in drug metabolism studies are of particular interest to pharmaceutical companies involved in testing new chemicals and therapeutic drugs. The animal model must mimic the metabolism of the compound as would be seen in man in order to achieve a suitable model of human toxicity (Gibson and Skett, 1994). Different animals may be used for different chemicals.

Species differences are seen for both phase I and phase II metabolism. The difference in metabolism is divided into two types, quantitative and qualitative. A quantitative difference occurs when a compound is metabolised by the same metabolic route in the two species but at a different rate. An example of a quantitative species difference is the metabolism of indinavir, a potent human immunodefieicency virus protease inhibitor (Lin *et al.*, 1996). Indinavir is metabolised by the CYP3A subfamily in the rat, monkey and dog; the plasma clearance of the drug is faster in the rat (107 ml/min/kg) than the monkey (36 ml/min/kg) than in the dog (16 ml/min/kg).

A qualitative species difference occurs when a compound is metabolised by different routes in different species. An example of a qualitative species difference is the metabolism of amphetamine (reviewed in Gibson and Skett, 1994; figure 1.4). Rats mainly hydroxylate amphetamine whereas rabbits, guinea-pigs and man largely deaminate the compound to the corresponding ketone. Rats excrete phenolic conjugated compounds. Guinea-pigs further oxidise the ketone to benzoic acid and excrete conjugates of the aromatic carboxylic acid. Rabbits reduce the ketone to an alcohol and excrete conjugates of this compound.

Species differences lead to difficulties in the extrapolation of toxicity data, concerning a new therapeutic drug, from the laboratory animal to human clinical trials.

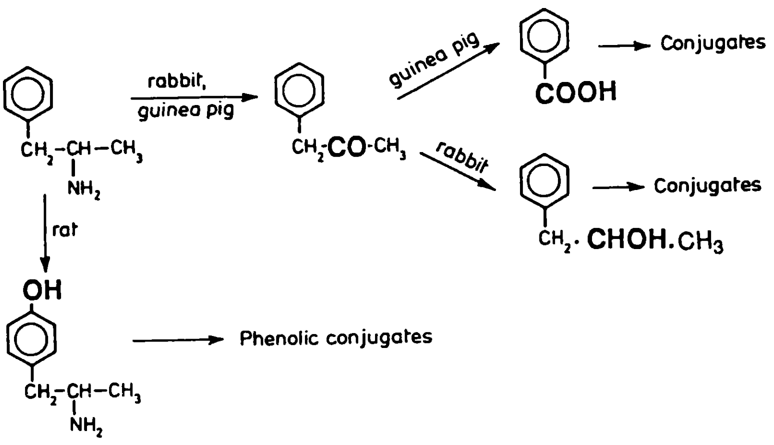

rabbit,
guinea pig
guinea pig
rabbit
rat
Conjugates
Conjugates
Phenolic conjugates
COOH
CH2-CH-CH3
NH2
CH2-CO-CH3
CH2. CHOH.CH3
OH

Figure 1.4: Species differences in the metabolism of amphetamine in rabbit, guinea-pig and rat (reproduced from Gibson and Skett, 1994).

## 1.10. HEPATOCYTE CULTURE SYSTEMS

An alternative to the use of animal models for drug metabolism studies is an *in vitro* system; such as a hepatocyte culture system. Information concerning the metabolic pattern, hepatotoxicity, genotoxicity and toxicological data of a novel drug can be gathered from such an *in vitro* situation. Inter-individual differences in the metabolism of a drug can also be assessed as each cell culture is derived from a single individual.

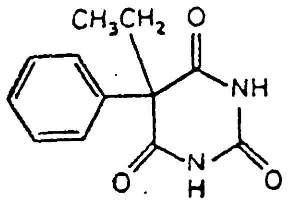
Many research groups, including our own laboratory have used rodent cell culture systems (Ciaramella, PhD. thesis, 1995). As has been previously mentioned in section 1.9, rodents exhibit differences in drug metabolism to that of man. Part of this thesis is concerned with the characterization of a human hepatocyte culture system developed in the laboratory of Prof. Vera Rogiers at the Vrije Universiteit in Brussels (section 4.1.) using polyclonal antibodies to various CYPs. Therefore a brief overview of hepatocyte culture systems is provided.

A problem often associated with hepatocyte culture systems is the loss of liver-specific functions and subsequent dedifferentiation of the hepatocytes. This is associated with the disruption of cell-cell and cell-matrix interactions during the isolation of the hepatocytes from the whole liver (Bissell and Guzelian, 1980). There is a rapid loss of expression of many CYPs (Steward *et al.*, 1985) and their response to well known inducers (Rogiers *et al.*, 1990; Akrawi *et al.*, 1993a and Akrawi *et al.*, 1993b) as the hepatocytes dedifferentiate. Guillouzo *et al.* (1985) have shown that cultured human hepatocytes contain 50% of the initial CYP content after eight days of culture. However, cultured human hepatocytes are able to metabolise drugs by various phase I and phase II reactions for several days (Berthou *et al.*, 1988; Bégué *et al.*, 1983 and Grant *et al.*, 1987). It has been shown that the use of a serum free hormonally defined media (Enat *et al.*, 1984) supplemented with glucocorticoids is important in the maintenance of the hepatocytes differentiated state (Sinclair *et al.*, 1990). Waxman *et* *al.* (1990) has shown that the use of type I collagen-coated tissue culture plates is also important in the maintenance of the differentiated state of the hepatocytes. These conditions attempt to mimic, as far as possible, the natural environment of the hepatocytes in the liver.

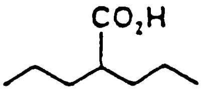
A successful human hepatocyte culture system can be used to investigate the metabolism and cytotoxicity of therapeutic drugs, carcinogens and other xenobiotics. The culture system can also be used to study the effects of therapeutic drugs on drug-metabolising enzymes and also investigate potential drug-drug and drug-xenobiotic interactions. The human hepatocyte culture system characterised in Chapter 4 was used to determine if two anti-epileptic drugs, phenobarbital and valproic acid, induce the expression of human CYPs. This has clinical relevance as both these drugs are metabolised by the CYP family (section 1.10.1. and 1.10.2.) along with a wide array of therapeutic drugs. Therefore potential drug-drug interactions may ensue after administration of either phenobarbital or valproic acid. Approximately 30% of epilepsy sufferers receive long term antiepileptic polytherapy (Riva *et al.*, 1996); the remaining 70% of patients are normally administered a single anti-epileptic drug. Drug-drug interactions present serious clinical implications.

### 1.10.1. PHENOBARBITAL

Phenobarbital (structure is shown in figure 1.5) is a widely used anti-convulsant drug that is a classical inducer of members of the CYP2A, CYP2B CYP2C and CYP3A subfamilies, amongst others, both in laboratory animals and in man (reviewed in Waxman and Azaroff, 1992). The mechanism of phenobarbital induction has not yet been clearly elucidated. However, the induction of the expression of the *CYP2B1* and *CYP2B2* genes in the rat is mediated by a twenty-fold induction in the amounts of the two CYP mRNAs (Phillips *et al.*, 1981; Hardwick *et al.*, 1983). The increase in CYP2B1 and CYP2B2 mRNAs is through an increase in transcription (Pike *et al.*,



Phenobarbital



Valproic acid

Figure 1.5: Structures of the anti-epileptic agents phenobarbital and valproic acid.

1985). It is possible that there is a receptor for phenobarbital which mediates induction of the phenobarbital responsive *CYP* genes. For instance an aromatic hydrocarbon (Ah) receptor plays a role in the induction of transcription of *CYP1A1* by polycyclic aromatic hydrocarbons (Nebert *et al.*, 1990). Clofibrate, a peroxisomal proliferator, induces the expression of *CYP4A* by a peroxisomal proliferator activated receptor (Hardwick *et al.*, 1987; Muerhoff *et al.*, 1992). Studies in our laboratory, have indicated that the transcription factors CCAAT/enhancer binding protein (C/EBP)$\alpha$ and C/EBP$\beta$ are involved in the phenobarbital response mechanism (Shephard *et al.*, 1994; Dell, PhD. Thesis, 1997).

The main metabolic pathway for elimination of phenobarbital is by aromatic hydroxylation to form *p*-hydroxy-phenobarbital. This reaction is mediated by CYPs, possibly including CYP2C19 (Anderson and Levy, 1995).

### 1.10.2. VALPROIC ACID

Valproic acid (structure is shown in figure 1.5) has been used as an anti-epileptic agent for over thirty years. Its use has been associated with altered endogenous metabolism and hepatoxicity (Eadi *et al.*, 1988; Dreifuss *et al.*, 1987). The exact mechanism for this adverse drug reaction is unclear, but the $\Delta^4$-metabolite of valproic acid is believed to be involved (reviewed in Rettie *et al.*, 1987). The $\Delta^4$-metabolite is produced by desaturation of valproic acid by CYPs (Rettie *et al.*, 1987; Rettie *et al.*, 1988). CYP2B1 is involved in the desaturation of valproic acid to the $\Delta^4$-metabolite in the rat (Baillie, 1988); CYP2C9 may be involved in the formation of this metabolite in man (Rettie, personal communication). Both the $\Delta^4$-metabolite and its main metabolite $\Delta^{2,4}$-valproate are potent inducers of microvesicular steatosis and inhibitors of fatty acid $\beta$-oxidation (Kesterson *et al.*, 1984; Granneman *et al.*, 1984). Valproic acid is often co-administered with phenobarbital; as has been mentioned in section 1.10.1. this drug is a classical inducer of many CYP subfamilies in rodents. The use of anti-epileptic polytherapy may contribute greatly to the potential hepatotoxicity of valproic acid.

Valproic acid increases the amounts of the CYP2B, CYP4A and P450-reductase mRNAs in co-cultured rat hepatocytes (Akrawi *et al.*, 1993b). The mechanism of induction of transcription of these genes has not yet been elucidated.

## 1.11. DRUG-DRUG AND DRUG-XENOBIOTIC INTERACTIONS

The clinical significance of drug-drug interactions has been well documented; treatment with one drug can either potentiate the action or diminish the efficacy of a subsequently administered drug (Beaune and Guengerich, 1988; Conney, 1982; Guengerich, 1988). For example pregnancies and menstrual problems have been observed in women who have been co-administered rifampicin (an inducer of CYP3A4) and the oral contraceptive 17α-ethynylestradiol, which is metabolised by CYP3A4 (Guengerich, 1988).

Co-administration of anti-epileptic agents leads to many drug-drug interactions; an example of which is the effect of administering valproic acid to a patient already prescribed phenobarbital (reviewed in Riva, *et al.*, 1996). Valproic acid inhibits the *p*-hydroxylation of phenobarbital. The metabolic and total clearance of phenobarbital is significantly reduced; this leads to an increase in concentration of phenobarbital. The dose of phenobarbital must be reduced by 30% if valproic acid is to be co-administered.

The phosphorothioate pesticide, parathion, is mostly oxidatively biotransformed by CYP3A4. Butler and Murray (1997) have shown that during parathion oxidation in human liver CYP3A4 undergoes significant inactivation. As CYP3A4 is involved in the metabolism of many therapeutic drugs, exposure to phosphorothioate pesticides may lead to clinically significant drug-xenobiotic interactions.

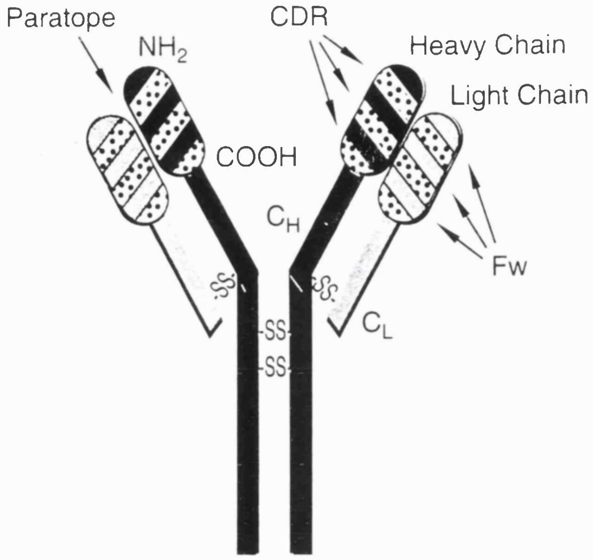
## 1.12. MONOCLONAL ANTIBODIES

Potential drug-drug interactions were investigated in Chapter 4 of this thesis by western blot analysis of human hepatocyte homogenates treated with either phenobarbital or valproic acid. Polyclonal antibodies were used to detect induction of expression of certain CYP subfamilies by these anticonvulsants. The polyclonal antibodies recognise all members of the subfamily concerned and are unable to distinguish between individual isoforms of that subfamily. The use of polyclonal antibodies to identify which CYP subfamilies are induced by anti-epileptic agents provides information on potential drug-drug interactions. However, monoclonal antibodies raised to individual CYPs would provide more detailed information of the individual CYPs affected by these drugs.

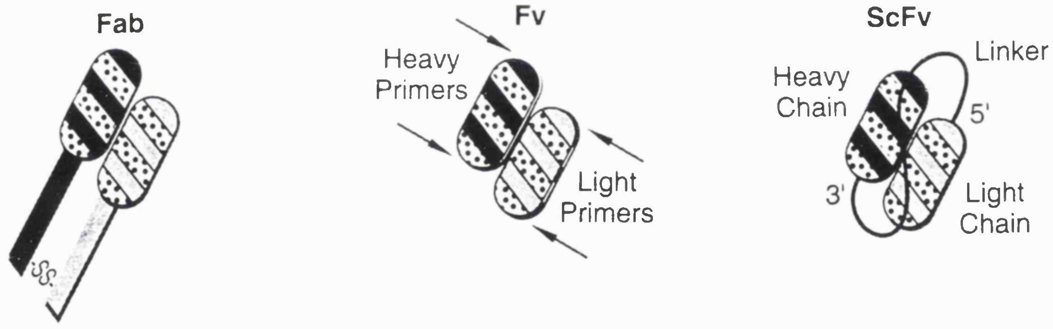
Monoclonal antibodies to CYP2E1 and CYP3A4 have been produced by the traditional hydridoma technology (Gelboin *et al.*, 1995; Gelboin *et al.*, 1996). Chapter 5 in this thesis is concerned with the production of monoclonal antibodies to CYP2A6 and CYP3A4 using an adaptation of the recombinant phage antibody system.

### 1.12.1. ANTIBODY STRUCTURE

All antibodies have a common structure consisting of two identical heavy (H) chain polypeptides (440 amino acids, apparent molecular weight of 55,000-70,000) and two identical light (L) chains (220 amino acids, apparent molecular weight of 24,000) held together by disulphide bridges and non-covalent bonds [figure 1.6; reviewed in Roitt *et al.* (1989) and in Harlow and Lane (1988)]. The heavy and light chains are divided into regions: variable (V), diversity (D, heavy chain only), joining (J) and constant (C). The DNA sequence of the C region is fairly well conserved between different animal species whereas the V region DNA sequence is antigen-dependent. An antigen-binding site


Paratope
NH$_2$
CDR
Heavy Chain
Light Chain
COOH
C$_H$
Fw
SS
-SS-
-SS-
C$_L$

Antibody


Fab
SS
Fv
Heavy Primers
Light Primers
ScFv
Linker
Heavy Chain
5'
3'
Light Chain

Fragment antibody

Fragment variable antibody

Single-chain fragment variable antibody

Figure 1.6: Structures of an antibody, a fragment antibody (Fab), a fragment variable (Fv) antibody and a single-chain fragment antibody (ScFv) [reproduced from the instructions booklet supplied with the recombinant phage antibody system].

The diagram of the antibody shows the subunit composition and domain distribution along the polypeptide chains. Fab and Fv fragments are generated by either proteolytic cleavage or recombinant technology.

(paratope) is created by the pairing of the V regions of the heavy and light chains. The antigen-binding site recognises a single antigenic determinant (epitope).

The C region of the heavy and light chains are located at the C-terminus of these chains. In the mouse, there are five classes of constant heavy ($C_H$) chain genes: $\alpha$, $\delta$, $\varepsilon$, $\gamma$ and $\mu$; and two classes of constant light ($C_L$) chain genes: $\kappa$ and $\lambda$. In man, there are the same classes of heavy chain as in the mouse but there are four $\gamma$ and two $\alpha$ chain subclasses; the light chain classes are identical to those found in the mouse. Antibodies are grouped into five major classes on the basis of their $C_H$ region: IgA, IgD, IgE, IgG and IgM. An antibody is further distinguished by the class of light chain paired with the heavy chain.

The V region is located at the N-terminus of the heavy and light chains. It is composed of alternating framework (FW) and hypervariable complementarity-determining regions (CDRs). The CDRs show great sequence diversity whereas the FW regions are more conserved. The CDRs interact with the antigen to form the antigen-binding site. The variability in the sequence of the CDRs determines the antigenic affinity and specificity of an antibody.

The J region (heavy and light chains) and the D region (heavy chain only) are located in between the C and V regions.

Two functional antigen-binding fragments can be produced from an antibody molecule either by recombinant techniques or by protease digestion (figure 1.6). The larger fragment antibody (Fab) contains the $V_L$-$C_L$ and $V_H$-$C_H$ chains linked by disulphide bonds. The smaller fragment variable (Fv) is composed of the $V_H$ and $V_L$ regions only (figure 1.6).

### 1.12.2. HYBRIDOMA TECHNOLOGY

The hybridoma technology was originally developed in 1975 by Kohler and Milstein (Kohler and Milstein, 1975). A mouse is immunised with the antigen of interest and once the animal has been shown to be producing antibodies to the antigen, the spleen is removed. The mouse B-lymphocytes are fused with myeloma tumour cells by the addition of polyethylene glycol (PEG), which promotes membrane fusion. The myeloma tumour cells are immortalised but do not secrete any antibodies. The hybridomas are selected from unfused spleen and myeloma cells by culturing the fusion mixture in a medium containing a mixture of hypoxanthine, aminopterin and thymidine (known as HAT medium).

Individual hybridomas are cloned and screened for the desired monoclonal antibody by either enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), immunoblotting analysis or a combination of the assays. The selected hybridoma clone is usually subcloned three times to assure monoclonality (Gelboin *et al.*, in press).

The monoclonal antibody produced from a hybridoma recognises a specific single antigenic site or epitope on the antigen. Hybridoma cells can be grown in culture indefinitely; however, some hybridomas are unstable and can stop producing the monoclonal antibody after a few generations. A frozen stock of antibody producing cells are always kept, but these are also prone to instability.

### 1.12.3. RECOMBINANT PHAGE ANTIBODY SYSTEM

Hybridoma technology immortalises the antibodies; the recombinant phage antibody system immortalises the genes for the monoclonal antibodies (reviewed in Winter and Milstein, 1991). This system is based on work carried out by McCafferty *et al.* (1990) and Marks *et al.* (1991); reviewed in Winter and Milstein (1991) where fragments of antibodies are expressed as fusion proteins displayed on the M13 phage particle's surface.

The immunoglobulin variable region genes (heavy and light) are amplified from either hybridomas or mouse spleen cells expressing the antibody of interest. The variable heavy ($V_H$) and variable light ($V_L$) genes are joined together at random by a flexible linker to yield single-chain fragment variable (ScFv) molecules (figure 1.6). The diverse array of ScFv fragments are cloned into a phagemid vector. The phagemid vector permits expression of the ScFvs on the surface of the M13 phage, phage-displayed, or as soluble antibodies secreted into the periplasm of bacteria (Marks *et al.*, 1991; reviewed in Plückthun, 1991). As the phage-displayed and soluble antibodies maintain their antigen-binding capabilities, antibodies of defined specificity and affinity can be selected from a population. The phage-displayed antibodies can be immobilised and used for affinity purification of the antigen to which they are raised; the soluble antibodies are used as immunological tools. This technology mimics the immune system by providing a diverse repertoire of antibodies and selecting out a structure with the correct binding activities.

The "immortalisation" of the antibody genes allows the quick production of soluble antibody genes in bacterial cultures. Rapid advances in the technology have resulted in antibodies being genetically manipulated to provide higher affinity antibodies for the antigen (Marks *et al.*, 1992).

## 1.13. TARGETING ANTI-PEPTIDE ANTIBODIES TO CYPs

Another approach to producing antibodies to CYPs has been taken by the research group of Dr. Edwards. They have immunised animals, mainly rabbits, with synthetic peptides that are directed towards unique regions of the target CYP (Edwards *et al.*, 1988). The advantages of this method are that there is no requirement for purified

CYPs and the resulting antibodies should not cross-react with other CYPs. Anti-peptide antibodies have been raised to the following rat CYPs: CYP1A1, CYP1A2, CYP2B1/2, CYP2D1, CYP2E1 and CYP3A1 (reviewed in Edwards, in press). Anti-peptide antibodies have been raised to the following human CYPs: CYP1A1, CYP1A2, CYP1B1, CYP2E1, and CYP3A4 (reviewed in Edwards, in press).

There are disadvantages to this technology. This method of antibody production is limited to those CYPs whose amino acid sequence is known. The protocols also rely on prediction of epitopes using computer analysis of known CYP protein sequences; this may not always produce an antibody that cross-reacts with the protein. Only a few anti-peptide antibodies can be produced and characterised at one time due to the expensive nature of this technology. Whereas, a wide array of monoclonal antibodies are produced from the hybridoma and recombinant phage antibody system technologies.

This thesis has mainly focused on human CYPs, in particular CYP2A6 and CYP3A4. However the expression of CYP2B6 and the CYP2C subfamily were also briefly examined in Chapter 4. The final part of this introduction will give a brief overview of the human CYP2A, CYP2B, CYP2C and CYP3A subfamilies.

## 1.14. HUMAN CYP2A SUBFAMILY

The human CYP2A subfamily consists of three members, CYP2A6, CYP2A7 and CYP2A13. CYP2A6 was previously denoted IIA3, IIA4 or P450(1) (Nelson *et al.*, 1993). In man, the *CYP2A* gene subfamily maps to chromosome 19q12→13.2 (Davis *et al.*, 1986), within a cluster of genes in the CYP2B and CYP2F subfamilies spanning a 240 kb region (Bale *et al.*, 1991).

### 1.14.1. CYP2A6

It is estimated that CYP2A6 accounts for a maximum of 1% of the total P450 present in human liver microsomes; the expression of this enzyme is subject to a wide inter-individual difference (reviewed in Fernandez-Salguero *et al.*, 1995). This inter-individual variation has been observed in the amounts of expression of CYP2A6 mRNA and the catalytic activity of CYP2A6 (Miles *et al.*, 1990; Yamano *et al.*, 1990). The variation in the amounts of CYP2A6 mRNA in human liver samples is thought to be due to the induction of transcription by administered therapeutic drugs. Whereas the differences in catalytic activity of the expressed protein are due to the occurrence of mutant *CYP2A6* alleles (Yamano *et al.*, 1990; reviewed in Fernandez-Salguero *et al.*, 1995). The *CYP2A6v1* allele has a single base mutation (T to A) that leads to an amino acid change of leucine to histidine. *CYP2A6v2* allele was formed by a gene conversion between the wild-type *CYP2A6* and *CYP2A7* in exons 3, 6 and 8.

CYP2A6 catalyses the 7-hydroxylation of coumarin and 7-ethoxycoumarin O-deethylation (Yun *et al.*, 1991). Coumarin is a plant alkaloid that is used as a sweetener, fixative, stabiliser and food additive as well as an anti-coagulant in the form of warfarin which CYP2A6 does not metabolise (Honkakoski *et al.*, 1992). In 1954 the use of coumarin as a food additive was banned after reports that it produced liver tumours in rats when administered at high concentrations. This effect is not paralleled in man and it is now thought that coumarin has a number of potential therapeutic uses including treatment of bacterial infections and cancer (Egan *et al.*, 1990). The metabolism of coumarin is an example of a quantitative species difference in xenobiotic metabolism (section 1.9.). A high microsomal coumarin 7-hydroxylase activity has been found in rabbits, guinea-pigs, hamsters and pigs as well as in humans (Juvonen *et al.*, 1988); in contrast there appears to be virtually no activity in the rat (Raunio *et al.*, 1988a). Miles *et al.* (1990) identified CYP2A6 as being the major coumarin 7-hydroxylase in the liver. Homozygotes for the *CYP2A6v1* allele are unable to 7-

hydroxylate coumarin; the activity of CYP2A6v2 towards coumarin is unknown (Fernandez-Salguero *et al.*, 1995). 7-hydroxycoumarin is the major metabolite in man although hydroxylation can also occur at carbon atoms 3,5,6 and 8. 7-hydroxycoumarin is rapidly converted by conjugation to glucuronide and excreted into the urine (Pelkonen *et al.*, 1993).

CYP2A6 metabolises several compounds, including tobacco-related nitrosamines, to genotoxic, cytotoxic or mutagenic products (Yamazaki *et al.*, 1992; Yun *et al.*, 1991; Crespi *et al.*, 1990). CYP2A6 is also thought to be expressed in nasal mucosa (Su *et al.*, 1996), this has important implications for the metabolism of nasal toxicants such as nicotine. Nicotine is present in inhaled cigarette smoke and is metabolised by several CYPs including CYP2A6 (Nakajima *et al.*, 1996).

### 1.14.2. CYP2A7

The *CYP2A7* gene is expressed in adult human liver and shares 96% nucleotide sequence identity with *CYP2A6*; the protein shares 94% amino acid sequence identity with CYP2A6 (Miles *et al.*, 1989; Yamano *et al.*, 1990). CYP2A7 is thought to be unable to metabolise coumarin (Yamano *et al.*, 1990; Ding *et al.*, 1995); the function(s) of CYP2A7 are currently unknown. CYP2A7 mRNA can be alternatively spliced to yield a mRNA missing exon 2, but containing the template for three amino acids derived from intron 1 (Ding *et al.*, 1995), this is designated CYP2A7S. cDNA-directed expression of CYP2A7S in COS-7 cells yielded a truncated protein of apparent molecular weight of 44,000 (Ding *et al.*, 1995). Both CYP2A6 and CYP2A7 are proteins of apparent molecular weight of approximately 49,000.

### 1.14.3. CYP2A13

A genomic clone containing the *CYP2A13* gene has been identified by Fernandez-Salguero *et al.* (1995). Computer analysis of the genomic clone has lead to the prediction of a cDNA sequence for CYP2A13 (Fernandez-Salguero *et al.*, 1995) which shares 95% nucleotide identity with the cDNA of CYP2A6 (Yamano *et al.*, 1990). The predicted amino acid sequence of CYP2A13 is 93% identical to the amino acid sequence of CYP2A6. The research group have not, as yet, been able to isolate a cDNA clone of CYP2A13 from human liver.

## 1.15. HUMAN CYP2B SUBFAMILY

The human CYP2B subfamily is thought to consist of two genes, *CYP2B6* and *CYP2B7*. *CYP2B7* contains an in-frame internal stop codon and is incapable of encoding a functional protein (Yamano *et al.*, 1989). A pseudogene of CYP2B7 was thought to exist but this is now recognised as being an alternative splicing product of CYP2B6 (Nelson *et al.*, 1996). At least four alternatively spliced mRNAs are derived from *CYP2B6* (Miles *et al.*, 1989). In man, the *CYP2B* gene subfamily maps to a 240 kb region of chromosome 19 within a cluster of genes of the CYP2A and CYP2F (Bale *et al.*, 1991). A positive correlation between the expression of CYP2A6 and CYP2B6 has been observed in human livers (Gonzalez, 1989), in other words the greater the amount of CYP2A6, the greater the amount of CYP2B6.

CYP2B6 metabolises coumarin (Miles *et al.*, 1990), several carcinogens (reviewed in Fernandez-Salguero and Gonzalez, 1995) and nicotine (McCracken *et al.*, 1992).

## 1.16. HUMAN CYP2C SUBFAMILY

Four human CYP2C genes have been identified clustered within 300 kb on chromosome 10q24; these are *CYP2C8*, *CYP2C9*, *CYP2C18* and *CYP2C19* (Gray *et al.*, 1995). A further sequence, *CYP2C10*, has been identified but is unclear if this is an allelic variant of *CYP2C9* (Umbenhauer *et al.*, 1987). Genetic polymorphisms have been identified at the *CYP2C9* and *CYP2C19* gene loci (Stubbins *et al.*, 1996; de Morais *et al.*, 1994a; de Morais *et al.*, 1994b).

CYP2C9 is the most abundantly expressed member of the CYP2C subfamily in human liver (Goldstein and de Morais, 1994) and metabolises numerous therapeutic drugs such as phenytoin, warfarin, ibuprofen and tamoxifen (Rettie *et al.*, 1992; Goldstein and de Morais, 1994; Crewe *et al.*, 1997). Polymerase chain reaction-based genotyping assays developed by Stubbins *et al.* (1996) will enable the importance of the *CYP2C9* polymorphism to be investigated in relation to the metabolism of new and old therapeutic drugs metabolised by CYP2C9.

## 1.17. HUMAN CYP3A SUBFAMILY

The glucocorticoid-inducible CYP3A subfamily are the most abundantly expressed group of CYPs in human liver (Gonzalez, 1992). The CYP3A subfamily is comprised of four members, CYP3A3, CYP3A4, CYP3A5 and CYP3A7. However, the existence of CYP3A3 is now in doubt (Nelson *et al.*, 1996). The *CYP3A* genes have been mapped to chromosome 7q22-qter (Spurr *et al.*, 1989).

### 1.17.1. CYP3A4

CYP3A4 is the most abundantly expressed CYP in man. The expression of CYP3A4 in human liver specimens is highly variable, some livers have up to 60% of their total P450 as CYP3A4 while others contain < 10% (Gonzalez, 1992).

### 1.17.2. CYP3A5

CYP3A5 is expressed in most foetal liver samples and is expressed polymorphically in livers of adolescents and adults with 25% of individuals producing the protein (Wrighton and Vandenbranden, 1989; Wrighton *et al.*, 1989; Wrighton *et al.*, 1990). When there is expression of CYP3A5 in adult livers the amount is approximately 30% that of CYP3A4 (Wrighton *et al.*, 1989; Wrighton *et al.*, 1990). CYP3A5 is 84% similar in amino acid sequence to CYP3A4 and shows similar substrate specificity to CYP3A4 but with a slower turnover rate with respect to some substrates (Aoyama *et al.*, 1989). Two *CYP3A5* pseudogenes have been identified, *CYP3A5P1* (Schuetz and Guzelian, 1995) and *CYP3A5P2* (Nelson *et al.*, 1996).

### 1.17.3. CYP3A7

CYP3A7 shares 95% nucleotide sequence identity and 87% amino acid sequence identity with CYP3A4 (Hashimoto *et al.*, 1993). CYP3A7 accounts for 36% of total CYP content in foetal liver (Kitada *et al.*, 1985); it is also expressed in the placenta (Schuetz *et al.*, 1993). CYP3A7 was originally thought to be only expressed in the foetus (Komori *et al.*, 1990). However, CYP3A7 mRNA has been found in the majority of adult livers studied by Hakkola *et al.* (1994) and Schuetz *et al.* (1994). However, it is unclear whether CYP3A7 protein is expressed in the adult liver.

The enzymes belonging to the CYP3A subfamily are involved in the metabolism of endogenous steroid substrates such as testosterone, progesterone and androsteredione (Aoyama *et al.*, 1989). They also metabolise numerous clinically important drugs, some examples of which are shown in table 1.2., and are able to metabolically activate several procarcinogens (Gonzalez, 1992; Aoyama *et al.*, 1990a; Aoyama *et al.*, 1990b). CYP3A4 also metabolises the carcinogenic environmental pollutant 1-nitropyrene (Howard *et al.*, 1990).

The CYP3A subfamily have also been found to be expressed in human extrahepatic tissues, including the gastrointestinal tract (reviewed in McKinnon *et al.*, 1995). There is considerable inter-individual heterogeneity in the expression of these enzymes in the gastrointestinal tract; this may lead to differences in susceptibility to cancer from dietary-derived carcinogens (McKinnon *et al.*, 1995).

| Therapeutic drug | Use | Reference |
|---|---|---|
| Cyclosporin | Immunosuppressant | Aoyama *et al.*, 1989; Kronbach *et al.*, 1988 |
| Erythromycin | Antibiotic | Delaforge *et al.*, 1983 |
| 17α-ethynylestradiol | Oral contraceptive | Guengerich *et al.*, 1986a |
| Indinavir | Human immunodeficiency virus protease inhibitor | Lin *et al.*, 1996 |
| Lidocaine | Local anaesthetic agent | Bargetzi *et al.*, 1989 |
| Midazolam | Benzodiazepine tranquilliser | Kronbach *et al.*, 1989 |
| Nifedipine | $Ca^{2+}$ channel blocker | Guengerich *et al.*, 1986a; Aoyama *et al.*, 1989 |
| Quinidine | Anti-arrhythmic agent | Guengerich *et al.*, 1986b |
| Troleandomycin | Antibiotic | Pessayre *et al.*, 1981 |

Table 1.2: Examples of therapeutic drugs metabolised by the CYP3A subfamily (compiled from the references listed in the table).

# *CHAPTER TWO*

# MATERIALS AND METHODS

# 2. MATERIALS AND METHODS

## 2.1. MATERIALS

### 2.1.1. BACTERIAL STRAINS

For the *Escherichia coli* (*E. coli*) strains listed below, the genes noted signify that the bacterium carries a mutant allele. The genes present on the F' episome, however, represent the wild-type alleles unless otherwise indicated.

| Strain | Genotype | Source |
|---|---|---|
| JM109 | e14$^-$ (McrA$^-$), *recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17, ($r_{k-}$, $m_{k+}$), *supE*44, *relA*1, $\Delta$(*lac-proAB*), [F' *traD*36, *proAB*, *lacI*$^q$Z$\Delta$M15] | Stratagene (Cambridge, Cambridgeshire, U.K.) |
| XL1-Blue | *recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17, *supE*44, *relA*1, *lac* [F' *proAB*, *lacI*$^q$Z$\Delta$M15, Tn*10* (*tet*$^r$)][a] | Stratagene |
| TG1 | K12 $\Delta$ (*lac-pro*), *supE*, *thi*, *hsd*$\Delta$5/F' [*traD*36, *proAB*, *lacI*$^q$, *lacZ*$\Delta$M15] | Pharmacia Biotech (St. Albans, Hertfordshire, U.K.) |
| HB2151 | K12 $\Delta$ (*lac-pro*), *ara*, *nal*$^r$, *thi*/F' [*proAB*, *lacI*$^q$, *lacZ*$\Delta$M15] | Pharmacia Biotech |

[a] An uncharacterized mutation enhances α-complementation to give a more intense blue colour on agar plates containing 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) and isopropyl-β-D-thiogalactopyranoside (IPTG).

### 2.1.2. SUPPLIES

#### 2.1.2.1. CHEMICAL REAGENTS

All chemicals were of analytical grade and were obtained from either Sigma Chemical Company (Poole, Dorset U.K.) or British Drug Houses [B.D.H. (Lutterworth, Leicestershire, U.K.)]. LB (Luria-Bertani), SOB media capsules and LB agar capsules were purchased from Stratech Scientific (Luton, Bedfordshire, U.K.). 0.2 μm "Minisart-plus" filters were acquired from Sartorius (Gottingen, Germany) and 0.45 μm filters were purchased from Millipore U.K. Limited (Watford, Hertfordshire). Polaroid Black-And-White Print Film Type 55 were bought from Sigma and X-ray medical films were supplied by Fuji (Japan). Gauze for a filtration step in the Qiagen DNA prep kit was obtained from Nybolt (Switzerland).

#### 2.1.2.2. ITEMS SPECIFICALLY FOR DNA MANIPULATIONS AND SEQUENCING

A 1 Kb molecular weight standard DNA ladder [1.0 μg/μl in 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.1 mM EDTA] was purchased from Gibco BRL (Paisley, Renfrewshire, U.K.). The ladder was diluted 1 in 10 with 8 parts autoclaved distilled water and 1 part DNA loading buffer (section 2.2.4.). The bands of the ladder each contain from 1 to 12 repeats of a 1,018 base pair (bp) DNA fragment. In addition to these 12 bands, the ladder contains DNA fragments in the range 75 to 1,636 bp produced from *Hinf* I digestion of the plasmid pBR322. The DNA band sizes are:
75; 134; 154; 201; 220; 298; 344; 396; 506/517; 1,018; 1,636; 2,036; 3,054; 4,072; 5,090; 6,108; 7,126; 8,144; 9,162; 10,180; 11,198 and 12,216.

A 100 base pair (bp) molecular weight standard DNA ladder [1.0 μg/μl in TE buffer (pH 7.5)] was obtained from Pharmacia Biotech. The ladder was generated from two plasmids, one plasmid was partially digested by *Ava* 1 and released multiples of 100 bp upon digestion; the other was digested to completion with *Hind* III and released a fragment of 800 bp. The digested plasmids are mixed in a proportion such that the 800 bp band appears at twice the intensity of the other bands after ethidium bromide staining.

All restriction enzymes and DNA modifying enzymes (including T4 DNA ligase) together with 100 mM ATP were acquired from Pharmacia Biotech . All enzymes were supplied with a 1 ml aliquot of 10 x One-Phor-All Buffer Plus [10 x OPA$^+$: 100 mM Tris-acetate (pH 7.5), 100 mM magnesium acetate, 500 mM potassium acetate]. Qiagen Tip-500 plasmid DNA prep kits were obtained from Hybaid (Teddington, Middlesex, U.K.) and RNaseA was purchased from Boehringer Mannheim (Mannheim, Germany). Sequagel and Protogel were acquired from National Diagnostics (Atlanta, Georgia, U.S.A.) and liquefied phenol washed with Tris buffer (pH 8.0) was obtained from Fisons (Loughborough, U.K.).

T7Sequencing$^{\text{TM}}$ Kit was purchased from Pharmacia Biotech.

Components:

| | |
|---|---|
| *"A" Mix-Short*: | ddATP in solution with dATP, dCTP, dGTP and dTTP. |
| *"C" Mix-Short*: | ddCTP in solution with dATP, dCTP, dGTP and dTTP. |
| *"G" Mix-Short*: | ddGTP in solution with dATP, dCTP, dGTP and dTTP. |
| *"T" Mix-Short*: | ddTTP in solution with dATP, dCTP, dGTP and dTTP. |
| *T7 DNA polymerase*: | In buffered glycerol solution. |
| *Enzyme dilution buffer*: | Provided by manufacturer. |

| | |
|---|---|
| *Universal primer*: | 5'-d[GTAAAACGACGGCCAGT]-3' in aqueous solution, 0.80 µM (4.44 µg/ml). |
| *Annealing buffer*: | Provided by manufacturer. |
| *Labelling mix-dATP*: | dCTP, dGTP and dTTP in solution. |
| *Labelling mix-dCTP*: | dATP, dGTP and dTTP in solution. |
| *Stop solution*: | Deionised formamide solution containing EDTA, xylene cyanol and bromophenol blue (concentrations not given by manufacturer). |
| *Control template*: | 10 µg of single-stranded M13mp18 DNA in 50 µl of Tris-EDTA buffer provided by manufacturer (concentrations and pH not given). |

Deaza G/A $^{T7}$Sequencing$^{TM}$ Mixes were purchased from Pharmacia Biotech; the mixes are designed to eliminate ambiguities in dideoxy sequencing using T7 DNA polymerase. The substitution of 7-deaza dGTP ($c^7$dGTP) and 7-deaza ATP ($c^7$dATP) for dGTP and dATP, respectively, prevent the formation of intra-strand secondary structures in chain-terminated DNA fragments during electrophoresis. This eliminates a large percentage of band compressions in sequencing gels.

Components

| | |
|---|---|
| *"A" Mix*: | 95 µM each $c^7$dATP and $c^7$dGTP;<br>840 µM dCTP and dTTP; 2 µM ddATP;<br>40 mM Tris-HCl (pH 7.6) and 50 mM NaCl. |

*"C" Mix*: 95 μM each $c^7$dATP, $c^7$dGTP and dCTP;
840 μM dTTP; 10 μM ddCTP;
40 mM Tris-HCl (pH 7.6) and 50 mM NaCl.

*"G" Mix*: 95 μM each $c^7$dATP and $c^7$dGTP;
840 μM each dCTP and dTTP; 4 μM ddGTP;
40 mM Tris-HCl (pH 7.6) and 50 mM NaCl.

*"T" Mix*: 95 μM each $c^7$dATP, $c^7$dGTP and dTTP;
840 μM dCTP; 6 μM ddTTP;
40 mM Tris-HCl (pH 7.6) and 50 mM NaCl.

*Annealing buffer*: 1 M Tris-HCl (pH 7.6), 100 mM magnesium chloride and 160 mM dithiothreitol (DTT).

*Deaza labelling mix-dATP*: 1.37 μM each dCTP, $c^7$dGTP and dTTP; 333.3 mM NaCl.

*Deaza labelling mix-dCTP*: 1.37 μM each $c^7$dATP, $c^7$dGTP and dTTP; 333.3 mM NaCl.

*Stop solution*: 0.3% each bromophenol blue and xylene cyanol; 10 mM EDTA (pH 7.5) and 97.5% deionised formamide.

[$\alpha$-$^{35}$S]dATP, 500 Curie per mmol (10 μCi/μl), was obtained from New England Nuclear Research Products (Stevenage, Hertfordshire, U.K.). Primers for sequencing were either synthesised in-house or purchased from Stratagene/Pharmacia Biotech. Figures 3.4A and 3.10A show schematic diagrams of the use of the primers.

The following primers were obtained from Stratagene:

*- 20 Reverse*: 5'-d[AACAGCTATGACCATG]-3' in aqueous solution, 2.5 ng/µl.

*- 40 Reverse*: 5'-d[AGCGGATAACAATTTCACACAGGA]-3' in aqueous solution, 2.5 ng/µl.

*- 40 Forward*: 5'-d[GTTTTCCCAGTCACGAC]-3' in aqueous solution, 2.5 ng/µl.

*T7*: 5'-d[AATACGACTCACTATAG]-3' in aqueous solution, 2.5 ng/µl.

The following primers were synthesised in-house:

*181*: 5'-d[CGGTCCCAAAGACAATGGA]-3' in aqueous solution, 2.94 ng/µl.

*447*: 5'-d[ACGGTGGTGAAGAAGAGAAA]-3' in aqueous solution, 3.09 ng/µl.

*H2A1*: 5'-d[CCGATCACTCTGTCAAT]-3' in aqueous solution, 2.62 ng/µl.

*SP6*: 5'-d[ATTTAGGTGACACTATAGAATAC]-3' in aqueous solution, 3.55 ng/µl.

*oligonucleotide (oligo) A*: 5'-d[GACCCCAGTTTCTTCTCCAAC]-3' in aqueous solution, 3.24 ng/µl.

| | |
|---|---|
| *oligo B*: | 5'-d[CATGAAGCACCCAGAGGTGGA]-3' in aqueous solution, 3.24 ng/μl. |
| *oligo C*: | 5'-d[CCCTGCGGGACTTCGGGGTGG]-3' in aqueous solution, 3.24 ng/μl. |
| *oligo D*: | 5'-d[TCCCGAATGCGGATGAGAAAG]-3' in aqueous solution, 3.24 ng/μl. |
| *oligo E*: | 5'-d[AGAACATCTCATAGAGCTGCC]-3' in aqueous solution, 3.24 ng/μl. |
| *oligo F*: | 5'-d[TCCTTTTTGACTCTGCGGGCC]-3' in aqueous solution, 3.24 ng/μl. |
| *oligo G*: | 5'-d[TCACTCTTCTTAAACTGCCCC]-3' in aqueous solution, 3.24 ng/μl. |

The following primers were synthesised by Pharmacia Biotech:

| | |
|---|---|
| *oligo H*: | 5'-d[CCTCATGAAGATCAGTGAGCG]-3' supplied lyophilised, adjusted to 3.24 ng/μl in aqueous solution. |
| *oligo I*: | 5'-d[CTTCAAGGAGCTGCAAGGGCT]-3' supplied lyophilised, adjusted to 3.24 ng/μl in aqueous solution. |
| *oligo J*: | 5'-d[CCCACCGAACTCCTCAGCCTG]-3' supplied lyophilised, adjusted to 3.24 ng/μl in aqueous solution. |

The concentrations of the synthetic oligonucleotides were determined by measuring an $O.D._{260}$; an O.D. of 1 at 260 nm ≡ 33 μg/ml of DNA.

The primers that were synthesised in-house and the ones that were purchased from Pharmacia Biotech were adjusted to a concentration that gave the equivalent of 0.5 pmol/μl using the equation:

$$\text{pmol}/\mu\text{l} = \frac{\mu\text{g/ml} \times 1{,}000}{309 \times \text{oligo length}}$$

An ultrapure dNTP set for Polymerase Chain Reaction (PCR) and RNase inhibitor (RNA guard, 31.2 units/μl) were purchased from Pharmacia Biotech. DyNAZyme™ (2 units/μl) was obtained from Finnzymes Oy (Finland) and AmpliTaq® DNA polymerase (5 units/μl) was acquired from Perkin-Elmer (Roche Molecular Systems Incorporated, New Jersey, U.S.A.). 200 units/μl Moloney-Murine Leukaemia Virus Reverse Transcriptase (M-MLVRT), 10 x DTT and 1 mg/ml nuclease-free Bovine Serum Albumin (BSA) were obtained from Gibco BRL.

Oligonucleotides for PCR were synthesised in-house:

*Forward primer*: 5'-d[GCTCAGTCTAGAACCATGGTGGCCTCAGGGATG]-3'
*(oligo 108)* in aqueous solution, 12.5 μmoles/μl.

*Reverse primer*: 5'-d[CGAGTCTCTAGATCAGCGGGGCAGGAAGCTCA]-3'
*(oligo 109)* in aqueous solution, 12.5 μmoles/μl.

Spin-X tubes for DNA fragment isolation from low melting-point agarose gels were purchased from Co-Star (Massachusetts, U.S.A.).

### 2.1.2.3. COMMONLY USED SOLUTIONS AND MEDIA

| | |
|---|---|
| Ampicillin (AMP): | A 50 mg/ml stock solution was prepared in distilled water. The solution was filter-sterilised (0.2 µm filter), aliquoted and stored at -20°C. |
| Nalidixic acid: (Sodium salt, Sigma) | A 100 mM (~25 mg/ml) stock was prepared in distilled water. The solution was filter-sterilised (0.2 µm filter), aliquoted and stored at -20°C. |
| LB medium: | Five LB media capsules were placed in 200 ml of distilled water and the medium was autoclaved. |
| LB AMP medium: | LB medium containing 50 µg/ml AMP. |
| SOB medium: | Three SOB media capsules were placed in 100 ml of distilled water and the medium was autoclaved. |
| SOC medium: | 9.9 ml of SOB medium + 100 µl of 2 M glucose (filter-sterilised, 0.2 µm filter). |
| SOBAG medium: | 900 ml of distilled water were added to 20 g of Bacto-tryptone (Difco Laboratories, Detroit, Michigan, U.S.A.), 5 g of Bacto-yeast extract (Difco Laboratories) and 0.5 g of NaCl. This was autoclaved and cooled to 50-60°C. 10 ml of sterile 1 M $MgCl_2$, 55.6 ml of sterile 2 M glucose and 2 ml of filter-sterilised 50 mg/ml AMP were added to the medium. |
| SOBAG-N medium: | SOBAG medium containing 100 µg/ml nalidixic acid. |

2 x YT medium: 900 ml of distilled water were added to 17 g of Bacto-tryptone, 10 g of Bacto-yeast extract and 5 g of NaCl. This was adjusted to 1 litre with distilled water and autoclaved.

2 x YT-AG medium: 2 x YT medium containing 100 µg/ml AMP and 2% glucose.

2 x YT-AI medium: 2 x YT medium containing 100 µg/ml AMP and 1 mM IPTG.

2 x YT-N medium: 2 x YT medium containing 100 µg/ml nalidixic acid.

2 x YT-G medium: 2 x YT medium containing 2% glucose.

LB agar: Four LB agar capsules were placed in 100 ml of distilled water and the agar was autoclaved. The agar was cooled to 40-50°C before the plates were poured. 100 ml of agar was sufficient for four Sterilin plates of 8.6 cm in diameter.

LB AMP agar: LB agar containing 50 µg/ml AMP.

LB AMP X-gal/IPTG agar: LB agar containing 50 µg/ml AMP. Once the agar had set, 40 µl of 2% X-gal in N, N-dimethylformamide (Sigma) and 40 µl of 0.1 M IPTG were placed on the surface of each agar plate. The mixture was dispersed evenly over the agar plate using a sterile glass spreader. The plates were dried for 10-15 minutes prior to use.

SOBAG agar: SOBAG medium supplemented with 15 g of Bacto-agar (Difco Laboratories) prior to autoclaving.

| | |
|---|---|
| SOBAG-N agar: | SOBAG-N medium supplemented with 15 g of Bacto-agar prior to autoclaving. |
| 2 x YT-AG agar: | 2 x YT-AG medium supplemented with 15 g of Bacto-agar prior to autoclaving. |
| 2 x YT-AI agar: | 2 x YT-AI medium supplemented with 15 g of Bacto-agar prior to autoclaving. |
| Minimal medium agar: | In a 500 ml bottle, 6 g of $Na_2HPO_4$, 3 g of $KH_2PO_4$ and 1 g of $NH_4Cl$ were dissolved in distilled water to a final volume of 500 ml. The pH was adjusted to 7.4 with NaOH. In a separate 1 litre bottle, distilled water was added to 15 g of Bacto-agar to a final volume of 500 ml. Both bottles were autoclaved simultaneously, cooled to 50-60$^{\circ}$C and then combined. The agar medium was supplemented with 1 ml of 1 M $MgCl_2 \bullet 6H_2O$, 1 ml of 1 M $CaCl_2 \bullet 2H_2O$, 1 ml of 1 M thiamine hydrochloride (Sigma) and 5 ml of sterile 20% glucose. |

All the media were stored at room temperature, whereas the agar plates and sterile glucose solutions were kept at 4$^{\circ}$C. The media and agar plates were used within two weeks if they contained antibiotics or within four weeks if no antibiotics were present.

## 2.2. **METHODS**

### 2.2.1. PREPARATION AND MAINTENANCE OF *E. COLI* STOCKS

Short-term storage

*E. coli* cells were stored for up to two weeks at 4°C on agar plates.

Long-term storage

5 ml of LB or 2 x YT medium were inoculated with a single colony from an agar plate. The culture was incubated overnight at 37°C with shaking. 1 ml of bacterial cells were taken from the stationary phase culture and mixed with 1 ml of media containing 30% glycerol. The glycerol stock was mixed thoroughly and stored at -80°C.

### 2.2.2. PLASMID DNA PREPARATION

#### 2.2.2.1. PLASMID DNA MINI-PREP, ALKALINE LYSIS [ADAPTED FROM SAMBROOK *ET AL.* (1989)]

*Harvesting of bacteria*

5 ml of LB AMP (50 μg/ml) media was inoculated with a single bacterial colony carrying the plasmid to be isolated. After overnight incubation at 37°C, with vigorous shaking, 1.5 ml of culture was pipetted into a sterile microcentrifuge tube and was centrifuged for 2 minutes in a microcentrifuge at 9,500 x g. The medium was removed by gentle aspiration, ensuring that the bacterial pellet was left as dry as possible.

The rpm required to obtain a force of 9,500 x g was calculated as follows:

$$RCF = 9{,}500 = (1.12)(r)(rpm/1{,}000)^2$$

where RCF = relative centrifugal force; r = radius in mm measured from centre of spindle to bottom of rotor bucket; and rpm = revolutions per minute

*Alkaline lysis*

Solutions:

Solution 1: 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl (pH 8.0).

Solution 2: 0.2 N sodium hydroxide, 1% w/v sodium dodecyl sulphate (SDS).

Solution 3: 60% v/v 5 M potassium acetate, 11.5% v/v glacial acetic acid.

The bacterial pellet was resuspended by vortexing in 100 μl of ice-cold solution 1, the tube was stored at room temperature for 5 minutes for cell lysis to proceed. 200 μl of freshly prepared solution 2 was added, the top of the tube was closed tightly and the contents were mixed by inverting the tube rapidly 2 or 3 times with vigour. The tube was stored on ice for 5 minutes to allow the DNA and proteins to denature. 150 μl of ice-cold solution 3 was added, the cap of the tube was shut tightly and the tube was vortexed in an inverted position for 10 seconds. The tube was placed on ice for 5 minutes to allow neutralisation of the contents to occur, under these conditions the supercoiled plasmid DNA renatures. The larger chromosomal DNA, denatured proteins, cellular debris and SDS were precipitated. The tube was centrifuged for 5 minutes in a microcentrifuge at 9,500 x g and the supernatant was transferred to a fresh sterile microcentrifuge tube. 0.5 volumes of phenol was added and the tube was vortexed for 20 seconds to ensure adequate mixing between the phenol and DNA. 0.5 volumes of chloroform was added and the tube was vortexed again for 20 seconds and then centrifuged for 5 minutes in a microcentrifuge at 9,500 x g. The top aqueous layer was transferred to a fresh sterile microcentrifuge tube and 2 volumes of absolute ethanol equilibrated to room temperature were added to precipitate the plasmid DNA.

The tube was vortexed for 1 second and left to stand at room temperature for 2 minutes. The plasmid DNA was pelleted in a microcentrifuge at 16,000 x g for 10 minutes; the supernatant was removed and any remaining fluid was allowed to drain away by standing the tube in an inverted position on a paper towel. The pellet was washed with 1 ml of 70% ethanol, the tube was vortexed briefly and centrifuged for 5 minutes. The supernatant was removed as described above and the pellet was dried briefly in a vacuum desiccator. The DNA pellet was resuspended in 20 μl of autoclaved distilled water. 1-2 μl of plasmid DNA were used for an appropriate restriction enzyme digest (section 2.2.3.). The resulting DNA fragments were analysed on a 1% agarose gel (section 2.2.4.) to confirm the presence of the correct plasmid DNA. A 1.5 ml bacterial culture normally yielded 2-3 μg DNA.

#### 2.2.2.2. PREPARATION OF PLASMID DNA FOR SEQUENCING (MIERENDORF AND PFEFFER, 1987)

This protocol is identical to the plasmid DNA mini-prep, alkaline lysis [adapted from Sambrook *et al.* (1989)] up to and including the addition of solution 3. The supernatant was transferred to a fresh sterile microcentrifuge tube and 2-3 μl of RNaseA (10 mg/ml) was added. The tube was incubated at 37°C for at least 20 minutes to ensure complete RNA degradation. The sample was phenol/chloroform extracted as described in the plasmid DNA min-prep, alkaline lysis protocol. 2.5 volumes of absolute ethanol were added and the tube was stored on dry ice for 10 minutes. The tube was centrifuged for 15 minutes in a microcentrifuge at 16,000 x g. The supernatant was removed as previously described and the DNA pellet was washed with 1 ml of 80% ethanol (no vortexing). The tube was centrifuged as before, the supernatant was carefully removed and the pellet was resuspended in 16 μl of autoclaved distilled water by vortexing. 4 μl of 4 M NaCl and 20 μl of 13% PEG (MW 8,000) were added to the microcentrifuge tube which was vortexed briefly after each addition.. The tube was stored on ice for 20 minutes and was then centrifuged at 16,000 x g for 10 minutes. The supernatant was carefully discarded and any residual fluid was removed with a capillary tube. The almost invisible pellet was washed with 200 µl of 80% ethanol (no vortexing) and the tube was centrifuged for 3 minutes. The wash was repeated and the resulting pellet was air-dried. It Is essential to remove all traces of PEG from the pellet as PEG inhibits T7 DNA polymerase, the enzyme used for sequencing reactions. The pellet was resuspended in 10 µl of autoclaved distilled water. 2-3 µl of DNA was digested with an appropriate restriction endonuclease (section 2.2.3.). The resulting DNA fragments were analysed on a 1% agarose gel (section 2.2.4.) to confirm the presence of the correct plasmid DNA before sequencing was carried out.

### 2.2.2.3. QIAGEN TIP-500 PLASMID DNA PREP *(HIGH COPY NO. PLASMID)*

Solutions provided with the Qiagen kit:

Solution P1: 50 mM Tris-HCl, 10 mM EDTA, 100 µl/ml RNaseA; adjusted to pH 8.0.

Solution P2: 200 mM sodium hydroxide, 1% w/v SDS.

Neutralisation buffer P3: 3.0 M potassium acetate (pH 5.5).

Equilibration buffer QBT: 750 mM NaCl, 50 mM 3-[*N*-morpholino] propanesulfonic acid buffer (MOPS), 15% v/v ethanol, 0.15% v/v Triton X-100; adjusted to pH 7.0.

Wash buffer QC: 1.0 M NaCl, 50 mM MOPS, 15% v/v ethanol; adjusted to pH 7.0.

Elution buffer QF: 1.25 M NaCl, 50 mM Tris-HCl, 15% v/v ethanol; adjusted to pH 8.5.

In the morning 5 ml of LB AMP (50 µg/ml) was inoculated with a single bacterial colony containing the plasmid to be isolated. The culture was incubated with vigorous shaking at 37°C. In the late afternoon 100-200 ml of LB AMP (50 µg/ml) was inoculated with the 5 ml culture. The culture was grown overnight at 37°C and the cells were harvested the next morning by centrifugation at 4°C for 15 minutes at 6,000 x g (6,000 rpm in Sorvall GSA rotor). All traces of the medium were removed and the bacterial pellet was resuspended in 10 ml of chilled P1 buffer, ensuring no bacterial clumps were present. The sample was transferred to a sterile plastic capped 30 ml centrifuge tube, to which 10 ml of lysis buffer P2 was added. The tube was capped and inverted gently 4-6 times to mix the contents without shearing the genomic DNA. The tube was stored at room temperature for 5 minutes. 10 ml of chilled neutralisation buffer P3 was added, the tube was capped and inverted immediately 5-6 times and placed on ice for 20 minutes. The tube was centrifuged at 4°C for 30 minutes at > 30,000 x g (14,500 rpm in Sorvall SS-34 rotor). Meanwhile the Qiagen Tip 500 column was equilibrated by applying 10 ml of equilibration buffer, QBT. The clear supernatant removed from the centrifuge tube was passed through a gauze into a sterile 50 ml tube. The filtrate was applied to the Qiagen Tip-500 column; the column was washed twice with 30 ml of QC buffer. The lower end of the Qiagen column was placed in a 30 ml Corex® tube. The plasmid DNA was eluted into the Corex® tube by applying 15 ml of QF buffer to the column. The DNA was precipitated with 0.7 volumes of isopropanol at room temperature. The Corex® tube was immediately centrifuged at 4°C for 30 minutes at 15,000 x g (9,500 rpm in a Sorvall SS-34 rotor). The supernatant was removed and the DNA pellet was washed with 15 ml of 70% ethanol. The Corex® tube was centrifuged as before for 15 minutes and the supernatant was removed. The pellet was air-dried and resuspended in 250 µl of autoclaved distilled water. The concentration of the DNA was evaluated by reading an O.D.$_{260}$; an O.D. of 1 at 260 nm ≡ 50 µg/ml of DNA. The O.D. at 280 nm was also measured to estimate the DNA : protein ratio. An appropriate restriction endonuclease digest was carried out (section 2.2.3.) and the resulting DNA fragments were analysed on a 1% agarose gel (section 2.2.4.). A yield of 500-1,500 µg of high copy number plasmid DNA was normally obtained from a 200 ml bacterial culture.

### 2.2.3. RESTRICTION ENDONUCLEASE DIGESTION OF PLASMID DNA

For a 20 µl reaction mixture the following were included:

- DNA (0.1-5 µg)
- Pharmacia 10 x OPA$^+$ buffer (final concentration of 1 x, 1.5 x or 2 x depending on restriction enzyme used)
- Restriction enzyme (1-4 units depending on amount of DNA)
- Autoclaved distilled water to give a final volume of 20 µl

If the DNA to be restricted was obtained from a mini-prep it was necessary to add 1 µl of RNaseA (10 mg/ml) to the digest mixture. The volumes of the constituents of the reaction mixture were adjusted accordingly for either smaller or larger digest volumes. The samples were incubated at 37$^o$C for at least 1 hour before an aliquot of each sample was loaded onto a 1-2% agarose gel (section 2.2.4.) to check that digestion of the DNA was complete. Digestion by the enzyme *Sma* I required incubation of the sample at 30$^o$C.

### 2.2.4. AGAROSE GEL ELECTROPHORESIS OF DNA

Solutions:

1 x Tris-borate EDTA buffer: 0.09 M Tris-borate, 0.002 M EDTA.
(1 x TBE)

| | |
|---|---|
| 6 x DNA loading buffer: | 0.25% w/v bromophenol blue [electrophoresis purity grade (BIO-RAD, Richmond, California; U.S.A.)], 40% w/v sucrose; filter-sterilised using a 0.2 μm filter prior to use. |

A 50 ml 1% agarose gel was prepared by adding 0.5 g of agarose and 50 ml of 1 x TBE to a conical flask. The flask was microwaved on high power for 2 minutes in order to dissolve the agarose. Approximately 1 μl of ethidium bromide [10 mg/ml (B.D.H.)] was added to the conical flask and mixed thoroughly. The gel was poured into the casting tray after the agarose had cooled to 50-60°C. The appropriate amount of DNA loading buffer was added to each of the samples which were to be loaded onto the gel. The comb which had been used to make the wells in the gel was removed and the samples were loaded. 1 μg (10 μl) of prepared 1 Kb molecular weight standard DNA ladder (section 2.1.2.2.) was loaded either side of the samples. The gel was electrophoresed at 10 Volts/cm using 1 x TBE as the electrophoresis buffer. The relative proportions of agarose and 1 x TBE were varied accordingly depending on the size and percentage of the gel to be set. The gel was photographed under U.V. light using Polaroid Black-And-White Print Film Type 55 (Sigma).

### 2.2.5. ISOLATION OF A DNA FRAGMENT FROM A 0.8% LOW MELTING POINT AGAROSE GEL

The sample containing the DNA fragment to be isolated was loaded onto a 0.8% agarose gel. The gel was electrophoresed at 5 Volts/cm to prevent the gel melting. The appropriate band was excised from the gel, under U.V. light, using a sterile scalpel and placed in the column part of a Co-Star Spin-X tube. The column was placed in a Co-Star microcentrifuge tube. The assembly was placed in liquid nitrogen for 5 minutes and then centrifuged in a microcentrifuge at 16,000 x g for 15 minutes. The freezing and centrifugation steps were repeated until no agarose could be seen in the column (usually twice). 100 μl of autoclaved distilled water was added to the column, the freezing and centrifugation steps were repeated as before. The column was removed from the microcentrifuge tube and the extracted DNA solution was phenol/chloroform extracted as described in section 2.2.2.1. The DNA was precipitated by the addition of 2.5 volumes of absolute ethanol and 0.1 volume of 3 M sodium acetate. The tube was stored on dry ice for 10 minutes or at -20°C overnight. The tube was centrifuged in a microcentrifuge at 16,000 x g for 20 minutes and the supernatant was removed. The pellet was washed with 200 μl of 70% ethanol, centrifuged for 10 minutes, air-dried and resuspended in 10-30 μl of autoclaved distilled water, depending on the size of the pellet.

### 2.2.6. FILLING-IN 3' OVERHANGING ENDS USING KLENOW DNA POLYMERASE

If a fragment produced by a 5'-cutting restriction enzyme, such as *Nco* I, was required to be cloned into a plasmid and there was no unique restriction site for that enzyme in the multiple cloning region of the plasmid, the overhanging ends were filled-in with Klenow DNA polymerase. This produced a blunt-ended fragment which could be inserted into the *Sma* I (or other blunt-ended restriction enzyme) site of the plasmid contained in the multiple cloning region. The appropriate restriction enzyme digest was allowed to go to completion and the enzyme was heat-inactivated at the correct temperature. The concentration of $OPA^+$ buffer was adjusted to 1 x for the filling-in reaction. For a 20 μl digest already in 1 x $OPA^+$ buffer, 2 μl 10 x $OPA^+$ buffer, 4 μl of 10 mM deoxynucleoside triphosphate (dNTP) mix and 2-10 units of Klenow DNA polymerase (10 units/μl) per μg of DNA were added. Sufficient autoclaved distilled water was added to give a final volume of 40 μl. The tube was incubated at 37°C for 30 minutes and then at 65°C for 10 minutes to inactivate the enzyme.

### 2.2.7. LIGATION REACTIONS

i. Religation of plasmid DNA following fragment excision

Religation of plasmid DNA following fragment excision was carried out in a final volume of 20 μl. An appropriate amount of plasmid DNA extracted from a low melting point agarose gel (section 2.2.5.) was added to a microcentrifuge tube with 2 μl of 10 x $OPA^+$ buffer, 4 μl of 5 mM ATP, 1 μl of T4 DNA ligase (6.7 units/μl) and autoclaved distilled water to adjust the volume to 20 μl.

ii. Ligation of an insert DNA to vector DNA

For ligation of an insert DNA to vector DNA a ratio of 3:1, insert DNA : vector DNA in a final volume of 10 μl was used. An appropriate amount of each of insert and vector DNA were added to a microcentrifuge tube with 1 μl of 10 x $OPA^+$ buffer, 2 μl of 5 mM ATP, 1 μl of T4 DNA ligase (6.7 units/μl) and autoclaved distilled water to adjust the volume to 10 μl.

A ligation control was set up with all ligation reactions. 1 μl of sticky-end or blunt-end linearised pUC19 was added to 1 μl of 10 x $OPA^+$ buffer, 2 μl of 5 mM ATP, 1 μl of T4 DNA ligase (6.7 units/μl) and autoclaved distilled water to adjust the volume to 10 μl.

Sticky-end ligations were incubated at 10$^o$C for 4-16 hours and blunt-end ligations were incubated at 10-16$^o$C overnight. The resulting ligated plasmids were phenol/chloroform extracted, ethanol precipitated in the presence of 3 M sodium acetate and the DNA pellet was washed with 70% ethanol (section 2.2.5.). The DNA pellet was resuspended in 2-10 μl of autoclaved distilled water.

### 2.2.8. PREPARATION OF FROZEN COMPETENT *E. COLI* FOR TRANSFORMATION [DEVELOPED BY HANAHAN (1983); ADAPTED BY SAMBROOK *ET AL.* (1989)]

Solutions:

| | |
|---|---|
| Transformation buffer: | 10 mM potassium acetate (pH 7.5), 45 mM manganese chloride, 10 mM calcium chloride, 100 mM potassium chloride, 3 mM hexaminecobalt chloride, 10% v/v glycerol. |

10 ml of LB (no antibiotic) was inoculated with a loopful of a glycerol stock or a single colony of an *E. coli* strain from an agar plate. The culture was incubated overnight at 37$^{o}$C with shaking and the next morning 5 ml was taken and used to inoculate 100 ml of SOB media in a 1 litre flask. The culture was incubated at 37$^{o}$C until an O.D.$_{550}$ of 0.45-0.55 was obtained i.e. log growth phase was reached. The culture was divided into 2 x 50 ml sterile Greiner® tubes [Greiner Labortechnik Limited (U.K.), Stonehouse, Gloucestershire] and the bacteria were harvested by centrifugation at 4,000 x g at room temperature in a bench-top centrifuge. The media was decanted from the cell pellets and the tubes were stood in an inverted position for 1 minute to allow the last traces of media to drain away. The pellets were resuspended, by gentle vortexing, in 20 ml (per 50 ml tube) of ice-cold transformation buffer and were stored on ice for 10 minutes. The cells were recovered by centrifugation at 4,000 x g for 10 minutes in a bench-top centrifuge. The buffer was decanted from the cell pellets as before, the cell pellets were resuspended by gentle vortexing in 4 ml (per 50 ml tube) of ice-cold transformation buffer. 140 µl of dimethyl sulphoxide (DMSO) per 4 ml of resuspended cells was added, the contents of the tubes were gently mixed and the suspensions was stored on ice for 15 minutes. 140 µl of DMSO was added to each suspension, these were mixed gently by swirling and then returned to ice. 200 µl aliquots of suspension were quickly

transferred to pre-chilled sterile microcentrifuge tubes. The tubes were immediately snap-frozen in liquid nitrogen and stored at -80°C until needed.

### 2.2.9. TRANSFORMATION OF *E. COLI* BY PLASMID DNA [DEVELOPED BY HANAHAN (1983); ADAPTED BY SAMBROOK *ET AL.* (1989)]

A tube of competent cells was removed from the -80°C freezer and thawed on ice. The tube was stored on ice for a further 10 minutes post-thawing. 100 µl of cells was transferred to a sterile polypropylene tube (Falcon 2059, 17 mm x 100 mm; Becton Dickinson Labware, New Jersey, U.S.A.) which had been pre-chilled on ice. An appropriate amount of DNA, not exceeding 5% of the volume of competent cells was added [generally 1 µl (~100 ng) of mini-prep DNA and 5 µl of purified ligated construct] to the tube. For each transformation, a negative and positive control were also prepared. The negative control consisted of cells only, no DNA was added. 5-100 ng of plasmid DNA known to be able to transform *E. coli*, for example pUC19, was added to 100 µl of cells to provide a positive control. The contents of the tubes were swirled gently and stored on ice for 30 minutes. The tubes were transferred to a 42°C waterbath for 90 seconds to allow "heat-shock" to proceed. The tubes were rapidly placed on ice for 1-2 minutes following which 800 µl of SOC medium was added to each tube. The cultures were warmed to 37°C and then incubated with shaking for 1 hour at 37°C to allow the transformed bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid. 100 µl and 200 µl aliquots of transformed cells were spread on LB AMP (50 µg/ml) X-gal/IPTG agar plates. Once the surface of the plates were dry, they were inverted and incubated at 37°C overnight.

### 2.2.10. PURIFICATION OF SYNTHETIC OLIGONUCLEOTIDES FOR SEQUENCING

Oligonucleotides for sequencing were synthesised in-house using an Applied Biosystems 391 DNA synthesiser by Dr. Colin Dolphin in the laboratory of Prof. I. R. Phillips. The oligonucleotides were produced such that the trityl group was removed from the synthesis column.

The oligonucleotides were removed from the column by attaching a 5 ml syringe to one end of the column, another 5 ml syringe containing 2 ml of concentrated ammonium hydroxide (ammonia solution) was carefully attached to the other end of the column. The ammonium hydroxide was slowly pushed backwards and forwards through the column for approximately 30 seconds, ensuring the column matrix was in contact with the solution. The column was left standing upright for 10 minutes and the push/pull routine was repeated. The column was left standing upright for 10 minutes. This was repeated 3 more times to ensure the ammonium hydroxide solution had cleaved the oligonucleotide from the column support matrix. The ammonium hydroxide containing the oligonucleotide was transferred to a glass vial with a teflon lined cap. The cap was sealed with Nescofilm and the vial was heated at $70^{o}C$ in a water bath for 1 hour. The vial was allowed to cool to room temperature before it was placed at $-20^{o}C$ for 10 minutes. 0.5 ml of the solution was transferred to a 2 ml microcentrifuge tube to which 55 µl of sodium acetate (pH 5.5) and 1.4 ml of 100% ethanol were added. The tube was mixed and the oligonucleotide was precipitated on dry ice for 30 minutes. The tube was centrifuged at 16,000 x g for 30 minutes and the supernatant was discarded. The pellet was washed with 200 µl of 80% ethanol, centrifuged as above and the supernatant was carefully removed. The pellet was air-dried or vacuum-desiccated briefly and was resuspended in 50 µl of autoclaved distilled water. 5 µl of oligonucleotide solution was used to obtain an $O.D._{260}$. The solution was diluted appropriately to give a concentration equivalent to 0.5 pmol/µl.

### 2.2.11. T7 DNA POLYMERASE DOUBLE-STRANDED DIDEOXY DNA SEQUENCING (SANGER *ET AL.*, 1980; MIERENDORF AND PFEFFER, 1987)

*Denaturation of plasmid DNA immediately prior to annealing*

7-8 μl of plasmid DNA from a PEG DNA prep (section 2.2.2.2.) or 2 μg of DNA from a Qiagen DNA prep (section 2.2.2.3.) was used per annealing reaction. The final volume was made up to 10 μl with autoclaved distilled water. 2 μl of 2 N sodium hydroxide was added to the side of the microcentrifuge tube containing the DNA, the tube was vortexed and centrifuged briefly. The tube was incubated at room temperature for no more than 5 minutes. 8 μl of 5 M ammonium acetate (pH 7.5) and 112 μl of absolute ethanol equilibrated to -20°C were added immediately; the tube was vortexed and centrifuged briefly. The tube was stored on dry ice for 10 minutes and then centrifuged in a microcentrifuge at 16,000 x g for 10 minutes. The pellet was washed with 200 μl of 80% ethanol, centrifuged and the supernatant was carefully removed. The pellet was air-dried or vacuum-desiccated briefly and was resuspended in 10 μl of autoclaved distilled water. The annealing reaction was proceeded to immediately.

*Annealing reaction*

To the denatured template DNA, 2 μl of annealing buffer and 2 μl of primer solution (concentration equivalent to 0.5 pmol/μl) were added to the side of the tube. The tube was vortexed, centrifuged briefly and was incubated at 37°C for 20 minutes to allow annealing of the primer to the template DNA. After 20 minutes, the tube was centrifuged briefly to collect any moisture at the top of the microcentrifuge tube. The contents of the tube were cooled at room temperature for 10 minutes before the sequencing reactions were performed.

*Sequencing reactions*

Short$^{T7}$Sequencing$^{TM}$ Mixes

2.5 µl respectively, of the "A" Mix-short, "C" Mix-short, "G" Mix-short and "T" Mix-short were pipetted into separate tubes. Use of the short-mixes enabled around 200-500 nucleotides to be sequenced. T7 DNA polymerase was diluted to 1.5 units/µl in cold enzyme dilution buffer; 2 µl of the diluted stock was required for each template. An "enzyme pre-mix" was prepared; the following components were added in the order shown per n templates: n µl of autoclaved distilled water, 3n µl of labelling mix-dATP, 2n µl of diluted T7 DNA polymerase and n µl of [$\alpha$-$^{35}$S]dATP (10 µCi/µl). The total volume was 7n µl; the tube was stored on ice during each addition. 6 µl of "enzyme pre-mix" was added to the tube containing the annealed template and primer. The contents were mixed by gentle agitation with a micropipette tip and were incubated at room temperature for 5 minutes. Meanwhile, the 4 sequencing mixes previously dispensed were warmed at 37$^{o}$C for at least 1 minute. After the 5 minute incubation of the labelling reaction, the termination reactions were carried out immediately.

Deaza G/A $^{T7}$Sequencing$^{TM}$ Mixes

2.5 µl respectively, of the "A" Mix, "C" Mix, "G" Mix and "T" Mix were pipetted into separate tubes. T7 DNA polymerase was diluted in cold enzyme dilution buffer; for n templates: 0.5n µl of enzyme was diluted in 2n µl of enzyme dilution buffer. To the tube containing the annealed template and primer, 3 µl of labelling mix-dATP, 1 µl of [$\alpha$-$^{35}$S]dATP (10 µCi/µl) and 2 µl of diluted T7 DNA polymerase were added. The sample was mixed by centrifuging the tube briefly in a microcentrifuge. The tube was incubated at room temperature for 5 minutes. Meanwhile, the 4 sequencing mixes previously dispensed were warmed at 37$^{o}$C for at least 1 minute. After the 5 minute incubation of the labelling reaction, the termination reactions were carried out immediately.

*Termination reactions for all mixes*

After the 5 minute labelling reaction incubation, 4.5 µl of this reaction was transferred into each of the 4 pre-warmed sequencing mixes. The samples were mixed by centrifuging briefly in a microcentrifuge and incubated at 37°C for 5 minutes. 5 µl of Stop solution was added to the side of each tube, and the contents were mixed by centrifuging briefly in a microcentrifuge. 4 µl of each stopped reaction was transferred to a fresh tube. The lids of the tubes were pierced and the 4 samples were boiled for 3 minutes. The 4 samples were immediately loaded onto a 6 or 8% acrylamide sequencing gel. The remaining stopped reactions were stored at -20°C until use.

### 2.2.12. POLYACRYLAMIDE GEL ELECTROPHORESIS FOR DNA SEQUENCING

Solutions:

| | |
|---|---|
| Sequagel buffer: | 50% urea (8.3 M) in 1.0 M Tris-borate-20 mM EDTA buffer (pH 8.3). |
| Sequagel diluent: | 500 g urea (8.3 M) in a 1 L solution. |
| Sequagel concentrate: | equivalent of 237.5 g acrylamide, 12.5 g of methylene bis-acrylamide and 500 g urea (8.3 M) in a 1 L solution. |

A 6% acrylamide sequencing gel was prepared by mixing 10 ml of Sequagel buffer, 66 ml of Sequagel diluent with 24 ml of Sequagel concentrate in a beaker in a fume hood. 800 µl of freshly prepared 10% ammonium persulphate and 40 µl of TEMED (N, N, N', N'-Tetramethylethylenediamine; BIO-RAD) were added to the beaker. The contents were thoroughly mixed and the gel mixture was poured carefully, but quickly, in between two taped-up glass sequencing plates. The glass plates and 0.4 mm plastic spacers had been thoroughly cleaned and the top plate had been siliconised with dimethylchlorosilane to aid its removal once the gel had been run. Electrophoresis combs were inserted in an inverted position; once the gel was set the combs were removed and inserted the correct way-up. The gel was pre-run for 0.5-1 hour at a constant power of 60 Watts (1,200-1,500 Volts); the electrophoresis buffer was 1 x TBE. The pre-boiled samples were loaded and the gel was electrophoresed until the xylene cyanol had run off; new pre-boiled aliquots of the same samples were loaded into new wells and the gel was run until the bromophenol blue of the new samples had run off. This constituted a "long" and "short" run respectively. The plates were removed and the gel was transferred onto 3MM Whatman paper. The gel was covered with cling film and dried in a Gel-drier for about 0.5 hour. The dried gel was placed in a cassette and was overlaid with an X-ray film. The film was developed after 12-24 hours.

### 2.2.13. REVERSE TRANSCRIPTION (RT) OF THE NOVEL MARMOSET CYP2A MESSENGER RNA (mRNA) FROM MARMOSET LIVER RNA

Solutions:

5 x enzyme buffer: 0.25 M Tris-HCl (pH 8.3), 0.375 M potassium chloride, 15 mM magnesium chloride.

Total liver RNA previously prepared from a phenobarbital-treated marmoset was available in the laboratory. 15 μg of this RNA (3.55 μg/μl) was added to 2.5 μmoles of the reverse primer oligo 109 (position of primer is defined in figure 3.1) and 18.67 μl of filter-sterilised distilled water in a sterile 500 μl microcentrifuge tube. A parallel control tube was set up containing filter-sterilised water in place of the marmoset liver RNA. 50 μl of sterile mineral oil was placed on top of the contents of the microcentrifuge tubes to prevent evaporation of the sample. The samples were incubated at 65°C for 10 minutes. The tubes were immediately snap-chilled on ice and made up to a final volume of 50 μl by the addition of: 2.5 μl of 10 mM dNTP mix, 5 x enzyme buffer, 50 units RNA guard, 200 units M-MLVRT, 10 mM DTT and 5 μl of

1 mg/ml nuclease-free Bovine serum albumin (BSA). The samples were incubated at 37°C for 90 minutes and then for a further 10 minutes at 65°C. The aqueous layers were transferred to fresh sterile 500 µl microcentrifuge tubes to which 2 volumes of 100% ethanol were added. The tubes were left on dry ice for 30 minutes before centrifuging at 16,000 x g for 20 minutes in a microcentrifuge. The supernatants were removed and the pellets were washed with 70% ethanol, vacuum-dried and resuspended in 50 µl of filter-sterilised distilled water.

### 2.2.14. AMPLIFICATION OF THE NOVEL MARMOSET CYP2A COMPLEMENTARY DNA (cDNA) BY POLYMERASE CHAIN REACTION (PCR)

Solutions:

10 x buffer: 100 mM Tris-HCl (pH 8.8), 15 mM magnesium chloride, 500 mM potassium chloride, 1% Triton X-100.

5 µl of the novel marmoset CYP2A cDNA, the product from the reverse transcription step, was placed in a sterile 500 µl microcentrifuge tube. A negative control tube for the PCR was set up containing 5 µl of filter-sterilised distilled water. 5 µl of the reverse transcription negative control was placed in a sterile microcentrifuge tube. To each of these tubes 5 µl of 10 x buffer, 1 µl of 10 mM dNTP, 5 µl of 1 mg/ml nuclease-free BSA, 2.5 µmoles each of forward and reverse primer (oligo 108 and 109 respectively; positions of primers are defined in figure 3.1) and 5 µl of DMSO were added. The final volume was made up to 49 µl with filter-sterilised distilled water. The contents of the tubes were overlaid with 50 µl of sterile mineral oil, the tubes were heated at 95°C for 5 minutes ("hot-start") in a Hybaid Thermal Reactor. 1 µl of DyNAZyme™ was added through the paraffin layer, the tubes were flicked and centrifuged briefly in an Appligene minicentrifuge. The tubes were returned to the thermocycler where 35 cycles of 90°C for 1 minute, 45°C for 1.5 minutes, 72°C for 3 minutes were carried out with 1 final cycle of 72°C for 15 minutes to ensure all the PCR products were fully

extended. 5 µl of each of the PCR products were taken from below the paraffin layer and were electrophoresed through a 1% agarose gel (section 2.2.4.) for analysis of the results of the PCR and reverse transcription protocols.

### 2.2.15. PROTEINASE K DIGESTION OF PCR PRODUCTS FOR IMPROVED CLONING EFFICIENCY (CROWE *ET AL.*, 1991)

Solutions:

A 25 µg/µl stock solution of proteinase K (Boeringher Mannheim, supplied lyophilised) was made in 50 mM Tris-HCl (pH 8.0), 1 mM calcium chloride. The solution was incubated at 37°C for 5 minutes, cooled to room temperature and stored at -20°C.

The PCR product to be cloned was removed from below the mineral oil layer and transferred to a sterile 1.5 ml microcentrifuge tube. The sample was phenol/chloroform extracted, 2.5 volumes of 100% ethanol and 0.1 volume of 3 M sodium acetate were added to the aqueous phase to precipitate the DNA. The sample was centrifuged at 16,000 x g in a microcentrifuge for 20 minutes. The DNA pellet was washed with 200 µl 80% ethanol and centrifuged as above. The resulting DNA pellet was air-dried and resuspended in 20-50 µl of water. 1 µl of 0.5 M EDTA, 1 µl of 1 M Tris-HCl (pH 8.0) and 5 µl of 10% SDS were added to the sample. Proteinase K was added to a final concentration of 50 µg/ml and sterile distilled water was added to adjust the volume to 100 µl. The sample was incubated at 37°C for 30 minutes. Proteinase K was heat-inactivated by placing the tube at 68°C for 10 minutes. The sample was phenol/chloroform extracted and the PCR product was precipitated with 2.5 volumes of ice-cold 100% ethanol and 0.1 volume of 3 M sodium acetate. The DNA pellet was washed in 80% ethanol, dried and was resuspended in 20 µl autoclaved distilled water which had been filter-sterilised (0.2 µm filter).

### 2.2.16. LIGATION OF THE NOVEL MARMOSET CYP2A INTO pGEM-T VECTOR

| | |
|---|---|
| 10 x T4 DNA ligase buffer: (Promega, Madison, Wisconsin, U.S.A.) | 300 mM Tris-HCl (pH 7.5), 100 mM magnesium chloride, 100 mM DTT, 10 mM ATP. |

1 µl of purified marmoset CYP2A PCR product (approximately 150 ng) was added to a microcentrifuge tube containing 1 µl of 10 x T4 DNA ligase buffer, 1 µl of pGEM-T vector (50 ng, Promega) and 1 µl of T4 DNA ligase (1 unit/µl, Promega). The volume was made up to 10 µl with filter-sterilised distilled water. An equivalent volume ligation control tube was set up replacing the purified marmoset CYP2A PCR product with 2 µl of pGEM-T vector control (8 ng, Promega). The tubes were left at room temperature overnight to enable optimal ligation to be achieved. The tubes were incubated at 72°C for 10 minutes in order to inactivate the T4 DNA ligase. The tubes were allowed to cool to room temperature before all 10 µl of ligated product were used to transform competent XL1-Blue *E. coli* cells.

### 2.2.17. ISOLATION OF TOTAL RNA FROM MICE SPLEENS USING ULTRASPEC™ RNA ISOLATION SYSTEM

Solutions:

| | |
|---|---|
| Ultraspec™ RNA reagent: | 14 M solution of guanidine salts and urea. |

Ultraspec™ RNA reagent was obtained from Biotecx (Houston, Texas, U.S.A.) and was stable for approximately nine months when stored in the dark at 4°C. The reagent was brought to room temperature and shaken thoroughly before use. The frozen mice spleens were weighed rapidly in sterile tubes and 1 ml of Ultraspec™ RNA reagent was added for each 10-100 mg of frozen tissue. To prevent RNA degradation, the spleens were homogenised while still frozen using a polytron homogeniser. The homogenate was stored at 4°C for 5 minutes to permit complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform/ml of Ultraspec™ RNA was added to the homogenate. The sample was covered tightly, shaken vigorously for 15 seconds and incubated on ice at 4°C for 5 minutes. The homogenates were split into 2 x 2 ml sterile microcentrifuge tubes due to the large volume of sample. The tubes were centrifuged at 12,000 x g at 4°C for 15 minutes. The aqueous phase from each sample (4/5th total volume) containing the RNA was carefully transferred to a fresh sterile microcentrifuge tube taking care not to disturb the interphase. The organic phase and interphase containing DNA and protein were discarded. If the aqueous phase was very small in volume the samples were stored at -20°C overnight to compact the interphase. The tubes were centrifuged as above to enable the RNA to be released from the interphase into the aqueous phase. An equal volume of isopropanol was added to the aqueous phase, the tube was mixed and stored at 4°C for 10 minutes. The samples were centrifuged at 12,000 x g at 4°C for 10 minutes and the supernatant was removed. The white RNA pellet was washed twice with 75% ethanol (1 ml of 75% ethanol/ml of initial solution used) by vortexing and subsequent centrifugation at 7,500 x g at 4°C for 5 minutes. The RNA pellet was air-dried for approximately 15 minutes. The pellet was not allowed to dry completely as this would have greatly decreased its solubility. The RNA pellet was resuspended in 50 μl of autoclaved distilled water which had been filter-sterilised (0.2 μm filter) by vortexing for 1 minute. The RNA was not resuspended in diethylpyrocarbonate (DEPC) treated water as the RNA was to be used for RT-PCR reactions and *Taq* polymerase is inhibited by DEPC. The sample was incubated at 50°C for 5 minutes and subsequently left on ice for 1 hour to ensure the RNA was completely dissolved.

The amount of RNA present was evaluated by reading an $O.D._{260}$; an O.D. of 1 at 260 nm ≡ 40 μg/ml of RNA. The O.D. at 280 nm was also measured to estimate the RNA : protein ratio. A good RNA preparation should have an RNA : protein ratio of 1.8-2.0.

The integrity of the RNA sample was checked by electrophoresing 1 µg of total RNA on an agarose gel in the presence of the denaturant formaldehyde (section 2.2.19.).

### 2.2.18. ISOLATION OF INTACT, TRANSLATIONALLY ACTIVE RNA FROM CULTURED HUMAN HEPATOCYTES (ADAPTED FROM CATHALA *ET AL.*, 1983)

The solutions required for this method were made up with autoclaved water in glassware which had been previously autoclaved. All the final solutions, except the lysis buffer, were autoclaved to destroy nucleases.

Lysis buffer

5 M guanidine monothiocyanate, 50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 8% v/v β-mercaptoethanol added before use. The guanidine monothiocyanate was dissolved in a small volume of water at 50°C on a heated stirrer. The EDTA and Tris-HCl were added once the guanidine monothiocyanate had completely dissolved.

RNA solubilisation buffer

0.1% SDS, 1 mM EDTA, 10 mM Tris-HCl (pH 7.5).

Phenol equilibration buffer

1 mM EDTA, 50 mM Tris-HCl (pH 7.5).

1 volume of phenol equilibration buffer was added to 1 volume of phenol (pH 8.0). The tube was shaken vigorously, centrifuged at 1,800 rpm for 5 minutes in a bench top centrifuge and the aqueous phase was discarded. This was repeated twice, the pH was checked with pH paper (pH Indicator strips, pH 0-6.0; B.D.H.) to ensure a pH of approximately 6.0 had been reached.

Human liver hepatocytes were cultured in Prof. Vera Rogier's laboratory in Brussels. Cell pellets, in 15 ml Greiner tubes, were sent to our laboratory on dry ice and were stored at -80°C. 7 ml of lysis buffer was added per 1 ml of packed cells, the cells were homogenised by vortexing. The homogenate was transferred to an ice-cold sterile Corex® tube. The RNA was precipitated with 5 volumes of 4 M lithium chloride (LiCl) per volume of homogenate overnight at 4°C. The sample was centrifuged at 11,000 x g at 4°C for 90 minutes. The supernatant was discarded and the RNA pellet was resuspended with vortexing in as small a volume as possible of 3 M LiCl. The RNA was pelleted as before, the supernatant was discarded and 1-5 ml of RNA solubilisation buffer was added. The solubilisation of RNA was facilitated by freezing the pellet in solubilisation buffer on dry ice and vortexing while the pellet was thawing. This was repeated several times until the RNA appeared to be in solution. The RNA was extracted with 1 volume of phenol (equilibrated to pH 6.0) and 1 volume of chloroform (containing 4% iso-amyl alcohol) in a sterile 50 ml Greiner tube. The sample was centrifuged at 1,800 rpm for 10 minutes in a bench top centrifuge and stored overnight at -20°C to compact the interphase. The sample was centrifuged again, the aqueous phase was transferred to a fresh sterile 50 ml Greiner tube and the RNA was phenol/chloroform extracted as above omitting the overnight incubation. The aqueous phase was removed and was chloroform extracted. The last aqueous phase was transferred to an ice-cold sterile Corex® tube and 0.05 volume of saturated ammonium acetate and 2 volumes of 100% ethanol were added. The sample was placed at -20°C overnight. The RNA was pelleted by centrifugation at 11,000 x g at 4°C for 70 minutes. The RNA was washed with 1 ml of cold 90% ethanol and centrifuged at 11,000 x g at 4°C for 60 minutes. The RNA pellet was dried in a vacuum desiccator and resuspended in 50-300 µl autoclaved water which had been filter-sterilised (0.2 µm filter). The concentration of the RNA was evaluated by reading an $O.D._{260}$; an O.D. of 1 at 260 nm ≡ 40 µg/ml of RNA. The O.D. at 280 nm was also measured to estimate the RNA : protein ratio. The integrity of the RNA was checked by electrophoresing 2-5 µg on an RNA agarose gel (section 2.2.19.).

### 2.2.19. AGAROSE GEL ELECTROPHORESIS OF RNA

Solutions:

| | |
|---|---|
| 10 x MOPS/EDTA: | 0.2 M MOPS, 50 mM sodium acetate, 10 mM EDTA; adjusted to pH 7.0. |
| RNA loading buffer: | 0.75 ml of deionised formamide, 0.15 ml of 10 x MOPS, 0.24 ml of formaldehyde, 0.1 ml of filter-sterilised distilled water, 0.1 ml of glycerol, 0.08 ml of 10% w/v bromophenol blue. |

Gel preparation

A spatula, all glassware and measuring cylinders were autoclaved before use. 1.0 g of agarose was weighed out in a sterilised 250 ml conical flask. The agarose was dissolved in 10 ml of 10 x MOPS/EDTA and 87 ml of autoclaved water. When the agarose solution had cooled to 50°C, 5.1 ml of 37% formaldehyde was added to the conical flask in a fume hood. The gel was poured into a thoroughly cleaned 11.0 cm x 14.0 cm gel tray situated in the fume hood. The gel was left to set for 1 hour. The gel was placed in a gel tank containing 1 x MOPS/EDTA, the comb was removed and the wells were flushed with 1 x MOPS/EDTA.

Sample preparation and electrophoresis

1 μg of total RNA was adjusted to a total volume of 5 μl with filter-sterilised distilled water. 25 μl of RNA loading buffer was added to the RNA sample which was then incubated at 65°C for 15 minutes. 1 μl of ethidium bromide solution (1 mg/ml) was mixed with the sample which was then loaded into a well of the agarose gel. The gel was electrophoresed at 100 V for 2-3 hours and photographed under U.V. light.

### 2.2.20. ISOLATION OF mRNA FROM SPLEEN TOTAL RNA BY OLIGO(dT)-CELLULOSE CHROMATOGRAPHY

An mRNA purification kit containing pre-packed oligo(dT)-cellulose spun columns was purchased from Pharmacia Biotech.

Components of mRNA purification kit:

*Oligo(dT)-cellulose columns*: Oligo(dT)-cellulose suspended in storage buffer containing 0.15% Kathon® CG.

*High-salt buffer*: 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.5 M NaCl.

*Sample buffer*: 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 3.0 M NaCl.

*Low-salt buffer*: 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.1 M NaCl.

*Elution buffer*: 10 mM Tris-HCl (pH 7.4), 1 mM EDTA.

*Glycogen solution*: Glycogen in solution at 5-10 mg/ml.

Preparation of spun columns

The spun columns were inverted several times to resuspend the oligo(dT)-cellulose. The top and bottom closures were removed in that order, the columns were placed in upright 15 ml Corex® tubes and the storage buffer was allowed to drain through the columns. 1 ml of high-salt buffer was added and allowed to drain through the columns under gravity. This wash was repeated. The columns were removed from the Corex® tubes, the buffer was discarded and the columns were replaced in the tubes.

### Sample preparation

Each spun column had the capacity to bind approximately 25 μg of polyadenylated ribonucleic acid [poly(A)$^+$] RNA. It was assumed that 2% of total RNA was polyadenylated, therefore 1.25 mg of total RNA (approximately 31 O.D.$_{260}$ units) was the maximum amount applied to each column.

The poly(A)$^+$ RNA sample was made up to a final volume of 1 ml with elution buffer and heat-denatured at 65°C for 5 minutes. The sample was placed on ice, 0.2 ml of sample buffer was added and the contents of the tube were mixed gently.

### Spun-column chromatography

The sample was applied to the top of the cellulose bed in the column and allowed to soak in under gravity. The column was centrifuged at 350 x g for 2 minutes in a bench-top centrifuge. 0.25 ml of high-salt buffer was added to the column which was centrifuged again for 2 minutes at 350 x g. The wash and centrifugation steps were repeated once. Using the same centrifugation routine, the column was washed three times with 0.25 ml aliquots of low-salt buffer. The column was removed from the Corex® tube and the buffer which had been collected in the latter was discarded. A sterile 1.5 ml microcentrifuge tube was placed inside the 15 ml tube to collect subsequent effluents from the column. The spun column was replaced in the Corex® tube with its lower end inside the microcentrifuge tube. The bound poly(A)$^+$ RNA was eluted by washing the column four times with successive 0.25 ml aliquots of elution buffer, prewarmed to 65°C. The spun column was removed and the microcentrifuge tube containing the 1 ml of eluate was recovered from the Corex® tube using flamed forceps.

Approximately 50% of the RNA recovered from the first spun column was poly(A)$^+$ RNA. A second round of spun-column chromatography increased the proportion of poly(A)$^+$ RNA to over 90% of the sample but there was approximately a 33% decrease in yield as compared to one round of purification.

For a second round of purification, the eluate from the first spun column was treated as unfractionated total RNA in elution buffer. The entire eluate was heat-denatured as before, cooled and 0.2 ml of sample buffer was added. The sample was applied to a freshly prepared spun column and a second round of spun-column chromatography was carried out as above.

The concentration of the mRNA was evaluated by making a 1 in 10 dilution (final volume of dilution was 200 µl) and measuring O.D.$_{260}$ using a path length of 0.1 cm; an O.D. of 1 at 260 nm ≡ 40 µg/ml of RNA.

### Concentration of mRNA

To provide a more concentrated sample of mRNA, the eluate was transferred to a sterile 15 ml Corex® tube. 100 µl of sample buffer, 10 µl of glycogen solution and 2.5 ml of ice-cold ethanol were added to the eluate. The tube was swirled gently and placed at -20°C for at least 2 hours. The precipitated mRNA was recovered by centrifugation at 4°C for 10 minutes at 12,000 x g. The pellet was washed with 1 ml of 80% ethanol and centrifuged for 5 minutes at 4°C at 12,000 x g. The pellet was resuspended in 20 µl of autoclaved water which had been filter-sterilised and the mRNA was stored at -80°C.

### 2.2.21. PURIFICATION OF HUMAN CYP3A4 FROM INSECT CELL MICROSOMES BY AFFINITY CHROMATOGRAPHY ON *P*-CHLOROAMPHETAMINE-COUPLED SEPHAROSE (ADAPTED FROM SUNDIN *ET AL.*, 1987)

Solutions for chromatography:

| | |
|---|---|
| Starting buffer: | 5 mM sodium phosphate (pH 7.5) containing 20% v/v glycerol, 0.125% w/v sodium cholate (Sigma), 0.05% v/v Emulgen 911 (Kao Atlas, Japan), 0.4 mM phenylmethylsulphonylfluoride (PMSF) dissolved in ethanol. |
| Solubilisation buffer: | 5 mM sodium phosphate buffer (pH 7.5) containing 20% v/v glycerol, 0.6% w/v sodium cholate, 0.24% v/v Emulgen 911, 0.48 mM PMSF, 1.2 mM DTT, 1.2 mM EDTA, 27.6 μM butylated hydroxy-toluene (BHT). |
| Running buffer: | 5 mM sodium phosphate buffer (pH 7.5) containing 20% v/v glycerol, 0.125% w/v sodium cholate, 0.05% v/v Emulgen 911, 100 mM NaCl, 0.4 mM PMSF. |
| Elution buffer: | 5 mM sodium phosphate buffer (pH 7.5) containing 20% v/v glycerol, 0.5% w/v sodium cholate, 0.2% v/v Emulgen 911, 0.4 mM PMSF. |

Coupling of ligand to support matrix

The ligand *p*-chloroamphetamine is a Class 1 listed drug belonging to the amphetamine family. Hence it was necessary to obtain a Home Office Drugs Licence prior to ordering the compound from Sigma. The support matrix, Affigel-10, was purchased from BIO-RAD. Affigel-10 is a sepharose matrix with a chemical binding capacity of 15 μmoles per ml of gel.

The amount of ligand (μmoles) to be coupled to the support was calculated by multiplying the volume of gel to be used (25 ml) by the binding capacity of the support. However, a ten-fold excess of ligand was used to ensure that all the possible ligand coupling sites were occupied.

770 mg of *p*-chloroamphetamine was dissolved in 25 ml of 0.1 M sodium hydrogen carbonate in a 50 ml sterile Greiner tube which was placed on ice. The 25 ml vial of Affigel-10 was shaken to achieve a uniform suspension. The gel was transferred to a Buchner funnel lined with slightly dampened 3MM Whatman filter paper; the funnel was connected to a Buchner flask situated in the fume cupboard. The solvent from the Affigel-10 was removed under vacuum and the gel was washed with 3 bed volumes (75 ml) of 4°C deionised water; it was essential that the gel was not allowed to dry out during this procedure. The entire wash step was carried out within 20 minutes to enable optimal ligand coupling to be achieved. The moist gel was placed in a 100 ml bottle to which the ice-cold ligand solution was added (at least 0.5 ml of ligand solution per ml of gel, but not exceeding 4.5 ml per ml of gel). The bottle was sealed with parafilm and agitated for 4 hours at 4°C to ensure a uniform suspension was obtained.

### Preparation and equilibration of the column

A 1.5 cm x 20 cm Pharmacia column was packed with the *p*-chloroamphetamine-coupled Sepharose. The column was left overnight at 4°C in 0.2% w/v sodium azide to allow the matrix to pack uniformly. Prior to use, the sodium azide solution was drained and the column was washed with 4 bed volumes of 10 mM Tris-HCl/100 mM NaCl (pH 8.0) and 4 bed volumes of 10 mM sodium acetate/100 mM NaCl (pH 4.5). The gel was equilibrated in starting buffer immediately before use.

### Solubilisation of insect cell microsomes

The microsomes prepared from *Trichoplusia ni* (*T ni*) insect cells expressing human CYP3A4 were donated by GlaxoWellcome. The microsomes had been resuspended in

300 mM potassium phosphate buffer (pH 7.7) containing 20% v/v glycerol. The microsomal suspension was diluted 1 in 6 with solubilisation buffer. The microsomal suspension was placed in a conical flask containing a magnetic stirring bar. The flask was placed inside a 2 litre beaker packed with ice which was situated on a magnetic stirrer. The microsomal suspension was stirred for 0.5-1 hour. 1.5 ml of the solubilised microsomes was taken for comparative study with the purified protein fractions to be collected and was stored at -80°. The solubilised microsomal solution was further diluted 1 in 4 with 20% v/v glycerol to lower the concentration of the detergents to that of the starting buffer used to equilibrate the column.

Affinity chromatography

The chromatography procedure was performed at 4°C. The solubilised microsomal solution was loaded onto the column at a rate of 0.5 ml/min. After all the sample had been loaded, the column was washed with 3 bed volumes (75 ml) of running buffer. The proteins were eluted with elution buffer at a rate of 1.0 ml/min and each fraction was collected over 3 minutes. The $O.D._{280}$ and $O.D._{417}$ were measured for each fraction and on the basis of these readings certain fractions were selected for electrophoresis on SDS-PAGE gels (section 2.2.26.) for silver staining (section 2.2.28.) and western blot analysis (section 2.2.29.). Fractions containing CYP3A4 were pooled and concentrated in an Amicon PM30 ultrafiltration cell [section 2.2.30., (Amicon, Stonehouse, Gloucestershire, U.K.). The sample was further concentrated in an Amicon centricon-30 unit (section 2.2.30.).

## 2.2.22. PURIFICATION OF HUMAN CYP3A4 FROM INSECT CELL MICROSOMES BY CHROMATOGRAPHY ON A *N*-OCTYLAMINO-SEPHAROSE COLUMN (ADAPTED FROM GUENGERICH AND MARTIN, IN PRESS)

### Solutions for chromatography:

Solubilisation buffer: 0.1 M potassium phosphate buffer (pH 7.25) containing 1 mM EDTA, 20% v/v glycerol, 20 μM BHT and 1 mM DTT.

Buffer A: 0.1 M potassium phosphate buffer (pH 7.25) containing 1 mM EDTA, 20% v/v glycerol, 0.6% w/v sodium cholate.

Buffer B: 0.1 M potassium phosphate buffer (pH 7.25) containing 1 mM EDTA, 20% v/v glycerol, 0.42% w/v sodium cholate.

Buffer C: 0.1 M potassium phosphate buffer (pH 7.25) containing 1 mM EDTA, 20% v/v glycerol, 0.33% w/v sodium cholate, 0.06% v/v Emulgen 911.

### Preparation of *n*-octylamino-sepharose

Sepharose 4B (Pharmacia Biotech) was suspended in distilled water, 1,200 ml per 400 ml packed gel volume, by stirring with a glass rod in a coarse sintered glass funnel. An aspirator vacuum was applied to a Buchner flask which was connected to the funnel. This was repeated several times in order to wash the gel free of preservatives. The 400 ml of washed Sepharose 4B was suspended in 400 ml of cold distilled water in a beaker containing a magnetic stirring bar. The beaker was placed in a fume hood and the electrode of a pH meter was placed in the gel suspension. A solution of 100 g cyanogen bromide (Sigma) dissolved in 100 ml of 1,4-dioxane (Sigma) was poured into the suspension all at once. The pH was maintained at 11 over a period of 20 minutes by the addition of 8 N NaOH. The reaction was quenched by the addition of approximately 400 g of ice. The gel was collected by pouring the suspension into a sintered glass funnel attached to a Buchner flask under vacuum in the fume hood. The collected gel was washed once with cold distilled water and placed in a beaker. 115 g of 1,8-diaminooctane (Aldrich, Milwaukee, Wisconsin, U.S.A.) dissolved in 400 ml of distilled water was added to the gel, the pH was adjusted to 10.0 with 6N HCl. The beaker was covered with plastic wrapping and the suspension was stirred overnight as slowly as possible at 4°C. The gel was recovered on a sintered glass funnel and washed repeatedly with distilled water and 2 L of 0.1 M potassium phosphate buffer (pH 7.25) containing 1 mM EDTA. The gel was removed from the funnel, suspended in 0.1 M potassium phosphate buffer (pH 7.25) containing 1 mM EDTA and stored at 4°C until use.

Preparation of chromatography column

A 2.5 cm x 36 cm Pharmacia column was packed with the gel to a height of 20 cm. The amount of gel sufficient to fill the column (100 ml) was equilibrated to room temperature and placed in a Buchner flask in 0.1 M potassium phosphate buffer (pH 7.25) and capped. Aspirator vacuum was applied for 10 minutes with intermittent swirling of the flask to disperse the air bubbles. The gel slurry was poured into the column and allowed to stand for 10 minutes after which time the outlet valve was opened. Sufficient gel slurry was added to the top column of the column until it was packed to the correct height, ensuring the top of the matrix did not dry out.

Solubilisation of microsomes

The microsomes prepared from *T ni* insect cells expressing human CYP3A4 were donated by GlaxoWellcome. The microsomes had been resuspended in 0.1 M potassium phosphate buffer (pH 7.4) containing 1 mM EDTA and 20% v/v glycerol. The microsomes were stirred at a protein concentration of 2 mg/ml in solubilisation buffer. Sodium cholate was added to give a final concentration of 0.6%; a 3 : 1 ratio of sodium cholate to protein w/w. The solution was stirred for 30 minutes at 4°C and then was centrifuged at 100,000 x g for 60 minutes. The supernatant was removed and the pellet was discarded. 500 µl of solubilised microsomes were removed and stored at -80°C.

*N*-octylamino-sepharose chromatography

The chromatography procedure was performed at 4°C. The column was equilibrated with 500 ml (5 bed volumes) of Buffer A at a flow rate of approximately 1 ml/min. The cholate-solubilised microsomes were applied to the column at the same flow rate. The column was washed with 320 ml (3.25 bed volumes) of Buffer B at 1 ml/min, the buffer was changed to Buffer C and 10 ml fractions were collected. The O.D.$_{280}$ and O.D.$_{417}$ were measured for each fraction and on the basis of these readings certain fractions were selected for electrophoresis on SDS-PAGE gels (section 2.2.26.) for Coomassie blue staining (section 2.2.27.) and western blot analysis (section 2.2.29.). Fractions containing CYP3A4 were pooled and concentrated in an Amicon PM30 ultrafiltration cell (section 2.2.30.). The CYP3A4 sample was then subjected to further purification by diethylaminoethyl (DEAE) anion exchange chromatography (section 2.2.23.). 200 µl of the sample was removed and stored at -80°C for further analysis.

### 2.2.23. FURTHER PURIFICATION OF CYP3A4 BY ANION EXCHANGE CHROMATOGRAPHY ON A DEAE-SEPHACEL COLUMN (ADAPTED FROM GUENGERICH AND MARTIN, IN PRESS)

Solutions for chromatography:

| | |
|---|---|
| Buffer D: | 5 mM potassium phosphate (pH 7.7) containing 0.1 mM EDTA, 20% v/v glycerol, 0.2% w/v sodium cholate. |
| Buffer E: | 5 mM potassium phosphate (pH 7.7) containing 0.1 mM EDTA, 20% v/v glycerol, 0.5% w/v sodium cholate, 0.2% v/v Emulgen 911. |

Buffer F: 5 mM potassium phosphate (pH 7.7) containing 0.1 mM EDTA, 20% v/v glycerol, 0.5% w/v sodium cholate, 0.2% v/v Emulgen 911, 0.2 M NaCl.

Preparation of dialysis tubing

A 4.5 cm x 30 cm strip of dialysis tubing was boiled twice for 5 minutes in a solution of 2% w/v sodium bicarbonate and 1 mM EDTA, changing the solution between boiling. The tubing was rinsed by boiling it several times in fresh autoclaved distilled water; it was stored until use in fresh autoclaved distilled water at room temperature.

Dialysis of sample containing CYP3A4 for DEAE chromatography

The concentrated sample containing CYP3A4 from the *n*-octylamino-sepharose column was dialysed extensively against Buffer D, 4 changes of 20 volumes, ≥ 8 hours per dialysis.

Preparation of DEAE column

A 2.5 cm x 20 cm Pharmacia chromatography column was used. DEAE-sephacel was supplied by Pharmacia as preswollen beads and was stored at 4°C. The matrix was equilibrated to room temperature, the column was filled to the top with gel and this was allowed to stand for 10 minutes. The outlet valve was opened and the storage buffer was removed, enough gel was added to fill the column ensuring room was left for the inlet valve. The pH of the gel was adjusted by washing the column with 4 bed volumes (400 ml) of 1.0 M potassium phosphate buffer (pH 7.7) at 1 ml/min.

DEAE chromatography

All steps were performed at room temperature. The column was connected to a Pharmacia FPLC system (Liquid Chromatography Controller LCC-500 Plus). The column was equilibrated with 1,000 ml of buffer E at 1 ml/min. The sample was applied at a flow rate of 1.5 ml/min and 10 ml fractions were immediately collected. The column was washed with 500 ml of buffer E. A linear gradient of 0-0.2 M NaCl was applied to the column by using 750 ml of Buffer E in one flask and 750 ml of Buffer F in a second flask, the gradient mixer was contained within the FPLC machine. The O.D.$_{280}$ and O.D.$_{417}$ were measured for each fraction and on the basis of these readings certain fractions were selected for electrophoresis on SDS-PAGE gels (section 2.2.26.) for Coomassie blue staining (section 2.2.27.) and western blot analysis (section 2.2.29.). Fractions containing CYP3A4 were pooled and concentrated in an Amicon PM30 ultrafiltration cell (section 2.2.30.) and were further concentrated in an Amicon centricon-30 unit (section 2.2.30.).

## 2.2.24. BRADFORD PROTEIN ASSAY (ADAPTED FROM BRADFORD, 1976)

Solutions supplied with the kit:

| | |
|---|---|
| 5 x dye reagent: | Coomassie brilliant blue G-250, phosphoric acid, methanol; relative proportions not stated. |

The BIO-RAD protein assay is based on the protein assay originally developed by Bradford (Bradford, 1976). It is a dye-binding assay based on the differential change of colour of a reagent dye depending on the concentration of the proteins being assayed. The absorbance maximum for an acidic solution of Coomassie brilliant blue G-250 was shifted from 465 nm to 595 nm when protein binding occurred.

*Standard assay procedure* (20-140 µg of protein; 200-1400 µg/ml)

Protein standards in the range of 20-140 µg were prepared in triplicate using a stock solution of BSA (1.42 µg/µl, BIO-RAD). 0.1 ml of standards and appropriately diluted samples (in triplicate) were placed in clean, dry test tubes; 0.1 ml of sample buffer was used as a zero marker. The dye reagent was supplied as 5 x , immediately before use the quantity of dye required for the assay was diluted with 4 volumes of high quality distilled water and filtered using a Costar bottle top filter unit. To each of the test tubes 5.0 ml of diluted dye reagent was added and the tubes were mixed several times by gentle inversion. The O.D.$_{595}$ versus reagent zero was measured and a standard curve of O.D.$_{595}$ versus amount of BSA (μg) was plotted. The amount of protein in each of the samples was determined from the calibration curve.

*Microassay procedure* (1-20 μg of protein; <25 μg/ml)

Protein standards in the range of 1-20 μg were prepared in triplicate using a stock solution of BSA (1.42 μg/μl). 0.8 ml of standards and appropriately diluted samples (in triplicate) were placed in clean, dry microcentrifuge tubes; 0.8 ml of sample buffer was used as a zero marker. To each tube 0.2 ml of dye reagent concentrate was added and the tubes were gently inverted to mix their contents. The O.D.$_{595}$ versus reagent blank were measured and a standard curve of O.D.$_{595}$ versus amount of BSA (μg) was plotted. The amount of protein in each of the samples was determined from the calibration curve.

### 2.2.25. LOWRY PROTEIN ASSAY (LOWRY *ET AL.*, 1951)

Solutions:

| | |
|---|---|
| Reagent A: | 1:1:100 ratio of solution (i) 2% v/v potassium sodium tartrate, solution (ii) 1% v/v copper sulphate, solution (iii) 2% v/v sodium carbonate in 0.1N sodium hydroxide respectively. Solutions (i) and (ii) were mixed first, then solution (iii) was added. |
| Reagent B: | Folin Ciocalteau reagent (Sigma) diluted in a ratio of 1:1.5 v/v with distilled water. |

The Lowry determination of protein content was used for samples estimated to contain between 0-100 μg of protein. Protein standards in the range of 2-100 μg were prepared in triplicate using a stock solution of BSA (1.42 μg/μl); three blank control tubes were also set up. 0.2 ml of protein standards, samples and controls (in triplicate) were placed in clean, dry test tubes to which 1 ml of reagent A was added to each tube. The tubes were left at room temperature for 20 minutes; if the samples contained detergents, 1 ml of 10% w/v SDS was added to the samples and standards (modification by Wang and Smith, 1975). To each tube 0.1 ml of reagent B was added, the tubes were mixed immediately and left at room temperature for 45 minutes. The O.D.$_{700}$ versus the blank control were measured and a standard curve of O.D.$_{700}$ versus amount of BSA (μg) was plotted. The amount of protein in each of the samples was determined from the calibration curve.

## 2.2.26. SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) OF PROTEINS (ADAPTED FROM LAEMMLI, 1970)

Solutions for SDS-PAGE:

| | |
|---|---|
| Protogel: | 300g of acrylamide and 8 g of methylene bisacrylamide in a 1 L solution. |
| pH 8.8 buffer: | 1.5 M Tris-HCl, 0.4% w/v SDS; adjusted to pH 8.8. |
| pH 6.8 buffer: | 0.5 M Tris-HCl, 0.4% w/v SDS; adjusted to pH 6.8. |
| Running buffer: | 0.025 M Tris-HCl, 0.192 M glycine, 0.1% w/v SDS. |
| 2% SDS protein loading buffer: | 2% w/v SDS, 10 mM EDTA, 10 mM sodium phosphate pH 7.0, 1% v/v β-mercaptoethanol, 15% v/v glycerol, 0.01% w/v bromophenol blue, 4 mM PMSF; filter-sterilised (0.2 μm filter). |

|  | *Components* | *Apparent MW* |
|---|---|---|
| Mid-range protein molecular weight (MW) standards (0.5 mg/ml Promega): | Phosphorylase B | 97 400 |
|  | Bovine serum albumin | 66 200 |
|  | Glutamate dehydrogenase | 55,000 |
|  | Ovalbumin | 42 700 |
|  | Aldolase | 40 000 |
|  | Carbonic anhydrase | 31,000 |
|  | Soybean trypsin inhibitor | 21,500 |
|  | Lysozyme | 14 400 |

Assembly of gel apparatus

SDS-PAGE required a 3% stacking gel and a variable % (7, 10 or 13%) separating gel to be set within the same clamped glass vertical plates; 14 cm x 9 cm, 1.5 mm thick. The plates were thoroughly cleaned prior to use along with the combs and spacers. The spacers were inserted between the top and bottom glass plates and the apparatus was clamped in a vertical position onto a BIO-RAD gel system casting stand.

Separating gels:

7%: 23.25 ml of Protogel, 34 .25 ml of autoclaved distilled water, 25 ml of pH 8.8 buffer, 76.6 µl of TEMED and 1.5 ml of fresh 1.5% w/v ammonium persulphate.

10%: 25 ml of Protogel, 27.5 ml of autoclaved distilled water, 18.75 ml of pH 8.8 buffer, 57.5 µl of TEMED and 3.75 ml of fresh 1.5% w/v ammonium persulphate.

13%: 21.6 ml of Protogel, 12.16 ml of autoclaved distilled water, 12.5 ml of pH 8.8 buffer, 38.36 µl of TEMED and 2.5 ml of fresh 1.5% w/v ammonium persulphate.

The separating gel was carefully poured into the gel apparatus ensuring enough room remained for the stacking gel to be set. Two large separating gels (14 cm x 9 cm) were able to be cast using the above mixtures; each gel surface was overlaid with 0.1% w/v SDS. Once the separating gel had set the 3% stacking gel was prepared.

| | |
|---|---|
| 3% Stacking gel: | 3 ml of Protogel, 7.5 ml of pH 6.8 buffer, 18 ml of autoclaved distilled water, 30 µl of TEMED and 1.5 ml of 1.5% w/v fresh ammonium persulphate. |

The 0.1% SDS overlay was poured off and the stacking gel was poured on top of the set separating gel. The comb was inserted into the stacking gel and clamped to prevent any movement. Once the stacking gel had set the apparatus was removed from the casting stand and placed in the electrophoresis tank. The bottom of the gel apparatus was submerged with running buffer and the top reservoir was filled with running buffer.

### Sample preparation and electrophoresis

An equal amount of the protein sample was added to an equal amount of 2% SDS protein loading buffer in a microcentrifuge tube. 5 µl of protein MW markers was aliquoted into a microcentrifuge tube. The lids of the tubes were pierced with a hot needle and the samples were boiled for 3 minutes. In the fume cupboard, 1 µl of β-mercaptoethanol per 10 µl of total sample was added and the tubes were centrifuged briefly. The samples were loaded into the wells of the stacking gel which was electrophoresed at 15 mA per gel in order to allow the proteins to stack. The current was increased to 30 mA per gel for the electrophoresis in the separating gel. The gel was run until the bromophenol blue was 1 cm away from the bottom of the separating gel. The apparatus was dismantled and the separating gel was removed for either staining (section 2.2.27 or 2.2.28.) or western blot analysis (section 2.2.29.).

### 2.2.27. COOMASSIE BRILLIANT BLUE STAINING OF SDS-PAGE GELS

Solutions:

Coomassie brilliant blue stain: 0.2% w/v Coomassie brilliant blue R in an aqueous solution of 45% methanol and 10% acetic acid.

Destain solution: An aqueous solution of 45% methanol and 10% acetic acid.

The SDS-PAGE separating gels to be stained were placed in a plastic container, a sufficient amount of Coomassie brilliant blue stain solution was added to completely cover the surface of the gel. The gels were left in the stain for 2-4 hours, the stain solution was poured off and the destain solution was added. The gels were destained overnight and washed in destain solution several times the next day to ensure all the background colour was removed. The stained gels were viewed and photographed with the aid of a light box.

### 2.2.28. SILVER STAINING OF SDS-PAGE GELS

The Silver Stain Plus kit was purchased from BIO-RAD, the composition of the solutions in the kit were not given.

Solutions provided with the kit:

Fixative enhancer solution: 50% v/v : 10% v/v : 10% v/v : 30% v/v ratio of methanol : acetic acid : fixative enhancer concentrate : deionised distilled water respectively.

Staining solution: 35% v/v deionised distilled water was placed in a beaker with a teflon coated stir bar. The following solutions were added in the order listed to the beaker:
5% v/v Silver complex solution
5% v/v Reduction moderator solution
5% v/v Image development reagent and immediately before use 50% v/v development accelerator solution.

Prior to carrying out the silver staining protocol all glass staining vessels, beakers and teflon coated stir bars were cleaned with 50% nitric acid and were thoroughly rinsed with high quality deionised water before use. This prevented silver deposits forming on the equipment and eliminated inconsistent staining. The SDS-PAGE separating gels (2 x 1.5 mm thick large gels) were fixed in 800 ml of fixative enhancer solution for 30 minutes with gentle agitation. The fixative enhancer solution was decanted from the vessel and the gels were rinsed twice in 800 ml of deionised distilled water for 20 minutes. Approximately 5 minutes before the staining step was due to be carried out 300 ml of staining solution was prepared. The staining solution was poured into the staining vessel which was gently agitated until the desired staining intensity had been achieved. The length of time required for staining was dependent on the nature of the protein sample and quantity of protein loaded onto the gel. The gels were placed in 400 ml of 5% v/v acetic acid for a minimum of 15 minutes to stop the reaction. The stained gels were viewed and photographed with the aid of a light box.

### 2.2.29. WESTERN BLOT ANALYSIS OF PROTEINS

Solutions:

Transfer buffer: 192 mM glycine, 25 mM Tris-HCl, 20% v/v methanol.

| | |
|---|---|
| 1 x Tris buffered saline:<br>(1 x TBS) | 20 mM Tris-HCl, 500 mM NaCl; adjusted to pH 7.5. |
| 1 x Tween-20 Tris buffered saline:<br>(1 x TTBS) | 1 x TBS containing 0.05% v/v Tween-20 (BIO-RAD). |
| Blocking solution: | 3% w/v gelatin (BIO-RAD) in 1 x TBS. |
| Primary ($1^{o}$) antibody solution: | 1% gelatin w/v in 1 x TTBS containing $1^{o}$ antibody at appropriate concentration. |
| Secondary ($2^{o}$) antibody solution: | 1 x TBS containing a Sigma antibody alkaline phosphatase (AP) conjugate (1 in 30,000 dilution) or a BIO-RAD antibody AP conjugate (1 in 3,000 dilution). |

AP colour development kit (BIO-RAD):

| | |
|---|---|
| 25 x AP colour development buffer: | Constituents not given. Diluted with distilled water to give a 1 x solution. |
| AP colour reagent A: | Nitroblue tetrazolium in aqueous N, N-dimethylformamide containing magnesium chloride. |
| AP colour reagent B: | 5-bromo-4-chloro-3-indoyl phosphate in N, N-dimethylformamide. |

Solutions for staining MW markers:

Amido black stain: 0.1% w/v amido black in an aqueous solution of 45% methanol and 10% acetic acid.

Destain solution: An aqueous solution of 90% methanol and 2% acetic acid.

The proteins of interest were transferred electrophoretically overnight in transfer buffer in a BIO-RAD Trans Blot Cell from an SDS-PAGE gel to either a Hybond-C extra nitrocellulose membrane (Amersham, Buckinghamshire, U.K.) or a supported nitrocellulose blotting membrane (B.D.H.). A current of 100 mA was used overnight; this was increased to 200 mA for 1 hour the next morning. The membrane was washed in distilled water and then immersed in blocking solution for 1 hour at room temperature with gentle agitation. The blocking solution was decanted and the membrane was washed in 1 x TTBS for 10 minutes. The wash solution was poured off and the $1^o$ antibody solution was added. The membrane was incubated with gentle agitation for 2 hours at room temperature; the $1^o$ antibody solution was removed and kept for use in subsequent experiments. Any residual unbound $1^o$ antibody was removed by washing the membrane twice in 1 x TTBS for 10 minutes. The wash solution was poured off and the $2^o$ antibody solution was added for 1 hour. The $2^o$ antibody solution was removed and the membrane was washed twice in 1 x TTBS for 10 minutes. A further 10 minute wash in 1 x TBS was carried out to remove residual Tween-20 from the membrane surface. Meanwhile the substrate for the colour reaction was prepared using the AP colour development kit. To 100 ml of 1 x AP colour development buffer at room temperature, 1 ml of AP colour reagent A and 1 ml of AP colour reagent B were added. This was poured over the membrane after the 1 x TBS had been decanted. The membrane was incubated at room temperature with gentle agitation until the colour development desired had been achieved. The colour reaction was halted by washing the membrane in distilled water for 10 minutes with gentle agitation; the water was changed within this period to remove any residual colour development solution. The strip of membrane containing the protein MW markers was cut off, immersed in amido black stain for 45 seconds and destained. The membrane was air-dried and then photographed together with the stained MW markers.

### 2.2.30. CONCENTRATION OF PURIFIED PROTEIN

Fractions containing CYP3A4 from protein purification methods (sections 2.2.21., 2.2.22. and 2.2.23.) were concentrated to approximately 5 ml using an Amicon PM30 ultrafiltration cell. The samples were further concentrated to 0.5-1.0 ml using Amicon Centricon-30 units.

#### Operating procedure for PM30 ultrafiltration cells

The PM30 membrane was removed from the protective envelope and was floated (glossy-side down) in a beaker of distilled water for at least 1 hour to remove the preservatives glycerin and sodium azide. The membrane was placed into the ultrafiltration device with the glossy side toward the solution to be concentrated. The sample was concentrated under pressure from a nitrogen cylinder at 55 pounds per square inch (3.7 atmosphere).

#### Operating procedure for Centricon units

The membrane support base was inserted into the filtrate cup. The centricon membranes contained trace amounts of glycerin; this was removed by prerinsing the membrane with 2 ml of distilled water. The concentrator unit was placed in a Corex® tube which was centrifuged in a Sorvall SS-34 rotor at 1,000-5,000 x g (~5,000 rpm) until at least half of the rinse had passed through into the filtrate cup. The device was inverted and spun at 300-1,000 x g (~1,500 rpm) for 3 minutes to remove the remaining distilled water. The centricon unit was now ready for use. A maximum of 2 ml of solution was added to the sample reservoir which was sealed with parafilm to minimise sample evaporation. The concentrator unit was centrifuged at 5,000 rpm until the desired concentration had been achieved. The filtrate cup was removed from the membrane support base, capped and stored at -20°C. The parafilm was removed from the sample reservoir and the retentate cup was attached. The concentrator unit was inverted and centrifuged at 1,500 rpm for 2 minutes to transfer the concentrated sample into the retentate cup. The sample was stored at -20°C; aliquots were subject to protein determination (sections 2.2.24. or 2.2.25.), staining techniques (sections 2.2.27. or 2.2.28.) and western blot analysis (section 2.2.29.).

### 2.2.31. PREPARATION OF WHOLE CELL EXTRACTS FROM CULTURED HUMAN HEPATOCYTES

Solutions:

| | |
|---|---|
| Cell resuspension buffer: | 10 mM sodium phosphate buffer (pH 7.25), 1 mM EDTA (pH 8.0), 20% v/v glycerol, 4 mM PMSF. |

Ice-cold filter-sterilised cell resuspension buffer was added to the samples as they were thawing (0.5 ml buffer/1 x $10^7$ cells). The cell pellets were disrupted by vortexing and each suspension was transferred to a sterile ice-cold glass homogeniser. The cell suspensions were gently homogenised with four strokes of the homogeniser. The samples were aliquoted and stored at -80°C. A Bradford microassay (section 2.2.24.) was used to determine the protein concentrations of the samples.

### 2.2.32. INJECTION OF MICE WITH CYP2A6 OR CYP3A4

Solutions:

Freunds complete and incomplete adjuvant were purchased from Sigma.

| | |
|---|---|
| Freunds complete adjuvant: | each ml contained 1 mg of *Mycobacterium Tuberculosis* heat killed and dried, 0.85 ml paraffin oil and 0.15 ml mannide monooleate. |
| Freunds incomplete adjuvant: | each ml contained 0.85 ml paraffin oil and 0.15 ml mannide monooleate. |

Five female mice per antigen were injected intra-peritoneally with a 1:1 emulsion of approximately 2 µg antigen : Freunds complete adjuvant. Two different strains of mice were used in case one strain became hyper-immunised to the antigen, they were Balb-c and $Cba_{ca}$. 10 days later the mice were given a similar injection, replacing Freunds complete adjuvant by Freunds incomplete adjuvant. Blood samples were taken from the mices' tails 10 days after the booster injection (day 21 of immunisation schedule) to check for the presence of antibodies against the injected antigen.

Mouse tail bleed

The mouse was isolated in a small cage, warmed with hot water and placed in a special restraint. An infra-red lamp was directed at the mouse's tail to increase the blood flow and a 1.5-2 inch section of tail was swabbed with ethanol. The underside of the tail was nicked with a sterile scalpel and a blood sample was collected in a 0.5 ml microcentrifuge tube.

The blood sample from the tail bleed was incubated at 37°C for 1 hour. The tube was flicked several times to dislodge the blood clot and was incubated for a minimum of 2 hours at 4°C. The tube was centrifuged at 10,000 x g for 10 minutes at 4°C in a microcentrifuge. The serum was removed carefully from the cell pellet and was transferred to a fresh microcentrifuge tube. The sample was centrifuged for 10 minutes at 10,000 x g at 4°C, the supernatant was removed and stored at -20°C. The serum obtained from the tail bleed was analysed by double immunodiffusion.

The mice were given a final booster injection on day 21. The mice were sacrificed 10 days later, their spleens were removed and heart-punctures were carried out. The sera obtained from the heart punctures were analysed by double immunodiffusion. The spleens were stored at -80°C until they were required.

## 2.2.33. DOUBLE IMMUNODIFFUSION ASSAY (OUCHTERLONY, 1953)

Solutions:

| | |
|---|---|
| Phosphate buffered saline: (PBS) | 2.68 mM KCl, 1.76 mM potassium dihydrogen orthophosphate, 0.14 M NaCl, 10 mM disodium hydrogen orthophosphate; adjusted to pH 7.4. |
| Physiological saline: | 0.9% w/v NaCl. |
| Amido black stain solution: | 0.5% w/v amido black 10B in an aqueous solution of 45% methanol and 10% acetic acid. |
| Destain solution: | An aqueous solution of 45% methanol and 10% acetic acid. |

0.2 g of Seakem agarose (FMC Corporation, Rockland, U.S.A.) was added to 20 ml of PBS in a conical flask. The contents of the flask were heated, with continuous stirring, to a light boil in order to dissolve the agarose. 0.1% w/v sodium azide and 2.5% w/v PEG 6,000 were added to the agarose solution. This was poured into the Ouchterlony plastic casting plate (95 cm x 45 cm, Miles Laboratory incorporated, U.S.A.) and was left undisturbed for at least 15 minutes. The plate was covered by its lid whilst the gel was allowed to harden for 1 hour. In order to cut the wells in the gel, the lid was removed and a plastic template was placed over the plate. A hollow, thin steel rod was inserted into a positioning hole in the template, pressed firmly down into the agarose gel and removed. This procedure was repeated for all the holes in the template to be cut. The circle of agarose remaining in the well was removed using a small needle. A maximum of 30 μl antigen, specimen and reference sera were carefully added to the appropriate wells with care. The plate was incubated in a moist chamber at room temperature for 48-72 hours to allow time for the samples to diffuse. After 72 hours the plate was held up to the light to enable any precipitin lines, which may have formed, to be seen. When antibody and antigen are combined in proportions at or near equivalence, the complex precipitates to form a precipitin line. The precipitin lines were often difficult to visualise, hence staining with amido black 10B was carried out to enable the lines to be seen more easily. The upper left hand corner of the gel was notched to indicate position for later orientation. The gel was removed from its plate and washed in physiological saline for 24-48 hours. During this time the saline was changed 2 or 3 times. The gel was washed in distilled water for 6 hours and then placed on a glass plate where the wells were filled with distilled water. The agarose gel was covered with moist 3MM Whatman paper, insuring there were no air bubbles. The Whatman paper was allowed to dry overnight. The next morning the paper was wetted with distilled water and gently removed. The gel was rinsed in a stream of distilled water to remove any debris from the surface of the gel before staining. The gel was immersed in amido black stain solution for 2-3 minutes. The gel was removed and washed in destain solution with occasional agitation for 5 minutes. Two additional distilled water washes were carried out in order to enable the desired staining intensity to be achieved. The gel was air-dried, placed in a sealed plastic bag for storage and finally photographed on a light box.

### 2.2.34. RECOMBINANT PHAGE ANTIBODY SYSTEM (RPAS)

The recombinant phage antibody system was purchased from Pharmacia Biotech. The system is designed in a three-module format:

- Mouse ScFv module
- Expression module
- Detection module

Only the mouse ScFv and expression modules were purchased as an alternative method of screening the antibodies for antigen recognition was used. It was necessary to extensively alter the protocols from those given in the instruction leaflets sent with the modules. The reasoning behind the alterations will be discussed in the Results and Discussion section (sections 5.3. and 5.4.). The protocols listed below are the final optimised versions actually used.

The recombinant phage antibody system was used to construct three different ScFv antibody libraries. One library was optimised for antibodies to CYP2A6; this was constructed from the mRNA isolated from spleens, which were removed from mice that had been injected with CYP2A6. A similar library was made for CYP3A4. A naive library was assembled from mRNA isolated from spleens, taken from mice that had not been injected with any antigen. To differentiate between libraries, mRNA, $V_H$, $V_L$ or ScFv, each sample will be referred to as: CYP3A4 mRNA, CYP2A6 mRNA or control (naive) mRNA etc.

MOUSE ScFv MODULE

Components of the kit:

| | |
|---|---|
| *Primed first-strand reaction mix*: | FPLC*pure*® cloned murine reverse transcriptase, random hexadeoxyribonucleotides [pd$(N)_6$], RNAguard®, RNase/DNase-free BSA, dATP, dCTP, dGTP and dTTP in aqueous buffer. |
| *RPAS control mRNA*: | Mouse spleen mRNA in RNase-free water, used to optimise the experimental conditions. |
| *DTT solution*: | 200 mM DTT solution. |
| *Heavy primer 1*: | 5' variable heavy chain primer in water (upstream primer). |
| *Heavy primer 2*: | 3' variable heavy chain primer in water (downstream primer). |
| *Light primer mix*: | Mixture of 10 variable light chain primers (both 5' and 3') in water. |
| *Linker-primer mix*: | Equimolar mixture of 3' heavy and 5' light linker primers in water. |
| *RS primer mix*: | Mixture of 5' heavy chain primer with *Sfi* I site and 3' light chain primer with *Not* I site in water. |
| $V_H$ *marker*: | pUC18/$V_H$, *Eco*R I/*Xba* I digested. |
| *ScFv marker*: | pUC18/A10B, *Sfi* I/*Not* I digested. |

*MicroSpin™ columns*: Polypropylene minicolumns, each fitted with a 10 μm frit.

### 2.2.34.1. FIRST-STRAND cDNA SYNTHESIS OF $V_H$ AND $V_L$

Between 200 ng to 1 μg of mouse spleen mRNA were used per reaction:

| *Sample* | *Amount/ng* | *Volume/μl* |
|---|---|---|
| Control mRNA | 906.5 | 2.5 |
| CYP2A6 mRNA | 972.4 | 1.2 |
| CYP3A4 | 906.5 | 1 |
| RPAS control mRNA | unknown | 20 |

The appropriate amount of mRNA was aliquoted into a sterile microcentrifuge tube and was heated at 65°C for 10 minutes to remove any secondary structure that could hinder the reaction. The tube was immediately placed on ice. The first-strand cDNA reaction was started within 2 minutes after placing the sample on ice.

The following mixes were prepared in duplicate in 500 μl microcentrifuge tubes, one tube was labelled as $V_L$ and the second as $V_H$: 11 μl of primed first-strand mix, 1 μl of DTT solution, X μl of mRNA (added last) and sterile distilled water to give a final volume of 33 μl. The samples were incubated for 90 minutes at 37°C.

### 2.2.34.2. PRIMARY PCR AMPLIFICATION

The first-strand antibody cDNA was used as the template for the primary PCR amplification. The reagents shown overleaf were added to the appropriate tubes containing the first-strand reactions from the reverse transcription step:

| Light chain PCR | |
|---|---|
| First-strand reaction | 33 μl |
| Light primer mix | 2 μl |
| Sterile distilled water | 64 μl |
| Total volume | 99 μl |

| Heavy chain PCR | |
|---|---|
| First-strand reaction | 33 μl |
| Heavy primer 1 | 2 μl |
| Heavy primer 2 | 2 μl |
| Sterile distilled water | 62 μl |
| Total volume | 99 μl |

The contents of each tube were mixed gently and spun briefly in a minicentrifuge. Each reaction was overlaid with 100 μl of mineral oil. The tubes were placed in a Hybaid Thermal Reactor and heated at 95°C for 5 minutes. 1 μl of AmpliTaq® DNA polymerase (5 units/μl; Perkin-Elmer, Roche Molecular Systems, New Jersey, U.S.A.) was added to each reaction below the mineral oil layer. The reactions were subjected to 30 cycles of: 94°C for 1 minute, 55°C for 2 minutes, 72°C for 2 minutes. 5 μl of each PCR product was mixed with 1 μl of 6 x DNA loading buffer and electrophoresed through a 2% agarose gel (section 2.2.4.). 2 μl of 100 bp molecular weight standard DNA ladder (made up to 5 μl with sterile distilled water) was also electrophoresed through the same gel to check for the presence of the correct size of PCR products, 340 bp for the heavy chain and 325 bp for the light chain.

### 2.2.34.3. PURIFICATION OF PRIMARY PCR PRODUCTS

Solutions:

TE buffer: 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA.

Agarose gel electrophoresis

A 1.5% agarose gel (agarose NA, Pharmacia Biotech) about 1 cm thick was prepared using 0.5 x TAE buffer with wells sufficient to accommodate 50 μl samples. 85 μl of the amplification reactions were removed from below the mineral oil layer and transferred to fresh sterile tubes. The remainder of the reactions were stored at -20°C.

15 µl of 6 x DNA loading buffer were added to each sample and mixed well. Two 50 µl aliquots of each reaction mix were loaded into adjacent wells. The gel was electrophoresed at 100 Volts until the bromophenol blue dye had migrated approximately two-thirds the length of the gel.

MicroSpin column purification

Using a different sterile scalpel blade for each reaction mix, the 340 bp heavy chain and the 325 bp light chain DNA bands were excised under U.V. light. Any excess agarose was carefully removed. Each piece of agarose containing DNA was lightly blotted with a paper towel and sliced into tiny pieces with a scalpel. All the pieces from the heavy chain sample were placed into two separate empty MicroSpin columns. This was repeated for the light chain sample. The bottom parts of the MicroSpin columns were broken off and discarded. Each column was placed in a 1.5 ml microcentrifuge tube and centrifuged at 735 x g for 2 minutes. The microcentrifuge tube containing the eluate was removed and replaced with a fresh tube. The centrifugation step was repeated and the eluates containing the purified DNA were pooled. The eluates were phenol/chloroform extracted and the DNA was precipitated with 2.5 volumes of cold 100% ethanol with 0.1 volume of 3 M sodium acetate. The samples were centrifuged at 16,000 x g in a microcentrifuge for 20 minutes, the DNA pellets were washed with 500 µl of cold 70% ethanol and spun as above. The washed DNA pellets were air-dried and then resuspended in 20 µl of TE buffer.

### 2.2.34.4. GEL QUANTIFICATION OF PURIFIED PCR PRODUCTS

Agarose gel electrophoresis of aliquots of the purified heavy and light chain products alongside a known amount of $V_H$ marker provided a visual estimate of the relative amounts of the fragments based on their band intensities.

Agarose gel electrophoresis

A 1.5% agarose gel, 0.5-0.75 cm thick, was prepared using 0.5 x TAE buffer with wells ~3 mm in width. Ethidium bromide was added to the gel at a final concentration of 0.5 μg/ml. 1 μl and 2 μl of each purified $V_H$ and $V_L$ were aliquoted into separate microcentrifuge tubes. The volume of each sample was adjusted to 5 μl with TE buffer, 1 μl of 6 x DNA loading buffer was added and the sample was mixed thoroughly. $V_H$ marker samples were prepared using 2.5 μl and 5 μl of marker DNA. The samples were loaded onto the agarose gel which was electrophoresed until the bromophenol blue had migrated approximately two-thirds the length of the gel. The gel was photographed under U.V. light

Estimation of amounts of $V_H$ and $V_L$ product

The intensities of the bands in the lanes containing the heavy and light chain products were compared with the 370 bp $V_H$ marker bands. The marker bands contained approximately 12.5 ng of DNA in the 2.5 μl aliquot and approximately 25 ng in the 5 μl aliquot. The amount of DNA in each aliquot of $V_H$ and $V_L$ were estimated and the concentrations of the original purified heavy and light chain products were calculated. The volume of purified heavy chain product that corresponded to 50 ng of DNA was determined, this was repeated for the purified light chain product.

### 2.2.34.5. ScFv ASSEMBLY REACTIONS

In the assembly reaction, the antibody heavy and light chain DNA were joined into a single chain with a linker DNA to create an ScFv fragment.

Solutions

| | |
|---|---|
| 10 x PCR buffer: | 100 mM Tris-HCl (pH 8.3), 15 mM magnesium chloride, 500 mM potassium chloride, 0.01% w/v gelatin. |

The following components were added to a 500 µl microcentrifuge tube: X µl of heavy chain product (50 ng), X µl of light chain product (50 ng), 4 µl of linker-primer mix, 5 µl of 10 x PCR buffer, 5 µl of dNTP mix (10 mM of each dNTP), 7.5 µl of 50 mM magnesium chloride, 1 µl of AmpliTaq® DNA polymerase (5 units/µl) and sterile distilled water to give a final volume of 50 µl. The sample was mixed, centrifuged briefly in a minicentrifuge and overlaid with 50 µl of mineral oil. The tube was placed in the thermocycler and subjected to 7 cycles of: 94°C for 1 minute, 63°C for 4 minutes.

### 2.2.34.6. AMPLIFICATION OF ScFv DNA USING PRIMERS CONTAINING *Sfi* I OR *Not* I RESTRICTION SITES

The assembled ScFv DNA was amplified using restriction site (RS) primers. The primer which annealed to the 5' end of the heavy chain contained a *Sfi* I restriction site whereas the primer which annealed to the 3' end of the light chain contained a *Not* I restriction site A bulk primer mix was prepared by adding the following components to a 500 µl microcentrifuge tube for each PCR: 1 µl of AmpliTaq® DNA polymerase (5 units/µl), 5 µl of 10 x PCR buffer, 2 µl of dNTP mix (10 mM of each dNTP), 4 µl of RS primer mix and sterile distilled water to give a final volume of 50 µl.

The contents of the tube were mixed briefly and the bulk primer mix was added to the assembly reaction product beneath the mineral oil layer. The reaction mix was overlaid with an additional 50 µl of mineral oil. The tube was placed in the thermocycler and subjected to 30 cycles of: 94°C for 1 minute, 55°C for 2 minutes, 72°C for 2 minutes. 5 µl of the PCR product and a sample of 100 bp ladder were loaded onto a 1.5% agarose gel (section 2.2.4.) to check for the presence of the 750 bp assembled ScFv DNA.

### 2.2.34.7. PROTEINASE K DIGESTION OF ASSEMBLED ScFv DNA FOR IMPROVED CLONING EFFICIENCY

100 μl of the assembled ScFv DNA was removed from below the mineral oil layer and treated with proteinase K as in section 2.2.15. The resulting DNA pellet was resuspended in 10 μl of sterile distilled water.

### 2.2.34.8. GEL QUANTIFICATION OF ASSEMBLED ScFv DNA

Agarose gel electrophoresis of aliquots of the proteinase K treated ScFv DNA alongside a known amount of ScFv marker provided a visual estimate of the relative amounts of the fragments based on their band intensities. The electrophoresis protocol was identical to that already described in section 2.2.34.4. except that the samples loaded were: 1 μl and 1.5 μl of each ScFv; 2.5 μl and 5 μl of ScFv marker DNA.

Estimation of amount of ScFv product

The intensities of the bands in the lanes containing ScFv products were compared with the 750 bp ScFv marker bands. The marker bands contained approximately 12.5 ng of DNA in the 2.5 μl aliquot and approximately 25 ng in the 5 μl aliquot. The amount of DNA in each aliquot of assembled ScFv was estimated and the concentration of the original assembled ScFv was calculated. The volume of assembled ScFv product that corresponded to 120-300 ng of DNA was determined.

### 2.2.34.9. *Sfi* I AND *Not* I RESTRICTION DIGESTS OF ScFv DNA

Solutions:

| | |
|---|---|
| 10 x buffer 1: | 100 mM Tris-HCl (pH 8.0), 500 mM NaCl, 100 mM magnesium chloride, 10 mM DTT. |

10 x buffer 2: 100 mM Tris-HCl (pH 8.0), 100 mM magnesium chloride, 10 mM DTT, 0.34% v/v Triton X-100.

The restriction enzyme buffers were not available commercially. They were prepared, filter-sterilised (0.2 µm filter) and stored at -20°C.

*Sfi* I digestion

The *Sfi* I restriction digest mix was prepared by adding the following components to a 500 µl microcentrifuge tube: up to 70 µl of assembled ScFv product (120-300 ng), 8.5 µl of 10 x buffer 1, 2 µl of *Sfi* I (10 units/µl) and sterile distilled water to give a final volume of 85 µl. The contents of the tube were mixed, spun briefly in a minicentrifuge and overlaid with 85 µl of mineral oil. The reaction mix was incubated at 50°C for 4 hours. After the 4 hour incubation, the digested sample was equilibrated to room temperature and spun briefly.

*Not* I digestion

A *Not* I digestion mix was prepared by adding the following components to a 500 µl microcentrifuge tube: 3.6 µl of 3 M NaCl, 1.5 µl of 10 x buffer 2, 3 µl of *Not* I (9 units/µl) and 6.9 µl of sterile distilled water. The *Not* I digest mix was pipetted below the mineral oil layer of the *Sfi* I reaction mix, the sample was mixed and spun briefly. The sample was incubated at 37°C for 4 hours. The digestion efficiency of commercially obtained *Not* I is unreliable. Control digests were carried out using the plasmid pGL3-control vector, which contained a *Not* I restriction site, to check that the enzyme was active. To ensure the ScFv product was digested to completion, 1 µl of *Not* I was added to the sample which was incubated for a further 4 hours at 37°C.

The 100 µl digest mix was removed from below the mineral oil layer and transferred to a sterile 1.5 ml microcentrifuge tube. The sample was phenol/chloroform extracted (with vigorous vortexing for 1 minute) and the DNA was precipitated with 2.5 volumes of cold 100% ethanol and 0.1 volume of 3 M sodium acetate. The tube was centrifuged at 16,000 x g for 20 minutes in a microcentrifuge, the DNA pellet was washed in 1 ml of cold 70% ethanol and spun as above. The pellet was air-dried and then resuspended in 4 μl of sterile distilled water.

### EXPRESSION MODULE

Components of the kit:

| | |
|---|---|
| *pCANTAB 5 E*: | Aqueous solution (50 ng/μl) in TE buffer (pH 7.6). |
| *M13KO7 helper phage*:<br>(not used in adapted protocol) | Titered phage in YT medium. |
| *E. coli TG1 cells*: | lyophilised; K12 Δ(*lac-pro*), *supE*, *thi*, *hsd*Δ5/F'[*traD*36, *proAB*, *lacI*$^{q}$, *lacZ*ΔM15]. |
| *E. coli HB2151 cells*: | lyophilised; K12 Δ(*lac-pro*), *ara*, *nal*$^{r}$, *thi*/F'[*proAB*, *lacI*$^{q}$, *lacZ*ΔM15]. |
| *10 x OPA*$^{+}$ *buffer*: | 100 mM Tris-acetate (pH 7.5), 100 mM magnesium acetate, 500 mM potassium acetate. |
| *Control insert*: | A10B, *Sfi* I/*Not* I digested. |
| *ScFv marker*: | pUC18/A10B, *Sfi* I/*Not* I digested. |

All of the above components of the kit were stored at 4$^{o}$C.

### 2.2.34.10. LIGATION OF ScFv GENE INTO pCANTAB 5 E

The ligation of ScFv DNA into pCANTAB 5 E was carried out along with two control reactions; a vector background reaction (vector DNA only, no insert DNA) and ligation of a control insert into the phagemid. The three ligation reactions were set up in 1.5 ml microcentrifuge tubes as described below.

Ligation of ScFv DNA into pCANTAB 5 E

4 µl of antibody ScFv gene fragment (60-150 ng), 1.5 µl of 10 x OPA$^+$ buffer, 4 µl of pCANTAB 5 E (50 ng/µl), 3 µl of 5 mM ATP, 1 µl of T4 DNA ligase (6.7 units/µl) and 1.5 µl of sterile distilled water.

Vector background reaction

1.5 µl of 10 x OPA$^+$ buffer, 1 µl of pCANTAB 5E (50 ng/µl), 3 µl of 5 mM ATP, 1 µl of T4 DNA ligase (6.7 units/µl) and 8.5 µl of sterile distilled water.

Ligation of control insert into pCANTAB 5 E

3 µl of control insert (10 ng/µl), 1.5 µl of 10 x OPA$^+$, 1 µl of pCANTAB 5 E (50 ng/µl), 3 µl of 5 mM ATP, 1 µl of T4 DNA ligase (6.7 units/µl) and 5.5 µl of sterile distilled water.

The reactions were incubated overnight at 16°C. The reaction mixes were prepared for transformation of *E. coli* cells by electroporation.

Preparation of DNA for electroporation

The samples were phenol/chloroform and chloroform extracted. The DNA constructs were precipitated with 2.5 volumes of cold 100% ethanol and 0.1 volume of 3 M sodium acetate. The samples were placed on dry ice for 30 minutes, centrifuged at

16,000 x g for 30 minutes in a microcentrifuge and the supernatant was transferred to a fresh tube. The supernatant was centrifuged as above to prevent any construct DNA being accidentally discarded. The DNA pellets were thoroughly washed in 1 ml of cold 70% ethanol to ensure as much salt was removed as possible. Each pair of DNA pellets were air-dried, resuspended in 5 μl of sterile distilled water and combined to give a total volume of 10 μl.

### 2.2.34.11. PREPARATION OF *E. COLI* STOCKS

Growth

*E. coli* TG1 and HB2151 were supplied as lyophilised cultures. Each lyophilised culture was resuspended in 1 ml of 2 x YT medium and incubated overnight at 37°C with shaking at 250 rpm. A minimal medium plate was streaked with a loopful of each of the overnight cultures. The plates were incubated overnight at 37°C.

Glycerol stocks

A single colony was taken from a minimal medium plate and used to inoculate 5 ml of 2 x YT medium. The culture was incubated overnight at 37°C with shaking at 250 rpm. 200 μl of sterile 80% glycerol was added to 800 μl of stationary phase cells to prepare a glycerol stock. The glycerol stock was mixed well and stored at -80°C.

### 2.2.34.12. PREPARATION OF ELECTRO-COMPETENT *E. COLI*

10 ml of 2 x YT medium was inoculated with a single colony of *E. coli* TG1/HB2151 from a minimal medium plate. The culture was incubated overnight at 37°C with shaking at 250 rpm. 1 litre of 2 x YT medium was inoculated with the 10 ml overnight culture, this was incubated at 37°C with shaking until an $O.D._{600}$ of 0.5-0.7 had been reached (approximately 1.5-2 hours). The culture was placed on ice for 15-30 minutes. The bacterial cells were sedimented by centrifugation at 4,000 x g (6,500 rpm in a

Sorvall SL GSA rotor) for 20 minutes at 4°C. The supernatant was discarded and the cells were resuspended in 1 litre of ice-cold sterile 1 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES; pH 7.0). The cells were spun as above and the supernatant was discarded. The cells were resuspended in 500 ml of ice-cold sterile 1 mM HEPES (pH 7.0), spun as above and the supernatant was discarded. The cells were washed in 20 ml of sterile 1 mM HEPES (pH 7.0) containing 10% glycerol, spun as above and the supernatant was discarded. The cells were resuspended in a total volume of 2 ml of sterile 10% glycerol. The electro-competent cells were dispensed into 100 µl aliquots, frozen on dry ice and stored at -80°C.

### 2.2.34.13. TRANSFORMATION OF *E. COLI* BY ELECTROPORATION WITH ScFv-pCANTAB 5 E CONSTRUCTS

The electro-competent cells were thawed on ice, 50 µl of thawed cells were transferred to a pre-chilled 0.2 cm electroporation cuvette (Flowgen, Sittingbourne, Kent, U.K.). 1 µl of salt-free DNA containing ScFv-pCANTAB 5 E was added and shaken to the bottom of the cuvette. 1 µl of pBluescript (5 ng/µl) was added to a cuvette to provide a positive control, a negative control was set up with 50 µl of cells only (no DNA added). 2 µl of each of control insert-pCANTAB 5 E and vector background control were added to separate cuvettes. The cuvettes were placed on ice for at least 1 minute before electroporation. The *Easyject* electroporator (Flowgen) was programmed to give 25 µF, 2.5 kV at 200 ohms. A cuvette was dried with a tissue, placed in the electroporation chamber and subjected to one pulse with a time constant of 4.5-5 mseconds. 1 ml of ice-cold 2 x YT-G medium was immediately added, the cuvette was capped and inverted to resuspend the cells. The electroporation procedure was repeated for the other samples. Each cuvette was placed inside a 30 ml universal bottle which was filled with enough water to cover the culture. The samples were incubated for 1 hour at 37°C with shaking at 250 rpm. 100 µl of the following cultures were plated out on SOBAG plates: negative control, positive control, control insert-pCANTAB 5 E and vector background control. 3 x 50 μl and 2 x 375 μl aliquots of ScFv-pCANTAB 5 E culture were plated out on SOBAG-N plates. Once the surface of the plates were dry, they were inverted and incubated at 37°C overnight. The remaining 100 μl of ScFv-pCANTAB 5 E culture was added to 27.78 μl of sterile 80% glycerol to prepare a glycerol stock. Once it had been shown that the ligation reactions and transformation protocols had been successful the experiment was repeated. 2 μl of ScFv-pCANTAB 5 E was placed in each of four electroporation cuvettes and 1 μl of ScFv-pCANTAB 5 E in another cuvette to produce the complete cDNA library from 10 μl of ligation mix.

### 2.2.34.14. ESTIMATION OF SIZE OF ScFv cDNA LIBRARY

The size of the ScFv cDNA library was roughly estimated by the following procedure: The number of single colonies was counted on each of the three SOBAG-N plates that had been spread with 50 μl of ScFv-pCANTAB 5 E culture. The average number of single colonies in 50 μl of culture was multiplied by 21 (total volume of culture was 1,050 μl). This was repeated four times for the other ScFv-pCANTAB 5 E cultures. The five figures were added to calculate the total size of the ScFv cDNA library created from 10 μl of ligation mix.

### 2.2.34.15. STORAGE OF ScFv cDNA LIBRARY AND TITRATION OF STORED LIBRARIES

Storage of ScFv cDNA library

The following procedure was carried out for the set of five SOBAG-N plates (3 x 50 μl and 2 x 375 μl) plated from the ScFv-pCANTAB 5 E culture from the electroporation protocol:

1 ml of 2 x YT medium was added to one of the plates that had been spread with 50 μl of ScFv-pCANTAB 5 E culture. The bacterial cells were scraped into the medium

using a sterile glass spreader. The medium was transferred to another plate that had been spread with 50 μl of ScFv-pCANTAB 5 E culture and the bacterial cells were again scraped into the medium. The process was repeated and the medium containing the bacterial cells were used to scrape the two plates that had been spread with 375 μl of ScFv-pCANTAB 5 E culture. The medium was collected and transferred to a sterile 2 ml microcentrifuge tube on ice. Another 1 ml of 2 x YT medium was added to the first plate that had been scraped, the protocol was repeated to ensure most of the bacterial colonies had been removed from the surface of the plates. The medium was transferred to the 2 ml microcentrifuge tube and an equal volume of 2 x YT medium containing 30% glycerol was added. The tube was inverted a few times and 30 μl of the suspension was removed for titration of the library. The glycerol stock of the library was frozen on dry ice and was stored at -80°C. The protocol was repeated five times for the other sets of ScFv-pCANTAB 5 E plates. The six libraries were labelled a-f.

Titration of stored ScFv cDNA libraries

The 30 μl of library glycerol stock was used to prepare serial dilutions in 2 x YT medium, the final dilution was $10^{-8}$. 100 μl of the $10^{-6}$, $10^{-7}$ and $10^{-8}$ dilutions were plated out in duplicate on SOBAG plates. The plates were incubated overnight at 30°C and the number of single colonies on each plate were counted the next morning. The average number of colonies for each dilution were calculated. The titre of the stored library was calculated as follows:

titre of library = average no. of colonies for $10^{-8}$ dilution x $10^{8}$ x 10
(no. of cells per ml)

### 2.2.34.16. SCREENING OF A ScFv LIBRARY BY DETECTION OF ANTIGEN BINDING WITH A TWO-MEMBRANE SYSTEM (ADAPTED FROM SKERRA *ET AL.*, 1991 AND DREHER *ET AL.*, 1991)

Solutions:

| | |
|---|---|
| Blocking buffer: | 1 x PBS containing 3% non-fat dry milk. |
| Wash buffer: | 1 x PBS containing 0.05% Tween-20. |
| Horseradish peroxidase (HRP)/Anti-E tag conjugate: | 1 in 1,000 dilution of HRP/Anti-E tag conjugate (Pharmacia) in blocking buffer. |
| Triethanolamine buffered saline: | 128 mM NaCl, 0.28% v/v triethanolamine; adjusted to pH 7.5. |
| 4-chloro-1-napthol (4-CN) stock solution: | One 4-CN tablet (Sigma) was dissolved in 10 ml of methanol. |
| 4-CN substrate: | 2 ml of stock 4-CN solution and 5 μl of 30% hydrogen peroxide solution were added to 10 ml of triethanolamine buffered saline. |

One of the six glycerol stocks containing *E. coli* HB2151 transformed with CYP3A4 ScFv-pCANTAB 5 E recombinant phagemid DNA (section 2.2.34.13.) was thawed on ice. A master plate was prepared by inoculating a 2 x YT-AG agar plate (14 cm diameter) with the transformed *E. coli*. The plate was incubated overnight at 30°C or until the colonies were 1-2 mm in diameter. It was important that the colonies were well isolated for this procedure.

### Preparation of antigen-coated filter

A Protran BA 85 nitrocellulose membrane (13.2 cm diameter, 0.45 μm pore size; Schleicher and Scheull, Dassel, Germany) was placed on cling-film on an orbital shaker. The membrane was completely soaked with 3 ml of PBS containing 50 μg/ml of antigen. The membrane was shaken gently for 1 hour at room temperature. Any excess liquid was drained by holding one edge of the filter with forceps and gently touching the opposite edge onto a paper towel. The filter (antigen-side up) was placed on a paper towel and air-dried for 5-10 minutes at room temperature. The filter was completely immersed in blocking buffer and incubated for 1 hour with gently shaking. Any excess fluid was drained as before. The filter was washed briefly with 100-200 ml of 1 x PBS to remove any excess blocking buffer; excess fluid was drained as before. The filter was air-dried as before. The blocked antigen-coated filter (antigen-side up) was transferred onto the surface of a 2 x YT-AI agar plate (14 cm diameter).

### Colony lift

A Millipore GVWP Durapore nitrocellulose membrane (cut to 13.2 cm diameter, 0.22 μm pore size) was carefully placed onto the master plate containing the ScFv-expressing colonies of *E. coli*. The master filter was carefully removed with forceps after it began to appear moist; ~1-5 minutes incubation period.

The master filter was placed colony-side up onto the surface of the antigen-coated filter on the 2 x YT-AI agar plate. The plate was inverted and incubated overnight at 30$^{o}$C. The next day, the plate was inverted and both filters were marked simultaneously with a 16-gauge needle. The markings were used at a later step to align the two filters for the purpose of identifying colonies expressing antigen-positive ScFvs. The master filter was removed and transferred (colony-side up) to a fresh 2 x YT-AG agar plate. This was stored at 4$^{o}$C until needed.

### Detection of antigen binding ScFvs

The antigen-coated filter was removed from the 2 x YT-AI agar plate and was immersed in 50-100 ml of blocking buffer. This was incubated for 1 hour at room temperature with gentle shaking. The filter was incubated in 100-200 ml of wash buffer for 30 minutes with gentle agitation. This step was repeated with fresh wash buffer. The filter was removed from the wash buffer with forceps and examined for the presence of any yellow shiny *E. coli* debris. If debris was seen, the filter was washed until no debris was visible. The filter was incubated in 10 ml of diluted HRP/Anti-E tag conjugate (1 in 1,000 dilution) for 1 hour with gentle shaking. The filter was transferred to a fresh container to which 100-200 ml of wash buffer had been added. The membrane was rinsed briefly, the wash buffer was poured off and the wash step was repeated for a total of six washes. The filter was briefly washed in distilled water and any excess water was drained as before. The membrane was placed antigen-side up onto a paper towel and air-dried briefly for 1-5 minutes. The membrane was placed antigen-side up into the freshly prepared 4-CN substrate and was incubated until a suitable purple/blue colour had developed (5-20 minutes). The colour reaction was halted by immersing the filter in distilled water for 10 minutes. The filter was air-dried and aligned with the master filter in order to identify the colonies expressing ScFvs reacting with the antigen. Positive colonies were removed using a sterile loop and streaked on fresh 2 x YT-AG agar plates. The plates were incubated overnight at 30°C. A single colony from each streaked plate was transferred to sterile tubes containing 5 ml of fresh 2 x YT-AG medium. The tubes were incubated overnight at 30°C with shaking at 250 rpm. Glycerol stocks were prepared using the broth cultures. The remainder of the cultures were used to produce soluble antibodies (section 2.2.34.17.).

### 2.2.34.17. PRODUCTION OF SOLUBLE ANTIBODIES

Solutions:

| | |
|---|---|
| 1 x Tris-EDTA sucrose buffer (1 x TES): | 0.2 M Tris-HCl (pH 8.0), 0.5 mM EDTA, 0.5 M sucrose; filter-sterilised and stored at 4°C. |
| 1/5 x TES buffer: | 1 volume of 1 x TES buffer added to 4 volumes of distilled water. |

The remainder of the 2 x YT-AG overnight cultures from section 2.2.34.16. were each added to 50 ml of freshly prepared 2 x YT-AG medium. The cultures were incubated at 30°C for 1 hour with shaking at 250 rpm. Each 50 ml culture was treated in an identical manner as described. The culture was centrifuged at 1,500 x g for 20 minutes at room temperature. The supernatant was carefully removed from the sedimented cells and the supernatant was discarded. The sedimented cells were resuspended in 50 ml of freshly prepared 2 x YT-AI medium and incubated in 250 ml conical flasks overnight at 30°C with shaking at 250 rpm. The culture was divided into two separate centrifuge tubes and centrifuged at 1,500 x g for 20 minutes at room temperature. The supernatants were carefully removed from both pellets and were combined. The combined sample was filtered through a 0.45 μm filter with a pre-filter and stored at -20°C. One cell pellet was used to prepare a periplasmic extract while the other pellet was used to prepare a whole cell extract.

Preparation of a periplasmic extract (adapted from Skerra and Plückthun, 1988)
One cell pellet was resuspended in 0.5 ml of ice-cold 1 x TES. 0.75 ml of ice-cold 1/5 x TES was added and the sample was vortexed. The cell suspension was incubated on ice for 30 minutes. The sample was transferred to a 1.5 ml microcentrifuge tube and centrifuged at full speed (~16,000 x g) for 10 minutes. The supernatant, which

contained soluble antibodies from the periplasm, was transferred to a fresh tube and stored at -20°C. The pellet was discarded.

Preparation of a whole cell extract

The second cell pellet was resuspended in 0.5 ml of 1 x PBS and boiled for 5 minutes. The cell debris was pelleted as above and the supernatant, which contained the intracellular soluble antibodies, was carefully transferred to a fresh tube. The antibodies were stored at -20°C and the pellet was discarded.

### 2.2.34.18. WESTERN BLOT ANALYSIS OF SOLUBLE ScFvs

30 μl of each of the supernatant, periplasmic and whole cell extracts containing E-tagged ScFv antibodies were electrophoresed through a 10% SDS-PAGE gel (section 2.2.26.). The proteins were transferred to a nitrocellulose membrane as in section 2.2.29.; blocking buffer and wash buffer were prepared as in section 2.2.34.16. The membrane was incubated for 1 hour in a pre-measured volume of blocking buffer (10 ml of blocking buffer per 50 $cm^2$ of nitrocellulose) with gentle shaking. The membrane was washed with 100 ml of wash buffer for 10 minutes with gentle agitation. The wash buffer was removed and replaced with a 1 in 1,000 dilution of HRP/Anti-E tag conjugate in blocking buffer (10 ml of solution per 50 $cm^2$ of nitrocellulose). The blot was incubated for 1 hour with gentle shaking. The diluted conjugate solution was removed and the membrane was washed six times (0.5-2 minutes/wash) with an excess of wash buffer. A final wash was carried out using distilled water and any excess water was drained from the blot after the wash. The blot was placed in 4-CN substrate (section 2.2.34.16.) until a suitable colour development had been achieved. The blot was rinsed with distilled water to stop the reaction and was air-dried prior to being photographed.

If the ScFv antibodies were unable to be detected in any of the three extracts, the samples were concentrated using 20% v/v trichloroacetic acid (diluted with distilled water). 250 µl of 20% v/v trichloroacetic acid were added to a microcentrifuge tube containing 250 µl of one of the extracts containing the ScFv antibodies; the contents of the tube were mixed well. The tube was placed on ice for at least 15 minutes. The sample was centrifuged at 12,000-14,000 x g for 10 minutes in a microcentrifuge. The supernatant was removed and the pellet was resuspended in 10-25 µl of filter-sterilised distilled water. The concentrated extracts were analysed for the presence of E-tagged ScFv antibodies by western blotting as described above. If, after the addition of an equal amount of 2% SDS protein loading buffer to the sample, the sample became acidic (yellow in colour) a small crystal of Tris-base was added to the tube to return the sample to an alkaline pH.

### 2.2.34.19. WESTERN BLOT ANALYSIS OF ANTIGEN USING AN E-TAGGED ScFv ANTIBODY

10 µg of antigen was electrophoresed through a 13% SDS-PAGE gel (section 2.2.26.). The proteins were transferred to a nitrocellulose membrane as in section 2.2.29. and the blotting analysis was carried out as in section 2.2.34.18. except for the following addition to the protocol. After the 1 hour blocking step, the blot was incubated with a diluted ScFv solution for 2 hours with gentle agitation. The E-tagged ScFv sample to be tested against the antigen was diluted with an equal volume of fresh blocking buffer. 10 ml of blocking buffer was used per 50 $cm^2$ of nitrocellulose. The diluted ScFv solution was poured off after the 2 hour incubation and the blot was washed six times (30 seconds/wash) with an excess of wash buffer. The HRP/Anti-E tag conjugate was prepared as in section 2.2.34.18. and all subsequent steps were identical to section 2.2.34.18.

# RESULTS AND DISCUSSION

# SCOPE OF THIS THESIS

The results and discussion section of this thesis is split into three chapters, they are:

Chapter 3 - Isolation of a novel marmoset CYP2A cDNA clone and its sequence comparison to a human CYP2A6 cDNA clone

Chapter 4 - Characterization of a human hepatocyte cell culture system with respect to the expression and induction of the CYP2As, CYP2B6, CYP2Cs, CYP3As and P450-reductase

Chapter 5 - Production of monoclonal antibodies to CYP2A6 and CYP3A4 using an adaptation of the recombinant phage antibody system (RPAS)

It must be stressed that the experimental work presented in these three chapters took place concurrently.

In chapter 3 the potential use of the marmoset as an alternative animal model to rodents was addressed. Chapter 4 describes the use of an *in vitro* human hepatocyte system as an alternative to other animal culture systems with respect to the effects of two anti-epileptic agents on certain drug metabolising enzymes. The human hepatocyte cell culture system was set up in Prof. Vera Rogiers' laboratory at the Vrije Universiteit in Brussels. The major aim of this thesis was to produce monoclonal antibodies to CYP2A6 and CYP3A4 as described in chapter 5. However, significant problems were encountered with the RPAS protocols. This led to lengthy delays in the advancement of the experimental work while the optimum conditions for the experiments were investigated and new protocols were devised. Monoclonal antibodies were expressed in chapter 5 but were unable to be characterised due to a lack of time. This meant that not all the experimental work initially planned for this thesis was able to be carried out. However, a satisfactory end-point was reached and the work described in chapter 5 will be continued by a new member of our laboratory. The monoclonal antibodies will be used to investigate the expression of CYP2A6 and CYP3A4 in the human hepatocyte cell culture system already characterised with polyclonal antibodies to CYP2As and CYP3As in chapter 4.

# CHAPTER THREE

# ISOLATION OF A NOVEL MARMOSET CYP2A cDNA CLONE AND ITS SEQUENCE COMPARISON TO A HUMAN CYP2A6 cDNA CLONE

Rodents are often the preferred laboratory animal model as they are abundantly available and easy to keep. As has been mentioned in section 1.9 there are many examples of species differences in drug metabolism. The properties of CYPs belonging to the same subfamily can differ among animal species (Shimada *et al.*, 1989a; Shimada *et al.*, 1989b; Oguri *et al.*, 1991). This leads to difficulties in extrapolating data from experiments using rodents to human clinical trials and toxicity studies. It is reasonable to conclude that the more closely evolutionarily linked the animal is to man, the more similar the drug metabolising pathways will be. Non-human primates, such as the marmoset, are genetically closer to humans than rodents. Marmosets should theoretically provide a better animal model for comparative drug metabolism studies. The similarity of DNA sequences between species can give an idea of how closely evolutionarily linked two species are.

At the start of this thesis I was provided with two cDNA clones; one encoding a human CYP2A, supposedly CYP2A6 and the other encoding an homologous novel marmoset CYP2A. The initial aim was to compare the sequence of the novel marmoset CYP2A cDNA to the human CYP2A6 cDNA.

## 3.1. CHARACTERIZATION OF A cDNA CLONE ENCODING HUMAN CYP2A6

The human CYP2A sequence had been amplified from total RNA isolated from a human liver sample using a reverse transcription PCR approach (Nanji, PhD. Thesis, 1995). The oligonucleotides used to prime the PCR reactions were based on sequences located at the ends of the coding region of the human CYP2A6 cDNA (Yamano *et al.*, 1990). The forward and reverse primers (figure 3.1) used to amplify the cDNA in the PCR reaction contained *Xba* I sites to facilitate cloning of the PCR product. The human CYP2A cDNA was cloned into pUC19 via the unique *Xba* I site in the polylinker. In addition, the forward primer contained a *Nco* I site 3' to the *Xba* I site to enable the PCR product to be inserted into the baculovirus transfer vector pAcC5. This enabled

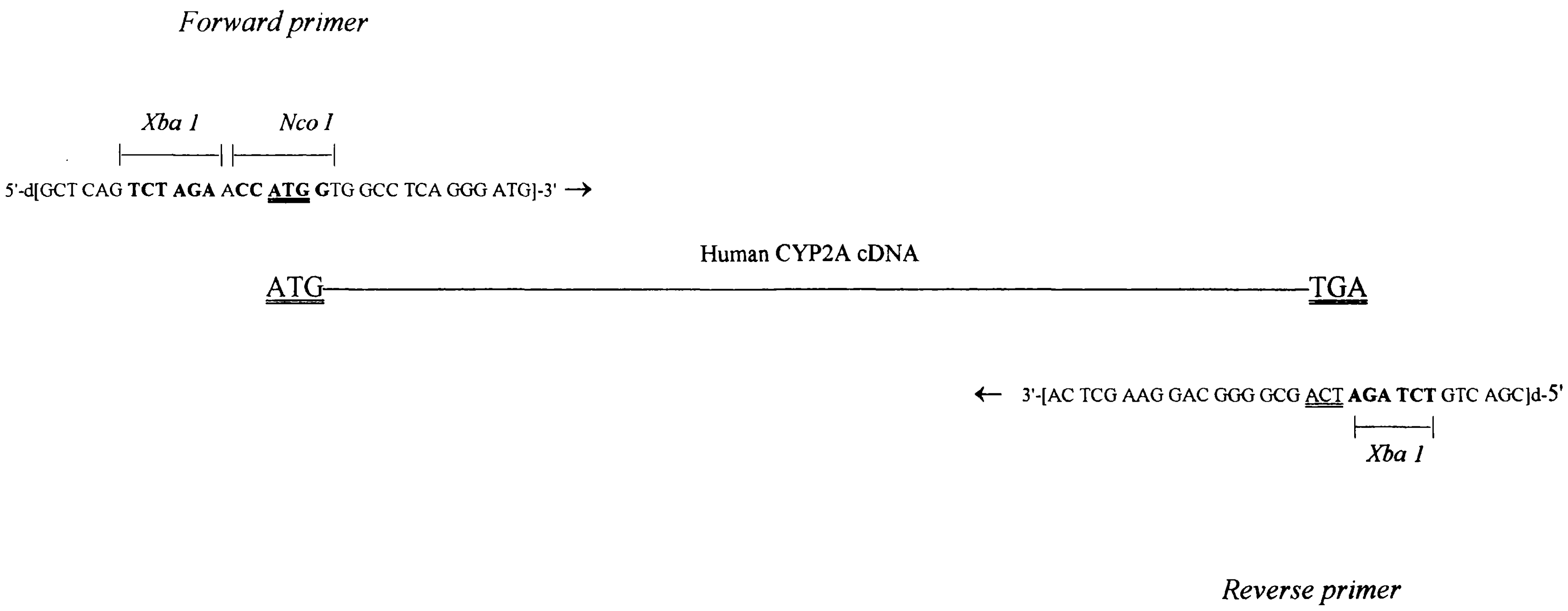

Forward primer
Xba 1
Nco 1
5'-d[GCT CAG TCT AGA ACC ATG GTG GCC TCA GGG ATG]-3'
Human CYP2A cDNA
ATG
TGA
3'-[AC TCG AAG GAC GGG GCG ACT AGA TCT GTC AGC]d-5'
Xba 1
Reverse primer

Figure 3.1: Schematic diagram showing the forward and reverse oligonucleotide primers with incorporated restriction endonuclease sites used to amplify the human CYP2A cDNA sequence from total RNA prepared from human liver.

eukaryotic protein expression studies, and eventually purification of the expressed protein to be carried out (Nanji, PhD. Thesis, 1995).

### 3.1.1. RESTRICTION ENDONUCLEASE MAPPING OF A PUTATIVE HUMAN CYP2A6 cDNA CLONE

pUC19-human CYP2A recombinant plasmid DNA was isolated from a cDNA clone of human CYP2A and used for restriction endonuclease mapping. The PCR product was not able to be excised from pUC19 by digestion with the restriction enzyme *Xba* I, the site into which it had been cloned. This was because the reverse primer contained a methylation sequence around the 3' *Xba* I site (figure 3.2). The recombinant plasmid had been used to transform the *E. coli* strain XL1-Blue which possesses the enzyme Dam methylase. This enzyme methylates the A residue in all GATC sequences, using *S*-adenosylmethionine as the methyl donor. The methylation of the 3' *Xba* I site prevented the restriction enzyme recognising the site.

5' CGA GTC *TCT AGA* TCA GCG GGG CAG GAA GCT CA 3'
←*Xba* I→

Figure 3.2: Reverse primer sequence illustrating the methylated A residue (bold type) within the *Xba* I restriction site.

The construct DNA was also used to transform DCM$^-$ which is a non-methylating strain of *E. coli*. The insert was released from pUC19 by digestion with *Xba* I and was estimated to be approximately 1,500 bp in length (Nanji, PhD. Thesis, 1995). The published cDNA sequence of CYP2A6 (Yamano *et al.*, 1990) shows the coding region is 1,485 bp in length (including the stop codon).

pUC19-human CYP2A recombinant plasmid DNA was digested with several restriction endonucleases. A sample of each digest was electrophoresed through a 1% agarose gel (figure 3.3) and a restriction endonuclease map was constructed from the data (figure 3.3). Computer analysis of the published human CYP2A6 cDNA sequence identified the following unique restriction enzyme sites: *Bam*H I at 777 bp; *Pst* I at 143 bp; *Sac* I at 1,346 bp; *Sph* I at 825 bp. The human CYP2A cDNA cloned in the laboratory was found to have the following unique restriction endonuclease sites: *Bam*H I at 705 bp; *Pst* I at 251 bp; *Sac* I at 1,266 bp; *Sph* I at 794 bp. The positions of the unique restriction enzyme sites within the cloned human CYP2A cDNA correlates with those of the published sequence +/- 100 bp. This result gave a good indication that the cloned cDNA may encode CYP2A6; the identity of the cDNA clone was confirmed by DNA sequence determination.

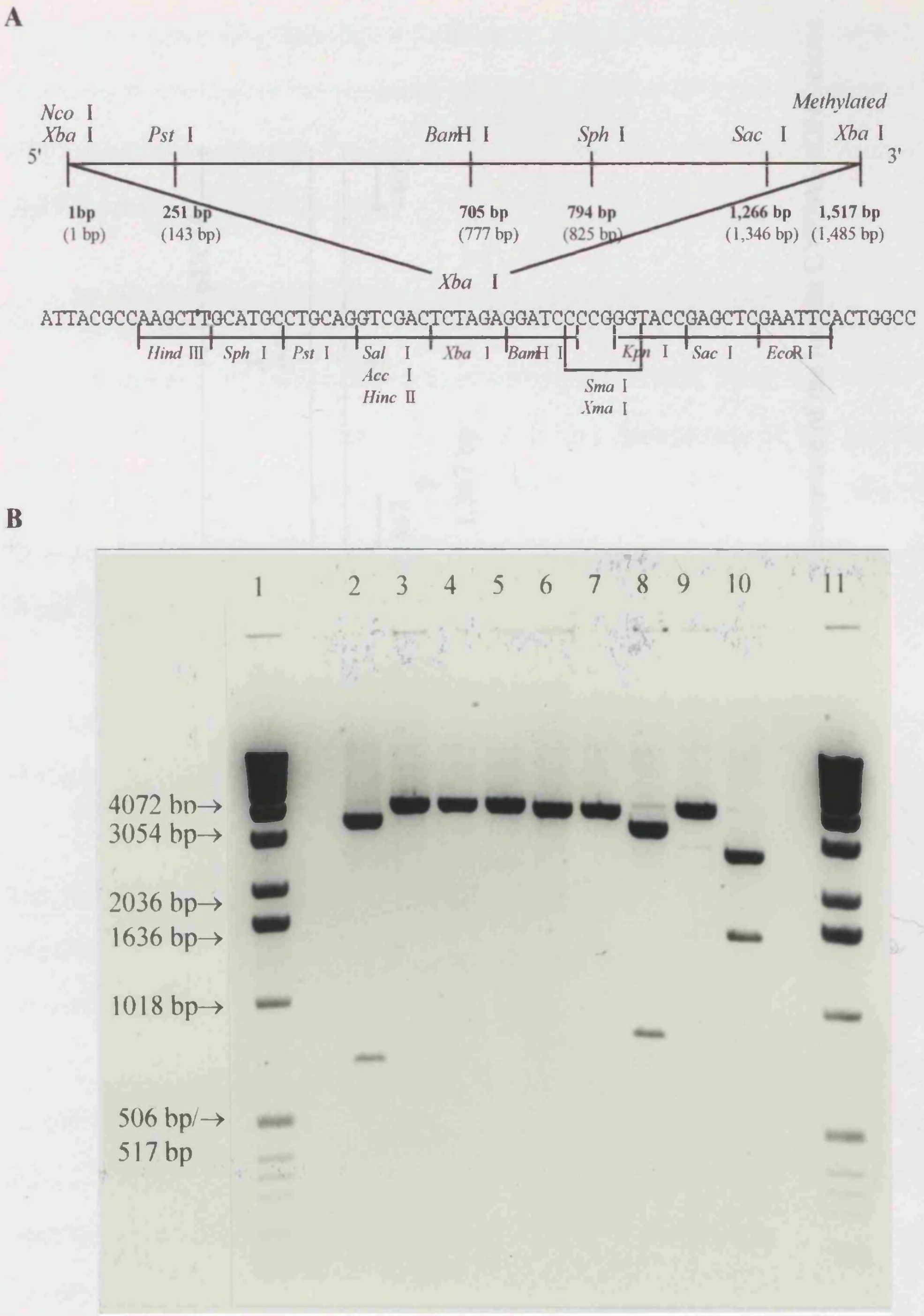
### 3.1.2. DNA SEQUENCE DETERMINATION OF A PUTATIVE HUMAN CYP2A6 cDNA CLONE

The putative human CYP2A6 cDNA was sequenced using the following primers: -40 reverse, -40 forward, -20 reverse, 181, 447 and oligo H2A1 (section 2.1.2.2.). In order to elucidate the complete sequence two subcloning events were necessary. The recombinant plasmid DNA was digested with *Bam*H I and the fragment containing pUC19+~700 bp cDNA was extracted from a 0.8% low melting point gel, purified and religated. The other subclone was created in a similar way but *Bam*H I was replaced by *Pst* I and contained ~1,340 bp of cDNA. The subcloned cDNAs were sequenced using the primers as listed above. The sequencing strategy is shown in figure 3.4A.

Figure 3.3: Restriction endonuclease analysis of the human CYP2A cDNA clone.

**A**: Schematic diagram showing the restriction endonuclease map of the human CYP2A cDNA clone and the DNA sequence of the polycloning site of pUC19. The restriction site positions in bold type are those determined from restriction endonuclease mapping. The numbers in brackets are the restriction site positions predicted by computer analysis of the published human CYP2A6 cDNA sequence (Yamano *et al.*, 1990).

**B**: The DNA samples were electrophoresed through a 1% agarose gel; the gel was photographed under U.V. light. Lanes 1 and 11 contain 1 µg of 1 Kb molecular weight standard DNA ladder (Gibco BRL). Lanes 2-10 contain the pUC19-human CYP2A recombinant plasmid DNA digested with: *Bam*H 1 (2); *Eco*R 1 (3); *Hind* III (4);.*Nco* 1 (5); *Pst* 1 (6); *Sac* 1 (7); *Sph* 1 (8); *Xba* 1 (9) and *Xba* I/*Eco*R 1 (10).


A
Nco I
Xba I
Pst I
BamH I
Sph I
Sac I
Methylated
Xba I
5'
3'
1bp
(1 bp)
251 bp
(143 bp)
705 bp
(777 bp)
794 bp
(825 bp)
1,266 bp
(1,346 bp)
1,517 bp
(1,485 bp)
Xba I
ATTACGCCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTCACTGGCC
Hind III
Sph I
Pst I
Sal I
Acc I
Hinc II
Xba I
BamH I
Kpn I
Sac I
EcoR I
Sma I
Xma I
B
1
2
3
4
5
6
7
8
9
10
11
4072 bp→
3054 bp→
2036 bp→
1636 bp→
1018 bp→
506 bp/→
517 bp

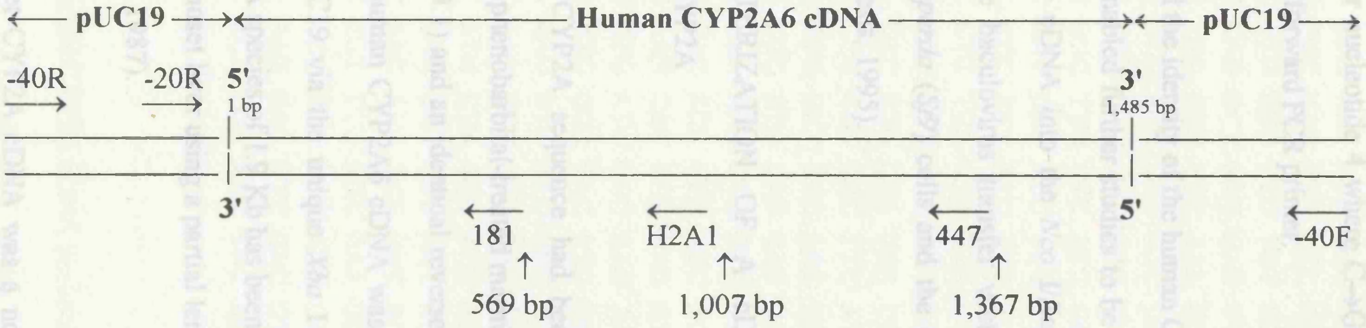

pUC19
Human CYP2A6 cDNA
pUC19
-40R
-20R
5'
1 bp
3'
1,485 bp
3'
5'
181
H2A1
447
-40F
569 bp
1,007 bp
1,367 bp

Figure 3.4A: Schematic diagram showing the sequencing strategy used for determining the cDNA sequence of the human CYP2A6 cDNA clone.

The collated sequencing data (figure 3.4B) shows that the CYP2A cDNA cloned in our laboratory is identical to the published cDNA sequence of CYP2A6 (Yamano *et al.*, 1990) except for nucleotide 4 where C→G. This was due to the incorporation of an *Nco* I site in the forward PCR primer.

As I had verified the identity of the human CYP2A cDNA clone as being that of human CYP2A6, this enabled further studies to be carried out. Manoj Nanji inserted the full length CYP2A6 cDNA into the *Nco* I/*Eco*R I sites downstream of the polyhedrin promoter in the baculovirus transfer vector, pAcC5. CYP2A6 was expressed in *Spodoptera fugiperda* (*Sf9*) cells and the expressed protein was eventually purified (Nanji, PhD. Thesis, 1995).

## 3.2. CHARACTERIZATION OF A cDNA CLONE ENCODING A NOVEL MARMOSET CYP2A

The marmoset CYP2A sequence had been amplified from total RNA previously prepared from a phenobarbital-treated marmoset (Nanji, PhD. Thesis, 1995). The same primers (figure 3.1) and an identical reverse-transcription PCR approach to that for the cloning of the human CYP2A6 cDNA was used. The marmoset CYP2A cDNA was cloned into pUC19 via the unique *Xba* I site in the polylinker. A phenobarbital-inducible mRNA species of 1.9 Kb has been identified by northern blot analysis of total RNA from marmoset liver using a partial length human CYP2A cDNA clone as a probe (Shephard *et al.*, 1987).

As the marmoset CYP2A cDNA was a novel cDNA, no information was available concerning unique restriction enzyme sites. However, it seemed highly probable that the restriction map would resemble that of the closely evolutionary linked human CYP2A6.

```
1
ATG GTG GCC TCA GGG ATG CTT CTG GTG GCC TTG CTG GTC TGC CTG
ACT GTG ATG GTC TTG ATG TCT GTT TGG CAG CAG AGG AAG AGC AAG
GGG AAG CTG CCT CCG GGA CCC ACC CCA TTG CCC TTC ATT GGA AAC
TAC CTG CAG CTG AAC ACA GAG CAG ATG TAC AAC TCC CTC ATG AAG
ATC AGT GAG CGC TAT GGC CCC GTG TTC ACC ATT CAC TTG GGG CCC
CGG CGG GTC GTG GTG CTG TGT GGA CAT GAT GCC GTC AGG GAG GCT
CTG GTG GAC CAG GCT GAG GAG TTC AGC GGG CGA GGC GAG CAA GCC
ACC TCC GAC TGG GTC TTC AAA GGC TAT GGC GTG GTA TTC AGC AAC
GGG GAG CGC GCC AAG CAG CTC CGG CGC TTC TCC ATC GCC ACC CTG
CGG GAC TTC GGG GTG GGC AAG CGA GGC ATC GAG GAG CGC ATC CAG
GAG GAG GCG GGC TTC CTC ATC GAC GCC CTC CGG GGC ACT GGC GGC
GCC AAT ATC GAT CCC ACC TTC TTC CTG AGC CGC ACA GTC TCC AAT
GTC ATC AGC TCC ATT GTC TTT GGG GAC CGC TTT GAC TAT AAG GAC
AAA GAG TTC CTG TCA CTG TTG CGC ATG ATG CTA GGA ATC TTC CAG
TTC ACG TCA ACC TCC ACG GGG CAG CTC TAT GAG ATG TTC TCT TCG
GTG ATG AAA CAC CTG CCA GGA CCA CAG CAA CAG GCC TTT CAG TTG
CTG CAA GGG CTG GAG GAC TTC ATA GCC AAG AAG GTG GAG CAC AAC
CAG CGC ACG CTG GAT CCC AAT TCC CCA CGG GAC TTC ATT GAC TCC
TTT CTC ATC CGC ATG CAG GAG GAG GAG AAG AAC CCC AAC ACG GAG
TTC TAC TTG AAA AAC CTG GTG ATG ACC ACG TTG AAC CTC TTC ATT
GGG GGC ACC GAG ACC GTC AGC ACC ACC CTG CGC TAT GGC TTC TTG
CTG CTC ATG AAG CAC CCA GAG GTG GAG GCC AAG CTC CAT GAG GAG
ATT GAC AGA GTG ATC GGC AAG AAC CGG CAG CCC AAG TTT GAG GAC
CGG GCC AAG ATG CCC TAC ATG GAG GCA GTG ATC CAC GAG ATC CAA
AGA TTT GGA GAC GTG ATC CCC ATG AGT TTG GCC CGC AGA GTC AAA
AAG GAC ACC AAG TTT CGG GAT TTC TTC CTC CCT AAG GGC ACC GAA
GTG TAC CCT ATG CTG GGC TCT GTG CTG AGA GAC CCC AGT TTC TTC
TCC AAC CCC CAG GAC TTC AAT CCC CAG CAC TTC CTG AAT GAG AAG
GGG CAG TTT AAG AAG AGT GAT GCT TTT GTG CCC TTT TCC ATC GGA
AAG CGG AAC TGT TTC GGA GAA GGC CTG GCC AGA ATG GAG CTC TTT
CTC TTC TTC ACC ACC GTC ATG CAG AAC TTC CGC CTC AAG TCC TCC
CAG TCA CCT AAG GAC ATT GAC GTG TCC CCC AAA CAC GTG GGC TTT
GCC ACG ATC CCA CGA AAC TAC ACC ATG AGC TTC CTG CCC CGC TGA
                                                       1485
```

Figure 3.4B : Human CYP2A cDNA sequence.

The cDNA sequence is identical to the published human CYP2A6 cDNA sequence (Yamano *et al.*, 1990) apart from nucleotide 4 where C→G, resulting in Leu→Val at amino acid 2; this is due to incorporation of a *Nco* I restriction site in the forward primer.

### 3.2.1. RESTRICTION ENDONUCLEASE MAPPING OF A NOVEL MARMOSET CYP2A cDNA CLONE

pUC19-marmoset CYP2A recombinant plasmid DNA was digested with several restriction endonucleases. A sample of each digest was electrophoresed through a 1% agarose gel (figure 3.5) and a restriction endonuclease map was constructed from the data (figure 3.5). The marmoset CYP2A cDNA was found to have the following unique restriction enzyme sites: *Bam*H I at 825 bp; *Hind* III at 677 bp; *Pst* I at 207 bp; *Sac* I at 1,429 bp. Two *Nco* I sites were located within the cDNA at 1,189 and 1,596 bp. The *Nco* I site incorporated into the forward primer had been mutated during the PCR amplification process. Restriction endonuclease analysis also showed that the cDNA was 1,825 bp, approximately 340 bp longer than the PCR product (Nanji, PhD. Thesis 1995).

### 3.2.2. DNA SEQUENCE DETERMINATION OF THE NOVEL MARMOSET CYP2A cDNA CLONE

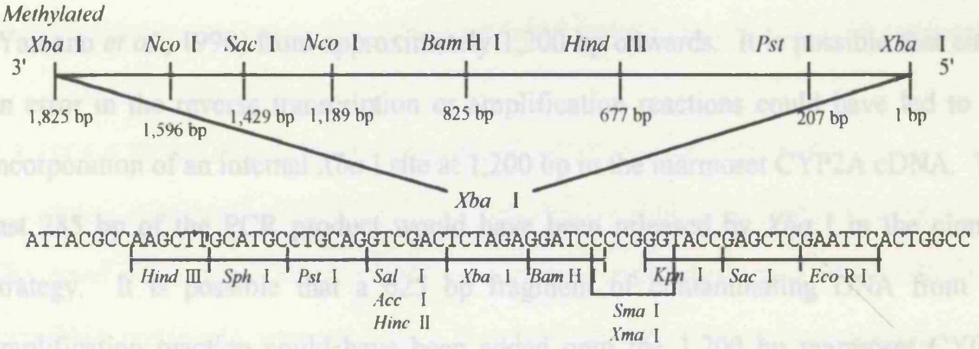
The novel marmoset CYP2A cDNA was sequenced using the following primers: -40 reverse, -40 forward, -20 reverse, 181, 447 and oligo H2A1 (section 2.1.2.2.). The recombinant plasmid DNA was digested with *Bam*H I and the fragment containing pUC19+~1,000 bp cDNA was extracted from a 0.8% low melting point gel, purified and religated. The DNA fragment of ~800 bp, also produced by digestion of the marmoset CYP2A cDNA with *Bam*H I, was cloned into the *Bam*H I site of pUC19. Two further subclones were created by digesting the marmoset CYP2A cDNA with *Pst* I; one subclone contained ~1,600 bp of cDNA whereas the other contained ~200 bp. A final subclone was generated by digesting the recombinant plasmid DNA with *Nco* 1. The fragment containing pUC19-*Nco* I digested cDNA (~1,400 bp) was religated. The sequencing strategy used was identical to that used for the sequencing of the human CYP2A6 cDNA (figure 3.4A).

Figure 3.5: Restriction endonuclease analysis of the marmoset CYP2A cDNA clone.

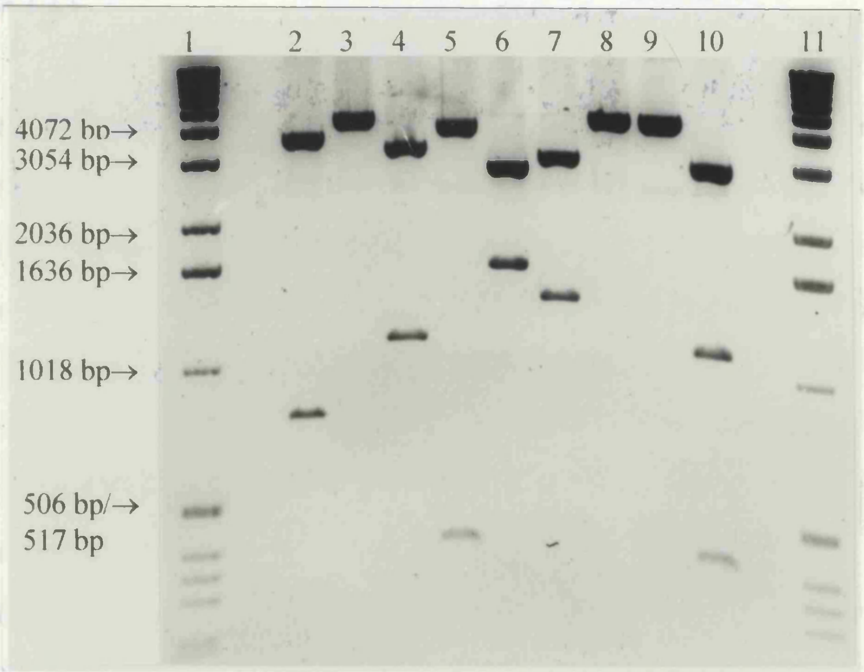
**A**: Schematic diagram showing the restriction endonuclease map of the marmoset CYP2A cDNA clone and the DNA sequence of the polycloning site of pUC19. The restriction sites are derived from restriction endonuclease mapping.

**B**: The DNA samples were electrophoresed through a 1% agarose gel; the gel was photographed under U.V. light. Lanes 1 and 11 contain 1 µg of 1 Kb molecular weight standard DNA ladder (Gibco BRL). Lanes 2-10 contain the pUC19-marmoset CYP2A recombinant plasmid DNA digested with: *Bam*H 1 (2); *Eco*R 1 (3); *Hind* III (4); *Nco* 1 (5); *Pst* 1 (6); *Sac* 1 (7); *Sph* 1 (8); *Xba* 1 (9) and *Xba* I/*Nco* 1 (10).

A


Methylated
Xba I
Nco I
Sac I
Nco I
Bam H I
Hind III
Pst I
Xba I
3'
5'
1,825 bp
1,596 bp
1,429 bp
1,189 bp
825 bp
677 bp
207 bp
1 bp
Xba I
ATTACGCCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTCACTGGCC
Hind III
Sph I
Pst I
Sal
Acc I
Hinc II
Xba
Bam H
Sma I
Xma I
Kpn I
Sac I
Eco R I

B


1
2
3
4
5
6
7
8
9
10
11
4072 bp→
3054 bp→
2036 bp→
1636 bp→
1018 bp→
506 bp/→
517 bp

Early results from DNA sequence analysis showed that the 3' end of the marmoset CYP2A cDNA bore no resemblance to the human CYP2A6 published cDNA sequence (Yamano *et al.*, 1990) from approximately 1,200 bp onwards. It is possible that either an error in the reverse transcription or amplification reactions could have led to the incorporation of an internal *Xba* I site at 1,200 bp in the marmoset CYP2A cDNA. The last 285 bp of the PCR product would have been released by *Xba* I in the cloning strategy. It is possible that a 625 bp fragment of contaminating DNA from the amplification reaction could have been added onto the 1,200 bp marmoset CYP2A cDNA in the ligation reaction. This would account for the 1,825 bp insert observed in section 3.2.1.

The reading frame of the cDNA had been changed due to a 1 bp deletion immediately adjacent to the start codon (figure 3.6). This frame shift mutation occurred where the last base (G) of the *Nco* I site should have been incorporated in the cDNA. The deletion was probably caused by the 3'→5' proofreading ability of the *Pyrococcus furiosis* (*Pfu*) DNA polymerase that had been used to amplify the cDNA.

| | | |
|---|---|---|
| **Published cDNA sequence of human CYP2A6**<br>(Yamano *et al.*, 1990) | 5' | ATG CTG GCC |
| **Marmoset CYP2A cDNA sequence** | 5' | ATG _TG GCC |

Figure 3.6: Diagram illustrating the frame shift mutation in the marmoset CYP2A cDNA sequence.

As this cDNA clone did not in fact encode a marmoset CYP2A, the total cDNA was not sequenced. A new cDNA clone was isolated from total RNA prepared from a liver of a phenobarbital-treated marmoset using a modified reverse transcription PCR approach.

## 3.3. ISOLATION OF A NOVEL MARMOSET CYP2A cDNA CLONE

The novel marmoset CYP2A mRNA was reverse transcribed using M-MLVRT from total RNA previously prepared from a liver of a phenobarbital-treated marmoset. The cDNA was amplified by PCR using DynaZyme, a thermostable DNA polymerase with a 5'→3' exonuclease activity. DynaZyme lacks the 3'→5' proofreading activity that *Pfu* DNA polymerase possesses. 5 μl of the PCR product and 5 μl of the PCR negative control were electrophoresed through a 1% agarose gel (figure 3.7). A DNA fragment corresponding to approximately 1,500 bp was observed in the lane containing the amplified marmoset CYP2A cDNA. DNA bands corresponding to unincorporated primers were seen in the lane containing the negative control. A sample of the negative control for the reverse transcription reaction was also amplified to ensure the validity of the PCR results. 5 μl of this PCR product was electrophoresed through a 1% agarose gel (data not shown), the only DNA band observed was that of unincorporated primers.

The marmoset CYP2A PCR product was treated with proteinase K as this has been shown to increase the cloning efficiency of PCR products (Crowe *et al.*, 1991). The proteinase K digested product was electrophoresed through a 0.8% low melting point agarose gel and the 1,500 bp DNA fragment was excised and purified. The DNA fragment, allegedly encoding marmoset CYP2A, was inserted into the plasmid vector pGEM-T (Promega). During the final extension cycle of 15 minutes at 72$^{o}$C in the PCR protocol, DynaZyme extended the PCR product by a single deoxyadenosine at the 3' ends. The pGEM-T vector is prepared by cutting the pGEM-5Zf(+) vector with *Eco*R V and adding a 3' terminal deoxythymidine to both ends. This enabled the PCR product to be easily ligated into this plasmid vector. The vector should also be unable to self-

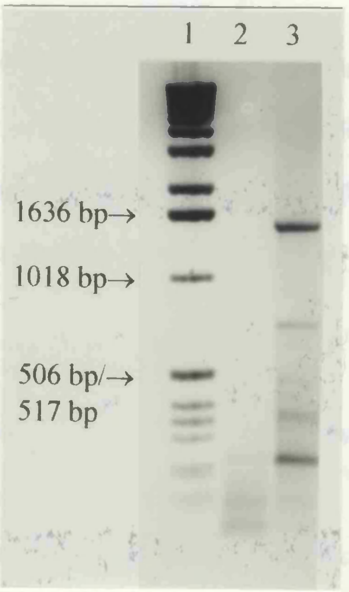


Figure 3.7: Amplification of the marmoset CYP2A cDNA sequence from total RNA isolated from the liver of a phenobarbital-treated marmoset.

1 µg of 1 Kb molecular weight standard DNA ladder (Gibco BRL, lane 1), 5 µl of the PCR negative control (lane 2) and 5 µl of the PCR product (lane 3) were electrophoresed through a 1% agarose gel. The gel was photographed under U.V. light.

ligate due to the incompatible 5' and 3' ends. A schematic diagram of the cloning strategy is shown in figure 3.8.

The pGEM-T-marmoset CYP2A construct DNA was used to transform XL1-Blue *E. coli* cells. The restriction endonuclease site used to clone the insert into the vector lay within a multiple cloning region situated in the α-peptide coding region of the enzyme β-galactosidase. This enabled recombinant clones to be directly selected by the blue/white assay.

36 white recombinant marmoset CYP2A cDNA clones were picked and were used to inoculate LB AMP media for a plasmid DNA mini-prep. All 36 recombinant plasmid DNA samples were cut with the restriction enzymes *Nco* 1 and *Sal* 1 to release the cloned insert. A sample of each digest was electrophoresed through a 1% agarose gel (data not shown). 31 out of 36 cDNA clones contained insert DNA fragments of 450 bp and 1,050 bp; the other 5 clones were false positives. The total 1,500 bp of insert DNA represented the marmoset CYP2A cDNA which contained either an internal *Nco* 1 restriction site or an internal *Sal* 1 restriction site. Three marmoset CYP2A cDNA clones were selected for further studies; they were clone (#) 4, #6 and #9.

### 3.3.1. RESTRICTION ENDONUCLEASE MAPPING OF THREE NOVEL MARMOSET CYP2A cDNA CLONES

Each pGEM-T-marmoset CYP2A recombinant plasmid DNA was digested with several restriction endonucleases. A sample of each digest was electrophoresed through a 1% agarose gel (figure 3.9). The three marmoset CYP2A cDNAs were found to have an identical restriction enzyme pattern. A restriction endonuclease map for the recombinant plasmid DNA was constructed from the data (figure 3.9). The marmoset CYP2A cDNAs were shown to have the following unique restriction enzyme sites: *Bam*H I at 989 bp; *Hind* III at 794 bp; *Nco* I at 1,106 bp; *Pst* I at 240 bp; *Sac* I at

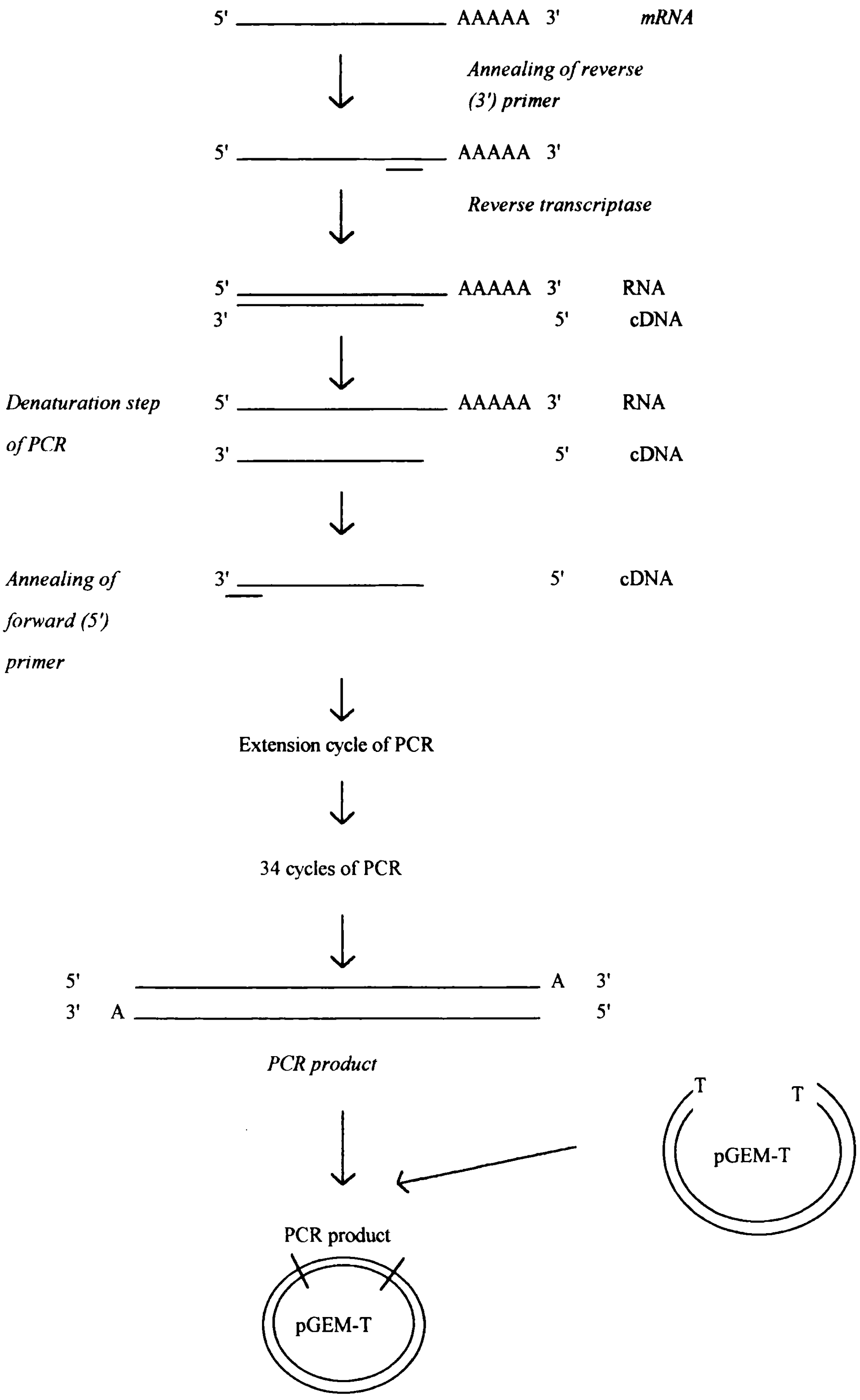

5' AAAAA 3'
*mRNA*
*Annealing of reverse (3') primer*
5' AAAAA 3'
*Reverse transcriptase*
5' AAAAA 3' RNA
3' 5' cDNA
*Denaturation step of PCR*
5' AAAAA 3' RNA
3' 5' cDNA
*Annealing of forward (5') primer*
3' 5' cDNA
Extension cycle of PCR
34 cycles of PCR
5' A 3'
3' A 5'
*PCR product*
T T
pGEM-T
PCR product
pGEM-T

Figure 3.8 : Schematic diagram illustrating the reverse transcription cloning strategy used to isolate cDNA clones of the novel marmoset CYP2A.

Figure 3.9: Restriction endonuclease analysis of #4, #6 and #9 marmoset CYP2A cDNA clones.

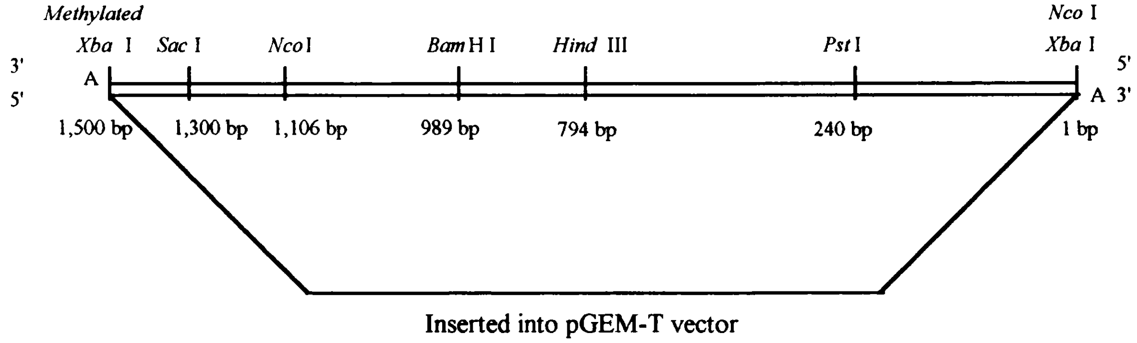
**A**: Schematic diagram showing the restriction endonuclease map of the three marmoset CYP2A cDNA clones and the DNA sequence of the of the multiple cloning site of the pGEM-T vector.

**B**: The DNA samples were electrophoresed through a 1% agarose gel; the gel was photographed under U.V. light. Lanes 1 and 12 contain 1 μg of 1 Kb molecular weight standard DNA ladder (Gibco BRL). Lanes 2-11 contain the pGEM-T-#4 marmoset CYP2A recombinant plasmid DNA digested with: *Nco* I/*Sal* I (2); *Nco* I (3); *Xba* I/*Sal* I (4); *Xba* I/ *Nco* I (5); *Pst* I (6); *Sph* I (7); *Sac* I (8); *Hind* III/*Sal* I (9); *Eco*R I/*Sal* I (10); *Bam*H I/*Sal* I (11).

**C**: As for figure **B** except the digested DNA sample was that of pGEM-T-#6 marmoset CYP2A recombinant plasmid DNA.

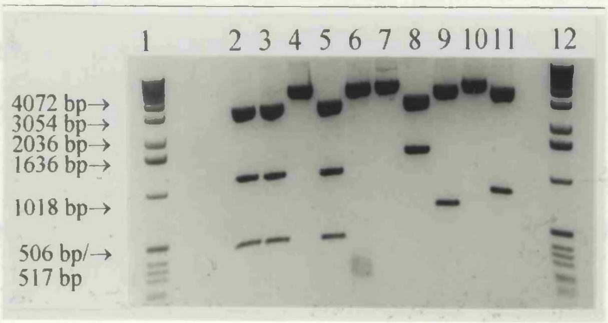
**D**: As for figure **B** except the digested DNA sample was that of pGEM-T-#9 marmoset CYP2A recombinant plasmid DNA.

A:

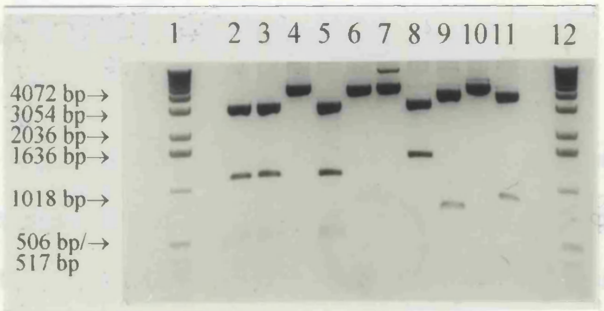

Methylated
Xba I
Sac I
NcoI
BamHI
Hind III
PstI
Nco I
Xba I
3'
5'
A
5'
A 3'
1,500 bp
1,300 bp
1,106 bp
989 bp
794 bp
240 bp
1 bp
Inserted into pGEM-T vector

```
                             ↑→ T7 transcription start
5'   TGT AAT ACG ACT CAC TAT AGG GCG AAT TGG GCC CGA CGT CGC ATG CTC
     ACA TTA TGC TGA GTG ATA TCC CGC TTA ACC CGG GCT GCA GCG TAC GAG
       |----------------------------------|  |-------||--------||-------|
                  T7 promoter                  Apa I     Aat II   Sph I

     CCG GCC GCC ATG GCC GCG GGATT  3'(cloned insert) AT CAC TAG TGC
     GGC CGG CGG TAC CGG CGC CCTA                  3'TTA GTG ATC ACG
             |--------||--------|                        |--------||-
                Nco I    Sac II                            Spe I

     GGC CGC CTG CAG GTC GAC CAT ATG GGA GAG CTC CCA ACG CGT TGG ATG
     CCG GCG GAC GTC CAG CTG GTA TAC CCT CTC GAG GGT TGC GCA ACG TAC
     ------| |-------| |-------| |-------|   |-------| |---------------| |--
      Not I   Pst I     Sal I     Nde I        Sac I        BstX I        Nsi I

        SP6 transcription start ←|
     CAT AGC TTG AGT ATT CTA TAG TGT CAC CTA AAT 3'
     GTA TCG AAC TCA TAA GAT ATC ACA GTG GAT TTA 5'
     ---|         |--------------------------------|
                           SP6 promoter
```

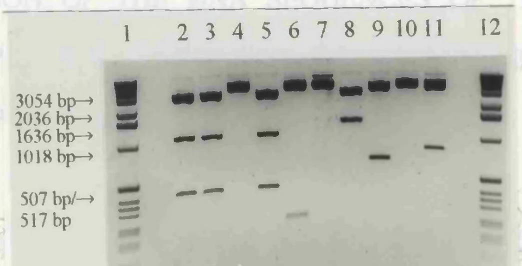
**B:**


1 2 3 4 5 6 7 8 9 10 11 12
4072 bp→
3054 bp→
2036 bp→
1636 bp→
1018 bp→
506 bp/→
517 bp

**C:**


1 2 3 4 5 6 7 8 9 10 11 12
4072 bp→
3054 bp→
2036 bp→
1636 bp→
1018 bp→
506 bp/→
517 bp

**D:**


1 2 3 4 5 6 7 8 9 10 11 12
3054 bp→
2036 bp→
1636 bp→
1018 bp→
507 bp/→
517 bp

1,300 bp. The DNA sequence was determined for each recombinant plasmid DNA to ascertain if the three marmoset CYP2A cDNAs were identical and to investigate the degree of similarity between the marmoset CYP2A cDNAs and CYP2A6 cDNA.

### 3.3.2. DNA SEQUENCE DETERMINATION OF THE THREE NOVEL MARMOSET cDNA CLONES

The three novel marmoset CYP2A cDNAs were sequenced using the following primers: SP6, T7, 181, H2A1, oligoA, oligo B, oligo C, oligo D, oligo E, oligo F, oligo G, oligo H, oligo I and oligo J (section 2.1.2.2.). The sequencing strategy is shown in figure 3.10A; figure 3.10B illustrates the alignment of the nucleotide sequences of #4, #6 and #9 marmoset CYP2A cDNA with CYP2A6 cDNA.

The nucleotide sequences of the three marmoset cDNAs were not identical, slight differences in sequence were observed. These differences in nucleotide sequence could have been due to mistakes in the reverse transcription or amplification reactions. It must be remembered that these reactions do not always take place with 100% fidelity with respect to the template sequence.

### 3.3.3. COMPARISON OF THE DNA SEQUENCES OF THE THREE NOVEL MARMOSET CYP2A cDNA CLONES TO THE NUCLEOTIDE SEQUENCES OF THE HUMAN CYP2A cDNAs

The nucleotide sequences of the three novel marmoset CYP2A cDNAs were also compared to the cDNA of CYP2A7 (Yamano *et al.*, 1990), data not shown. The marmoset CYP2A cDNAs were unable to be compared to the CYP2A13 cDNA, the other member of the human CYP2A subfamily, as only a genomic clone has been isolated (Fernandez-Salguero *et al.*, 1995). The percentage nucleotide identities

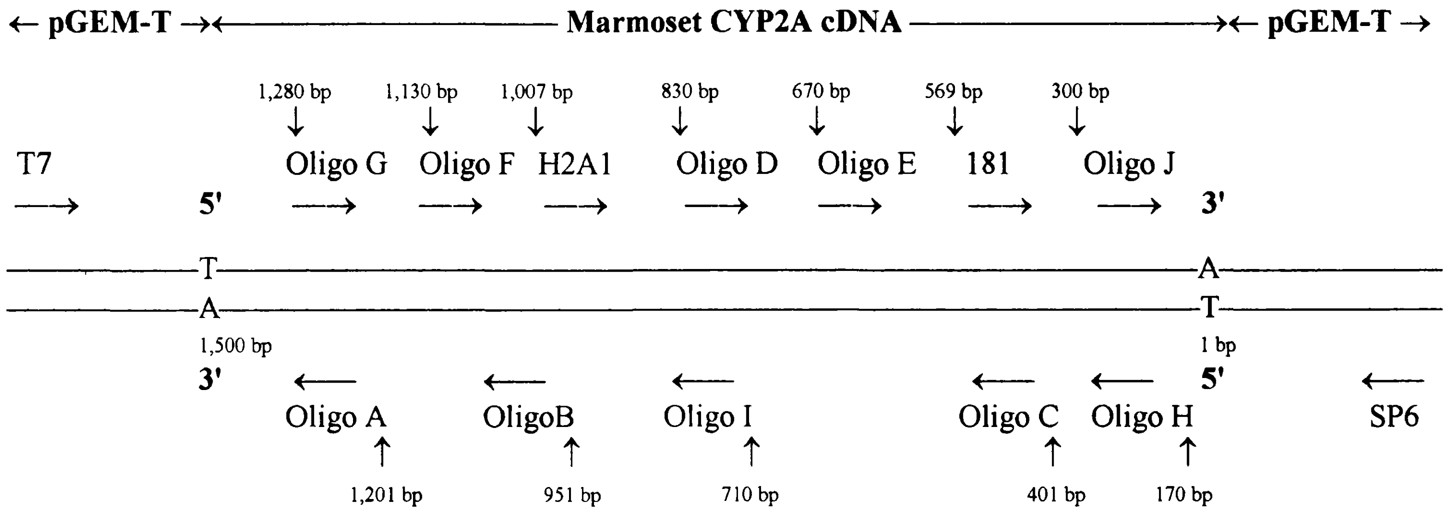

← pGEM-T →← Marmoset CYP2A cDNA →← pGEM-T →
1,280 bp
1,130 bp
1,007 bp
830 bp
670 bp
569 bp
300 bp
T7
Oligo G
Oligo F
H2A1
Oligo D
Oligo E
181
Oligo J
5'
3'
T
A
A
T
1,500 bp
1 bp
3'
5'
Oligo A
OligoB
Oligo I
Oligo C
Oligo H
SP6
1,201 bp
951 bp
710 bp
401 bp
170 bp

Figure 3.10A: Schematic diagram showing the sequencing strategy used for determining the cDNA sequences of the three marmoset CYP2A cDNA clones.

Figure 3.10B: Alignment of the nucleotide sequences of #4, #6 and #9 marmoset CYP2A cDNA with CYP2A6 cDNA (Yamano *et al.*, 1990).

The nucleotide sequence of CYP2A6 cDNA is displayed above and listed below are those of #4, #6 and #9 marmoset CYP2A cDNA respectively. The nucleotide sequence of the marmoset CYP2A cDNAs are identical to that of CYP2A6 where the symbol – is shown.

```
        1
CYP2A6  ATG CTG GCC TCA GGG ATG CTT CTG GTG GCC TTG CTG GTC TGC
#4      --- G-- --- --- --- --- --- --- --- --- --- --- -C- ---
#6      --- G-- --- --- --- --- --- --- --- --- --- --- -C- ---
#9      --- G-- --- --- --- --- --- --- --- --- --- --- -C- ---
        43
CYP2A6  CTG ACT GTG ATG GTC TTG ATG TCT GTT TGG CAG CAG AGG AAG
#4      --- --- --- --- --- --- --- --- --C --- AG- --- --- ---
#6      --- --- --- --- --- --- --- --- --C --- AG- --- --- ---
#9      --- --- --- --- --- --- --- --- --C --- AG- --- --- -G-
        85
CYP2A6  AGC AAG GGG AAG CTG CCT CCG GGA CCC ACC CCA TTG CCC TTC
#4      --- --- --- --- --- --- --- --- --- --- --G --- --- ---
#6      --- --- --- --- --- --- --- --- --- --- --G --- --- ---
#9      --- --- --- --- --- --- --- --- --- --- --G --- --- ---
        127
CYP2A6  ATT GGA AAC TAC CTG CAG CTG AAC ACA GAG CAG ATG TAC AAC
#4      --- --- --- --T --- --- --- --T --- --- --- --- --- ---
#6      --- --- --- --T --- --- --- --T --- --- --- --- --- ---
#9      --- --- --- --T --- --- --- --T --- --- --- --- --- ---
        169
CYP2A6  TCC CTC ATG AAG ATC AGT GAG CGC TAT GGC CCC GTG TTC ACC
#4      --- --- --- --- --- --- --- --- -T- --- --- --- --- ---
#6      --- --- --- --- --- --- --- --- --- --- --- --- --- ---
#9      --- --- --- --- --- --- --- --- --- --- --- --- --- ---
        211
CYP2A6  ATT CAC TTG GGG CCC CGG CGG GTC GTG GTG CTG TGT GGA CAT
#4      --- --- C-- --- TT- --- --- --- --- --- --- --C --- T-C
#6      --- --- C-- --- TT- --- --- --- --- --- --- --C --- T-C
#9      --- --- C-- --- TT- --- --- --- --- --- --- --C --- T-C
        253
CYP2A6  GAT GCC GTC AGG GAG GCT CTG GTG GAC CAG GCT GAG GAG TTC
#4      --- --- --- -A- --- --- --- C-- --- --- --- --- --- ---
#6      --- --- --- -A- --- --- --- C-- --- --- --- --- --- ---
#9      --- --- --- -A- --- --- --- C-- --- --- --- --- --- ---
        295
CYP2A6  AGC GGG CGA GGC GAG CAA GCC ACC TTC GAC TGG GTC TTC AAA
#4      G-T --- --C --- --- --G --- --- --- A-- --- C-- --- -T-
#6      G-T --- --C --- --- --G --- --- --- A-- --- C-- --- ---
#9      G-T --- --C --- --- --G --- --- --- A-- --- C-- --- ---
        337
CYP2A6  GGC TAT GGC GTG GTA TTC AGC AAC GGG GAG CGC GCC AAG CAG
#4      --- --- --- T-- -CG --G --- --- --- --- --- --- --- ---
#6      --- --- --- T-- -CG --G --- --- --- --- --- --- --- ---
#9      --- --- --- T-- -CG --G --- --- --- --- --- --- --- ---
        379
CYP2A6  CTC CGG CGC TTC TCC ATC GCC ACC CTG CGG GAC TTC GGG GTG
#4      --- --- --- --- --- --- A-- --- --- --- --- --- --- ---
#6      --- --- --- --- --- --- A-- --- --- --- --- --- --- ---
#9      --- --- --- --- --- --- A-- --- --- --- --- --- --- ---
        421
CYP2A6  GGC AAG CGA GGC ATC GAG GAG CGC ATC CAG GAG GAG GCG GGC
#4      --- --- --C --- --- --- --- --- --- --- --- --- --- ---
#6      --- --- --C --- --- --- --- --- --- --- --- --- --- ---
#9      --- --- --C --- --- --- --- --- --- --- --- --- --- ---
```

```
        463
CYP2A6  TTC CTC ATC GAC GCC CTC CGG GGC ACT GGC GGC GCC AAT ATC
#4      --- --- --- --G -T- --- --- --- --G C-- --- --- --- ---
#6      --- --- --- --G -T- --- --- --- --G C-- --- --- --- ---
#9      --- --- --- --G -T- --- --- --- --G C-- --- --- --- ---
        505
CYP2A6  GAT CCC ACC TTC TTC CTG AGC CGC ACA GTC TCC AAT GTC ATC
#4      --- --- --- --- --- --- --- --- --G --- --- --- --- ---
#6      --- --- --- --- --- --- --- --- --G --- --- --- --- ---
#9      --- --- --- --- --- --- --- --- --G --- --- --- --- ---
        547
CYP2A6  AGC TCC ATT GTC TTT GGG GAC CGC TTT GAC TAT AAG GAC AAA
#4      --- --T --C --- --- --- --- --- --- --- --- G-- --- ---
#6      --- --T --C --- --- --- --- --- --- --- --- G-- --- ---
#9      --- --T --C --- --- --- --- --- --- --- --- G-- --- ---
        589
CYP2A6  GAG TTC CTG TCA CTG TTG CGC ATG ATG CTA GGA ATC TTC CAG
#4      --- --T --- --- --- C-- --- --- --- --G --- -G- --- ---
#6      --- --T --- --- --- C-- --- --- --- --G --- -G- --- ---
#9      --- --T --- --- --- C-- --- --- --- --G --- -G- --- ---
        631
CYP2A6  TTC ACG TCA ACC TCC ACG GGG CAG CTC TAT GAG ATG TTC TCT
#4      --- --C G-- --- --T -T- --- --- --- --- --- --- --- C--
#6      --- --C G-- --- --T -T- --- --- --- --- --- --- --- C--
#9      --- --C G-- --- --T -T- --- --- --- --- --- --- --- C--
        673
CYP2A6  TCG GTG ATG AAA CAC CTG CCA GGA CCA CAG CAA CAG GCC TTT
#4      --A --- --- --- --- --- --- --C --- --- --- --- --- --C
#6      --A --- --- --- --- --- --- --C --- --- --- --- --- --C
#9      --A --- --- --- --- -C- --- --C --- --- --- --- --- --C
        715
CYP2A6  CAG TTG CTG CAA GGG CTG GAG GAC TTC ATA GCC AAG AAG GTG
#4      A-- GA- --- --- --- --C A-- --- --- G-- --- --- --- ---
#6      A-- GA- --- --- --- --C A-- --- --- G-- --- --- --- ---
#9      A-- GA- --- --- --- --C A-- --- --- G-- --- --- --- ---
        757
CYP2A6  GAG CAC AAC CAG CGC ACG CTG GAT CCC AAT TCC CCA CGG GAC
#4      --- --- -G- --- --- --A --- --- --- --- --- --- --- ---
#6      --- --- -G- --- --- --A --- --- --- --- --- --- --- ---
#9      --- --- -G- --- --- --A --- --- --- --- --- --- --- ---
        799
CYP2A6  TTC ATT GAC TCC TTT CTC ATC CGC ATG CAG GAG GAG GAG AAG
#4      --- --C --- --- --- --- --- --- --T -G- --- --- --- ---
#6      --- --C --- --- --- --- --- --- --T -G- --- --- --- ---
#9      --- --C --- --- --- --- --- --- --T -G- --- --- --- ---
        841
CYP2A6  AAC CCC AAC ACG GAG TTC TAC TTG AAA AAC CTG GTG ATG ACC
#4      --- --- C-- --- --- --- --- --- --G --- --- --- C-- ---
#6      --- --- C-- --- --- --- --- --- --G --- --- --- C-- ---
#9      --- --- C-- --- --- --- --- --- --G --- --- --- C-- ---
        883
CYP2A6  ACG TTG AAC CTC TTC ATT GGG GGC ACC GAG ACC GTC AGC ACC
#4      --- C-- --- --- --- T-- -C- --- --T --- --- --- --- --G
#6      --- C-- --- --- --- T-- -C- --- --T --- --- --- --- --G
#9      --- C-- --- --- --- T-- -C- --T --T --- --- --- --- --G
```

```
            925
CYP2A6      ACC CTG CGC TAT GGC TTC TTG CTG CTC ATG AAG CAC CCA GAG
#4          --- --- --- --- --- --- C-- --- --- --- --T --- --- --A
#6          --- --- --- --- --- --- C-T --- --- --- --- --- --- --A
#9          --- --- --- --- --- --- C-- --- --- --- --T --- --- --A
            967
CYP2A6      GTG GAG GCC AAG GTC CAT GAG GAG ATT GAC AGA GTG ATC GGC
#4          --- --- --- --- --- --- --- --- --- --- --- --- --- ---
#6          --- --- --- --- --- --- --- --- --- --- --- --- --- ---
#9          --- --- --- --- --- --- --- --- --- --- --- --- --- ---
            1009
CYP2A6      AAG AAC CGG CAG CCC AAG TTT GAG GAC CGG GCC AAG ATG CCC
#4          --- --- --- --- --- --- --- --- --- --- --- --- --- ---
#6          --- --- --- --- --- --- --- --- --- --- --- --- --- ---
#9          --- --- --- --- --- --- --- --- --- --- --- --- --- ---
            1051
CYP2A6      TAC ATG GAG GCA GTG ATC CAC GAG ATC CAA AGA TTT GGA GAC
#4          --- -C- --- --G --- --- --- --- --G --- --- --- -C- ---
#6          --- -C- --- --G --- --- --- --- --G --- --- --- -C- ---
#9          --- -C- --- --G --- --- --- --- --G --- --- --- -C- ---
            1093
CYP2A6      GTG ATC CCC ATG AGT TTG GCC CGC AGA GTC AAA AAG GAC ACC
#4          A-A --- --- --- G-- C-- --- --- --G --- -CT --- --- ---
#6          A-A --- --- --- G-- C-- --- --- --G --- -CT --- --- ---
#9          A-A --- --- --- G-- C-- --- --- --G --- -CT --- --- ---
            1135
CYP2A6      AAG TTT CGG GAT TTC TTC CTC CCT AAG GGC ACC GAA GTG TAC
#4          --- --- --- --C --- A-- --- --- --- --- --- --- --- -T-
#6          --- --- --- --C --- A-- --- --- --- --- --- --- --- -T-
#9          --- --- --- --C --- A-- --- --- --- --- --- --- --- -T-
            1177
CYP2A6      CCT ATG CTG GGC TCT GTG CTG AGA GAC CCC AGT TTC TTC TCC
#4          --C C-- --- --- --- --- --A --- --- T-- --G --- --- ---
#6          --- C-- --- --- --- --- --A --- --- T-- --G --- --- ---
#9          --- C-- --- --- --- --- --A --- --- T-- --G --- --- ---
            1219
CYP2A6      AAC CCC CAG GAC TTC AAT CCC CAG CAC TTC CTG AAT GAG AAG
#4          --T --- -GT --- --- --C --- --- --- --- --- G-- --- ---
#6          --T --- -GT --- --- --C --- --- --- --- --- G-- --- ---
#9          --T --- -GT --- --- --C --- --- --- --- --- G-- --- ---
            1261
CYP2A6      GGG CAG TTT AAG AAG AGT GAT GCT TTT GTG CCC TTT TCC ATC
#4          --- --- --- --- --- --- --- --A --- --- --- --C --- ---
#6          --- --- --- --- --- --- --- --A --- --- --- --C --- ---
#9          --- --- --- --- --- --- --- --A --- --- --- --C --- ---
            1303
CYP2A6      GGA AAG CGG AAC TGT TTC GGA GAA GGC CTG GCC AGA ATG GAG
#4          --- --- --- T-- --- --- --- --- --- --- --- --- --- ---
#6          --- --- --- T-- --- --- --- --- --- --- --- --- --- ---
#9          --- --- --- T-- --- --- --- --- --- --- --- --- --- ---
            1345
CYP2A6      CTC TTT CTC TTC TTC ACC ACC GTC ATG CAG AAC TTC CGC CTC
#4          --- --- --G --- C-- --- --- A-- --- --- --- --- --- T--
#6          --- --- --G --- C-- --- --- A-- --- --- --- --- --- T--
#9          --- --- --G --- C-- --- --- A-- --- --- --- --- --- T--
```

| | |
|---|---|
| | 1387 |
| CYP2A6 | AAG TCC TCC CAG TCA CCT AAG GAC ATT GAC GTG TCC CCC AAA |
| #4 | --- --G C-G --- --G --C --- --- --C --- --- --- --- --- |
| #6 | --- --G C-G --- --G --C --- --- --C --- --- --- --- --- |
| #9 | --- --G C-G --- --G --C --- --- --C --- --- --- --- --- |
| | 1429 |
| CYP2A6 | CAC GTG GGC TTT GCC ACG ATC CCA CGA AAC TAC ACC ATG AGC |
| #4 | --- --- --- --- -G- --A --- --- -C- --- --- --- --- --- |
| #6 | --- --- --- --- -G- --A --- --- -C- --- --- --- --- --- |
| #9 | --- --- --- --- -G- --A --- --- -C- --- --- --- --- --- |
| | 1471 |
| CYP2A6 | TTC CTG CCC CGC TGA |
| #4 | --- --- --- --- --- |
| #6 | --- --- --- --- --- |
| #9 | --- --- --- --- --- |

between the marmoset CYP2A cDNAs and those of the human CYP2A6 and CYP2A7 cDNAs are shown in table 3.1.

| cDNA | % nucleotide identity between cDNAs | |
|---|---|---|
| | CYP2A6 | CYP2A7 |
| #4 marmoset CYP2A | 92.4 (112) | 91.4 (127) |
| #6 marmoset CYP2A | 92.7 (109) | 91.6 (124) |
| #9 marmoset CYP2A | 92.4 (112) | 91.4 (127) |

Table 3.1: This table illustrates the percentage nucleotide identities (to 1 decimal place) between the three marmoset CYP2A cDNAs and those of the human CYP2A6 and CYP2A7 cDNAs (Yamano *et al.*, 1990). The figures in brackets represent the number of nucleotide changes between the two cDNAs.

### 3.3.4. COMPARISON OF THE DEDUCED PROTEIN SEQUENCES OF THE THREE NOVEL MARMOSET CYP2As TO THE PROTEIN SEQUENCES OF THE HUMAN CYP2As

The protein sequence of each marmoset CYP2A cDNA clone was deduced using the genetic code. Figure 3.11 shows the alignment of the amino acids of these deduced protein sequences with the amino acid sequence of CYP2A6. The protein sequences of the three marmoset CYP2As were not 100% identical. As slight differences in the nucleotide sequences of the three marmoset CYP2A cDNAs had been observed in section 3.3.2., slight differences in the deduced protein sequences are to be expected.

The protein sequences of the three novel marmoset CYP2As were also compared to those of CYP2A7 (Yamano *et al.*, 1990) and CYP2A13 (Fernandez-Salguero *et al.*, 1995), data not shown. As has been mentioned in section 3.3.3. only a genomic clone

Figure 3.11: Alignment of the amino acid sequences of the three deduced marmoset CYP2A protein sequences with the amino acid sequence of CYP2A6 (Yamano *et al.*, 1990).

The amino acid sequence of CYP2A6 is displayed above and listed below are those of #4, #6 and #9 marmoset CYP2A respectively. The amino acid sequences of the marmoset CYP2As are identical to that of CYP2A6 where the symbol - is shown. A single point mutation in any of the marmoset CYP2A cDNAs that specifies the same amino acid as in CYP2A6 are indicated by xxx; whereas a change of two nucleotides in a codon is indicated by xxx. The conserved cysteine residue at position 439, that serves as the thiolate ligand to the haem iron, is underlined in the CYP2A6 sequence.

```
           1
CYP2A6     Met Leu Ala Ser Gly Met Leu Leu Val Ala Leu Leu Val Cys Leu Thr
#4         --- Val --- --- --- --- --- --- --- --- --- --- Ala --- --- ---
#6         --- Val --- --- --- --- --- --- --- --- --- --- Ala --- --- ---
#9         --- Val --- --- --- --- --- --- --- --- --- --- Ala --- --- ---
           17
CYP2A6     Val Met Val Leu Met Ser Val Trp Gln Gln Arg Lys Ser Lys Gly Lys
#4         --- --- --- --- --- --- Val --- Arg --- --- --- --- --- --- ---
#6         --- --- --- --- --- --- Val --- Arg --- --- --- --- --- --- ---
#9         --- --- --- --- --- --- Val --- Arg --- --- Arg --- --- --- ---
           33
CYP2A6     Leu Pro Pro Gly Pro Thr Pro Leu Pro Phe Ile Gly Asn Tyr Leu Gln
#4         --- --- --- --- --- --- Pro --- --- --- --- --- --- Tyr --- ---
#6         --- --- --- --- --- --- Pro --- --- --- --- --- --- Tyr --- ---
#9         --- --- --- --- --- --- Pro --- --- --- --- --- --- Tyr --- ---
           49
CYP2A6     Leu Asn Thr Glu Gln Met Tyr Asn Ser Leu Met Lys Ile Ser Glu Arg
#4         --- Asn --- --- --- --- --- --- --- --- --- --- --- --- --- ---
#6         --- Asn --- --- --- --- --- --- --- --- --- --- --- --- --- ---
#9         --- Asn --- --- --- --- --- --- --- --- --- --- --- --- --- ---
           65
CYP2A6     Tyr Gly Pro Val Phe Thr Ile His Leu Gly Pro Arg Arg Val Val Val
#4         Phe --- --- --- --- --- --- --- Leu --- Phe --- --- --- --- ---
#6         --- --- --- --- --- --- --- --- Leu --- Phe --- --- --- --- ---
#9         --- --- --- --- --- --- --- --- Leu --- Phe --- --- --- --- ---
           81
CYP2A6     Leu Cys Gly His Asp Ala Val Arg Glu Ala Leu Val Asp Gln Ala Glu
#4         --- Cys --- Tyr --- --- --- Lys --- --- --- Leu --- --- --- ---
#6         --- Cys --- Tyr --- --- --- Lys --- --- --- Leu --- --- --- ---
#9         --- Cys --- Tyr --- --- --- Lys --- --- --- Leu --- --- --- ---
           97
CYP2A6     Glu Phe Ser Gly Arg Gly Glu Gln Ala Thr Ser Asp Trp Val Phe Lys
#4         --- --- Gly --- Arg --- --- Gln --- --- Phe Asn --- Leu --- Ile
#6         --- --- Gly --- Arg --- --- Gln --- --- Phe Asn --- Leu --- ---
#9         --- --- Gly --- Arg --- --- Gln --- --- Phe Asn --- Leu --- ---
           113
CYP2A6     Gly Tyr Gly Val Val Phe Ser Asn Gly Glu Arg Ala Lys Gln Leu Arg
#4         --- --- --- Leu Ala Leu --- --- --- --- --- --- --- --- --- ---
#6         --- --- --- Leu Ala Leu --- --- --- --- --- --- --- --- --- ---
#9         --- --- --- Leu Ala Leu --- --- --- --- --- --- --- --- --- ---
           129
CYP2A6     Arg Phe Ser Ile Ala Thr Leu Arg Asp Phe Gly Val Gly Lys Arg Gly
#4         --- --- --- --- Thr --- --- --- --- --- --- --- --- --- Arg ---
#6         --- --- --- --- Thr --- --- --- --- --- --- --- --- --- Arg ---
#9         --- --- --- --- Thr --- --- --- --- --- --- --- --- --- Arg ---
           145
CYP2A6     Ile Glu Glu Arg Ile Gln Glu Glu Ala Gly Phe Leu Ile Asp Ala Leu
#4         --- --- --- --- --- --- --- --- --- --- --- --- --- Glu Val ---
#6         --- --- --- --- --- --- --- --- --- --- --- --- --- Glu Val ---
#9         --- --- --- --- --- --- --- --- --- --- --- --- --- Glu Val ---
           161
CYP2A6     Arg Gly Thr Gly Gly Ala Asn Ile Asp Pro Thr Phe Phe Leu Ser Arg
#4         --- --- Thr Arg --- --- --- --- --- --- --- --- --- --- --- ---
#6         --- --- Thr Arg --- --- --- --- --- --- --- --- --- --- --- ---
#9         --- --- Thr Arg --- --- --- --- --- --- --- --- --- --- --- ---
```

```
          177
CYP2A6    Thr Val Ser Asn Val Ile Ser Ser Ile Val Phe Gly Asp Arg Phe Asp
#4        Thr --- --- --- --- --- --- Ser Ile --- --- --- --- --- --- ---
#6        Thr --- --- --- --- --- --- Ser Ile --- --- --- --- --- --- ---
#9        Thr --- --- --- --- --- --- Ser Ile --- --- --- --- --- --- ---
          193
CYP2A6    Tyr Lys Asp Lys Glu Phe Leu Ser Leu Leu Arg Met Met Leu Gly Ile
#4        --- Glu --- --- --- Phe --- --- --- Leu --- --- --- Leu --- Ser
#6        --- Glu --- --- --- Phe --- --- --- Leu --- --- --- Leu --- Ser
#9        --- Glu --- --- --- Phe --- --- --- Leu --- --- --- Leu --- Ser
          209
CYP2A6    Phe Gln Phe Thr Ser Thr Ser Thr Gly Gln Leu Tyr Glu Met Phe Ser
#4        --- --- --- Thr Ala --- Ser Met --- --- --- --- --- --- --- Pro
#6        --- --- --- Thr Ala --- Ser Met --- --- --- --- --- --- --- Pro
#9        --- --- --- Thr Ala --- Ser Met --- --- --- --- --- --- --- Pro
          225
CYP2A6    Ser Val Met Lys His Leu Pro Gly Pro Gln Gln Gln Ala Phe Gln Leu
#4        Ser --- --- --- --- --- --- Gly --- --- --- --- --- Phe Lys Glu
#6        Ser --- --- --- --- --- --- Gly --- --- --- --- --- Phe Lys Glu
#9        Ser --- --- --- --- Pro --- Gly --- --- --- --- --- Phe Lys Glu
          241
CYP2A6    Leu Gln Gly Leu Glu Asp Phe Ile Ala Lys Lys Val Glu His Asn Gln
#4        --- --- --- Leu Lys --- --- Val --- --- --- --- --- --- Ser ---
#6        --- --- --- Leu Lys --- --- Val --- --- --- --- --- --- Ser ---
#9        --- --- --- Leu Lys --- --- Val --- --- --- --- --- --- Ser ---
          257
CYP2A6    Arg Thr Leu Asp Pro Asn Ser Pro Arg Asp Phe Ile Asp Ser Phe Leu
#4        --- Thr --- --- --- --- --- --- --- --- --- Ile --- --- --- ---
#6        --- Thr --- --- --- --- --- --- --- --- --- Ile --- --- --- ---
#9        --- Thr --- --- --- --- --- --- --- --- --- Ile --- --- --- ---
          273
CYP2A6    Ile Arg Met Gln Glu Glu Glu Lys Asn Pro Asn Thr Glu Phe Tyr Leu
#4        --- --- Ile Arg --- --- --- --- --- --- His --- --- --- --- ---
#6        --- --- Ile Arg --- --- --- --- --- --- His --- --- --- --- ---
#9        --- --- Ile Arg --- --- --- --- --- --- His --- --- --- --- ---
          289
CYP2A6    Lys Asn Leu Val Met Thr Thr Leu Asn Leu Phe Ile Gly Gly Thr Glu
#4        Lys --- --- --- Leu --- --- Leu --- --- --- Phe Ala --- Thr ---
#6        Lys --- --- --- Leu --- --- Leu --- --- --- Phe Ala --- Thr ---
#9        Lys --- --- --- Leu --- --- Leu --- --- --- Phe Ala Gly Thr ---
          305
CYP2A6    Thr Val Ser Thr Thr Leu Arg Tyr Gly Phe Leu Leu Leu Met Lys His
#4        --- --- --- Thr --- --- --- --- --- --- Leu --- --- --- Asn ---
#6        --- --- --- Thr --- --- --- --- --- --- Leu --- --- --- --- ---
#9        --- --- --- Thr --- --- --- --- --- --- Leu --- --- --- Asn ---
          321
CYP2A6    Pro Glu Val Glu Ala Lys Val His Glu Glu Ile Asp Arg Val Ile Gly
#4        --- Glu --- --- --- --- --- --- --- --- --- --- --- --- --- ---
#6        --- Glu --- --- --- --- --- --- --- --- --- --- --- --- --- ---
#9        --- Glu --- --- --- --- --- --- --- --- --- --- --- --- --- ---
          337
CYP2A6    Lys Asn Arg Gln Pro Lys Phe Glu Asp Arg Ala Lys Met Pro Tyr Met
#4        --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- Thr
#6        --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- Thr
#9        --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- Thr
```

```
        353
CYP2A6  Glu Ala Val Ile His Glu Ile Gln Arg Phe Gly Asp Val Ile Pro Met
#4      --- Ala --- --- --- --- Met --- --- --- Ala --- Ile --- --- ---
#6      --- Ala --- --- --- --- Met --- --- --- Ala --- Ile --- --- ---
#9      --- Ala --- --- --- --- Met --- --- --- Ala --- Ile --- --- ---
        369
CYP2A6  Ser Leu Ala Arg Arg Val Lys Lys Asp Thr Lys Phe Arg Asp Phe Phe
#4      Gly Leu --- --- Arg --- Thr --- --- --- --- --- --- Asp --- Ile
#6      Gly Leu --- --- Arg --- Thr --- --- --- --- --- --- Asp --- Ile
#9      Gly Leu --- --- Arg --- Thr --- --- --- --- --- --- Asp --- Ile
        385
CYP2A6  Leu Pro Lys Gly Thr Glu Val Tyr Pro Met Leu Gly Ser Val Leu Arg
#4      --- --- --- --- --- --- --- Phe Pro Leu --- --- --- --- Leu ---
#6      --- --- --- --- --- --- --- Phe --- Leu --- --- --- --- Leu ---
#9      --- --- --- --- --- --- --- Phe --- Leu --- --- --- --- Leu ---
        401
CYP2A6  Asp Pro Ser Phe Phe Ser Asn Pro Gln Asp Phe Asn Pro Gln His Phe
#4      --- Ser Arg --- --- --- Asn --- Arg --- --- Asn --- --- --- ---
#6      --- Ser Arg --- --- --- Asn --- Arg --- --- Asn --- --- --- ---
#9      --- Ser Arg --- --- --- Asn --- Arg --- --- Asn --- --- --- ---
        417
CYP2A6  Leu Asn Glu Lys Gly Gln Phe Lys Lys Ser Asp Ala Phe Val Pro Phe
#4      --- Asp --- --- --- --- --- --- --- --- --- Ala --- --- --- Phe
#6      --- Asp --- --- --- --- --- --- --- --- --- Ala --- --- --- Phe
#9      --- Asp --- --- --- --- --- --- --- --- --- Ala --- --- --- Phe
        433
CYP2A6  Ser Ile Gly Lys Arg Asn Cys Phe Gly Glu Gly Leu Ala Arg Met Glu
#4      --- --- --- --- --- Tyr --- --- --- --- --- --- --- --- --- ---
#6      --- --- --- --- --- Tyr --- --- --- --- --- --- --- --- --- ---
#9      --- --- --- --- --- Tyr --- --- --- --- --- --- --- --- --- ---
        449
CYP2A6  Leu Phe Leu Phe Phe Thr Thr Val Met Gln Asn Phe Arg Leu Lys Ser
#4      --- --- Leu --- Leu --- --- Ile --- --- --- --- --- Phe --- Ser
#6      --- --- Leu --- Leu --- --- Ile --- --- --- --- --- Phe --- Ser
#9      --- --- Leu --- Leu --- --- Ile --- --- --- --- --- Phe --- Ser
        465
CYP2A6  Ser Gln Ser Pro Lys Asp Ile Asp Val Ser Pro Lys His Val Gly Phe
#4      Pro --- Ser --- --- --- Ile --- --- --- --- --- --- --- --- ---
#6      Pro --- Ser --- --- --- Ile --- --- --- --- --- --- --- --- ---
#9      Pro --- Ser --- --- --- Ile --- --- --- --- --- --- --- --- ---
        481
CYP2A6  Ala Thr Ile Pro Arg Asn Tyr Thr Met Ser Phe Leu Pro Arg
#4      Gly Thr --- --- Pro --- --- --- --- --- --- --- --- ---
#6      Gly Thr --- --- Pro --- --- --- --- --- --- --- --- ---
#9      Gly Thr --- --- Pro --- --- --- --- --- --- --- --- ---
```

of CYP2A13 has been isolated. Fernandez-Salguero *et al.* (1995) showed that the gene presented a typical *CYP2* subfamily structure consisting of 9 exons. The intron/exon boundaries were located and the amino acid sequence of the protein was deduced by computer analysis. Table 3.2 shows the percentage amino acid identities between the three marmoset CYP2A and the three human CYP2A protein sequences.

| Protein sequence | % amino acid identity | | |
|---|---|---|---|
| | CYP2A6 | CYP2A7 | CYP2A13 |
| #4 marmoset CYP2A | 88.2 (58) | 86.2 (68) | 88.2 (58) |
| #6 marmoset CYP2A | 88.9 (55) | 86.8 (65) | 88.9 (55) |
| #9 marmoset CYP2A | 88.2 (58) | 86.2 (68) | 88.2 (58) |

Table 3.2: This table illustrates the percentage amino acid identities (to 1 decimal place) between the three marmoset CYP2A protein sequences and those of the human CYP2A6 (Yamano *et al.*, 1990), CYP2A7 (Yamano *et al.*, 1990) and CYP2A13 (Fernandez-Salguero *et al.*, 1995) protein sequences. The figures in brackets indicate the number of amino changes between the two protein sequences.

The three marmoset CYP2As showed the same percentage identity of protein sequence to CYP2A6 and CYP2A13. In order to confirm which human CYP2A the marmoset CYP2As were most closely related to, the protein sequences of the marmoset CYP2As were examined for potential coumarin hydroxylase activity.

Purified human CYP2A6 catalyses coumarin 7-hydroxylation (Yun *et al.*, 1991). Negishi *et al.* (1989) have characterised allelic variants of mouse CYP2A5, CYP2A5-coh$^h$ and CYP2A5-coh$^l$, having high and low coumarin 7-hydroxylase activity respectively. The allelic variants differ at only a single nucleotide associated with codon 117 that leads to an amino acid substitution of Val→Ala in CYP2A5-coh$^l$. Lindberg and Negishi (1989) showed that amino acid positions 209 and 365 were also important for coumarin hydroxylase activity in site-directed mutagenesis studies of mouse CYP2A4 and CYP2A5.

The deduced amino acid sequence of the human CYP2A6 cDNA cloned in our laboratory showed Val at position 117. This implies the expressed protein would be expected to show coumarin 7-hydroxylase activity; this was confirmed in the PhD. Thesis of Manoj Nanji (1995).

The protein sequence of CYP2A13 (Fernandez-Salguero *et al.*, 1995) shows Ala at position 117, implying that CYP2A13 will show low coumarin hydroxylase activity. An additional amino acid change at position 209 of Phe→Arg in CYP2A13 could also lower the catalytic activity of CYP2A13 towards coumarin. The above research group are currently attempting to isolate the cDNA for CYP2A13. Once this has been isolated, it is presumed that the protein will be expressed and tested for coumarin hydroxylase activity.

The three marmoset CYP2A deduced protein sequences show Ala at position 117, the amino acids at positions 209 and 365 are identical to CYP2A6. This would imply that the expressed proteins would show low coumarin 7-hydroxylase activity, intermediate between that shown by CYP2A6 and that expected to be shown by CYP2A13.

The three marmoset CYP2As show equal identity in protein sequences to CYP2A6 and CYP2A13 but are expected to show a different coumarin hydroxylase activity to the two human CYP2As.

### 3.3.5. COMPARISON OF THE N-TERMINAL AMINO ACID SEQUENCES OF CYP2As FROM A VARIETY OF SPECIES

The first twenty amino acids of human and non-human CYP2A sequences were compared and are shown in figure 3.12. It may be concluded from figure 3.12 that the marmoset, monkey and baboon CYP2As are more similar to the human CYP2As than mouse or rat CYP2As. This indicates that non-human primates may provide a better animal model for drug toxicology studies than rodents.

The nucleotide sequences of three more marmoset CYP2A cDNA clones will be determined by another member of our laboratory. Once two marmoset CYP2A cDNA clones have been shown to have identical cDNA sequences, one of these CYP2As will be expressed in *Sf9* cells using the baculovirus expression system. The expressed protein will be purified and characterised.

|  | 1 |  |  |  |  |  |  |  |  |  |  |  |  |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Human 2A6 | Met | Leu | Ala | Ser | Gly | Met | Leu | Leu | Val | Ala | Leu | Leu | Val |
| Human 2A7 | --- | --- | --- | --- | --- | Leu | --- | --- | --- | --- | --- | --- | Ala |
| Human 2A13 | --- | --- | --- | --- | --- | Leu | --- | --- | --- | Thr | --- | --- | Ala |
| Marmoset 2As | --- | Val | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | Ala |
| Baboon F1 | --- | --- | --- | --- | --- | Leu | --- | --- | --- | --- | --- | --- | Ala |
| Monkey CMLb | --- | --- | --- | --- | --- | Leu | --- | --- | --- | --- | --- | --- |  |
| Mouse 2A4, 2A5 | --- | --- | Thr | --- | --- | Leu | --- | --- | --- | --- | Ala | Val | Ala |
| Rat 2A1 | --- | --- | Asp | Thr | --- | Leu | --- | --- | --- | Val | Ile | --- | Ala |
| Rat 2A2 | --- | --- | Asp | Thr | --- | Leu | --- | --- | --- | Val | Ile | --- | Ala |
| Rat 2A3 | --- | --- | --- | --- | --- | Leu | --- | --- | --- | --- | Ser | Val | Ala |

|  | 14 |  |  |  |  |  |  |
|---|---|---|---|---|---|---|---|
| Human 2A6 | Cys | Leu | Thr | Val | Met | Val | Leu |
| Human 2A7 | --- | --- | --- | --- | --- | --- | --- |
| Human 2A13 | --- | --- | --- | --- | --- | --- | --- |
| Marmoset 2As | --- | --- | --- | --- | --- | --- | --- |
| Baboon F1 | XXX | --- | --- | --- | --- | --- | --- |
| Mouse 2A4, 2A5 | Phe | --- | Ser | --- | Leu | --- | --- |
| Rat 2A1 | Ser | --- | Ser | --- | Met | Leu | --- |
| Rat 2A2 | Ser | --- | Ser | --- | Met | Phe | --- |
| Rat 2A3 | Phe | --- | Ser | --- | Leu | --- | --- |

Figure 3.12: Alignment of the N-terminal amino acid sequences of CYP2As from a variety of species.

CYP2A6 and CYP2A7 sequence were compiled from Yamano *et al.* (1990); CYP2A13 from Fernandez-Salguero *et al.* (1995); baboon F1 from Dalet-Beluche *et al.* (1992); mouse CYP2A4 and CYP2A5 from Lindberg *et al.* (1989); rat CYP2A1 and CYP2A2 from Matsunaga *et al.* (1990); rat CYP2A3 from Ueno and Gonzalez (1990). Amino acid 14 in the baboon F1 sequence was unable to be positively identified by Edman degradation analysis (Dalet-Beluche *et al.*, 1992).

## *CHAPTER FOUR*

# CHARACTERIZATION OF A HUMAN HEPATOCYTE CULTURE SYSTEM

The major aim of this thesis was to produce monoclonal antibodies to CYP2A6 and CYP3A4. These antibodies will be used as immunological tools, particularly for studying the expression of these isoforms in a human hepatocyte culture system. In preparation for the use of these tools, the culture system was characterized with polyclonal antibodies that recognise all members of the CYP2A and CYP3A subfamily. This will enable a direct comparison to be made between the use of monoclonal and polyclonal antibodies. Interesting information regarding potential drug-xenobiotic and drug-drug interactions was gained from the characterization of the cell culture system with polyclonal antibodies raised to several different CYPs.

Primary toxicology studies for new pharmaceutical drugs for use by man are currently performed by *in vivo* testing using experimental animal models, in particular rodents. Human volunteers are given the drug in the next stage of clinical trials. As the safety demands of new drugs progressively increase, so does the consumption of experimental animals. An alternative to an animal model is the use of an *in vitro* system. As has been mentioned in section 1.9. and Chapter 3 there are species variations in drug metabolising enzymes leading to difficulty in extrapolating data from animal studies to man. Although a rat culture system provides an alternative to rodent *in vivo* experimentation, a human hepatocyte culture system would provide a better *in vitro* model for man.

## 4.1. HUMAN HEPATOCYTE CULTURE SYSTEM

A human hepatocyte culture system has been set up in the laboratory of Prof. Vera Rogiers at the Vrije Universiteit in Brussels. Hepatocytes are isolated from a piece of liver obtained from a patient undergoing a partial hepatectomy procedure or from an individual who has just died. The liver sample is subjected to a two-step collagenase perfusion; aliquots of $1 \times 10^7$ cells are washed twice with PBS, each aliquot is plated on a 15 cm diameter Petri dish in 25 ml of media. An aliquot of cells from the day of isolation is stored at -80$^{o}$C until further use.

The day of isolation of hepatocytes is designated day 0. The cells are incubated for 24 hours in T0 media (75% minimal essential medium and 25% medium 199 containing 1 mg/ml BSA, 10 μg/ml bovine insulin, 10% foetal bovine serum, 2.2 mg/ml $NaHCO_3$, 7.3 international units/ml benzyl penicillin, 50 μg/ml streptomycin sulphate, 50 μg/ml kanamycin monosulphate and 10 μg/ml sodium ampicillin).

From day 1 the human hepatocytes are cultured under three different conditions: untreated (control), supplemented with 2 mM phenobarbital (PB) or supplemented with 200 μg/ml of valproic acid (VP). T0 media supplemented with 7.1-5 M hydrocortisone hemisuccinate is given daily to the cells from day 1 of culture. The media is further supplemented with either phenobarbital or valproic acid where necessary.

A sample of each of the three cultures, control, phenobarbital-treated, and valproic acid-treated, is harvested at days 4 and 7 of culture. The cells are washed in PBS and sedimented by centrifugation at 5,000 x g for 5 minutes. The cultured hepatocyte pellets were sent on dry ice, to myself, for further analysis. Figure 4.1 shows a schematic diagram of the isolation of human hepatocytes from liver.

A major problem is the dedifferentiation of the hepatocytes during culturing. Attempts to minimise this process include i) the use of a serum free hormonally defined media (Enat *et al.*, 1984) supplemented with hydrocortisone [glucocorticoids have been shown to be important in the maintenance of the hepatocyte differentiated state] (Sinclair *et al.*, 1990); ii) plating cells on a type I collagen-coated tissue culture plate (Waxman *et al.*, 1990); iii) co-culturing hepatocytes with primitive biliary epithelial cells (Bégué *et al.*, 1984). These conditions attempt to mimic, as far as possible, the natural environment of the hepatocytes in the liver.

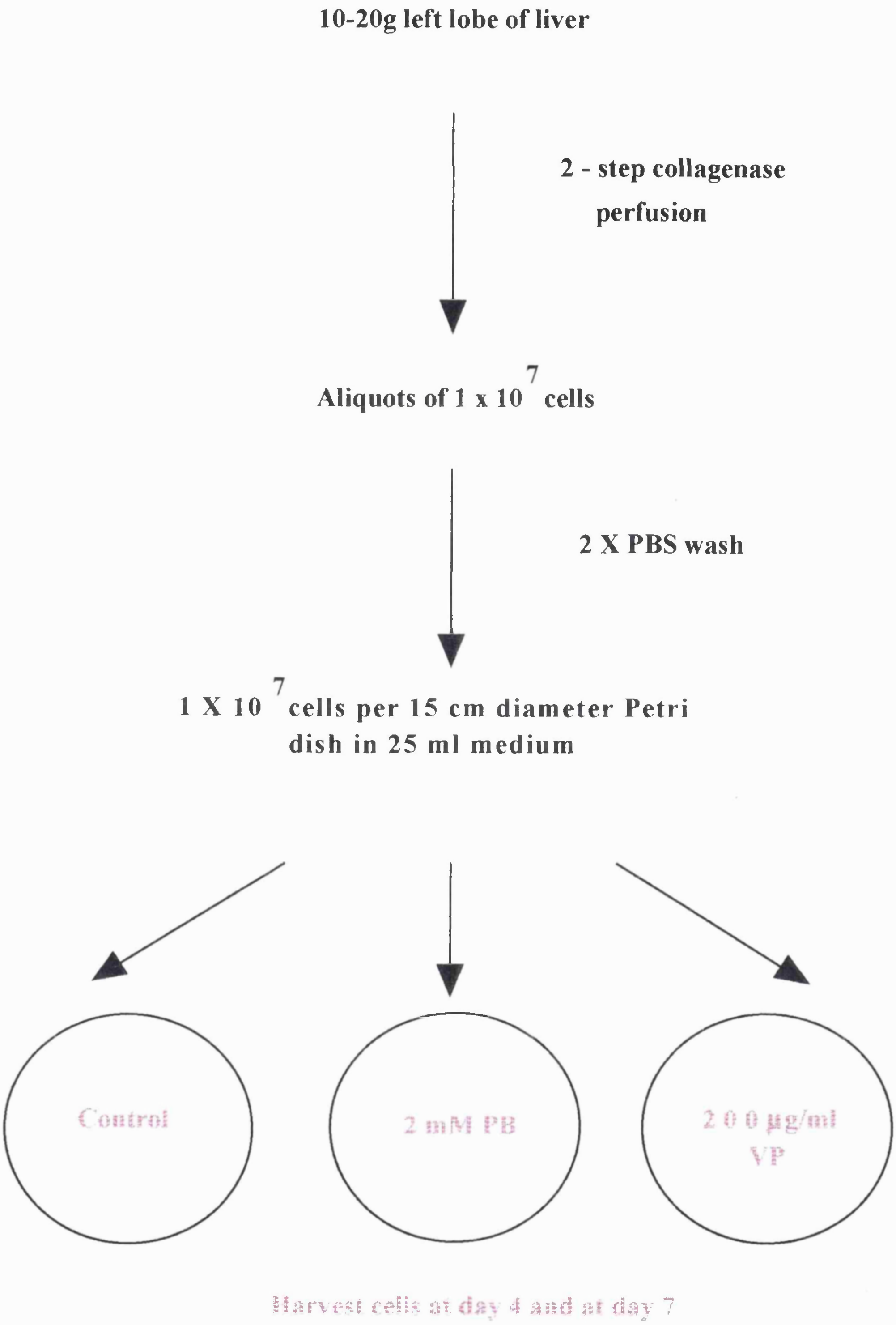

10-20g left lobe of liver
2 - step collagenase perfusion
Aliquots of 1 x 10^7 cells
2 X PBS wash
1 X 10^7 cells per 15 cm diameter Petri dish in 25 ml medium
Control
2 mM PB
2 0 0 µg/ml VP
Harvest cells at day 4 and at day 7

Figure 4.1: Schematic diagram of the isolation of human hepatocytes from liver.

The human hepatocytes are cultured under three different conditions: untreated (control), supplemented with 2 mM phenobarbital (PB) or supplemented with 200 µg/ml valproic acid.

After a few days of culturing, hepatocytes should still be their native hexagonal shape and bile canaliculi should be able to be distinguished by observation with a microscope. The bile canaliculi drain secreted bile from the hepatocytes to the bile ducts in a liver section. They are simply spaces between adjacent liver cells and can be used as a differentiation marker. Figure 4.2 shows photographs of control, phenobarbital-treated and valproic acid-treated human hepatocytes taken after five days of culturing using a camera attached to a light microscope. The hepatocytes shown in the three photographs are hexagonal in shape and bile canaliculi can be distinguished. The phenobarbital-treated culture appears to have more cells than the other two types of culture. This could be due to hepatocyte proliferation or that phenobarbital, in someway, increases the lifetime of the cells. Studies conducted during the 1960s suggested that phenobarbital produces liver enlargement due to both hyperplasia and hypertrophy of the liver cells (Ruttimann, 1972). The hepatocytes that have been cultured in the presence of valproic acid are hexagonal in shape but there is evidence of cell death having occurred. This is probably due to the known toxic effect of valproic acid on these cells (section 1.10.2.).

## 4.2. CHARACTERIZATION OF THE HUMAN HEPATOCYTE CULTURE SYSTEM

Whole cell extracts were prepared from hepatocyte cultures from four different individuals, A, B, C, and D. The expression of CYP2As, CYP3As, CYP2B6, CYP2Cs, hGSTP1-1 and NADPH-cytochrome P450-reductase (P450-reductase) were analysed by western blotting analysis, using polyclonal antibodies. The data for two individuals, individual A and B, are shown in the following sections.

Several factors must be kept in mind when interpreting results from experiments using human cultures. There is inter-individual variation in the expression of CYPs in humans; individuals have variable medical histories and social habits such as drinking and smoking. The medical histories of individuals A and B are shown in table 4.1.

C

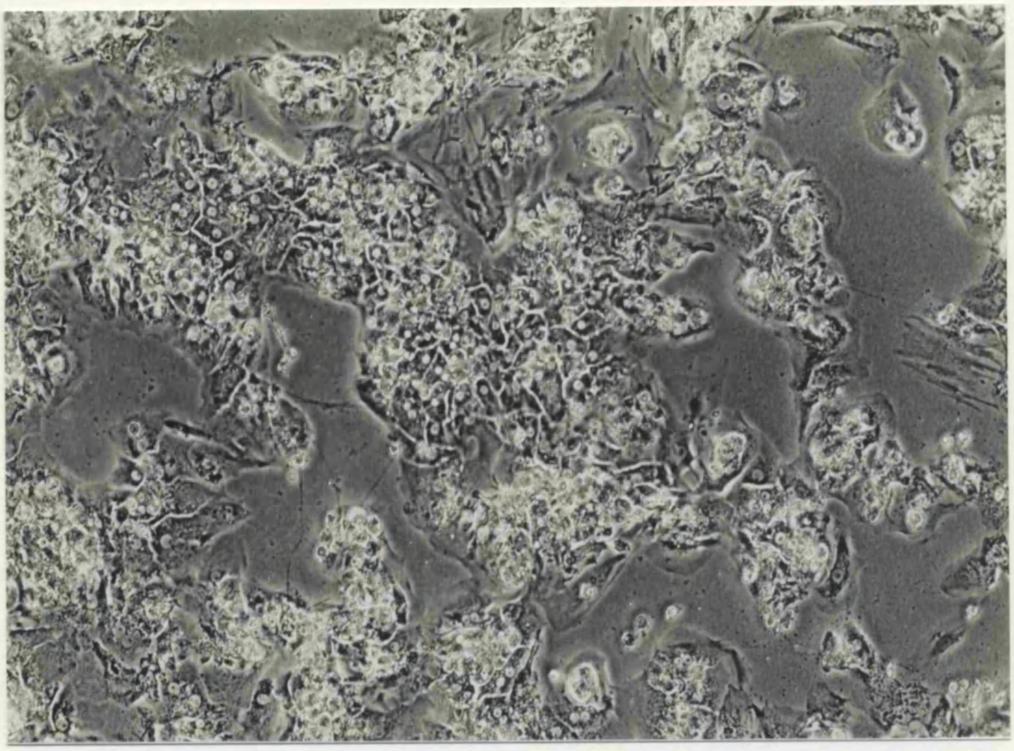


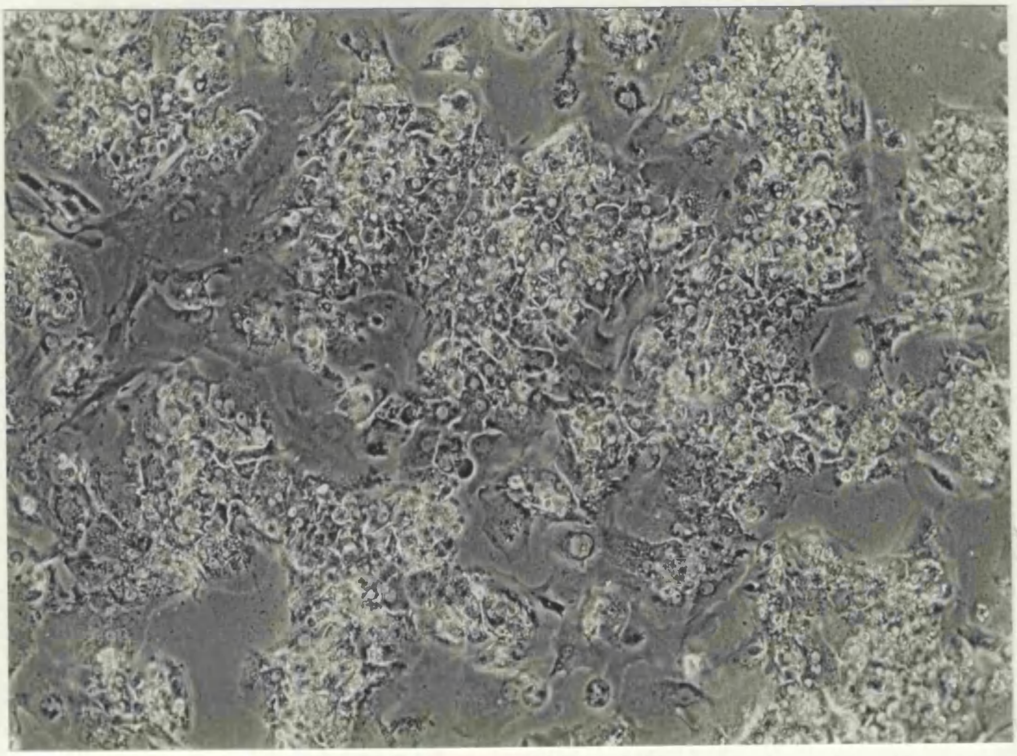
Figure 4.2: Photographs of control, phenobarbital-treated and valproic acid-treated human hepatocytes taken after five days of culturing.

**A**: Control human hepatocytes.

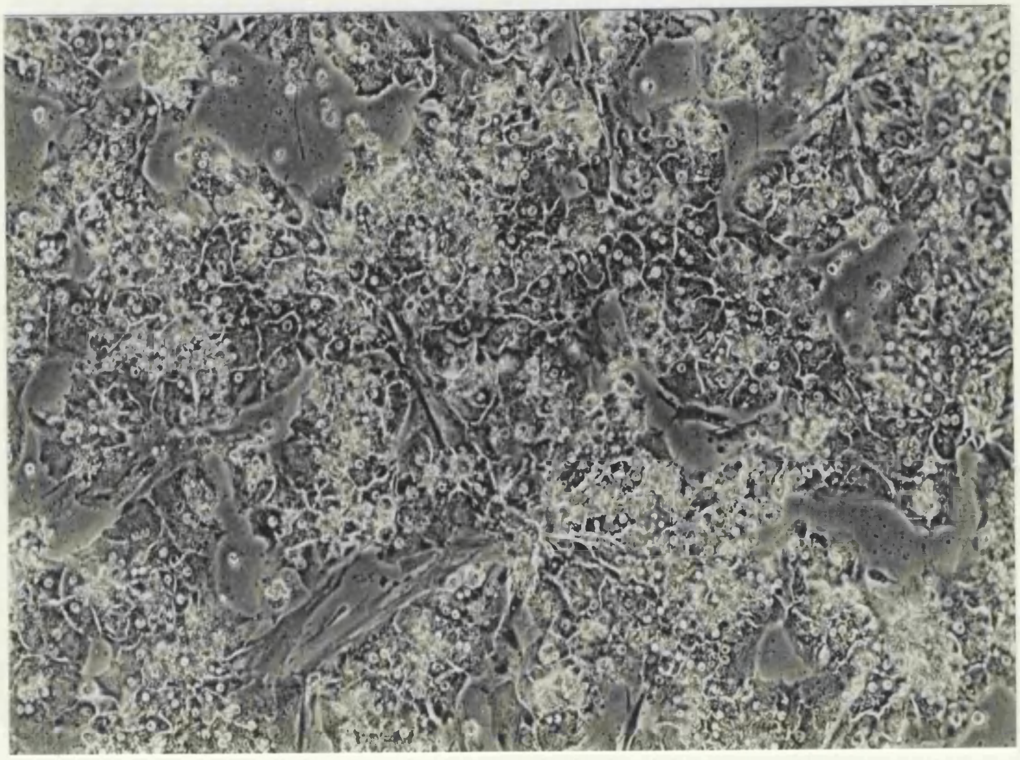
**B**: Cultured human hepatocytes supplemented with 2 mM phenobarbital.

**C**: Cultured human hepatocytes supplemented with 200 μg/ml valproic acid.

A



B



| | Individual A | Individual B |
|---|---|---|
| **Age**: | 45 years | 37 years |
| **Sex**: | female | male |
| **Cause of death**: | stroke | subarachnoid bleeding following an aneurism |
| **Drugs**: | dopamine<br>minotop<br>nodropil<br>lepakine<br>lasix<br>geloplasma<br>solumedrol<br>humiline regular<br>thyroxine | zantac<br>nesdanal<br>amucin<br>zinacef<br>dolacin<br>minotop<br>dobutrex<br>lexophed<br>mannitol<br>delakine |

Table 4.1: Medical histories of individuals A and B.

The age, sex, cause of death and therapeutic drugs administered to each patient are listed in the above table.

It has already been mentioned in section 4.1. that the human hepatocytes were cultured under three different conditions: untreated (control), supplemented with 2 mM phenobarbital (PB) or supplemented with 200 μg/ml of valproic acid (VP). Comparison of the expression of CYPs between the control sample and those treated with the anti-epileptic agents, phenobarbital or valproic acid, at the same day of culture (4 or 7) may provide information on potential drug-xenobiotic and drug-drug interactions.

### 4.2.1. EXPRESSION OF CYP2As IN A HUMAN HEPATOCYTE CULTURE SYSTEM

The expression of the CYP2As were investigated as CYP2A6, the major expressed isoform of this subfamily in adult human liver, has been shown to activate several compounds to cytotoxic and mutagenic products (Crespi *et al.*, 1990). Hence, any induction of expression of the CYP2As by phenobarbital or valproic acid might increase the susceptibility of epilepsy sufferers to these dangerous chemicals.

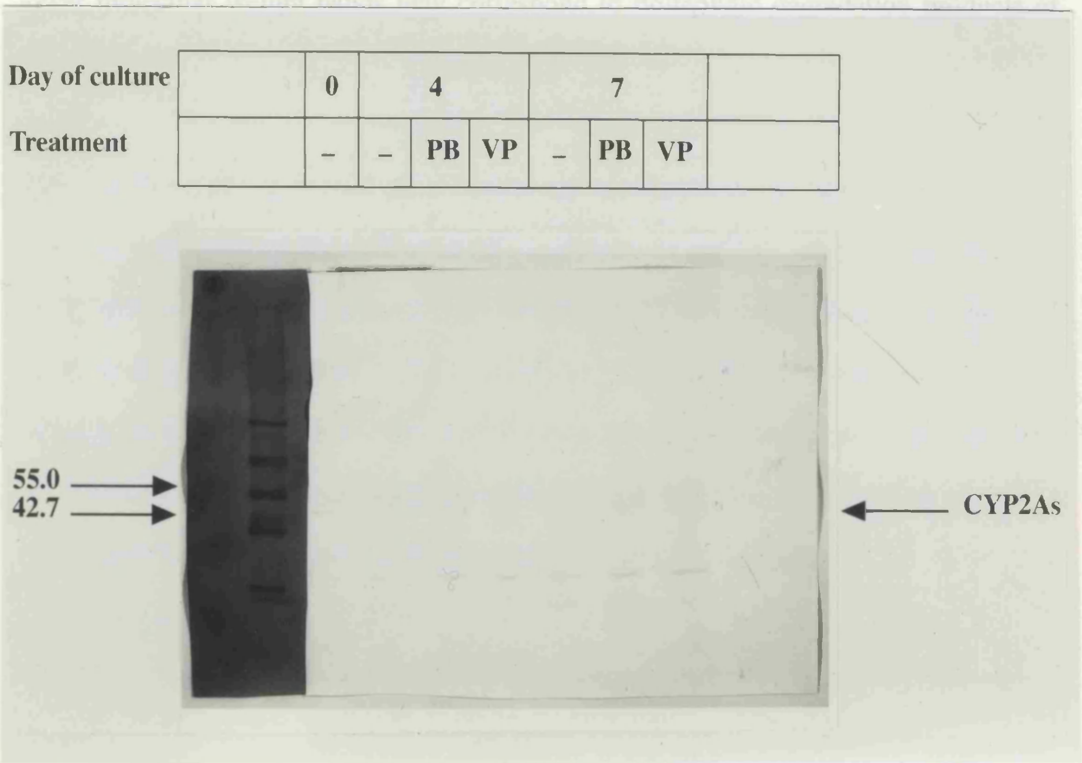
Whole cell homogenates were prepared from each culture sample for individual A and B. 10 μg and 20 μg of whole cell homogenates, for each individual, were analysed for the expression of CYP2As by western blotting analysis using an antisera to baboon CYP2A (1 in 2,000 dilution) that had been raised in a sheep. This antibody had been prepared in the laboratory of Dr. Patrick Maurel by Dr. C. Bonfils (Dalet-Beluche *et al.*, 1992) and a sample was kindly given to our laboratory. The polyclonal antibody cross-reacts with the human CYP2As but is unable to distinguish between the isoforms of the CYP2A subfamily. The blots were developed by using an alkaline phosphatase conjugated donkey anti-sheep immunoglobulin G (IgG; 1 in 30,000 dilution). The photographs of the blots for individual A (20 μg whole cell homogenate) and individual B (10 μg whole cell homogenate) are shown in figure 4.3. The CYP2As were detected as a band at the expected apparent molecular weight of 49,000. Other bands are also visible but the identities of the higher molecular weight proteins are not known. The

Figure 4.3: Western blot analysis of hepatocyte homogenate samples from cultures derived from individuals A and B using a polyclonal antisera to CYP2As.

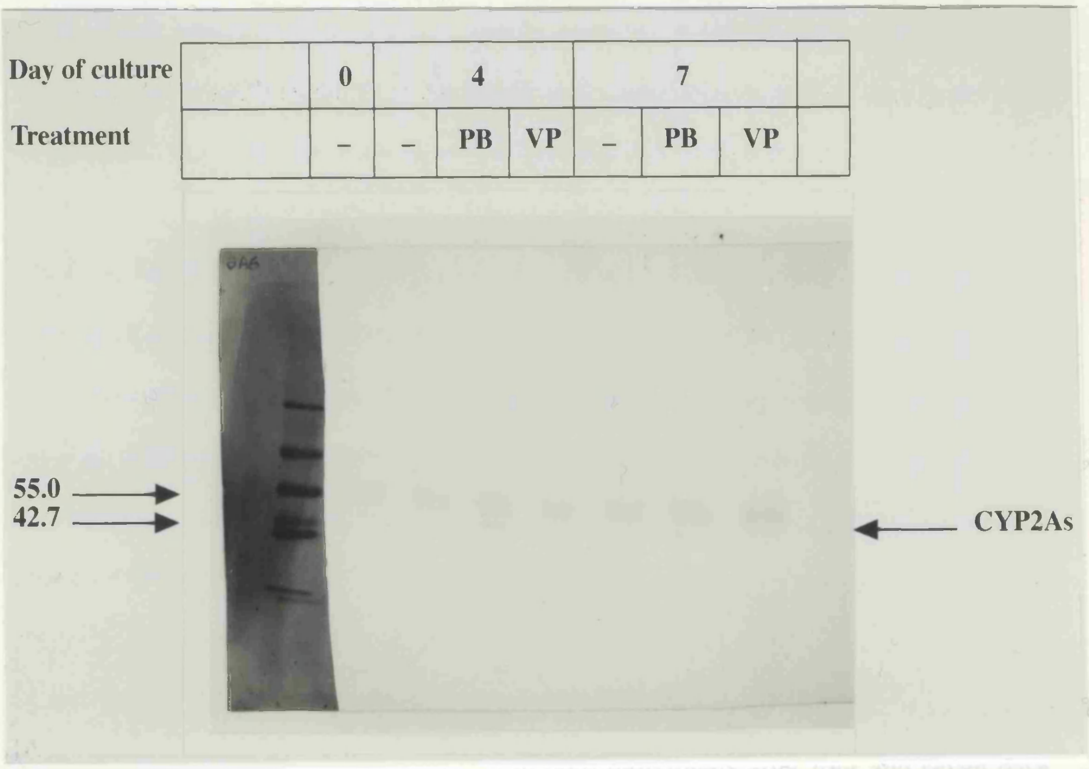
**A**: 20 µg of hepatocyte homogenate from the culture samples derived from individual A were electrophoresed through a 7% SDS-PAGE gel and transferred to nitrocellulose. The blot was probed with an antisera to baboon CYP2A (1 in 2,000 dilution) that had been raised in a sheep. The blot was developed by using an alkaline phosphatase conjugated donkey anti-sheep IgG (1 in 30,000 dilution). 2.5 µg of Promega mid-range molecular weight protein standards are shown x $10^{-3}$ in the first lane. The other lanes contain whole cell homogenate. The day of culture (0, 4 or 7) and culture conditions: control (-), phenobarbital-treated (PB) or valproic acid-treated (VP) are indicated above each lane.

**B**: As for figure **A** except that 10 µg of hepatocyte homogenate from the culture samples derived from individual B are shown.

**A**


Day of culture
0
4
7
Treatment
–
–
PB
VP
–
PB
VP
55.0
42.7
CYP2As

**B**


Day of culture
0
4
7
Treatment
–
–
PB
VP
–
PB
VP
55.0
42.7
CYP2As

lower molecular weight bands may correspond to proteolytic degradation products of the CYP2As.

The four blots, 10 μg and 20 μg of hepatocyte homogenates for individuals A and B, were scanned using a BIO-RAD GS-670 densitometer and the results were analysed using Molecular Analyst software. An absorbance value (relative units) was obtained for each hepatocyte sample. The absorbance values (relative units) for the 20 μg samples of control, phenobarbital and valproic acid-treated hepatocyte homogenates (individual A) and the 10 μg samples (individual B) were plotted against day of culture and inducer present. The results are presented graphically in figure 4.4.

The fold inductions of expression of the CYP2As in the hepatocyte homogenates treated with either phenobarbital or valproic acid were calculated relative to the control sample at the appropriate day of culture using the absorbance values (relative units). The average fold induction between the 10 μg and 20 μg of each phenobarbital and valproic acid-treated hepatocyte homogenate was calculated for each individual. The results are presented in table 4.2. Although it is interesting to look at fold inductions, no firm conclusions can be made as will be discussed in section 4.3.

It must be noted that it is not possible to carry out statistical analysis of these results and of those in the following sections. This is because the results are from two separate individuals who have different medical histories (table 4.1) and genetic identities. The two blots analysed for each individual are also unable to be statistically compared. This is because the experiments were carried out with different amounts of sample at separate times and were subject to unequal lengths of colour development.

As can be seen from figure 4.4 and table 4.2, both phenobarbital and valproic acid induce expression of the human CYP2As in both individuals after four and seven days of hepatocyte culture (3 and 6 days treatment with the anti-epileptic drugs,

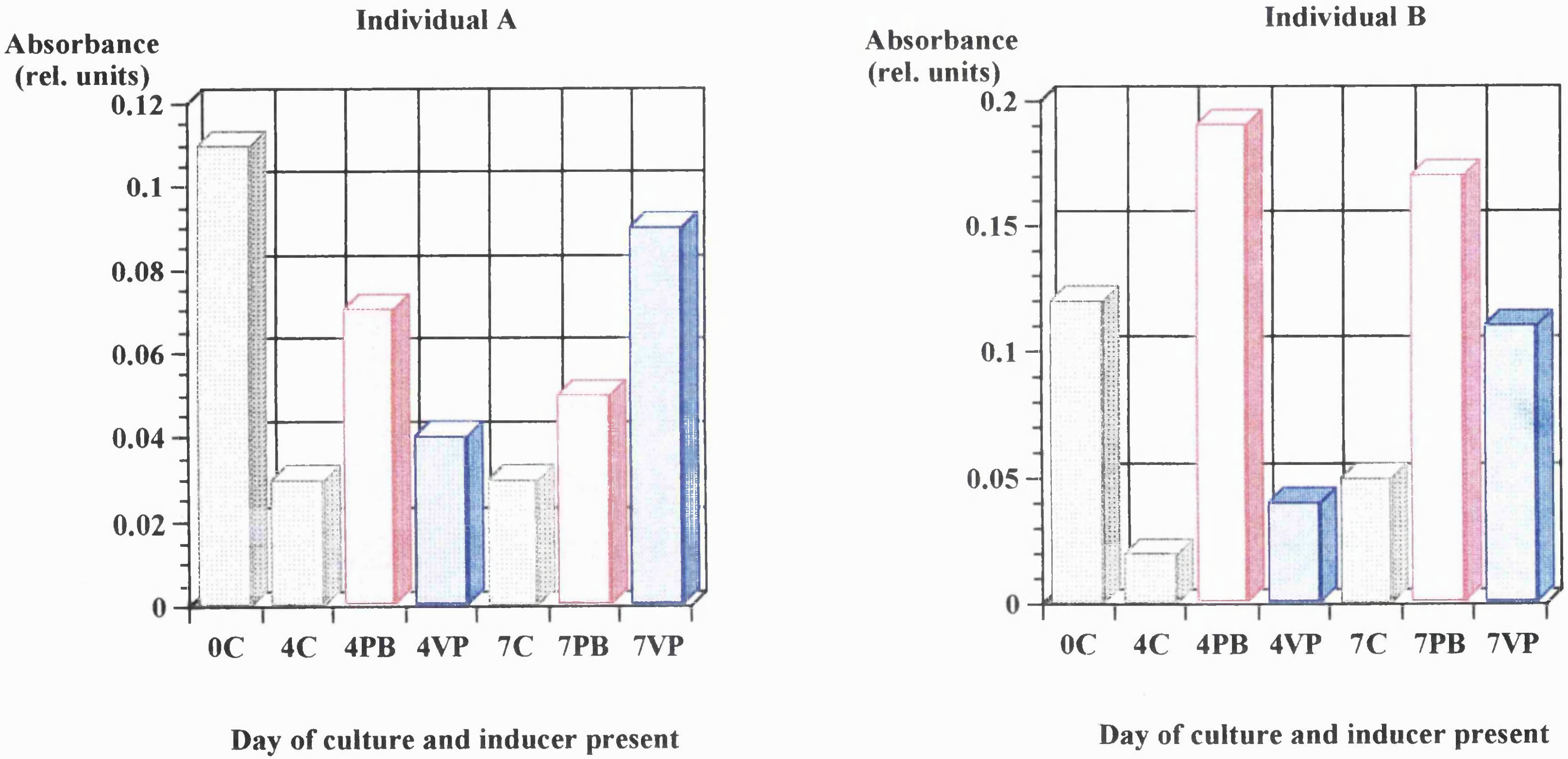


Figure 4.4: Induction of the expression of CYP2As by phenobarbital (PB) and valproic acid (VP).

The above graphs show the absorbance in relative (rel.) units of each control, PB- or VP-treated hepatocyte homogenate sample plotted against day of culture and inducer present.

| Individual | Day of culture | Inducer present | Average fold induction of expression of human CYP2As |
|---|---|---|---|
| A | 4 | phenobarbital | 2.23 |
| | 4 | valproic acid | 1.04 |
| | 7 | phenobarbital | 1.87 |
| | 7 | valproic acid | 2.77 |
| B | 4 | phenobarbital | 4.79 |
| | 4 | valproic acid | 1.33 |
| | 7 | phenobarbital | 2.09 |
| | 7 | valproic acid | 1.75 |

Table 4.2: Average fold induction of expression of the human CYP2As in the hepatocyte homogenate samples treated with either phenobarbital or valproic acid relative to the control sample at the appropriate day of culture.

The fold induction of expression of the human CYP2As in the hepatocyte homogenates treated with either phenobarbital or valproic acid was calculated relative to the control sample at the appropriate day of culture using the absorbance values (relative units) for all samples (10 μg and 20 μg of whole cell extracts for individuals A and B). The average fold induction was calculated for each phenobarbital and valproic acid-treated hepatocyte homogenate using the two values of fold induction already obtained.

respectively). Phenobarbital induced expression of the CYP2As to a greater extent after four days of culture whereas valproic acid produced a greater response after seven days. The above results were observed for both individuals A and B. The amount of expression of the CYP2As in the control sample markedly decreased, in both individuals, after four days of culture. The amount of expression was maintained in the day 7 control sample for individual A and was slightly elevated in individual B. This will be further discussed in section 4.3.

The results of these experiments are supported by other research groups work. Phenobarbital has been observed to increase coumarin hydroxylase activity in mice (Raunio *et al.*, 1988b), cynomologus monkeys (Pearce *et al.*, 1992) and in cultured human hepatocytes (Maurice *et al.*, 1991). The baboon P450 F1, that is closely related to the human CYP2A subfamily, is also inducible by phenobarbital (Dalet-Beluche *et al.*, 1992). There is no comparable research for valproic acid.

### 4.2.2. EXPRESSION OF CYP2B6 IN A HUMAN HEPATOCYTE CULTURE SYSTEM

CYP2B6 is the only known member of the human CYP2B subfamily that produces a functionally active enzyme. The expression of this protein was examined as the rat CYP2Bs, CYP2B1 and CYP2B2, are greatly induced by phenobarbital and valproic acid (Rogiers *et al.*, 1995). CYP2B1 is involved in the desaturation of valproic acid to the $\Delta^4$-metabolite (Baillie, 1988). This compound is a potent inducer of microvesicular steatosis and an inhibitor of fatty acid β-oxidation (Kesterson *et al.*, 1984 and Granneman *et al.*, 1984). Valproic acid is often co-administered with phenobarbital. If CYP2B6 metabolises valproic acid and is induced by both these drugs, the potential hepatoxicity of valproic acid will be increased.

20 μg (individual A and B), 30 μg (individual B) and 40 μg (individual A) of whole cell homogenates were analysed for the expression of CYP2B6 by western blotting analysis using an antisera to rabbit CYP2B4 (1 in 1,200 dilution) that had been raised in a sheep. This antibody had been prepared in the laboratory of Dr. Patrick Maurel (Bonfils *et al.*, 1990) and a sample was kindly given to our laboratory. The polyclonal antibody cross-reacts with human CYP2B6. The blots were developed by using an alkaline phosphatase conjugated donkey anti-sheep IgG (1 in 30,000 dilution). The photographs of the blots for individual A (20 μg whole cell homogenate) and individual B (20 μg whole cell homogenate) are shown in figure 4.5. CYP2B6 was detected as a band at the apparent molecular weight of 49,000.

The 20 μg (individual A and B), 30 μg (individual B) and 40 μg (individual A) blots of whole cell homogenates were scanned using a BIO-RAD GS-670 densitometer and the results were analysed using Molecular Analyst software. An absorbance value (relative units) was obtained for each hepatocyte sample. The absorbance values (relative units) for the 20 μg samples of control, phenobarbital and valproic acid-treated hepatocyte homogenates (individual A) and the 20 μg samples (individual B) were plotted against day of culture and inducer present. The results are presented graphically in figure 4.6.

The average fold induction between the 20 μg and 40 μg of each phenobarbital and valproic acid-treated hepatocyte homogenate was calculated for individual A as in section 4.2.1. A similar calculation was performed for the 20 μg and 30 μg samples of individual B. The results are presented in table 4.3.

As can be seen from figure 4.6 and table 4.3, both phenobarbital and valproic acid induce expression of CYP2B6 in both individuals after four and seven days of hepatocyte culture (3 and 6 days treatment with the anti-epileptic drugs, respectively). The expression of CYP2B6 was induced the greatest amount by phenobarbital on day 4 of culture for individuals A and B. Whereas valproic acid induced the expression of

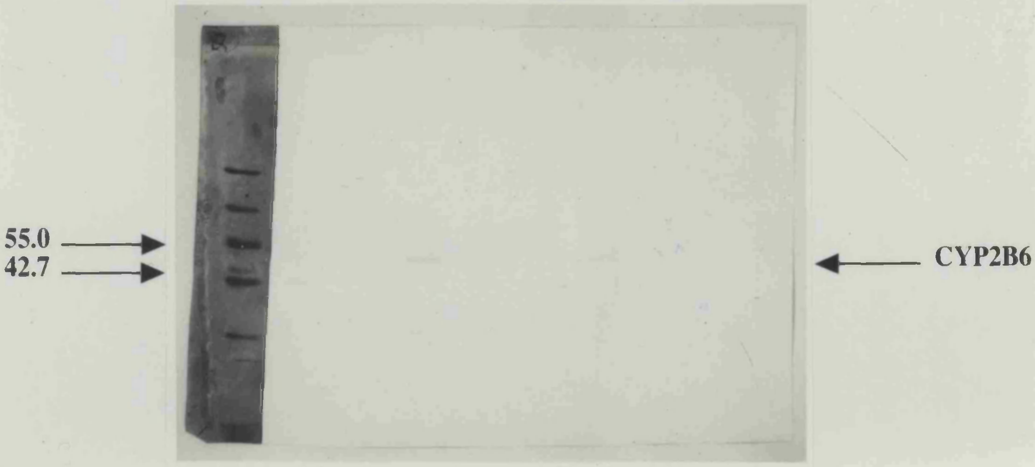
Figure 4.5: Western blot analysis of hepatocyte homogenate samples from cultures derived from individuals A and B using a polyclonal antisera to CYP2B4.

**A**: 20 µg of hepatocyte homogenate from the culture samples derived from individual A were electrophoresed through a 7% SDS-PAGE gel and transferred to nitrocellulose. The blot was probed with an antisera to rabbit CYP2B4 (1 in 1,200 dilution) that had been raised in a sheep. The blot was developed by using an alkaline phosphatase conjugated donkey anti-sheep IgG (1 in 30,000 dilution). 2.5 µg of Promega mid-range molecular weight protein standards are shown x $10^{-3}$ in the first lane. The other lanes contain whole cell homogenate. The day of culture (0, 4 or 7) and culture conditions: control (-), phenobarbital-treated (PB) or valproic acid-treated (VP) are indicated above each lane.

**B**: As for figure **A** except that 20 µg of hepatocyte homogenate from the culture samples derived from individual B are shown.

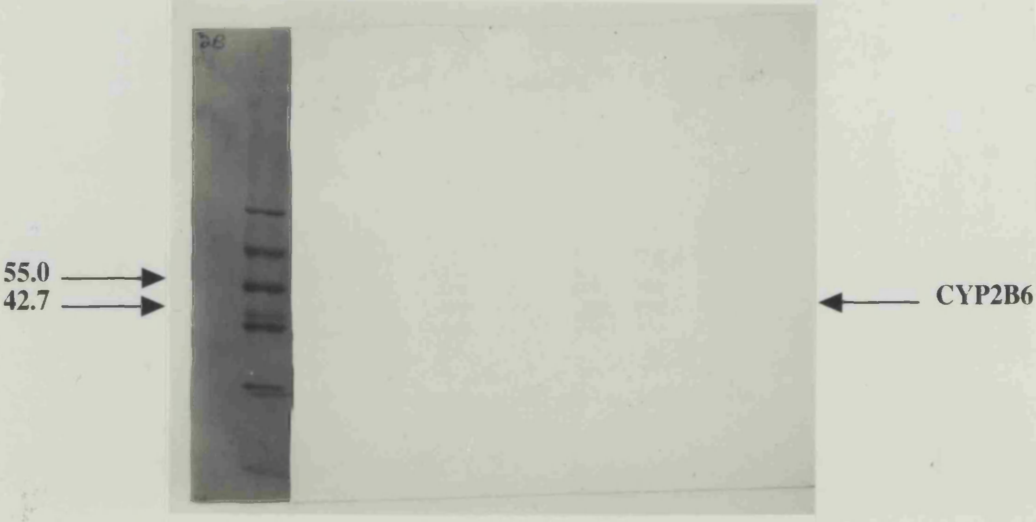
**A**

| Day of culture |  | 0 | 4 |  |  | 7 |  |  |  |
|---|---|---|---|---|---|---|---|---|---|
| Treatment |  | – | – | PB | VP | – | PB | VP |  |


55.0
42.7
CYP2B6

**B**

| Day of culture |  | 0 | 4 |  |  | 7 |  |  |  |
|---|---|---|---|---|---|---|---|---|---|
| Treatment |  | – | – | PB | VP | – | PB | VP |  |


55.0
42.7
CYP2B6

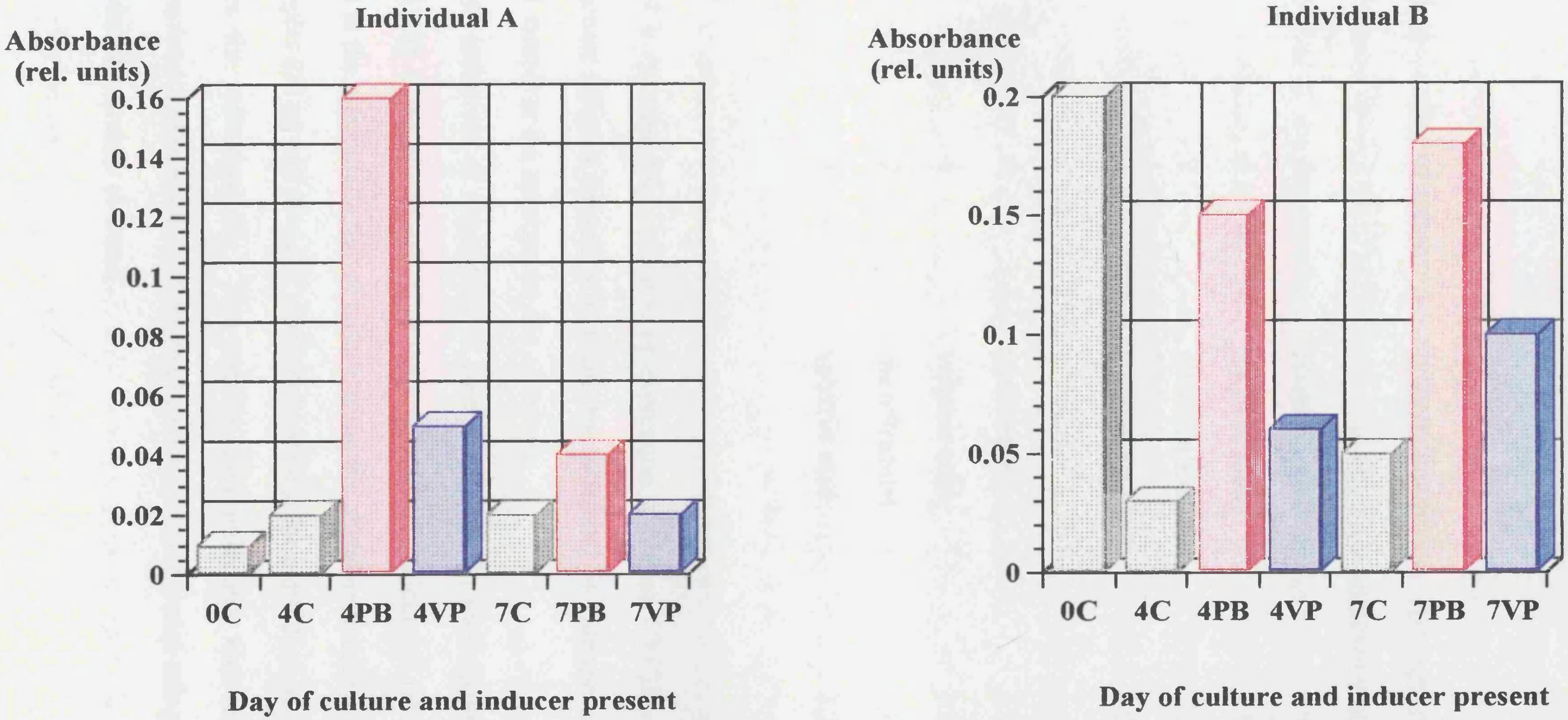


Figure 4.6: Induction of the expression of CYP2B6 by phenobarbital (PB) and valproic acid (VP).

The above graphs show the absorbance in relative (rel.) units of each control, PB- or VP-treated hepatocyte homogenate sample plotted against day of culture and inducer present.

| Individual | Day of culture | Inducer present | Average fold induction of expression of human CYP2B6 |
|---|---|---|---|
| A | 4 | phenobarbital | 6.95 |
|  | 4 | valproic acid | 1.89 |
|  | 7 | phenobarbital | 2.14 |
|  | 7 | valproic acid | 2.04 |
| B | 4 | phenobarbital | 3.21 |
|  | 4 | valproic acid | 1.28 |
|  | 7 | phenobarbital | 2.89 |
|  | 7 | valproic acid | 2.24 |

Table 4.3: Average fold induction of expression of human CYP2B6 in the hepatocyte homogenate samples treated with either phenobarbital or valproic acid relative to the control sample at the appropriate day of culture.

The fold induction of expression of human CYP2B6 in the hepatocyte homogenates treated with either phenobarbital or valproic acid was calculated relative to the control sample at the appropriate day of culture using the absorbance values (relative units) for all samples (20 μg and 40 μg of whole cell extracts for individual A; 20 μg and 30 μg of samples for individual B). The average fold induction was calculated for each phenobarbital and valproic acid-treated hepatocyte homogenate using the two values of fold induction already obtained.

CYP2B6 by the greatest amount on day 7 of culture for individual B. No apparent induction in expression of CYP2B6 by valproic acid was observed for individual A in figure 4.6. However, table 4.3 shows that there was an induction of expression of this enzyme; this could be due to errors introduced in the scanning of the blots. There was a slight increase in expression of CYP2B6 after four days of culture in the control sample of individual A; the amount of expression was maintained in the day 7 sample. There was a marked drop in expression of CYP2B6 in the day 4 control sample compared to the day 0 control sample for individual B; the expression of CYP2B6 rose slightly in the day 7 control sample. It is possible that the drug regime of individual B resulted in the high amount of expression of CYP2B6 detected in the day 0 sample or conversely, the drug regime of individual A suppressed CYP2B6 expression.

Phenobarbital has been shown to induce CYP2B isoforms in mice (Huang *et al.*, 1976), rat (Ryan *et al.*, 1976; Phillips *et al.*, 1981) guinea-pig (Oguri *et al.*, 1991) and non-human primates (Ohmori *et al.*, 1993). The mRNA's of the CYP2B subfamily have been observed to be induced by phenobarbital and valproic acid in rat hepatocytes co-cultured with non-parenchymal cells (Akrawi *et al.*, 1993b; Rogiers *et al.*, 1992; Rogiers *et al.*, 1995).

### 4.2.3. EXPRESSION OF CYP2Cs IN A HUMAN HEPATOCYTE CULTURE SYSTEM

CYP2C19 may be one of the CYP isoforms involved in the aromatic hydroxylation of phenobarbital (Anderson and Levy, 1995). CYP2C9 may also be involved in the metabolism of valproic acid (Allan Rettie, personal communication). Induction of either of these enzymes by phenobarbital or valproic acid would have clinical implications for anti-epileptic drug polytherapy.

10 μg and 20 μg of whole cell homogenates for individual B were analysed for the expression of CYP2Cs by western blotting analysis using an antisera to human CYP2C9 (1 in 1,600 dilution) that had been raised in a rabbit. This antibody had been prepared in the laboratory of Dr. Allan Rettie and a sample was kindly given to our laboratory. The polyclonal antibody cross-reacts with the human CYP2Cs but is unable to distinguish between the isoforms of the CYP2C subfamily. The blots were developed by using an alkaline phosphatase conjugated goat anti-rabbit IgG (1 in 3,000 dilution). The photograph of the 20 μg blot for individual B is shown in figure 4.7. The CYP2Cs were detected as a band at the apparent molecular weight of 55,000. The identity of the low molecular weight protein observed in the day 0 control sample is unknown.

The western blot analysis of the whole cell extracts using the CYP2C antisera was the last experiment carried out in these series of experiments (sections 4.2.1.-4.2.6.) The hepatocyte homogenate samples from individual A had been used up in previous experiments. Individual A was therefore unable to be screened for CYP2C expression.

The blots were scanned using a BIO-RAD GS-670 densitometer and the results were analysed using Molecular Analyst software. An absorbance value (relative units) was obtained for each hepatocyte sample. The absorbance values (relative units) for the 10 μg and 20 μg samples of control, phenobarbital and valproic acid-treated hepatocyte homogenates for individual B were plotted against day of culture and inducer present. The results are presented graphically in figure 4.8.

The average fold induction between the 10 μg and 20 μg of each phenobarbital and valproic acid-treated hepatocyte homogenate was calculated for individual B as in section 4.2.1. The results are presented in table 4.4.

As can be seen from figure 4.8 and table 4.4, both phenobarbital and valproic acid markedly induce the expression of the CYP2Cs in individual B after seven days of

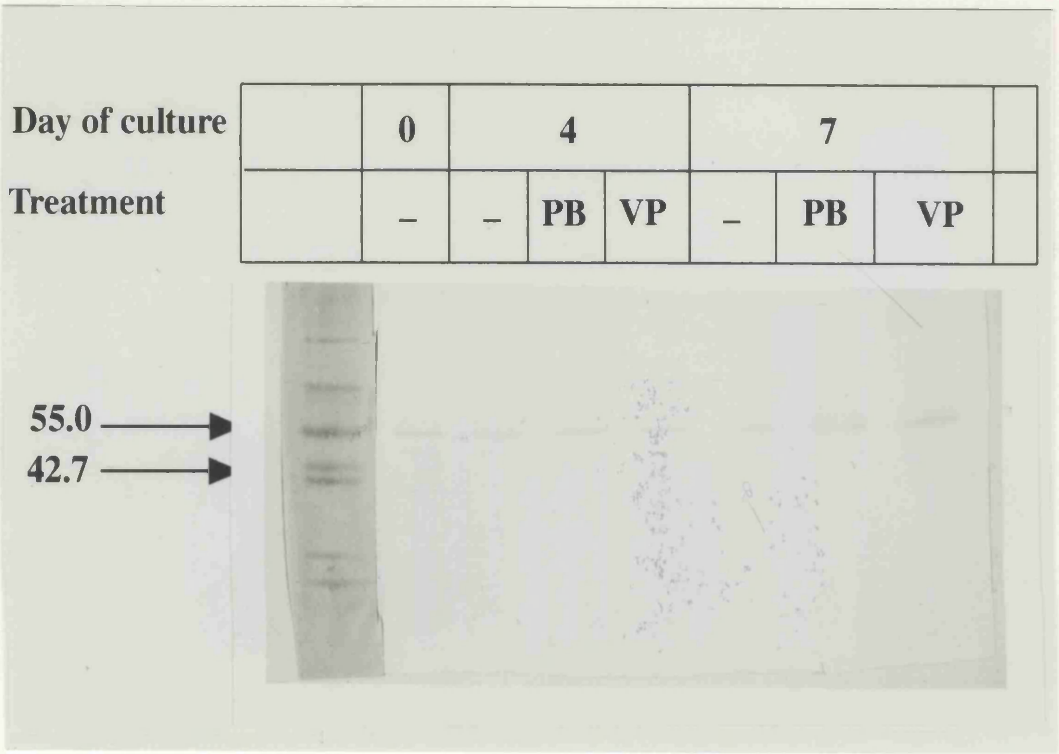


Figure 4.7: Western blot analysis of hepatocyte homogenate samples from a culture derived from individual B using a polyclonal antisera to CYP2Cs.

20 µg of hepatocyte homogenate from the culture samples derived from individual B were electrophoresed through a 7% SDS-PAGE gel and transferred to nitrocellulose. The blot was probed with an antisera to human CYP2C9 (1 in 1,600 dilution) that had been raised in a rabbit. The blot was developed by using an alkaline phosphatase conjugated goat anti-rabbit IgG (1 in 3,000 dilution). 2.5 µg of Promega mid-range molecular weight protein standards are shown x $10^{-3}$ in the first lane. The other lanes contain whole cell homogenate. The day of culture (0, 4 or 7) and culture conditions: control (-), phenobarbital-treated (PB) or valproic acid-treated (VP) are indicated above each lane.

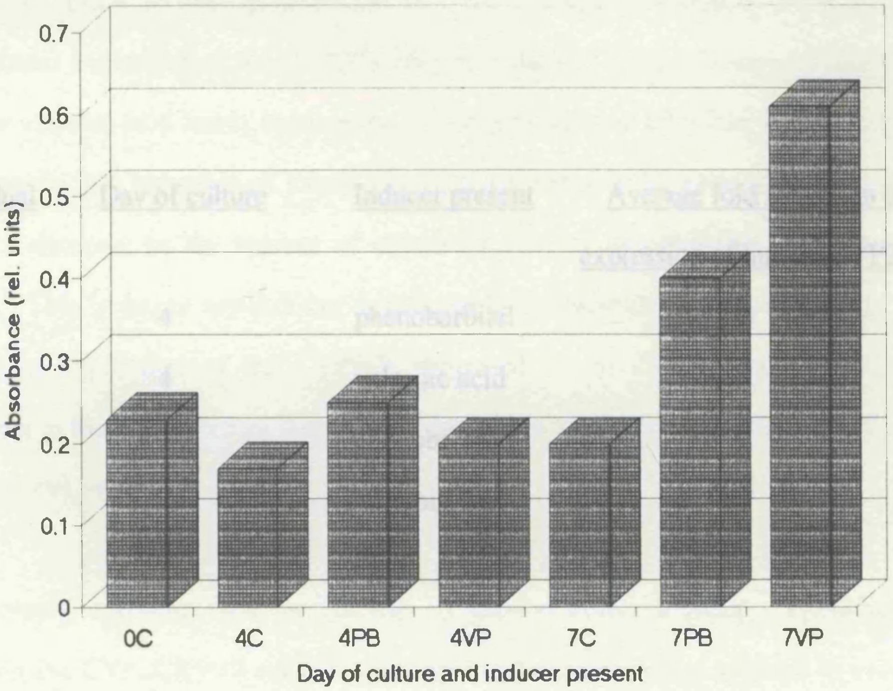


Fig 4.8: Induction of expression of the CYP2Cs by phenobarbital (PB) and valproic acid (VP).

The above graph shows the absorbance in relative (rel.) units of each control, PB- or VP-treated hepatocyte homogenate sample plotted against day of culture and inducer present.

| Individual | Day of culture | Inducer present | Average fold induction of expression of human CYP2Cs |
|---|---|---|---|
| B | 4 | phenobarbital | 1.38 |
|  | 4 | valproic acid | 0.75 |
|  | 7 | phenobarbital | 2.82 |
|  | 7 | valproic acid | 5.07 |

Table 4.4: Average fold induction of expression of the human CYP2Cs in the hepatocyte homogenate samples treated with either phenobarbital or valproic acid relative to the control sample at the appropriate day of culture.

The fold induction of expression of the human CYP2Cs in the hepatocyte homogenates treated with either phenobarbital or valproic acid was calculated relative to the control sample at the appropriate day of culture using the absorbance values (relative units) for all samples (10 μg and 20 μg of whole cell extracts for individual B). An average fold induction was calculated for each phenobarbital and valproic acid-treated hepatocyte homogenate using the two values of fold induction already obtained.

hepatocyte culture (6 days of treatment with the anti-epileptic drugs). Phenobarbital also induces expression of the CYP2Cs after four days of culture (3 days of treatment) whereas valproic acid barely induces the CYP2Cs expression after four days of culture. The average fold induction of expression of these enzymes by valproic acid at day 4 shows a decrease in the amount of expressed enzyme as compared to the control sample. This is due to small errors introduced by the scanning of the two blots. The amount of expression of the CYP2Cs decreased in the day 4 control sample as compared to the day 0 control sample; this amount of expression was maintained in the day 7 control sample.

These results correlate with the findings of another research group. Phenobarbital increases the CYP2C8/9/10 mRNA transcripts and corresponding proteins in cultured human hepatocytes (Morel *et al.*, 1990).

### 4.2.4. EXPRESSION OF CYP3As IN A HUMAN HEPATOCYTE CULTURE SYSTEM

The expression of the CYP3As were examined as collectively they metabolise >60% of therapeutic drugs administered to man. If the expression of these enzymes are induced by either phenobarbital or valproic acid this would lead to potentially serious drug-drug interactions.

5 μg (individual A and B), 2.5 μg (individual B) and 20 μg (individual A) of whole cell homogenates were analysed for the expression of CYP3As by western blotting analysis using an antisera to rabbit CYP3A6 (1 in 260 dilution) that had been raised in a goat. This antibody had been prepared in the laboratory of Dr. Patrick Maurel (Bonfils *et al.*, 1990) and a sample was kindly given to our laboratory. The polyclonal antibody cross-reacts with the human CYP3As but is unable to distinguish between the isoforms of the CYP3A subfamily. The blots were developed by using an alkaline phosphatase

conjugated rabbit anti-goat IgG (1 in 30,000 dilution). The photographs of the blots for individual A (5 μg whole cell homogenate) and individual B (5 μg whole cell homogenate) are shown in figure 4.9. The CYP3As were detected as a band at the expected apparent molecular weight of 51,000.

The 5 μg (individual A and B), 2.5 μg (individual B) and 20 μg (individual A) blots of hepatocyte homogenates were scanned using a BIO-RAD GS-670 densitometer and the results were analysed using Molecular Analyst software. An absorbance value (relative units) was obtained for each hepatocyte sample. The absorbance values (relative units) for the 5 μg samples of control, phenobarbital and valproic acid-treated hepatocyte homogenates (individual A) and the 5 μg samples (individual B) were plotted against day of culture and inducer present. The results are presented graphically in figure 4.10.

The average fold induction between the 5 μg and 20 μg of each phenobarbital and valproic acid-treated hepatocyte homogenate was calculated for individual A as in section 4.2.1. A similar calculation was performed for the 2.5 μg and 5 μg samples of individual B. The results are presented in table 4.5.

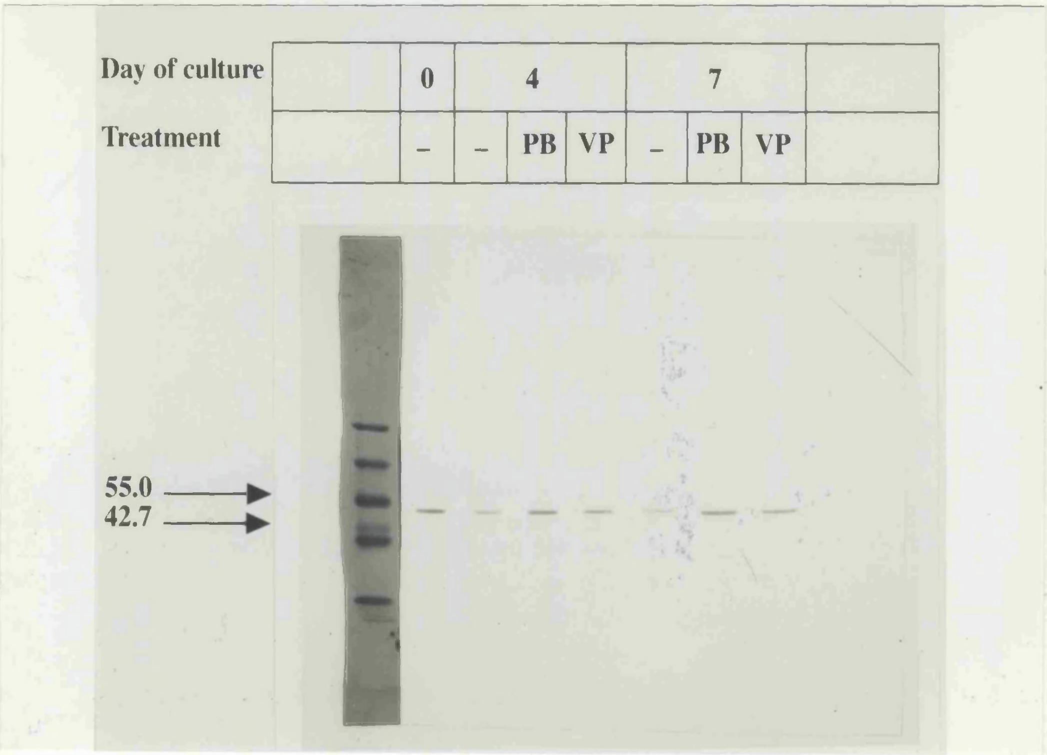
As can be seen from figure 4.10 and table 4.5, both phenobarbital and valproic acid induce expression of the human CYP3As in both individuals after four and seven days of hepatocyte culture (3 and 6 days treatment with the anti-epileptic drugs, respectively). However, for individual B, the induction of these enzymes by valproic acid after 4 days of culture is very small. The average fold induction for individual B shows a decrease in the amount of expressed CYP3As compared to the control sample. This is due to small errors introduced by the scanning of the two blots. For individual A, phenobarbital produced the greatest induction of the CYP3As at day 7; the same pattern of induction was observed for valproic acid. There was a marked decrease in expression of the CYP3As in the day 4 control homogenates for individuals A and B compared to the day 0 control sample. The expression of the CYP3As fell slightly in

Figure 4.9: Western blot analysis of hepatocyte homogenate samples from cultures derived from individuals A and B using a polyclonal antisera to CYP3As.

**A**: 5 µg of hepatocyte homogenate from the culture samples derived from individual A were electrophoresed through a 7% SDS-PAGE gel and transferred to nitrocellulose. The blot was probed with an antisera to rabbit CYP3A6 (1 in 260 dilution) that had been raised in a goat. The blot was developed by using an alkaline phosphatase conjugated rabbit anti-goat IgG (1 in 30,000 dilution). 2.5 µg of Promega mid-range molecular weight protein standards are shown x $10^{-3}$ in the first lane. The other lanes contain whole cell homogenate. The day of culture (0, 4 or 7) and culture conditions: control (-), phenobarbital-treated (PB) or valproic acid-treated (VP) are indicated above each lane.

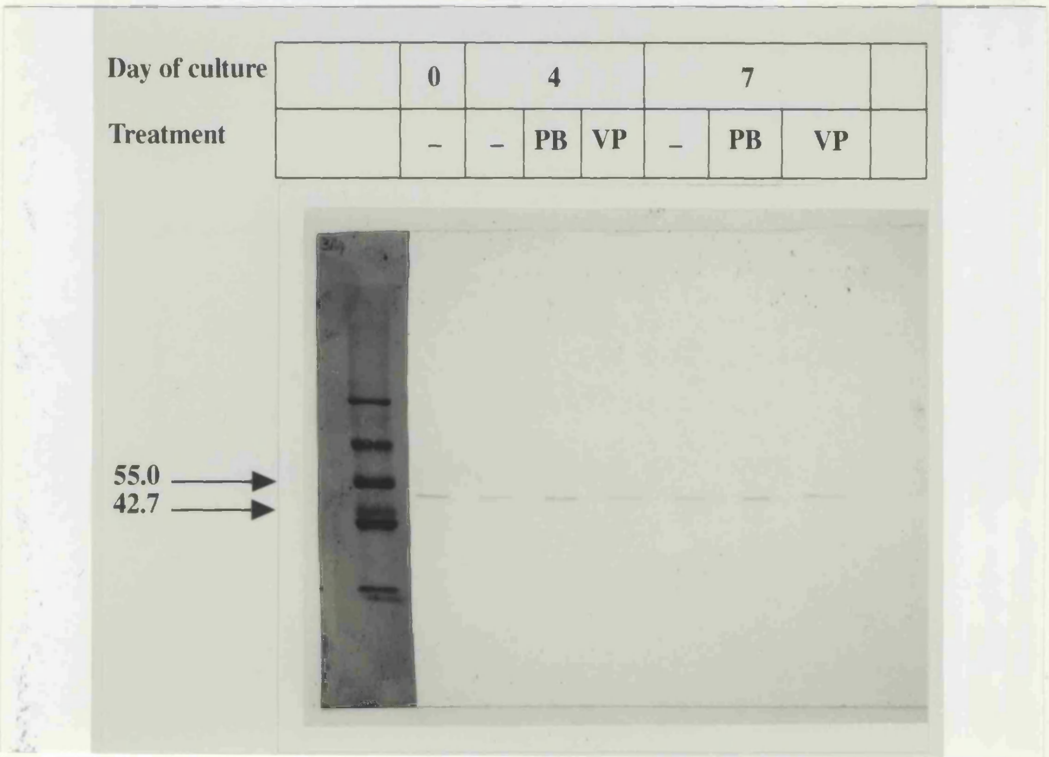
**B**: As for figure **A** except that 5 µg of hepatocyte homogenate from the culture samples derived from individual B are shown.

A



| Day of culture |  | 0 | 4 |  |  | 7 |  |  |  |
|---|---|---|---|---|---|---|---|---|---|
| Treatment |  | – | – | PB | VP | – | PB | VP |  |

B



| Day of culture |  | 0 | 4 |  |  | 7 |  |  |  |
|---|---|---|---|---|---|---|---|---|---|
| Treatment |  | – | – | PB | VP | – | PB | VP |  |

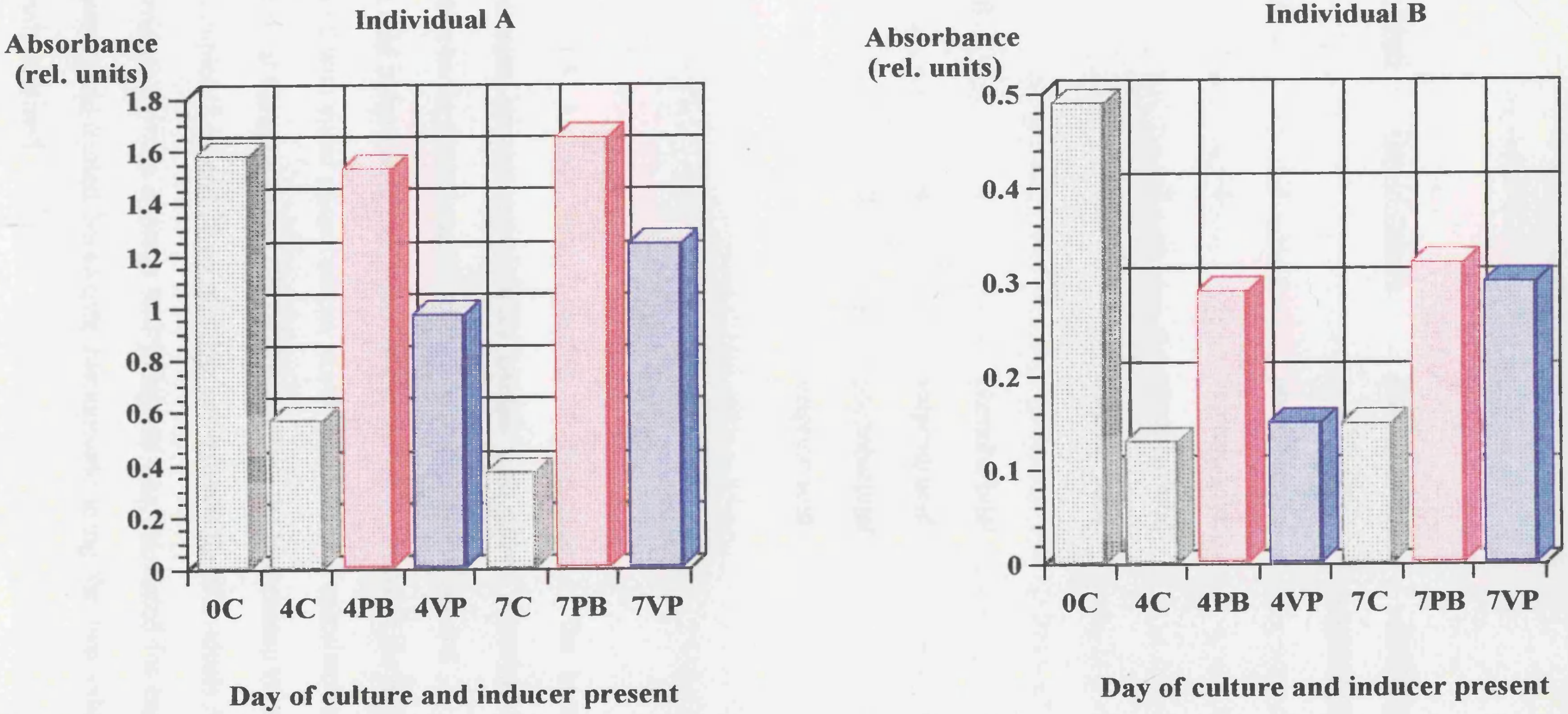


Figure 4.10: Induction of the expression of CYP3As by phenobarbital (PB) and valproic acid (VP).

The above graphs show the absorbance in relative (rel.) units of each control, PB- or VP-treated hepatocyte homogenate sample plotted against day of culture and inducer present.

| Individual | Day of culture | Inducer present | Average fold induction of expression of human CYP3As |
|---|---|---|---|
| A | 4 | phenobarbital | 2.38 |
| | 4 | valproic acid | 1.52 |
| | 7 | phenobarbital | 3.96 |
| | 7 | valproic acid | 3.42 |
| B | 4 | phenobarbital | 1.61 |
| | 4 | valproic acid | 0.80 |
| | 7 | phenobarbital | 1.53 |
| | 7 | valproic acid | 1.66 |

Table 4.5: Average fold induction of expression of the human CYP3As in the hepatocyte homogenate samples treated with either phenobarbital or valproic acid relative to the control sample at the appropriate day of culture.

The fold induction of expression of the human CYP3As in the hepatocyte homogenates treated with either phenobarbital or valproic acid was calculated relative to the control sample at the appropriate day of culture using the absorbance values (relative units) for all samples (5 μg and 20 μg of whole cell extracts for individuals A; 2.5 μg and 5 μg for individual B). An average fold induction was calculated for each phenobarbital and valproic acid-treated hepatocyte homogenate using the two values of fold induction already obtained.

the day 7 control sample for individual A but was slightly elevated in the day 7 control sample for individual B.

Mahnke *et al.* (1997) have shown that the *CYP3A2* gene is induced by phenobarbital in the male rat. P450hA7, a member of the CYP3A subfamily, has been purified from the liver of an epileptic who had been receiving long-term anticonvulsant therapy of phenobarbital, phenytoin, carbamazepine and valproic acid (Shaw *et al.*, 1989). P450hA7 was found at a higher percentage of total CYPs in individuals who had been receiving long-term anti-convulsant therapy than those who had not been administered therapy. Interestingly, Morel *et al.* (1990) found that the expression of the CYP3As in cultured human hepatocytes was unaffected by phenobarbital after three days of culture. This was due to the fact that they were comparing the amount of expression at day three to the amount of expression found in the hepatocytes before culturing and not to a control sample at day 3.

### 4.2.5. EXPRESSION OF NADPH-CYTOCHROME P450-REDUCTASE IN A HUMAN HEPATOCYTE CULTURE SYSTEM

Phenobarbital and valproic acid have been observed to induce the expression of certain CYPs (sections 4.2.1.-4.2.4.). P450-reductase is an essential component of the mixed function oxygenase system and is closely associated with the CYPs in the endoplasmic reticulum. Therefore it is reasonable to predict that if certain CYPs are induced by the anti-epileptic agents, then P450-reductase may also be induced.

The western blots of 5 μg and 20 μg of hepatocyte homogenates from individual A, previously analysed with an antisera to CYP3A6, were used to analyse the expression of P450-reductase. The blot of 20 μg of hepatocyte homogenates from individual B, previously analysed with an antisera to CYP2B4, was used to analyse expression of P450-reductase. A further analysis of P450-reductase expression in individual B was carried out using the 5 µg blot of samples that had been previously probed with the antisera to CYP3A6.

A polyclonal antibody prepared to rat P450-reductase which was raised in a rabbit (Shephard, PhD. Thesis, 1982) cross-reacts with human P450-reductase. The blots were probed with a 1 in 800 dilution of the antisera and were developed by using an alkaline phosphatase conjugated goat anti-rabbit IgG (1 in 3,000 dilution). The photographs of the blots for individual A (5 µg whole cell homogenate) and individual B (20 µg whole cell homogenate) are shown in figure 4.11. P450-reductase was detected as a band at the apparent molecular weight of 78,000.

The four blots, 5 µg and 20 µg of hepatocyte homogenates for individuals A and B, were scanned using a BIO-RAD GS-670 densitometer and the results were analysed using Molecular Analyst software. An absorbance value (relative units) was obtained for each hepatocyte sample. The absorbance values (relative units) for the 5 µg samples of control, phenobarbital and valproic acid-treated hepatocyte homogenates (individual A) and the 20 µg samples (individual B) were plotted against day of culture and inducer present. The results are presented graphically in figure 4.12.

The average fold induction between the 5 µg and 20 µg of each phenobarbital and valproic acid-treated hepatocyte homogenate was calculated for individuals A and B as in section 4.2.1. The results are presented in table 4.6.

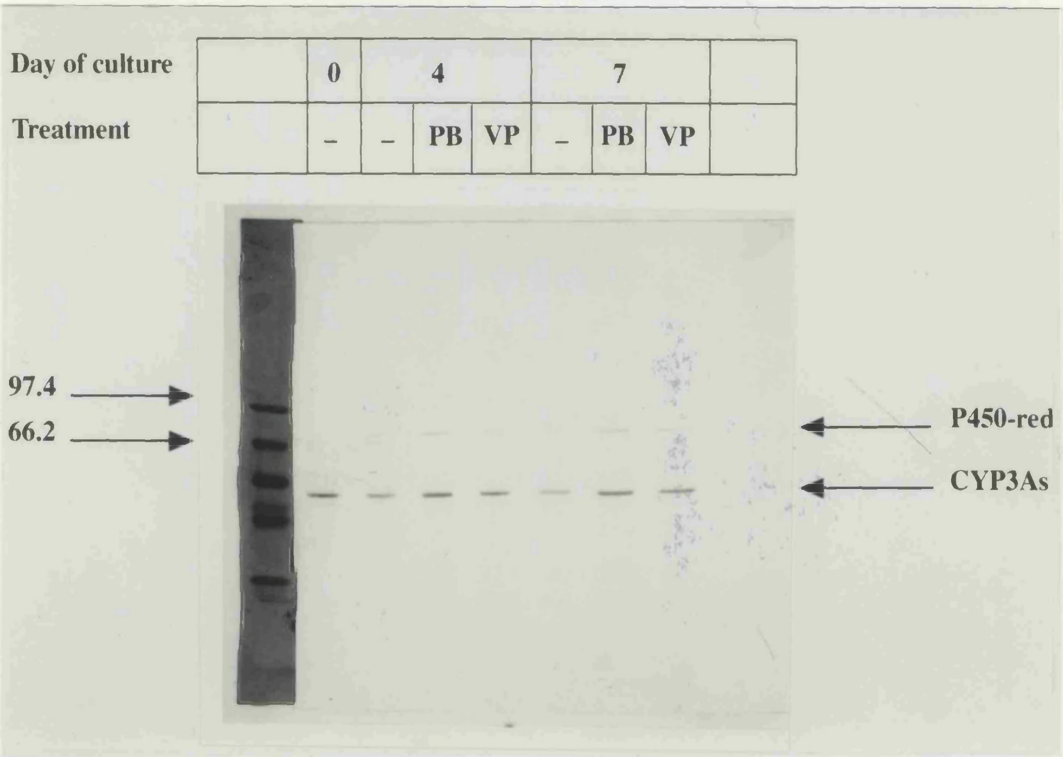
As can be seen from figure 4.12 and table 4.6, both phenobarbital and valproic acid induce expression of P450-reductase in both individuals after four and seven days of hepatocyte culture (3 and 6 days treatment with the anti-epileptic drugs, respectively). For individual A, phenobarbital induces the expression of P450-reductase by the greatest amount after seven days of culture. Whereas, the amount of induction of expression of this enzyme is similar for the two culture samples from individual B.

Figure 4.11: Western blot analysis of hepatocyte homogenate samples from cultures derived from individuals A and B using a polyclonal antisera to rat P450-reductase.

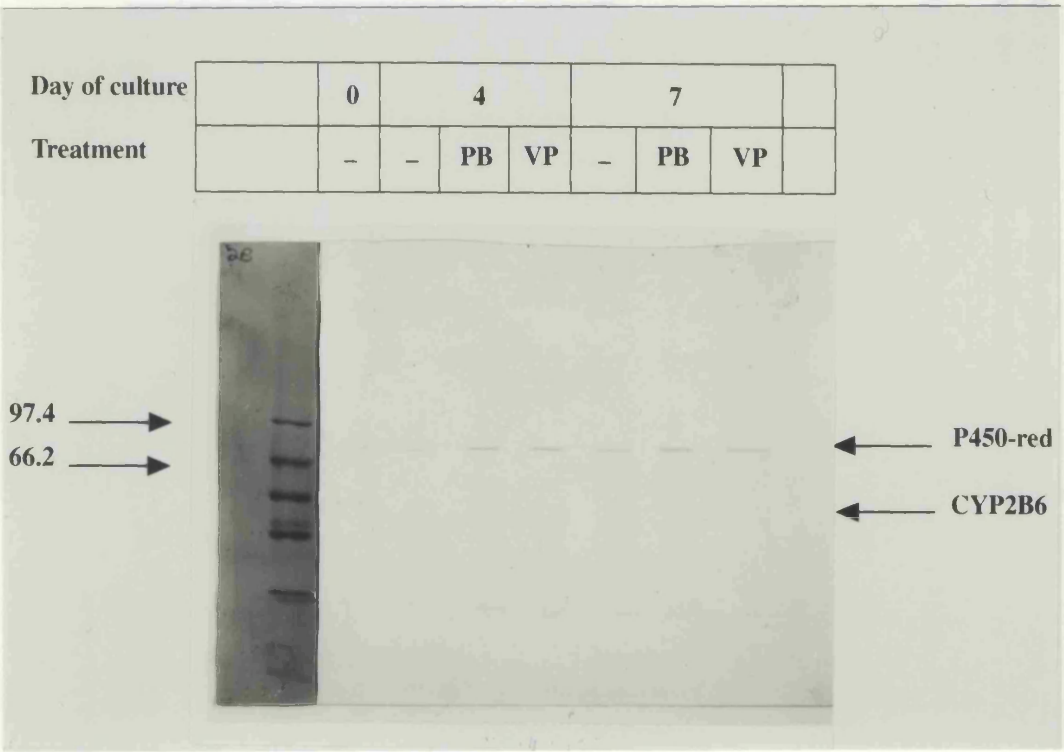
**A**: The western blot of 5 µg of hepatocyte homogenates from individual A, previously analysed with an antisera to CYP3A6, was reprobed with an antisera to rat P450-reductase (1 in 800 dilution) that had been raised in a rabbit. The blot was developed by using an alkaline phosphatase conjugated goat anti-rabbit IgG (1 in 3,000 dilution). 2.5 µg of Promega mid-range molecular weight protein standards are shown x $10^{-3}$ in the first lane. The other lanes contain whole cell homogenate. The day of culture (0, 4 or 7) and culture conditions: control (-), phenobarbital-treated (PB) or valproic acid-treated (VP) are indicated above each lane.

**B**: As for figure **A** except that the western blot of 20 µg of hepatocyte homogenates from individual B is shown. This blot had been previously analysed with an antisera to CYP2B4 and was reprobed with an antisera to rat P450-reductase (1 in 800 dilution).

A


Day of culture
0
4
7
Treatment
–
–
PB
VP
–
PB
VP
97.4
66.2
P450-red
CYP3As

B


Day of culture
0
4
7
Treatment
–
–
PB
VP
–
PB
VP
97.4
66.2
P450-red
CYP2B6

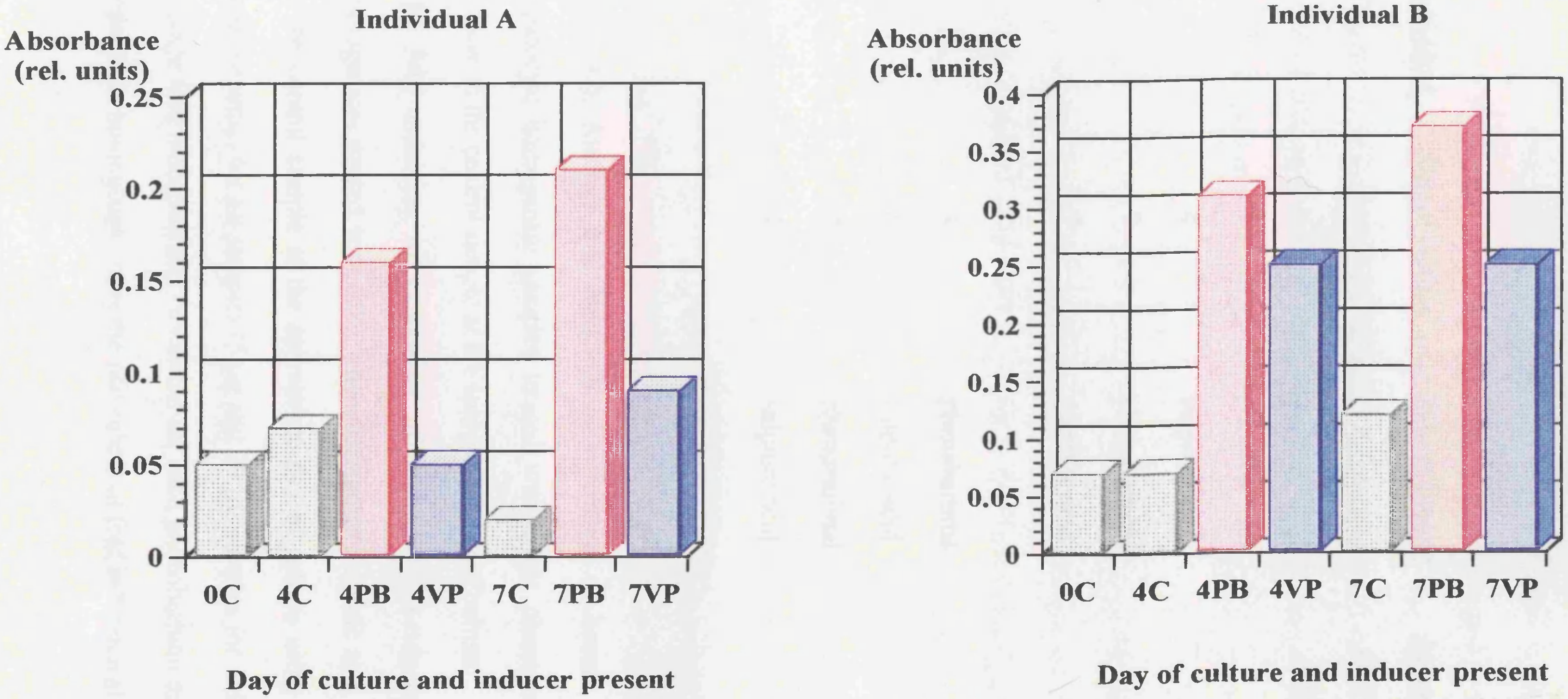


Figure 4.12: Induction of the expression of P450-reductase by phenobarbital (PB) and valproic acid (VP).

The above graphs show the absorbance in relative (rel.) units of each control, PB- or VP-treated hepatocyte homogenate sample plotted against day of culture and inducer present.

| Individual | Day of culture | Inducer present | Average fold induction of expression of human P450-reductase |
|---|---|---|---|
| A | 4 | phenobarbital | 2.06 |
| | 4 | valproic acid | 1.21 |
| | 7 | phenobarbital | 8.52 |
| | 7 | valproic acid | 4.24 |
| B | 4 | phenobarbital | 2.89 |
| | 4 | valproic acid | 2.27 |
| | 7 | phenobarbital | 2.39 |
| | 7 | valproic acid | 3.24 |

Table 4.6: Average fold induction of expression of human P450-reductase in the hepatocyte homogenate samples treated with either phenobarbital or valproic acid relative to the control sample at the appropriate day of culture.

The fold induction of expression of human P450-reductase in the hepatocyte homogenates treated with either phenobarbital or valproic acid was calculated relative to the control sample at the appropriate day of culture using the absorbance values (relative units) for all samples (5 μg and 20 μg samples for individuals A and B). An average fold induction was calculated for each phenobarbital and valproic acid-treated hepatocyte homogenate using the two values of fold induction already obtained.

Valproic acid induces the expression of P450-reductase by the greatest amount in both individuals, after seven days of culture. For individual A, the expression of P450-reductase in the day 4 control sample was slightly elevated as compared to the day 0 sample; the amount of expressed enzyme fell in the day 7 control homogenate. Whereas for individual B, the amount of expressed P450-reductase was similar in both the day 0 and day 4 control homogenates; the amount was elevated slightly in the day 7 whole cell extract.

Shephard *et al.* (1983) have shown that the expression of P450-reductase is induced by phenobarbital in rats; this is due to an increase in translatable mRNAs coding for the protein (Shephard *et al.*, 1982). Clark *et al.* (1996) observed that when cultured rat hepatocytes were exposed to phenobarbital for 24 hours there was an induction in expression of P450-reductase. Waxman *et al.* (1990) showed that if cultured rat hepatocytes were exposed to phenobarbital for a further 96 hours, there was again induction of expression of P450-reductase. Akrawi *et al.* (1993b) have shown that the gene for P450-reductase is induced by phenobarbital and valproic acid in co-cultured rat hepatocytes. The amount of P450-reductase in cultured hepatocytes is nearly equivalent to that found in freshly isolated cells (Akrawi *et al.*, 1993a). Just why this protein is maintained in culture but some CYPs are not remains a mystery.

### 4.2.6. EXPRESSION OF hGSTP1-1 IN A HUMAN HEPATOCYTE CULTURE SYSTEM

The rat GSTπ class, rGSTP1-1 (previously known as GST7-7) is expressed in very low amounts in adult rat liver but is greatly elevated in primary hepatomas (Meyer *et al.*, 1985); rGSTP1-1 is also expressed in foetal hepatocytes (Tee *et al.*, 1992). Abramovitz *et al.* (1989) have shown that rGSTP1-1 is expressed in cultured rat hepatocytes. The π class of GSTs appear to be highly conserved in all mammalian species (Hayes and Pulford, 1995). hGSTP1-1 is not normally expressed in the adult liver; it has been purified from many extrahepatic organs such as the lung, kidney and placenta (reviewed in Hayes and Pulford, 1995). The expression of rGSTP1−1 and the human equivalent form, hGSTP1-1, can be used as a marker for dedifferentiation of hepatocytes in rat and human culture systems, respectively.

20 μg (individual A and B) and 30 μg (individual B) of whole cell homogenates were analysed for expression of hGSTP1-1 by western blotting analysis using an antisera to rGSTP1-1 (1 in 1,000 dilution) that had been raised in a rabbit. The polyclonal antibody that cross-reacts with the human GSTπ class was purchased from Biotrin International. The blots were developed by using an alkaline phosphatase conjugated goat anti-rabbit IgG (1 in 3,000 dilution). The photographs of the blots for individual A (20 μg whole cell homogenate) and individual B (20 μg whole cell homogenate) are shown in figure 4.13.

hGSTP1-1 was not detected in the day 0 samples of hepatocyte homogenate for either individual. Very low expression of this enzyme was detected all in the hepatocyte homogenates after 4 days of culture for both individuals. The protein was detected as a band at the apparent molecular weight of 25,000. This amount of expression was maintained in the day 7 control sample for both individuals. A greater amount of expression of hGSTP1-1 was detected in the phenobarbital- and valproic acid-treated samples after seven days of culture. This result was observed for both individuals and was confirmed by the results from the 30 μg blot (individual B, data not shown).

hGSTP1-1 would not be expected to be seen in the day 0 samples. The appearance of GSTP1-1 in the four and seven day samples suggest that the hepatocytes are gradually undergoing dedifferentiation.

After analysing the expression of the CYPs, P450-reductase and hGSTP1-1 using western blotting, the abundance of these enzymes' mRNA transcripts in the cultured

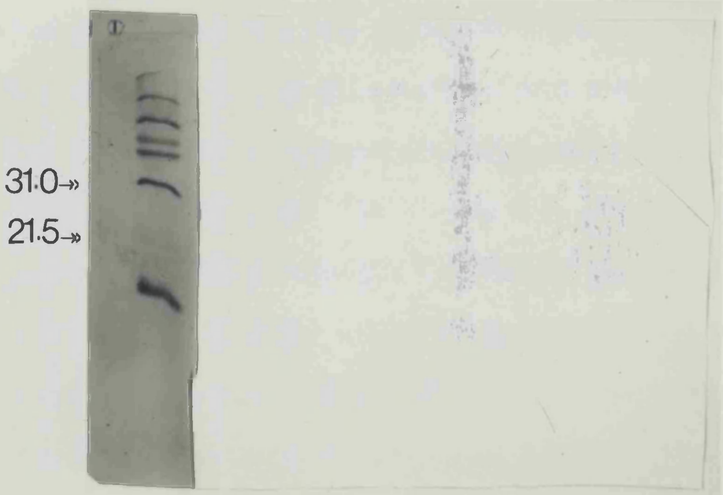
Figure 4.13: Western blot analysis of hepatocyte homogenate samples from cultures derived from individuals A and B using a polyclonal antisera to rGSTP1-1.

**A**: 20 µg of hepatocyte homogenate from the culture samples derived from individual A were electrophoresed through a 13% SDS-PAGE gel and transferred to nitrocellulose. The blot was probed with an antisera to rGSTP1-1 (1 in 1,000 dilution) that had been raised in a rabbit. The blot was developed by using an alkaline phosphatase conjugated goat anti-rabbit IgG (1 in 3,000 dilution). 2.5 µg of Promega mid-range molecular weight protein standards are shown x $10^{-3}$ in the first lane. The other lanes contain whole cell homogenate. The day of culture (0, 4 or 7) and culture conditions: control (-), phenobarbital-treated (PB) or valproic acid-treated (VP) are indicated above each lane.

**B**: As for figure **A** except that 20 µg of hepatocyte homogenate from the culture samples derived from individual B are shown.

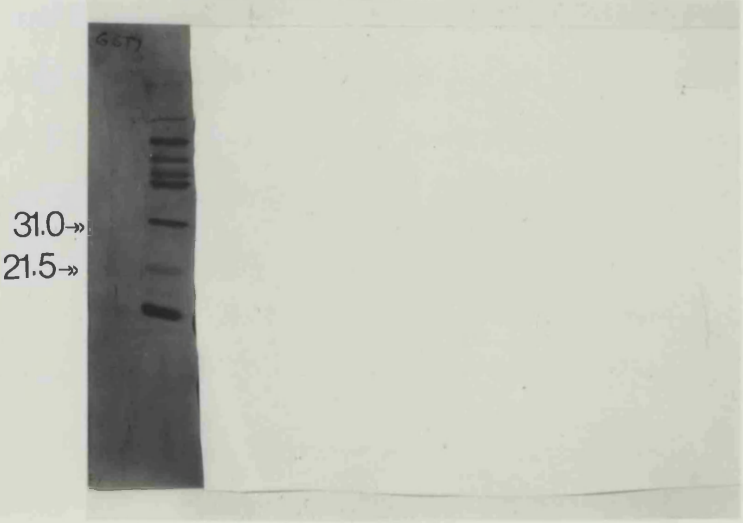
**A**

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Day of culture** | | 0 | 4 | | | 7 | | | |
| **Treatment** | | – | – | PB | VP | – | PB | VP | |



**B**

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Day of culture** | | 0 | 4 | | | 7 | | | |
| **Treatment** | | – | – | PB | VP | – | PB | VP | |



human hepatocytes should be investigated. Only one set of human hepatocyte pellets was available for individual B, this was used to make the whole extracts for the western blotting analysis. However, two sets of cell pellets were available for individual A. One was used to make whole cell extracts and the other was used to prepare total RNA by a method adapted from Cathala *et al.* (1983). This method has been previously used in our laboratory to prepare total RNA from rat hepatocyte cultures, HaCat cells (a transformed human keratinocyte cell line) and a number of other tissue samples. Total RNA was prepared from the human hepatocyte cell pellets and as a control for the isolation procedure, also from HaCat cells. The integrity of the RNA was checked by electrophoresing a sample of each RNA through a 1% denaturing agarose gel. 28S and 18S bands were observed for the HaCat cell RNA (figure 4.14A) and the human hepatocyte day 0 control RNA sample (figure 4.14B). It was observed that the total RNA prepared from the cultured human hepatocytes day 4 and day 7 samples were degraded (figure 4.14B). The yields of total RNA were low for all the samples, 2.5-4.4 $\mu g/1 \times 10^7$ cells. RNA degradation of other culture samples sent to our laboratory from Brussels has been encountered. The degradation of RNA in samples obtained from human hepatocyte cultures is problematic; it is essential to harvest the cells quickly and to freeze the cell pellets immediately on dry ice.

I was unable to analyse the effect of the anti-epileptic drugs on the abundance of the CYPs, P450-reductase and hGSTP1-1 mRNAs. An analysis of the mRNAs using an RNase protection assay would have permitted the identification of specific mRNA species induced by phenobarbital and valproic acid. As human liver is very difficult to obtain, it was not possible to repeat the cultures.

## 4.3. CONCLUSIONS

The important point to note concerning the results of the human hepatocyte culture experiments was that the expression of the CYP2As, CYP2B6, CYP3As, CYP2Cs and

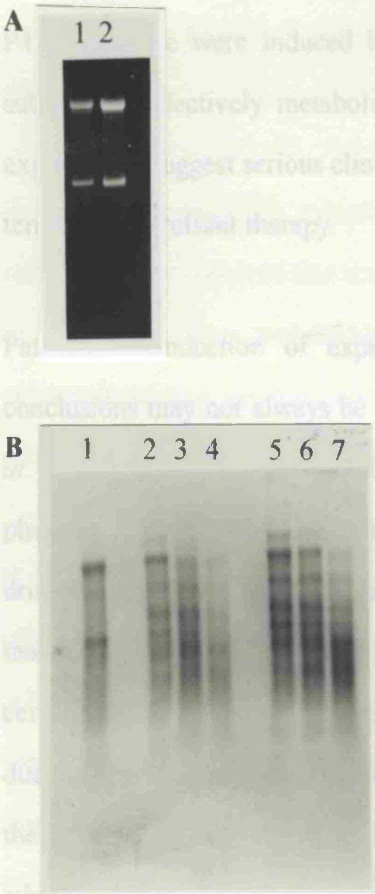


Figure 4.14: Preparation of total RNA from HaCat cells and cultured human hepatocytes by a method adapted from Cathala *et al.* (1983).

**A**: Total RNA was extracted from untreated HaCat cells. 0.9 µg (lane 1) and 2.2 µg (lane 2) of total RNA were electrophoresed through a 1% denaturing agarose gel. The gel was photographed under U.V. light.

**B**: Total RNA was extracted from cultured human hepatocytes from individual A. The samples were electrophoresed through a 1% denaturing agarose gel; the gel was photographed under U.V. light. Lanes 1-7 contain the following total RNA samples: 5 µg of control from day 0 (lane 1); 5 µg of control from day 4 (lane 2); 4.8 µg of phenobarbital- (PB) treated from day 4 (lane 3); 2.3 µg of valproic acid- (VP) treated from day 4 (lane 4); 5 µg of control from day 7 (lane 5); 5 µg of PB-treated from day 7 (lane 6) and 5 µg of VP-treated from day 7 (lane 7).

P450-reductase were induced by phenobarbital and valproic acid. As the CYP3A subfamily collectively metabolise >60% of therapeutic drugs, the findings of these experiments suggest serious clinical implications for epilepsy sufferers prescribed long-term anti-convulsant therapy.

Patterns in induction of expression of various CYPs were observed but valid conclusions may not always be able to be drawn from these results. Dalet-Beluche *et al.* (1992) and Diaz *et al.* (1990) have observed interculture variations in response to phenobarbital and other inducers of CYPs. Patients will have been prescribed different drug regimes which may well have affected the results of these experiments. A patient may have been previously administered a drug which had induced the expression of certain CYPs. This would mask the true fold induction of expression of those CYPs due to phenobarbital and valproic acid treatment. The real induction of expression of the CYPs due to the anti-epileptic agents will not be seen until the previously administered drug has been cleared from the hepatocytes.

The day 0 sample of whole cell extracts showed a greater expression of CYPs as compared to the control samples at day 4 or day 7; this was observed for individuals A (except for CYP2B6 expression) and B. This could be for two reasons:

1) Expression of CYPs has been shown to decrease during culture time (Steward *et al.*, 1985) as the hepatocytes dedifferentiate.

2) The expression of the CYPs may have already been induced in the patient by a previously administered drug. When the drug has been cleared from the hepatocytes, the CYPs are expressed at their native amounts.

It was observed that the morphology of the hepatocytes at the day of isolation was very important. If the morphology was not good, in other words the hepatocytes had started dedifferentiating, the induction responses of the CYPs to the anti-epileptics were not observed. This was found with the cultures derived from individuals C and D (data not shown).

Hepatocyte homogenate samples were originally analysed up to fourteen days of culture. It was observed that the morphology of the hepatocytes was not good, even in cultures derived from individuals A and B. There was loss of induction of the CYPs by phenobarbital and valproic acid (data not shown). This implies that this hepatocyte culture system can only be used for short-term experiments of up to seven days.

If hepatocyte cell culture systems are to be used for drug development by the pharmaceutical industry, the hepatocytes should be able to express CYPs and remain differentiated for periods of several weeks. This is clearly not the case with the conventional hepatocyte monolayer culture that has been analysed in this Chapter. However, this system is ideal for short-term analysis of the expression of drug metabolising enzymes and can be used to investigate drug-drug and drug-xenobiotic interactions.

Prof. Vera Rogiers's laboratory are investigating two other types of human hepatocyte cell culture. In one model, the hepatocytes are sandwiched between two layers of rat tail tendon collagen type I (Dunn *et al.*, 1989; Dunn *et al.*, 1991; Dunn *et al.*, 1992). This technique is referred to as a collagen sandwich culture. A variation of this model is the collagen gel immobilisation culture system. The hepatocytes are trapped within a collagen gel matrix (Koebe *et al.*, 1994).

The characterization of the hepatocyte culture system in this chapter was carried out by western blotting analysis using polyclonal antibodies. The polyclonal antibodies are unable to distinguish between different isoforms of the subfamily to which they are raised. Characterization of the cell culture system with monoclonal antibodies to individual CYPs would provide more detailed information on which CYPs in each subfamily are induced by particular drugs and toxic chemicals. This is especially important for the CYP3A subfamily which metabolise many therapeutic drugs. The most abundantly expressed member of this subfamily is CYP3A4. CYP2A6, the major isoform of the CYP2A subfamily in adult liver, has been shown to metabolically activate several compounds to cytotoxic and mutagenic products (Crespi *et al.*, 1990). CYP2A6 also catalyses tobacco-related nitrosamines in human liver microsomes to genotoxic products (Yamazaki *et al.*, 1992).

For the above reasons, CYP2A6 and CYP3A4 were chosen as antigens for the production of monoclonal antibodies. Chapter 5 is concerned with the production of monoclonal antibodies to CYP2A6 and CYP3A4 using an adaptation of phage display. These antibodies will eventually be used to examine the expression of these isoforms in the human hepatocyte culture system.

## *CHAPTER FIVE*

# PRODUCTION OF MONOCLONAL ANTIBODIES TO CYP2A6 AND CYP3A4 USING AN ADAPTATION OF THE RECOMBINANT PHAGE ANTIBODY SYSTEM

Chapter 4 of this thesis described the characterization of a human hepatocyte culture system by western blot analysis. The antibodies used for the western blotting experiments were polyclonal and raised to non-human CYPs (except for the antisera to the CYP2Cs). These polyclonal antibodies are unable to distinguish between different isoforms of CYPs in the same subfamily. Monoclonal antibodies that are isoform-specific would be far superior reagents for most analytical purposes.

Genetic polymorphisms with functional effects occur in many of the genes encoding CYPs in the human population (reviewed in Gonzalez and Idle, 1994 and in Daly, 1995). Affected individuals will be susceptible to toxic effects of drugs metabolised by the polymorphic CYP(s). The molecular basis of several polymorphisms have been determined and this has resulted in the development of genotyping assays using PCR (reviewed in Daly, 1995). Monoclonal antibodies to a polymorphic CYP could be used to assess the enzyme's capabilities of metabolising a substrate, particularly a therapeutic drug. Results from these kinds of experiments and genotyping assays would lead to more rational drug doses being administered to these individuals.

Very few monoclonal antibodies raised to human CYPs are available. Gelboin *et al.* (1996) have produced inhibitory and non-inhibitory monoclonal antibodies to human CYP2E1. The same group of researchers have also produced an inhibitory monoclonal antibody to CYP3A4 (Gelboin *et al.*, 1995). An anti-peptide antibody has been now been produced to CYP3A4 by the research group of Dr. Robert Edwards (reviewed in Edwards, in press). The monoclonal and anti-peptide antibodies to CYP3A4 had not been produced at the outset of this thesis.

This chapter is concerned with the production of monoclonal antibodies to CYP2A6 and CYP3A4 using an adaptation of the recombinant phage antibody system marketed by Pharmacia Biotech. I was hoping to produce isoform-specific monoclonal antibodies to different epitopes of the CYPs. These could then be used to recharacterize the human hepatocyte culture system with respect to the spectrum of expressed CYPs.

The recombinant phage antibody system is based on research work carried out by McCafferty *et al.* (1990) and was developed in conjunction with Cambridge Antibody Technology Limited, U.K.; the use of the system is subject to licensing restrictions. Fragments of antibodies, single chain fragment variables (ScFvs), are expressed as fusion proteins displayed on the surface of a phage or as soluble antibodies secreted into the periplasm of bacteria (Marks *et al.*, 1991; reviewed in Plückthun, 1991).

Phage-displayed/soluble recombinant antibodies offer several advantages over hybridoma-derived monoclonals:

- they are more easily cloned and screened
- they do not require ascites production in animals or large-scale cell culture
- they offer a stable genetic source
- they can be genetically manipulated to improve antigen binding.

The recombinant phage antibody system has been briefly discussed in section 1.12.3.; a more comprehensive overview is given in sections 5.3. and 5.4. This chapter has been divided into seven main sections; these are:

5.1. Isolation of purified CYP2A6 and CYP3A4
5.2. Injection of mice with antigen
5.3. Production of ScFv fragments
5.4. Expression of ScFv antibodies in bacteria.
5.5. Isolation of further purified CYP2A6 and CYP3A4.
5.6. Screening the ScFv antibody library.
5.7. Production and analysis of soluble ScFv antibodies.

Purified antigen is required as the starting point of the recombinant phage antibody system. The purified antigen is injected into mice and once the mice have been shown to have produced antibodies to the antigen, their spleens are removed and RNA isolated. The recombinant ScFv cDNA library is generated by PCR and the ScFv antibodies are expressed in bacteria.

## 5.1. ISOLATION OF PURIFIED CYP2A6 AND CYP3A4

CYP2A6 was expressed in *Sf9* insect cells using the baculovirus expression system; the enzyme was purified by affinity chromatography on *p*-chloroamphetamine-coupled sepharose (Nanji, PhD. Thesis, 1995).

Microsomes prepared from *T ni* insect cells expressing CYP3A4 were donated by GlaxoWellcome (Lee *et al.*, 1995). The recombinant baculovirus containing the CYP3A4 cDNA also carried the cDNA for P450-reductase. P450-reductase was included in the recombinant construct as GlaxoWellcome were interested in performing kinetic studies on CYP3A4. Kinetic studies on this enzyme have been previously hampered by low activity of CYP3A4 obtained from recombinant gene expression systems. Lee *et al.* (1995) observed that this problem was overcome by the inclusion of P450-reductase in the expression system.

### 5.1.1. PURIFICATION OF CYP3A4 FROM INSECT CELL MICROSOMES BY AFFINITY CHROMATOGRAPHY ON *P*-CHLOROAMPHETAMINE-COUPLED SEPHAROSE

The concentration of expressed CYP3A4 (0.055 nmole/ml) in the microsomes supplied by GlaxoWellcome was very low. Approximately 8 nmoles (≈408 μg) of CYP3A4 was contained in 150 ml of sample. In the initial stages of this section of research, only a maximum of 100 μg of purified CYP was required. This amount would be sufficient for subsequent injection of mice and analytical studies on the mice sera.

The microsomes were solubilised as in section 2.2.21. and CYP3A4 was purified by affinity chromatography on *p*-chloroamphetamine-coupled sepharose (adapted from Sundin *et al.*, 1987) as described in section 2.2.21. The $O.D._{280}$ and $O.D._{417}$ were measured for each fraction and on the basis of these readings certain fractions were selected for electrophoresis on 10% SDS-PAGE gels for silver staining (figure 5.1.) and western blot analysis (figures 5.2 and 5.3). The protein bands appeared distorted on the SDS-PAGE gels, and subsequent blots, this could be due to the presence of a high concentration of detergent in these fractions (0.5% w/v sodium cholate and 0.2% v/v Emulgen 911).

The western blots were probed with an antisera to rabbit CYP3A6 (1 in 260 dilution) that had been raised in a goat (figure 5.2). The blots were developed by using an alkaline phosphatase conjugated rabbit anti-goat IgG (1 in 30,000 dilution). The western blots were reprobed with an antisera to rat P450-reductase (1 in 800 dilution) that had been raised in a rabbit (figure 5.3). The blot was developed by using an alkaline phosphatase conjugated goat anti-rabbit IgG (1 in 3,000 dilution).

The initial volume of sample loaded on the column was very large, this meant a great number of fractions were collected for the flow-through and wash samples. If CYP3A4 was present in these fractions it would be at too low a concentration to be detected by either silver staining or western blot analysis. Therefore these fractions were not analysed.

Figure 5.1 shows that CYP3A4 was substantially enriched by affinity chromatography on *p*-chloroamphetamine-coupled sepharose in comparison to the sample of solubilised microsomes. A protein band corresponding to CYP3A4 was observed in fractions 3-48

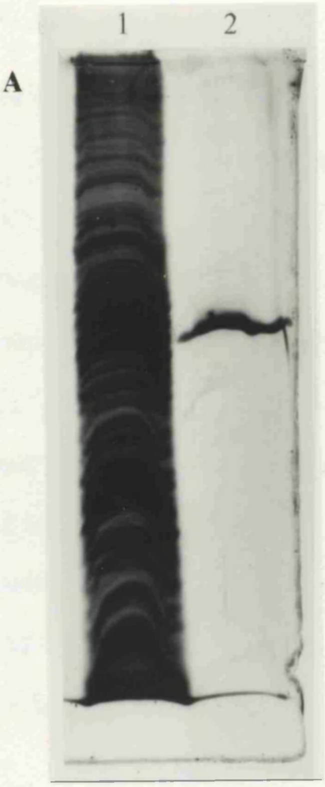


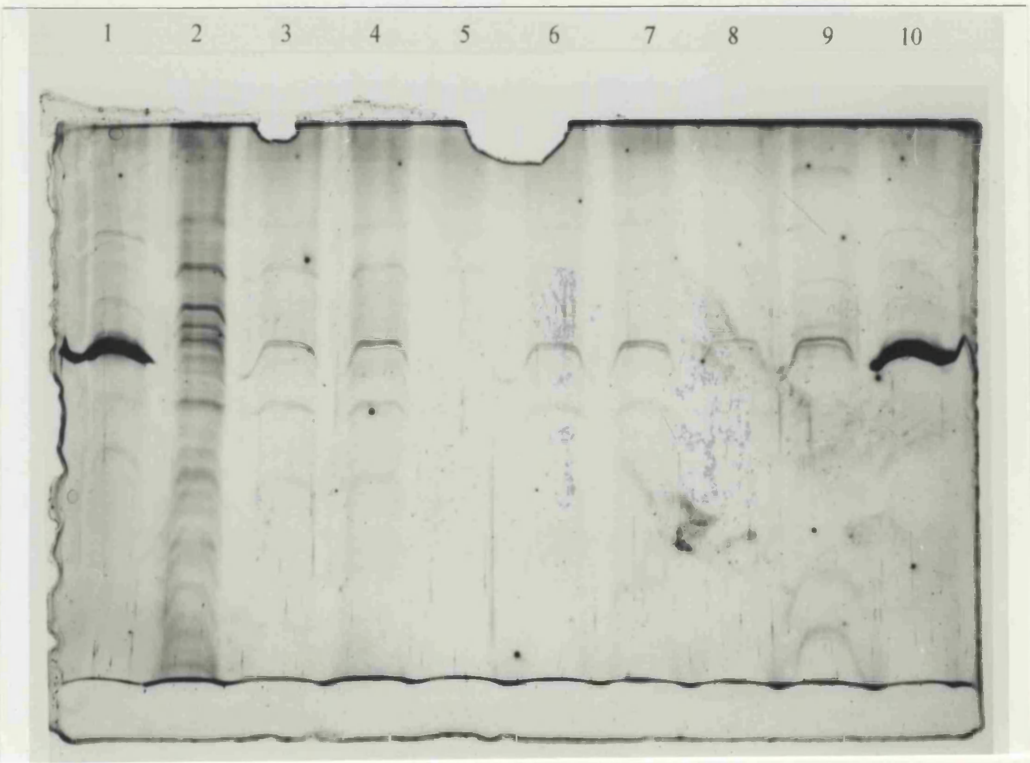
Figure 5.1: Silver stained SDS-PAGE gels showing fractions collected from a *p*-chloroamphetamine-coupled sepharose column during the purification of CYP3A4 from insect cell microsomes.

**A**: The samples were electrophoresed through a 10% SDS-PAGE gel; the gels were silver stained and photographed with the aid of a light box. Lane 1 contains 50 μl of solubilised microsomes and lane 2 contains 100 ng of purified rat CYP2B.

**B**: As for figure **A** except the following samples are shown: 100 ng of purified rat CYP2B (lanes 1 and 10); lanes 2-9 contain 50 μl of the following fractions: fraction 3 (lane 2); fraction 20 (lane 3); fraction 23 (lane 4); 12 ng of purified rat P450-reductase (lane 5); fraction 27 (lane 6); fraction 29 (lane 7); fraction 32 (lane 8) and fraction 39 (lane 9).

**C**: As for figure **A** except the following samples are shown: 100 ng of purified rat CYP2B (lane 1); lanes 2-8 contain 50 μl of the following fractions: fraction 42 (lane 2); fraction 45 (lane 3); fraction 48 (lane 4); 12 ng of purified rat P450-reductase (lane 5); fraction 91 (lane 6); fraction 93 (lane 7) and fraction 96 (lane 8).

**B**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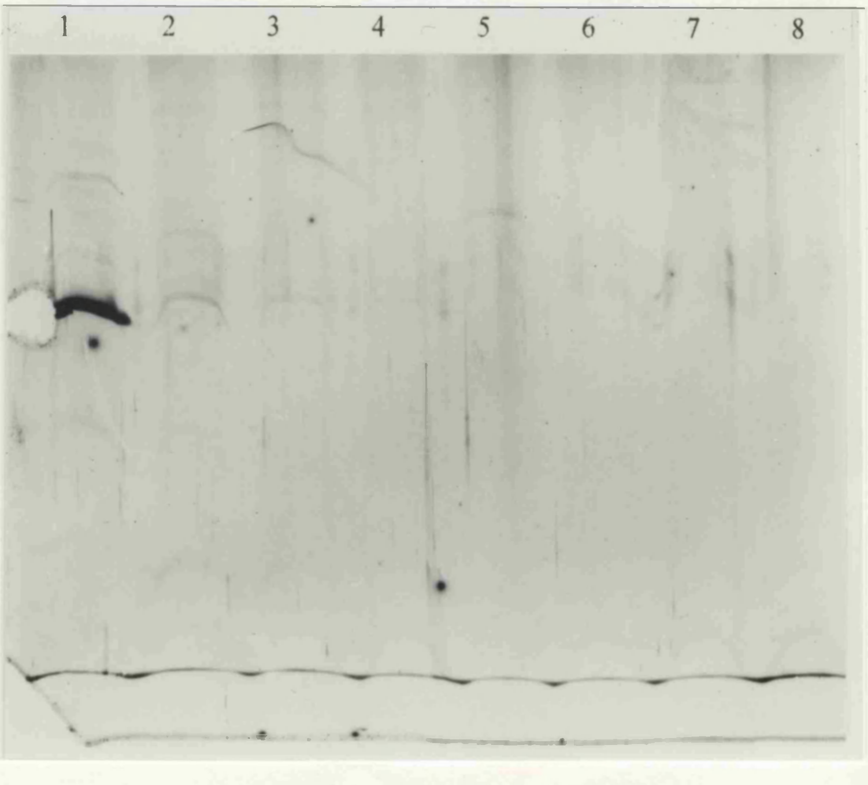
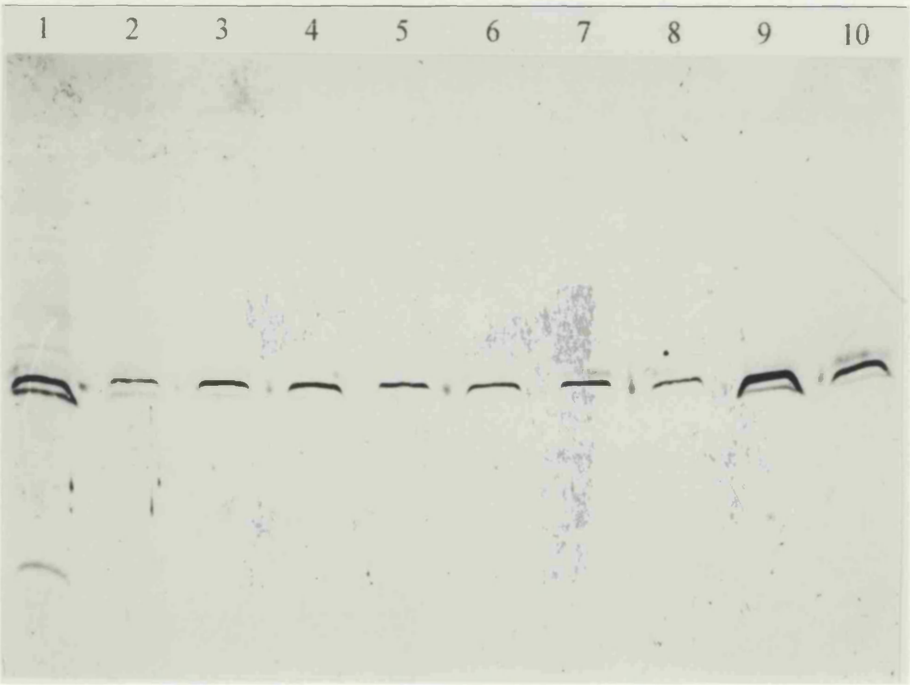


Figure 5.2: Western blot analysis of fractions collected from a *p*-chloroamphetamine-coupled sepharose column during the purification of CYP3A4 from insect cell microsomes using a polyclonal antisera to CYP3A6.

**A**: 50 µl of each sample was electrophoresed through a 10% SDS-PAGE gel and transferred to nitrocellulose. The blots were probed with an antisera to rabbit CYP3A6 (1 in 260 dilution) that had been raised in a goat. The blot was developed by using an alkaline phosphatase conjugated rabbit anti-goat IgG (1 in 30,000 dilution). The following samples are shown: solubilised microsomes (lane 1); fraction 3 (lane 2); fraction 20 (lane 3); fraction 23 (lane 4); fraction 24 (lane 5); fraction 27 (lane 6); fraction 29 (lane 7); fraction 32 (lane 8); fraction 36 (lane 9) and fraction 39 (lane 10).

**B**: As for figure **A** except the following samples are shown: fraction 42 (lane 1); fraction 45 (lane 2); fraction 48 (lane 3); fraction 49 (lane 4); fraction 51 (lane 5); fraction 91 (lane 6); fraction 93 (lane 7); fraction 96 (lane 8); fraction 99 (lane 9) and fraction 103 (lane 10).

**A**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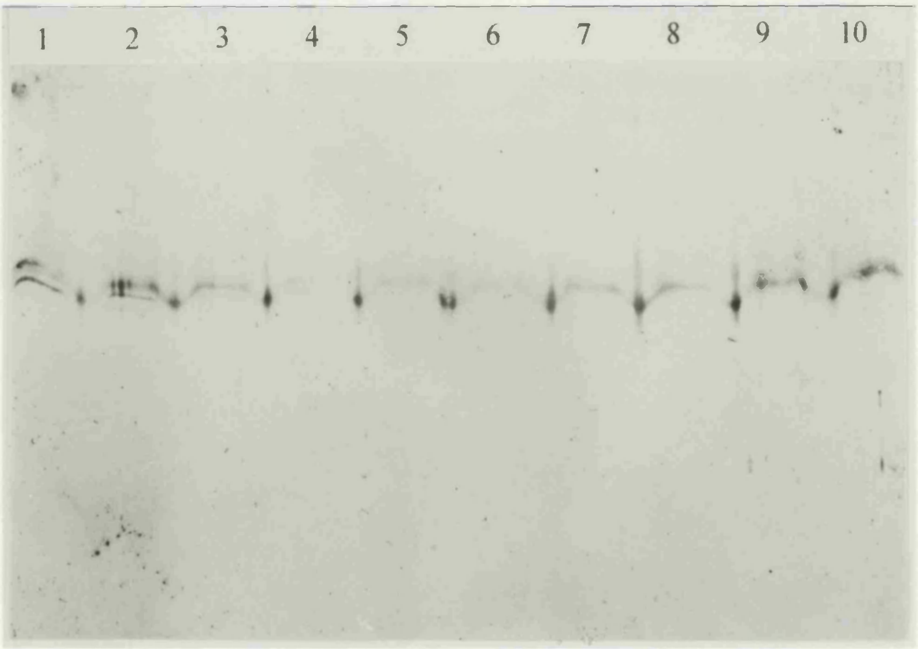
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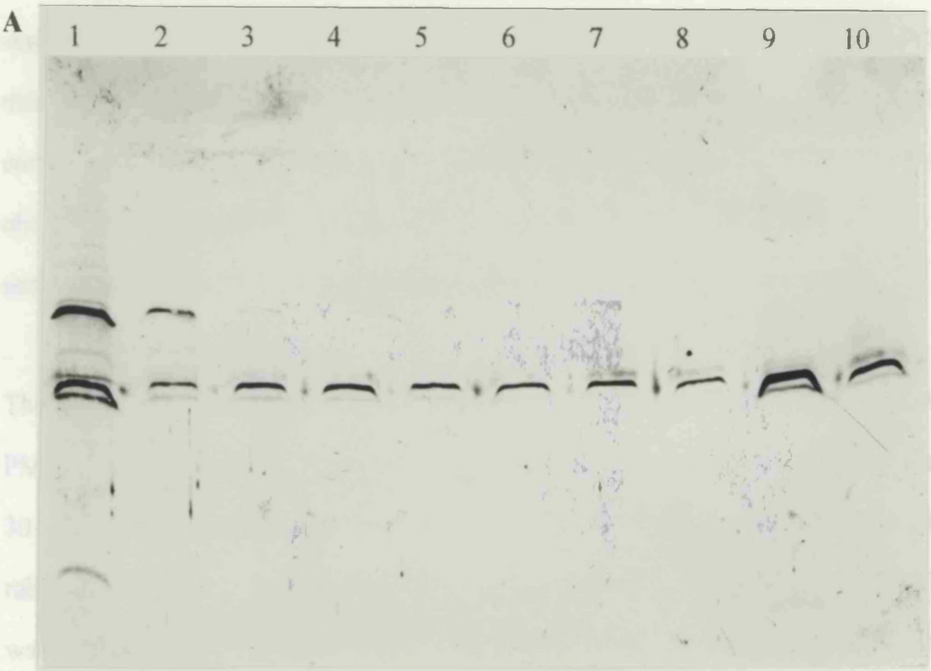
Figure 5.3: Western blot analysis of fractions collected from a *p*-chloroamphetamine-coupled sepharose column during the purification of CYP3A4 from insect cell microsomes using a polyclonal antisera to P450-reductase.

**A**: The western blot previously analysed with an antisera to CYP3A6, was reprobed with an antisera to rat P450-reductase (1 in 400 dilution) that had been raised in a rabbit. The blot was developed by using an alkaline phosphatase conjugated goat anti-rabbit IgG (1 in 3,000 dilution). The following samples are shown: solubilised microsomes (lane 1); fraction 3 (lane 2); fraction 20 (lane 3); fraction 23 (lane 4); fraction 24 (lane 5); fraction 27 (lane 6); fraction 29 (lane 7); fraction 32 (lane 8); fraction 36 (lane 9) and fraction 39 (lane 10).

**B**: As for figure **A** except the following samples are shown: fraction 42 (lane 1); fraction 45 (lane 2); fraction 48 (lane 3); fraction 49 (lane 4); fraction 51 (lane 5); fraction 91 (lane 6); fraction 93 (lane 7); fraction 96 (lane 8); fraction 99 (lane 9) and fraction 103 (lane 10).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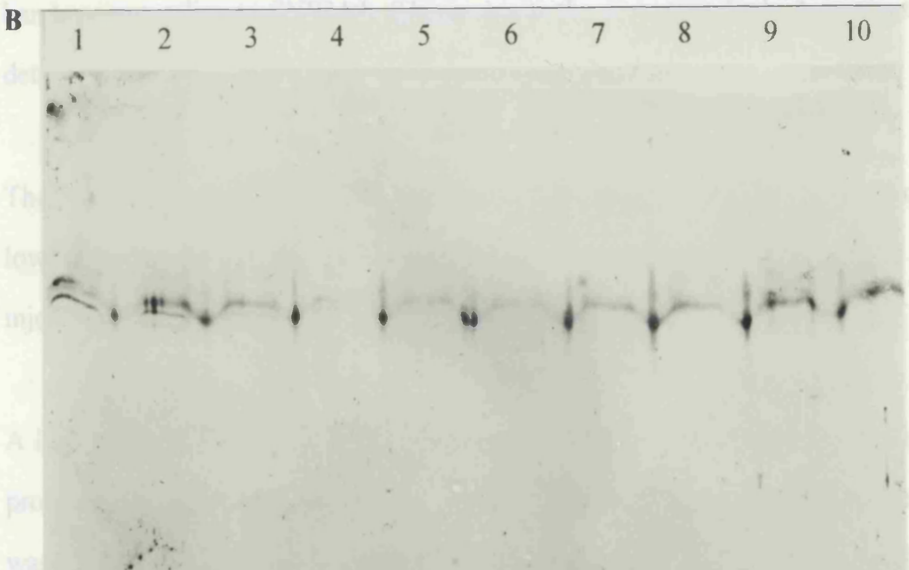
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at a slightly elevated apparent molecular weight to that of a purified rat CYP2B standard. P450-reductase was observed in fractions 3-29 at the same apparent molecular weight as that of a purified rat P450-reductase standard. These results were confirmed by western blot analysis (figures 5.2 and 5.3). The apparent smeared lines observed in the western blots for gel C (figures 5.2 and 5.3) are artifactual. These samples contained a high concentration of detergent but very little protein.

The fractions containing CYP3A4 were pooled and concentrated using an Amicon PM30 ultrafiltration cell, the sample was further concentrated in an Amicon centricon-30 unit. The concentrated sample was analysed by western blotting using an antisera to rabbit CYP3A6 (1 in 260 dilution) that had been raised in a goat (figure 5.4). The blot was developed in the same manner as already described in this section. A band corresponding to CYP3A4 was observed in figure 5.4 along with a faint band above the band corresponding to CYP3A4. This is due to the presence of a high concentration of detergents in the concentrated sample.

The amount of purified CYP3A4 obtained from this chromatography procedure was low, approximately 50 μg. However this amount was sufficient to proceed with the injection of the mice.

A low amount of purified CYP2A6 was recovered using an identical chromatography protocol (Nanji, PhD. Thesis, 1995). In this case the volume of the flow-through and wash samples were smaller and therefore fractions collected from these samples were able to be analysed by western blotting. It was observed that a large amount of CYP2A6 was present in these fractions. This suggested that the binding of this enzyme to the column was weak. Although the fractions containing the flow-through and wash samples were not tested in the purification of CYP3A4, it may be hypothesised that a similar situation arose. If the binding of CYP3A4 to the column was weak, this would account for the loss of about half of the CYP3A4 present in the starting material.

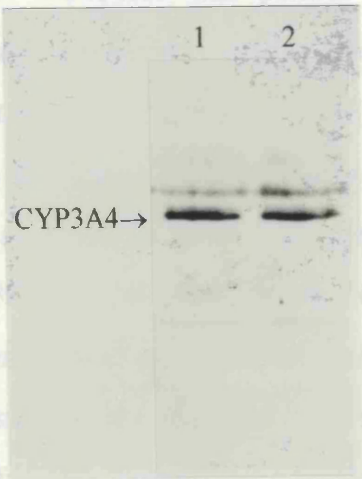


Figure 5.4: Western blot analysis of the concentrated sample of purified CYP3A4 obtained from chromatography on a *p*-chloroamphetamine-coupled sepharose column using a polyclonal antisera to CYP3A6.

Two aliquots of 20 µl of purified CYP3A4 obtained from chromatography on a *p*-chloroamphetamine-coupled sepharose column were electrophoresed through a 7% SDS-PAGE gel and transferred to nitrocellulose. The blot was probed with an antisera to rabbit CYP3A6 (1 in 260 dilution) that had been raised in a goat. The blot was developed by using an alkaline phosphatase conjugated rabbit anti-goat IgG (1 in 30,000 dilution).

## 5.2. INJECTION OF MICE WITH ANTIGEN

### 5.2.1. INJECTION REGIME

Five female mice per antigen were injected with approximately 2 µg of either purified CYP2A6 or CYP3A4. CYP3A4 was purified by chromatography on a *p*-chloroamphetamine-coupled sepharose column as described in section 2.2.21. CYP2A6 was purified in an identical manner by Manoj Nanji (Nanji, PhD. Thesis, 1995). Two strains of mice, Balb-c and $Cba_{ca}$, were used in case one strain was susceptible to hyper-immunisation by an antigen. A booster injection was administered to the mice 10 days later (section 2.2.32.). On day 21 of the injection schedule, a blood sample was taken from the tail of each mouse and the serum was extracted (section 2.2.32.). The polyclonal sera were tested for the presence of antibodies raised to the injected antigen by a double immunodiffusion assay (section 2.2.33.; data not shown). Antibody-antigen complexes precipitate when they are combined at or near equivalence to form precipitin bands. The assay showed that the majority of the mice had produced antibodies to the injected antigen. The mice were given a further booster injection on day 21. The mice were sacrificed 10 days later, their spleens were removed and heart-punctures were carried out. The polyclonal sera was extracted from each blood sample.

Throughout the immunisation procedure, the mice were housed in four different cages depending on the strain of the mouse and the antigen injected. Individual mice were identified by the presence/absence of ear clips.

Two balb-c and one $Cba_{ca}$ mice were not injected with any antigen; they were housed separately from the immunised animals. The sera from these mice were used as experimental controls (section 5.2.2.).

### 5.2.2. DOUBLE IMMUNODIFFUSION ANALYSIS OF MICE SERA

The polyclonal sera obtained from the heart punctures were analysed against the injected antigen by double immunodiffusion (figures 5.5A and B). Figure 5.5A illustrates that all five mice injected with CYP3A4 have raised antibodies to the antigen. The polyclonal sera from a balb-c mouse shown in well **c** did not give as strong a precipitin band as the other samples. This indicates that this mouse has not shown a good immunogenic response to CYP3A4. Spur precipitin lines were observed in the samples shown in wells **a**, **b**, **c** and **d**. This indicated that antibodies may have been raised to minor protein contaminants in the antigen preparation.

Figure 5.5B illustrates that all five mice injected with CYP2A6 have produced antibodies to the antigen. The mice whose polyclonal sera are shown in wells **c** and **e** did not produce as good an immune response to the antigen as the other mice. Spur precipitin lines were observed in the samples shown in wells **a**, **b**, **c**, **d** and to a lesser extent in **e**. This again indicated that antibodies may have been raised to minor protein contaminants in the antigen preparation.

A double immunodiffusion analysis with sera from uninjected mice from both strains against both antigens was carried out (data not shown). No precipitin lines were observed; this indicated that neither strain of mice possessed antibodies to either CYP2A6 or CYP3A4 before injection with these proteins.

Antibodies may have been raised to minor protein contaminants in the antigen preparations. This was further investigated by western blot analysis of the purified CYP3A4 sample, obtained from chromatography on a *p*-chloroamphetamine-coupled sepharose column, using the mice antisera allegedly raised to CYP3A4. A similar experiment was unable to be carried out for CYP2A6 as all the purified sample had been used for the injection regime and subsequent double immunodiffusion analysis.

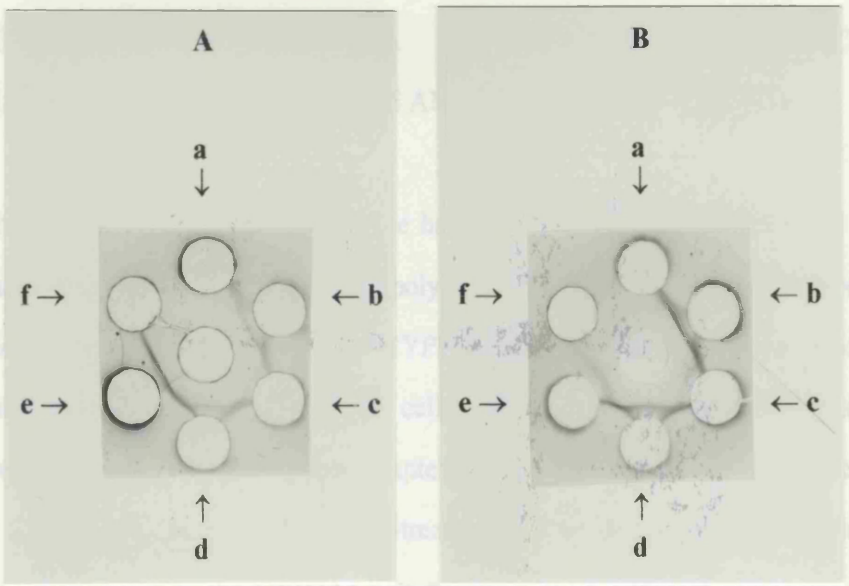


Figure 5.5**A**: Double immunodiffusion assay analysis using mice antisera raised to CYP3A4.

30 µl (≈1.5 µg) of CYP3A4 obtained from chromatography on a *p*-chloroamphetamine-coupled sepharose column was placed in the centre well. Well **a** contains 30 µl of antisera produced from a $\mathrm{Cba}_{\mathrm{ca}}$ mouse that was injected with CYP3A4 obtained from chromatography on a *p*-chloroamphetamine-coupled sepharose column. Wells **b-e** contain 30 µl of antisera produced from mice (balb-c strain) that were similarly injected. Well **f** contains 30 µl of a control sera; the mouse ($\mathrm{Cba}_{\mathrm{ca}}$ strain) was not injected with any antigen.

**B**: Double immunodiffusion assay analysis using mice antisera raised to CYP2A6.

15 µl (≈1 µg) of CYP2A6 obtained from chromatography on a *p*-chloroamphetamine-coupled sepharose column was placed in the centre well. Wells **a** and **b** contain 30 µl of antisera produced from $\mathrm{Cba}_{\mathrm{ca}}$ mice that were injected with CYP2A6 obtained from chromatography on a *p*-chloroamphetamine-coupled sepharose column. Wells **c-e** contain 30 µl of antisera produced from mice (balb-c strain) that were similarly injected. Well **f** contains 30 µl of a control sera; the mouse (balb-c strain) was not injected with any antigen.

### 5.2.3. WESTERN BLOT ANALYSIS OF PURIFIED CYP3A4 OBTAINED FROM CHROMATOGRAPHY ON A *P*-CHLOROAMPHETAMINE-COUPLED SEPHAROSE COLUMN USING MICE ANTISERA RAISED TO CYP3A4

To determine if the antibodies the mice had raised to the injected CYP3A4 produced the same western blot pattern as the polyclonal antisera to CYP3As (figure 4.9) the following samples were analysed, the CYP3A4 preparation that had been used to inject the mice and two samples of whole cell extracts from the human hepatocyte cell cultures previously characterised in chapter 4. The hepatocyte samples were from the day 0 control and day 4 valproic acid-treated cultures derived from individual A. A band corresponding to the CYP3As was observed at an apparent molecular weight of 51,000 in figure 4.9 in both these samples.

The polyclonal antisera, allegedly raised to CYP3A4, from the mouse whose sera was shown in well **e** in figure 5.5A was used to probe the western blot shown in figure 5.6. The sera of this mouse was chosen as an intense precipitin band was produced in the double immunodiffusion assay. mRNA prepared from the spleen of this mouse was used in the reverse transcription reaction (section 5.3.3) for the construction of the CYP3A4 ScFv cDNA library. A 1 in 250 dilution of the antisera was used; the blot was developed by using a 1 in 30,000 dilution of an alkaline phosphatase conjugated goat anti-mouse IgG.

A band of apparent molecular weight of 50,000-53,000 was observed in the 5 μl sample of the CYP3A4 preparation (figure 5.6); this band was darker in intensity in the 10 μl sample. A very intense band was detected on the blot at the level corresponding to the ion front of the gel in both the CYP3A4 samples. Another band of approximate apparent molecular weight of 33,000-35,000 was seen in both samples. This indicated that the mouse had raised antibodies to low molecular weight contaminants in the purified CYP3A4 preparation. A band of apparent molecular weight of 51,000,

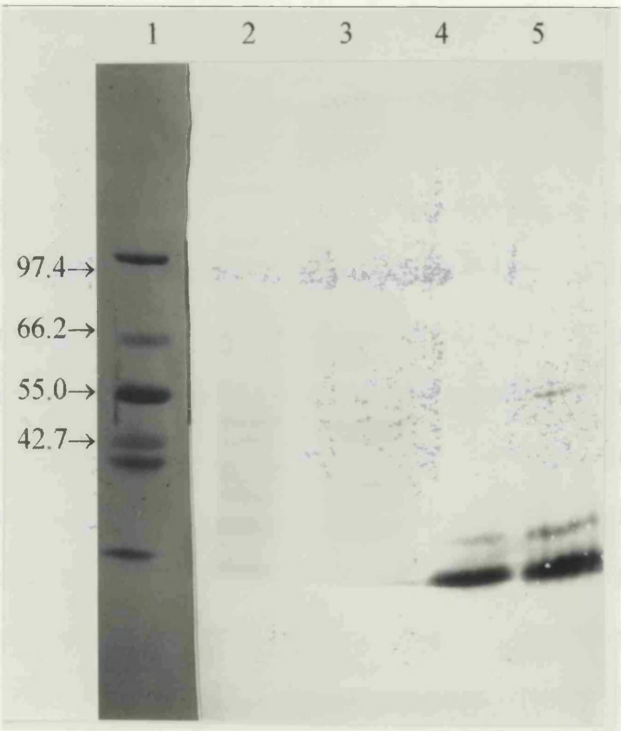


Figure 5.6: Western blot analysis of purified CYP3A4 obtained from chromatography on a *p*-chloroamphetamine-coupled sepharose column and human hepatocyte whole cell extracts using mouse polyclonal antisera to CYP3A4.

The samples were electrophoresed through a 7% SDS-PAGE gel and transferred to nitrocellulose. The blot was probed with the mouse polyclonal antisera allegedly raised to CYP3A4 (1 in 250 dilution). The blot was developed by using an alkaline phosphatase conjugated goat anti-mouse IgG (1 in 30,000 dilution). The following samples are shown: 2.5 µg of Promega mid-range molecular weight protein standards shown x $10^{-3}$ (lane 1); 40 µg of day 0 control (lane 2) and 40 µg of day 4 valproic acid-treated (lane 3) human hepatocyte homogenates from individual A (section 4.2.); 5 µl (lane 4) and 10 µl (lane 5) of purified CYP3A4 obtained from chromatography on a *p*-chloroamphetamine-coupled sepharose column.

corresponding to CYP3A4, was not detected in either of the hepatocyte homogenate samples.

The mouse had preferentially raised antibodies to lower molecular weight contaminating proteins in the purified CYP3A4 preparation. These highly immunogenic proteins are either insect cell or baculovirus proteins that have many antigenic epitopes. The band of apparent molecular weight of 50,000-53,000 detect in the purified CYP3A4 preparation may correspond to CYP3A4. If this is the case, then a very low titre of antibodies to CYP3A4 was produced by the mouse. It is possible that this band may represent an insect cell or baculovirus protein. To investigate this possibility the western blot was reprobed with an antisera to rabbit CYP3A6 (1 in 260 dilution) that had been raised in a goat. The blot was developed by using an alkaline phosphatase conjugated rabbit anti-goat IgG (1 in 30,000 dilution; data not shown).

A band of apparent molecular weight of 51,000 was detected in the hepatocyte homogenate samples; no other band was observed to have developed in the CYP3A4 preparations due to the reprobing of the blot. The band of apparent molecular weight of 50,000-53,000 in the purified CYP3A4 preparation showed the same intensity of colour development. The band of apparent molecular weight of 51,000 detected in the hepatocyte homogenates corresponded to isoforms of the CYP3A subfamily, including CYP3A4. This band was observed to be of a similar electrophoretic mobility as the bands of apparent molecular weight of 51,000 detected in the CYP3A4 samples. Therefore initial indications were that the mouse had produced a low titre of antibodies to CYP3A4. If the band corresponding to the CYP3As in the hepatocyte homogenates had been observed at a different electrophoretic mobility to the band detected in the purified CYP3A4 preparations and a new band had appeared in the CYP3A4 preparations then the 50,000-53,000 band would have represented an insect cell or baculovirus protein. This subject is further discussed in section 5.5.3.

Although CYP3A4 is the most abundantly expressed CYP in adult human liver, the concentration of CYP3A4 contained in a hepatocyte homogenate sample is low due to the vast numbers of other proteins contained in the hepatocytes. If the mouse had produced a low titre of antibodies to CYP3A4 a band representing CYP3A4 may not be detected in the homogenate samples.

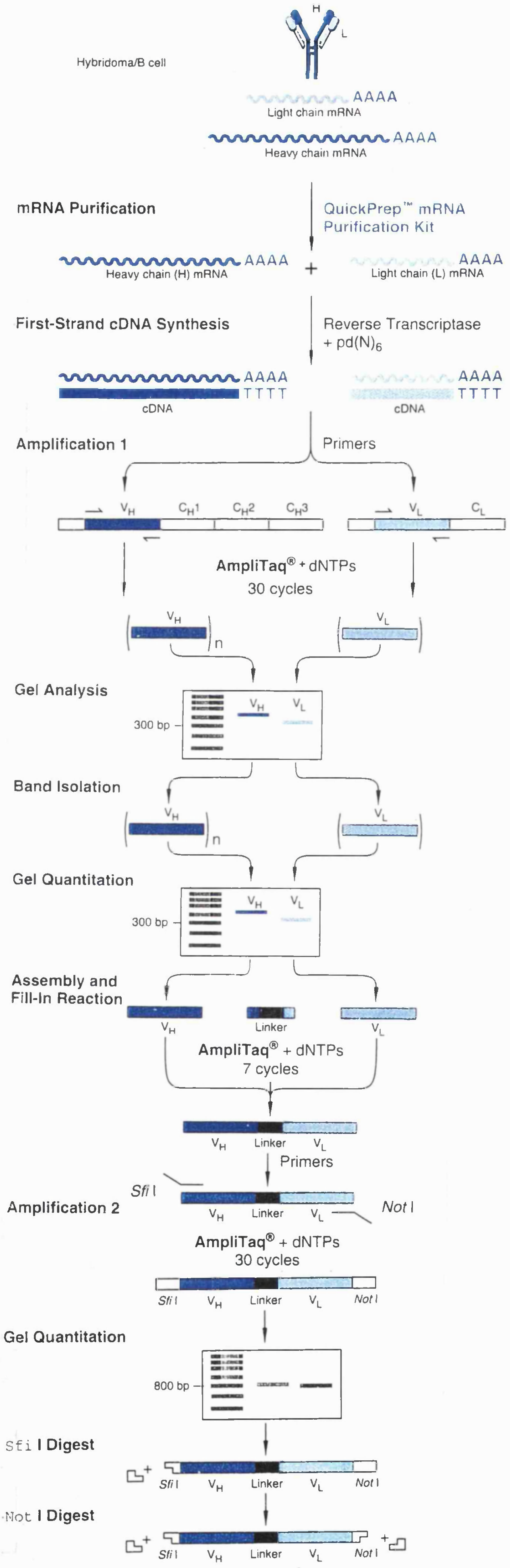
## 5.3. PRODUCTION OF ScFv FRAGMENTS

The ScFv fragments were produced using the mouse ScFv module of the recombinant phage antibody system (Pharmacia Biotech) as described in section 2.2.34.1.-2.2.34.9. The technology used in this system is based on research work by McCafferty *et al.* (1990) and Marks *et al.* (1991); reviewed in Winter and Milstein (1991). Additional technical information was provided with the protocols contained in the module. The module contained sufficient primers for the generation of five ScFv fragments. The protocols were extensively altered from those given in the instruction leaflets sent with the modules. The reasons behind these alterations will be discussed in the appropriate sections.

### 5.3.1. OVERVIEW OF PRODUCTION OF ScFv FRAGMENTS

A schematic diagram of the production of ScFv fragments is shown in figure 5.7. Total RNA is isolated from mice spleen cells, from which mRNA is extracted by affinity chromatography on oligo(dT)-cellulose. Reverse transcriptase synthesises the first-strand cDNA of the $V_H$ and $V_L$ chains from mRNA primed with random hexamers. The $V_H$ and $V_L$ chains are amplified in two separate reactions and then purified separately. The purified $V_H$ and $V_L$ DNA products are assembled at random into a single ScFv gene using a DNA linker fragment. The ScFv DNA fragment is amplified with a set of primers that introduce *Sfi* I and *Not* I restriction sites for cloning into the pCANTAB 5 E phagemid vector.

Figure 5.7: Schematic diagram showing the production of ScFv fragments (reproduced from the protocol booklet supplied with the mouse ScFv module).


H
L
Hybridoma/B cell
AAAA
Light chain mRNA
AAAA
Heavy chain mRNA
mRNA Purification
QuickPrep™ mRNA Purification Kit
AAAA
Heavy chain (H) mRNA
+
AAAA
Light chain (L) mRNA
First-Strand cDNA Synthesis
Reverse Transcriptase + pd(N)6
AAAA
TTTT
cDNA
AAAA
TTTT
cDNA
Amplification 1
Primers
VH
CH1
CH2
CH3
VL
CL
AmpliTaq® + dNTPs
30 cycles
VH
n
VL
Gel Analysis
300 bp
VH
VL
Band Isolation
VH
n
VL
Gel Quantitation
300 bp
VH
VL
Assembly and Fill-In Reaction
VH
Linker
VL
AmpliTaq® + dNTPs
7 cycles
VH
Linker
VL
Primers
Sfi I
Amplification 2
VH
Linker
VL
Not I
AmpliTaq® + dNTPs
30 cycles
Sfi I
VH
Linker
VL
Not I
Gel Quantitation
800 bp
Sfi I Digest
Sfi I
VH
Linker
VL
Not I
Not I Digest
Sfi I
VH
Linker
VL
Not I

### 5.3.2. ISOLATION OF TOTAL RNA FROM MICE SPLEEN CELLS AND PURIFICATION OF mRNA

Total RNA was isolated from the CYP2A6 and CYP3A4 immunised mice spleens using the Ultraspec$^{TM}$ RNA isolation system as described in section 2.2.17. Total RNA was also extracted from spleens of mice that had not been injected with any antigen; this was to enable a naive ScFv library to be generated. The total RNA samples from each spleen were divided into two or three aliquots. The integrity of the RNA samples were checked by electrophoresing 10 μg of each sample through a 1% denaturing agarose gel (a few examples are shown in figure 5.8). The yield of RNA was 5-8 μg/mg of spleen tissue. The 28S and 18S bands are visible but there is some evidence of RNA degradation (figure 5.8). Similar results were observed for the rest of the RNA samples (data not shown). A large amount of ribonuclease is present in the spleen; although the protocol was carried out as quickly as possible and with great care, some RNA degradation still occurred.

Total RNA samples were selected as the starting point of the recombinant phage antibody system on the basis of the results from the double immunodiffusion assay (figures 5.5A and B) and integrity of the RNA (figure 5.8 and data not shown).

The total RNA samples of the balb-c mice whose sera were used in wells **d** and **e** shown in figure 5.5A were selected for generation of the ScFv antibody library to CYP3A4. Whereas, the total RNA samples of the balb-c mice whose sera were used in wells **c** and **d** in figure 5.5B were selected for generation of the ScFv antibody library to CYP2A6. The total RNA samples isolated from the two unimmunised balb-c mice were used to generate the naive ScFv library. The two samples selected for generation of each type of ScFv library were pooled.

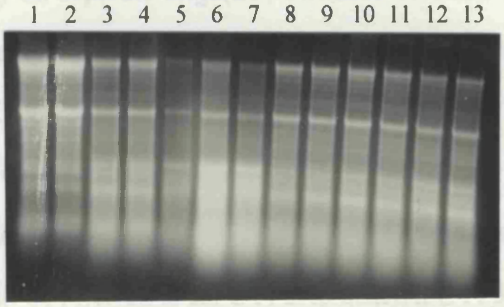


Figure 5.8: Analysis of total RNA from CYP2A6-, CYP3A4-immunised and unimmunised mice spleens using the Ultraspec$^{TM}$ RNA isolation system.

Total RNA was isolated from the mice spleens and 10 µg of each sample was electrophoresed through a 1% denaturing agarose gel. The gel was photographed under U.V. light. The following samples are shown: samples 1 and 2 of total RNA isolated from a balb-c unimmunised mouse (lanes 1-2, respectively); samples 1 and 2 of total RNA isolated from a balb-c unimmunised mouse (lanes 3-4, respectively); samples 1, 2 and 3 of total RNA isolated from a balb-c mouse immunised with CYP3A4 (lanes 5-7, respectively); samples 1 and 2 of total RNA isolated from a balb-c mouse immunised with CYP3A4 (lanes 8-9, respectively); samples 1 and 2 of total RNA isolated from a balb-c mouse immunised with CYP2A6 (lanes 10-11, respectively) and samples 1 and 2 of total RNA isolated from a balb-c mouse immunised with CYP2A6 (lanes 12-13, respectively).

mRNA was purified from the three pooled total RNA samples by affinity chromatography on separate oligo(dT)-cellulose columns (section 2.2.20.) and used to construct three ScFv cDNA libraries. One library was enriched for cDNAs of antibodies for CYP2A6, one for cDNAs of antibodies to CYP3A4 and the final one was a naive library which represents most of the possible ScFv cDNA combinations present in unimmunised mice. To differentiate between libraries, mRNA, $V_H$, $V_L$, or ScFv, each sample will be referred to as: CYP2A6 mRNA, CYP3A4 mRNA or control (naive) mRNA etc.

### 5.3.3. FIRST-STRAND cDNA SYNTHESIS OF $V_H$ AND $V_L$ AND PRIMARY PCR AMPLIFICATION

The first-strand cDNA $V_H$ and $V_L$ are synthesised using random hexamers by reverse transcriptase. The use of random hexamers eliminates the need for immunoglobulin-specific primers or oligo(dT) primers which would require the synthesis of a long cDNA, encoding the entire heavy or light chain. The cDNAs produced using hexamer primers are long enough to clone the V regions from both the heavy and light chain genes. These genes are amplified to generate enough DNA for cloning as the genes represent a very small fraction of the total cDNA.

The $V_H$ and $V_L$ genes are amplified by two sets of primers in two separate reactions. The primers are designed to hybridise to the opposite ends of the variable region of each chain (described at the beginning of section 2.34.). The primers anneal to the conserved framework sequences in the antibody variable regions. This enables all classes of antibodies from the mouse to be amplified.

A control mRNA sample was provided with the recombinant phage antibody system (RPAS) mouse ScFv module. The mRNA had been isolated from a mouse hybridoma; details of which were not provided. To avoid any confusion with the control mRNA used to generate the naive library, the control mRNA provided with the kit will be referred to as RPAS mRNA.

The reverse transcription reactions were carried out using between 200 ng to 1 µg of the four samples of mouse mRNA as described in section 2.2.34.1. The amplification reactions of the first-strand antibody cDNA were performed as described in section 2.2.34.2. The protocol provided with the kit suggested an incubation of 60 minutes at 37°C for the reverse transcription reaction. This incubation time was originally used for the reverse transcription.

5 µl of each of the amplified PCR products (RPAS control $V_H$ and $V_L$, control $V_H$ and $V_L$, CYP2A6 $V_H$ and $V_L$ and CYP3A4 $V_H$ and $V_L$) were analysed by electrophoresis through a 2% agarose gel (figure 5.9). The $V_H$ amplification reaction is expected to yield a DNA fragment of approximately 340 bp, whereas the $V_L$ amplification product should be approximately 325 bp. The RPAS control $V_H$ and $V_L$ were amplified successfully along with the control $V_H$ and $V_L$, CYP2A6 $V_L$ and CYP3A4 $V_L$ as the expected DNA fragments were observed in figure 5.9. All the $V_H$ and $V_L$ fragments (apart from the RPAS ones) were observed as doublets in figure 5.9. This may be because the three mRNA samples used for the RT-PCR reactions were pooled from two mice. The RPAS mRNA was obtained from a single mouse hybridoma clone. The CYP2A6 $V_H$ and CYP3A4 $V_H$ amplification reactions produced were not as successful; only a small quantity of the expected DNA fragments were observed (figure 5.9). Very small DNA fragments (<100 bp) were observed in these samples; these corresponded to unincorporated primers. 0.5 µl of AmpliTaq® DNA polymerase (5 units/µl) was added, below the mineral oil layer, to the two unsuccessful reaction tubes. The tubes were placed in the thermocycler and subjected to 30 cycles of the PCR programme described in section 2.2.34.2. 5 µl of each amplification product were analysed by electrophoresis through a 2% agarose gel (data not shown); there was no increase in the amount of expected amplification products. As other amplified $V_H$

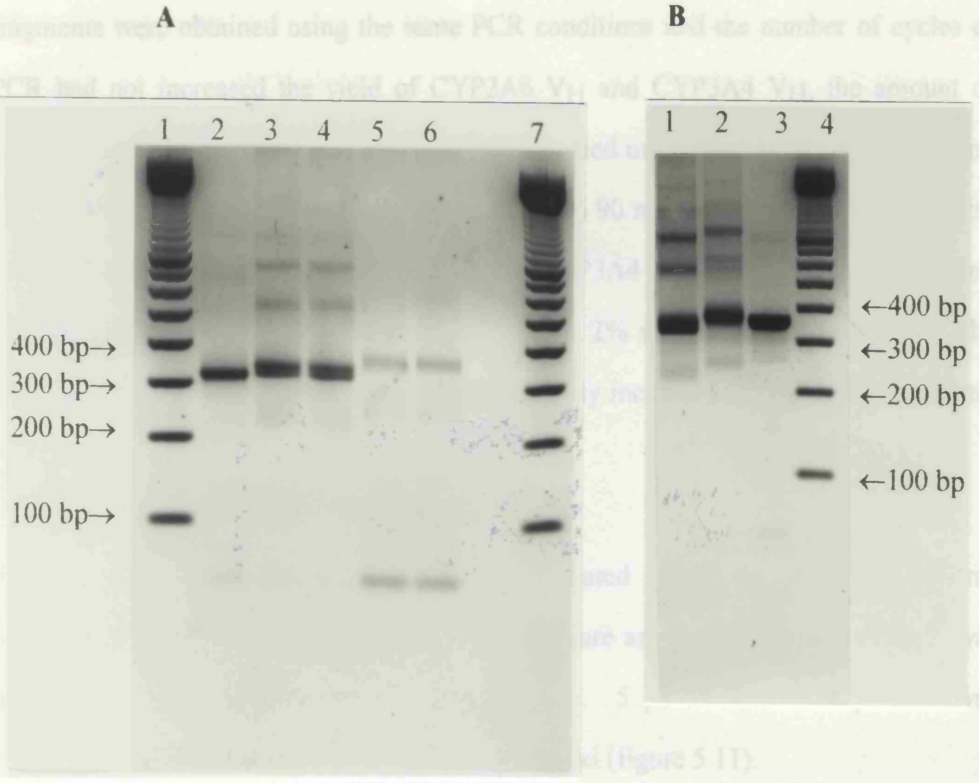

A
1
2
3
4
5
6
7
400 bp→
300 bp→
200 bp→
100 bp→
B
1
2
3
4
←400 bp
←300 bp
←200 bp
←100 bp

Figure 5.9: Primary amplification of $V_H$ and $V_L$ cDNA.

**A**: 5 µl of each amplified PCR product was electrophoresed through a 2% agarose gel along with 2 µg of 100 bp molecular weight standard DNA ladder (Pharmacia Biotech); the gel was photographed under U.V. light. The following samples are shown: 100 bp ladder (lanes 1 and 7); RPAS control $V_L$ (lane 2); CYP3A4 $V_L$ (lane 3); CYP2A6 $V_L$ (lane 4); CYP3A4 $V_H$ (lane 5) and CYP2A6 $V_H$ (lane 6).

**B**: As for figure **A** except the following samples are shown: control $V_L$ (lane 1); control $V_H$ (lane 2); RPAS control $V_H$ (lane 3) and 100 bp ladder (lane 4).

fragments were obtained using the same PCR conditions and the number of cycles of PCR had not increased the yield of CYP2A6 $V_H$ and CYP3A4 $V_H$, the amount of template for the PCR was increased. This was carried out by increasing the incubation time of the reverse transcription reaction from 60 to 90 minutes. The increased reverse transcription time was used in the RT-PCR of CYP3A4 $V_H$; 5 μl of the amplification product was analysed by electrophoresis through a 2% agarose gel (figure 5.10). The yield of the CYP3A4 $V_H$ fragment was substantially increased by the elevated amount of first-strand cDNA.

The RT-PCR of all eight samples were repeated using the increased reverse transcription incubation time of 90 minutes to ensure an adequate supply of each was available for the following stages of the module. 5 μl of each PCR product was analysed by electrophoresis through a 2% agarose gel (figure 5.11).

### 5.3.4. GEL PURIFICATION AND GEL QUANTIFICATION OF PRIMARY PCR PRODUCTS

The amplified $V_H$ and $V_L$ chain fragments were purified separately from a 1.5% agarose NA gel as described in section 2.2.34.3. This procedure removed the majority of primers and extraneous amplification products. The protocol supplied with the kit suggested that the $V_H$ and $V_L$ DNA bands excised from the 1.5% agarose gel should be placed in MicroSpin columns which were subsequently centrifuged once for 2 minutes at 735 x g. The resulting eluate of purified DNA was claimed to be pure enough to use in the assembly ScFv reactions. However, pieces of agarose were observed in the eluate; the membranes in the MicroSpin columns were thought to have cracked. Large amounts of DNA still remained in the agarose in the MicroSpin columns; this was determined by examination of the agarose pieces under U.V. light. A new set of columns were supplied by Pharmacia Biotech and the gel purification procedure was repeated with a new set of RT-PCR products. The MicroSpin columns were

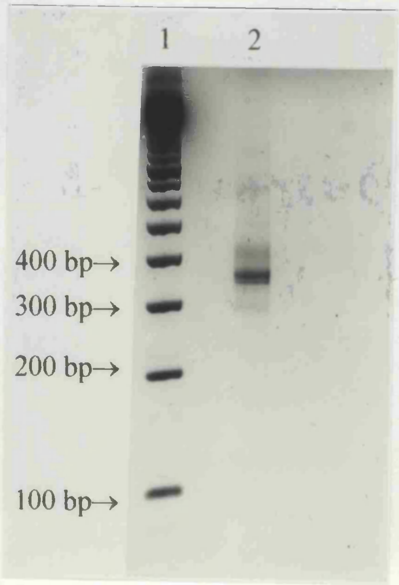


Figure 5.10: Primary amplification of increased amount of CYP3A4 $V_H$ cDNA.

5 μl of the amplified CYP3A4 $V_H$ (lane 1) was electrophoresed through a 2% agarose gel along with 2 μg of 100 bp molecular weight standard DNA ladder (Pharmacia Biotech; lane 2); the gel was photographed under U.V. light.

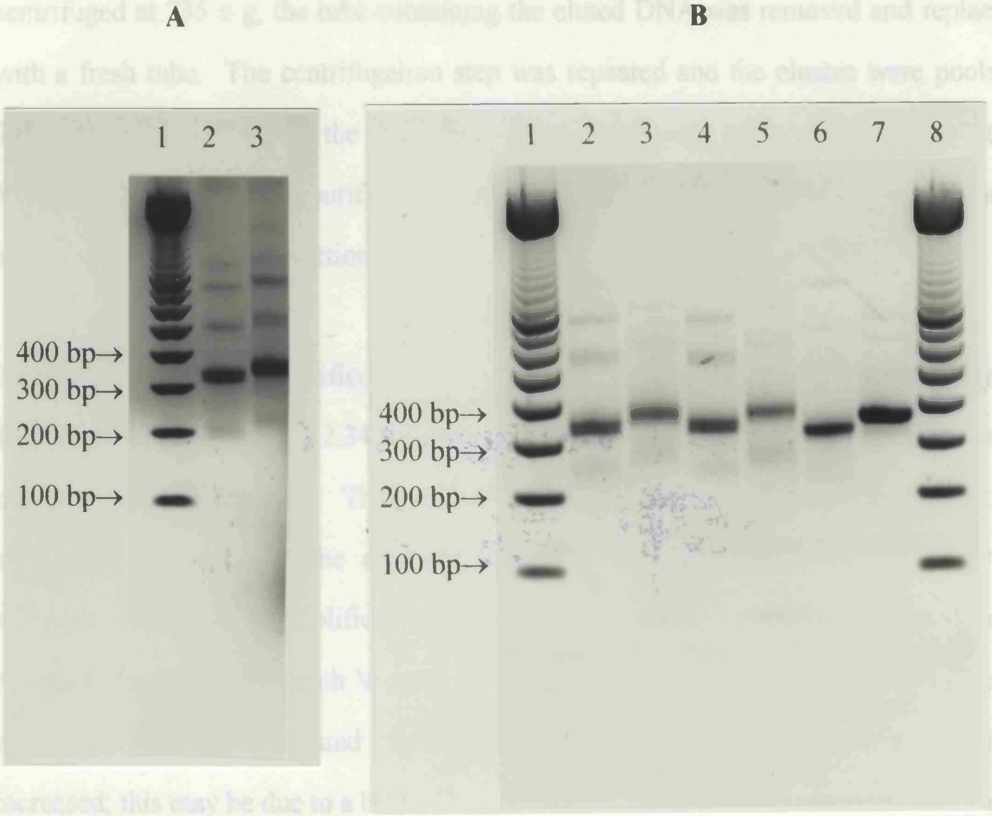

A
B
1 2 3
400 bp→
300 bp→
200 bp→
100 bp→
1 2 3 4 5 6 7 8
400 bp→
300 bp→
200 bp→
100 bp→

Figure 5.11: Primary amplification of increased amounts of $V_H$ and $V_L$ cDNA.

**A**: 5 µl of each amplified PCR product was electrophoresed through a 2% agarose gel along with 2 µg of 100 bp molecular weight standard DNA ladder (Pharmacia Biotech); the gel was photographed under U.V. light. The following samples are shown: 100 bp ladder (lane 1); control $V_L$ (lane 2) and control $V_H$ (lane 3).

**B**: As for figure **A** except the following samples are shown: 100 bp ladder (lanes 1 and 8); CYP3A4 $V_L$ (lane 2); CYP3A4 $V_H$ (lane 3); CYP2A6 $V_L$ (lane 4); CYP2A6 $V_H$ (lane 5); RPAS control $V_L$ (lane 6) and RPAS $V_H$ (lane 7).

centrifuged at 735 x g, the tube containing the eluted DNA was removed and replaced with a fresh tube. The centrifugation step was repeated and the eluates were pooled. This substantially reduced the amount of DNA left in the pieces of agarose in the MicroSpin columns. The purified $V_H$ and $V_L$ DNA samples were phenol/chloroform extracted as described in section 2.2.34.3. in order to increase the purity of the DNA.

2.5 µl and 5 µl of each purified $V_H$ and $V_L$ chain DNA product were quantified on a 1.5% agarose gel (section 2.2.34.4.) alongside 2.5 µl and 5 µl of a $V_H$ marker of known concentration (figure 5.12). This enabled the determination of the correct amount of each chain required for the assembly reaction. The first-strand antibody cDNA synthesis, primary PCR amplification, gel purification and gel quantification steps were repeated many times for each $V_H$ and $V_L$ product during the construction of the ScFv cDNA libraries. It was found that the amount of the purified $V_H$ and $V_L$ products increased; this may be due to a better yield of amplification product or an elevated yield of purified PCR product. This enabled smaller quantities (1 µl and 2 µl) of each PCR product to be used for gel quantification.

The intensities of the bands in the lanes containing the $V_H$ and $V_L$ products were compared to the 370 bp $V_H$ marker bands. The marker bands contained approximately 12.5 ng of DNA in the 2.5 µl aliquot and approximately 25 ng in the 5 µl aliquot. The amount of DNA in each $V_H$ and $V_L$ sample was estimated and the concentrations of the original purified DNA products were calculated (table 5.1). The $V_H$ marker also contained a larger DNA fragment; this corresponded to the plasmid into which the $V_H$ fragment had been cloned.

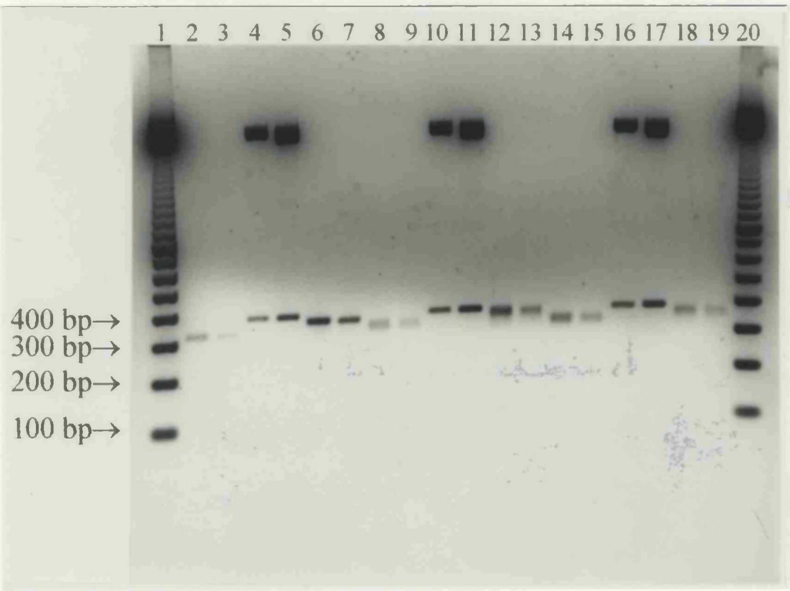
The volumes of purified $V_H$ and $V_L$ product that corresponded to 50 ng of DNA were determined; 50 ng of each product was required for the ScFv assembly reactions (section 5.3.5.).

Figure 5.12: Gel quantification of purified primary PCR products.

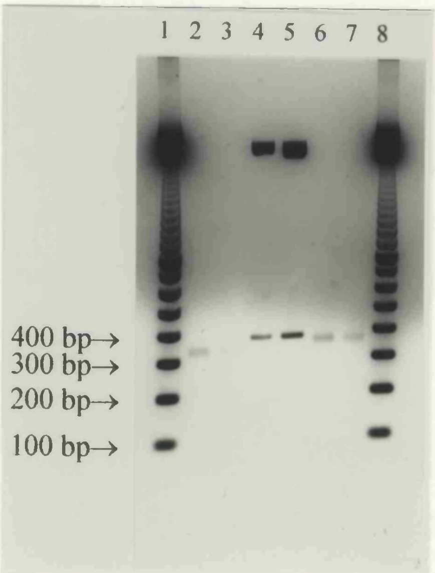
**A**: 2.5 µl and 5 µl of each purified $V_H$ and $V_L$ PCR product, 2.5 µl and 5 µl of a $V_H$ marker were electrophoresed through a 1.5% agarose gel along with 2 µg of 100 bp molecular weight standard DNA ladder (Pharmacia Biotech). The gel was photographed under U.V. light. The following samples are shown: 100 bp ladder (lanes 1 and 20); 5 µl and 2.5 µl of RPAS control $V_L$ (lanes 2 and 3); 2.5 µl and 5 µl of $V_H$ marker (lanes 4 and 5); 5 µl and 2.5 µl of RPAS control $V_H$ (lanes 6 and 7); 5 µl and 2.5 µl of control $V_L$ (lanes 8 and 9); 2.5 µl and 5 µl of $V_H$ marker (lanes 10 and 11); 5 µl and 2.5 µl of control $V_H$ (lanes 12 and 13); 5 µl and 2.5 µl of CYP3A4 $V_L$ (lanes 14 and 15); 2.5 µl and 5 µl of $V_H$ marker (lanes 16 and 17) and 5 µl and 2.5 µl of CYP3A4 $V_H$ (lanes 18 and 19).

**B**: As for figure **A** except the following samples are shown: 100 bp ladder (lanes 1 and 8); 5 µl and 2.5 µl of CYP2A6 $V_L$ (lanes 2 and 3); 2.5 µl and 5 µl of $V_H$ marker (lanes 4 and 5) and 5 µl and 2.5 µl of CYP2A6 $V_H$ (lanes 6 and 7).

**A**


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
400 bp→
300 bp→
200 bp→
100 bp→

**B**


1 2 3 4 5 6 7 8
400 bp→
300 bp→
200 bp→
100 bp→

| DNA product | Estimated concentration, ng/µl |
|---|---|
| RPAS $V_H$ | 5.0 |
| RPAS $V_L$ | 1.25 |
| | |
| Control $V_H$ | 2.5 |
| Control $V_L$ | 1.25 |
| | |
| CYP2A6 $V_H$ | 1.25 |
| CYP2A6 $V_L$ | 1.25 |
| | |
| CYP3A4 $V_H$ | 1.25 |
| CYP3A4 $V_L$ | 2.5 |

Table 5.1: Estimated concentrations of purified $V_H$ and $V_L$ DNA products.

### 5.3.5. ASSEMBLY OF THE ScFv FRAGMENTS AND AMPLICIFCATION OF ScFv DNA USING PRIMERS CONTAINING *Sfi* I OR *Not* I RESTRICTION SITES

The purified $V_H$ and $V_L$ chain DNA products were assembled into a single gene using a DNA linker fragment (section 2.2.34.5.). This was the most time-consuming step of the entire recombinant phage antibody system as the conditions had to be varied in order for the assembly to be successful. The linker fragment is constructed such that one end anneals to the 3' end of the $V_H$ chain while the other end hybridises to the 5' end of the $V_L$. The DNA of the linker fragment encodes $(Gly_4\ Ser)_3$, this maintains the correct reading frame of the ScFv gene. The assembled ScFv gene is approximately 750 bp. The linker peptide is very flexible, it allows the $V_H$ and $V_L$ regions to associate intramolecularly and stabilises the antigen binding site. The affinity and stability of ScFv antibodies containing the fifteen amino acid residue linker are generally comparable to those of the native antibody (Huston *et al.*, 1988).

The assembly reaction produces a small amount of the ScFv gene which would be undetectable on an ethidium bromide stained agarose gel. In order to acquire more ScFv DNA and to determine if the assembly reaction had been successful, the ScFv gene was amplified by PCR. The assembled antibody ScFv DNA fragment was amplified with a set of primers that added *Sfi* I and *Not* I restriction sites to the 5' and 3' ends of the gene, respectively (section 2.2.34.6.) for cloning into the pCANTAB 5 E vector. These restriction sites were used as they occur very infrequently in most genes due to their eight base pair recognition sequence. This should allow most ScFv genes to be cloned as a single *Sfi* I/*Not* I fragment.

The conditions for the assembly reaction were optimised using the RPAS control $V_H$ and $V_L$ purified DNA products and subsequently the control $V_H$ and $V_L$ purified DNA products when the supply of the former was exhausted. The optimised conditions are shown in section 2.2.34.5.

The assembly reaction was originally carried out using the conditions given in the protocol book which was supplied with the ScFv module; these were: 50 ng of $V_H$ and $V_L$ product, 4 µl of linker-primer mix, a final concentration of 1 x PCR buffer and 4 mM magnesium chloride, 2.5-5 units of AmpliTaq® DNA polymerase in a volume of 50 µl. The assembly reaction product was amplified and analysed by electrophoresis through a 1.5% agarose gel, DNA bands of 340 bp and 325 bp were observed (data not shown). These DNA bands corresponded to unassembled $V_H$ and $V_L$ products. Small DNA fragments, corresponding to unincorporated linker and restriction site primers, were also observed. The assembly and amplification reactions were performed many times; each time a new condition was varied. The following conditions were separately varied: amount of $V_H$ and $V_L$ products, amount of AmpliTaq® DNA polymerase, amount of dNTP mix, number of cycles for the assembly reaction and the number of cycles for the amplification reaction. All of these experiments failed to generate any detectable 750 bp ScFv DNA product (data not shown).

The final condition that was varied was the concentration of magnesium chloride present in the assembly reaction mix. The protocol provided with the ScFv module suggested that a final concentration of 4 mM magnesium chloride would allow a successful ScFv assembly reaction to proceed. The effect of increasing the final magnesium chloride concentration to 6.5 mM and 9 mM was investigated using the control $V_H$ and $V_L$ DNA products. 5 µl of each amplified DNA product was analysed by electrophoresis through a 1.5% agarose gel (figure 5.13). Both the modified protocols resulted in the generation of a 750 bp ScFv DNA band. A final concentration of 9 mM magnesium chloride yielded a greater amount of the ScFv DNA product. A final concentration of 9 mM magnesium chloride was used for all future assembly reactions (section 2.2.34.5.).

CYP2A6 and CYP3A4 ScFv products were assembled and amplified along with more control ScFv DNA. 5 µl of each PCR product was analysed by electrophoresis through

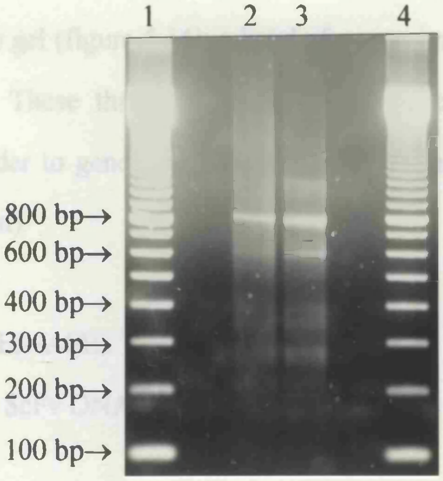


Figure 5.13: Assembly and amplification of control ScFv DNA.

The assembly reaction was performed in the presence of either 6.5 mM or 9 mM magnesium chloride. 5 µl of each amplified ScFv product was electrophoresed through a 1.5% agarose gel along with 2 µg of 100 bp molecular weight standard DNA ladder (Pharmacia Biotech). The gel was photographed under U.V. light. The following samples are shown: 100 bp ladder (lanes 1 and 4); control ScFv assembled in the presence of 6.5 mM magnesium chloride (lane 2) and control ScFv assembled in the presence of 9 mM magnesium chloride (lane 3).

a 1.5% agarose gel (figure 5.14); a band of approximately 750 bp was detected in all of the samples. These three assembly and amplification reactions were successfully repeated in order to generate sufficient DNA for the subsequent steps of this module (data not shown).

### 5.3.6. PROTEINASE K DIGESTION AND GEL QUANTIFICATION OF ASSEMBLED ScFv DNA

Pharmacia Biotech recommended spun-column purification of the amplified ScFv gene to remove unincorporated primers and extraneous PCR products. This was not carried out as problems with spun-column purification had already been encountered and the amount of ScFv DNA was limiting. Instead the ScFv products were treated with proteinase K (sections 2.2.34.7. and 2.2.15.) as this has been shown to increase the cloning efficiency of PCR products (Crowe *et al.*, 1991). The purity of the DNA was increased during this procedure by the phenol/chloroform extractions carried out. The resulting DNA pellets were resuspended in 10 μl of sterile distilled water.

1 μl and 1.5 μl of each proteinase K treated ScFv product were gel quantified on a 1.5% agarose gel (section 2.2.34.8.) alongside 2.5 μl and 5 μl of a ScFv marker of known concentration (figure 5.15). The intensities of the bands in the lanes containing ScFv products were compared to the 750 bp marker bands. The marker bands contained approximately 12.5 ng of DNA in the 2.5 μl aliquot and approximately 25 ng in the 5 μl aliquot. The amount of DNA in each of the ScFv products was estimated and the concentration of the original ScFv DNA was calculated (table 5.2). This allowed determination of the volume of ScFv product that corresponded to 120-300 ng of DNA. At least 100 ng of ScFv DNA was required for the *Sfi* I and *Not* I restriction digests (section 5.3.7.).

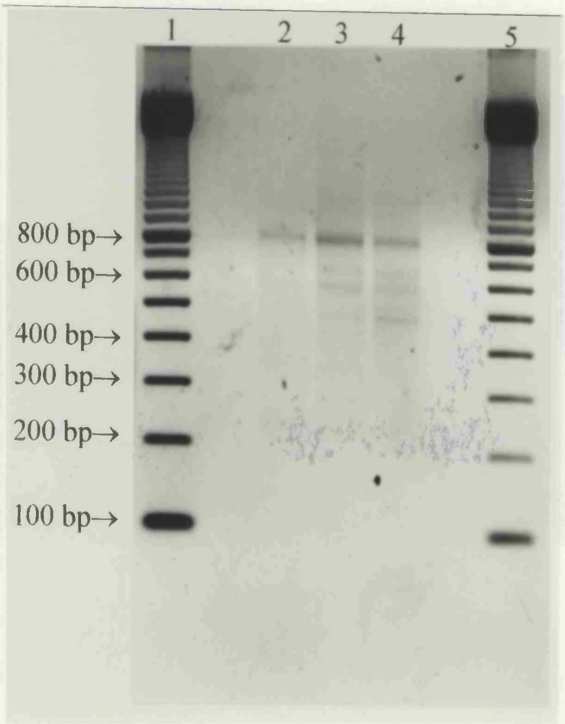


Figure 5.14: Assembly and amplification of control ScFv DNA, CYP2A6 ScFv DNA and CYP3A4 ScFv DNA.

The assembly reactions were performed in the presence of 9 mM magnesium chloride. 5 µl of each amplified ScFv product was electrophoresed through a 1.5% agarose gel along with 2 µg of 100 bp molecular weight standard DNA ladder (Pharmacia Biotech). The gel was photographed under U.V. light. The following samples are shown: 100 bp ladder (lanes 1 and 5); control ScFv DNA (lane 2); CYP3A4 ScFv DNA (lane 3) and CYP2A6 ScFv DNA (lane 4).

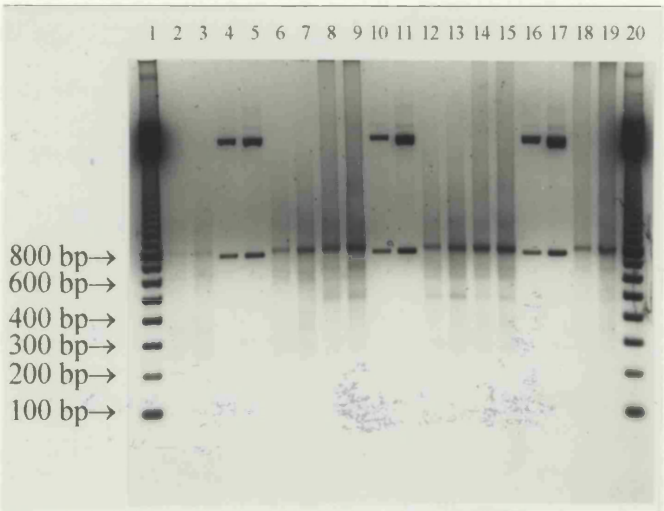


Figure 5.15: Gel quantification of assembled ScFv DNA.

1 μl and 1.5 μl of each ScFv DNA product, 2.5 μl and 5 μl of a ScFv marker were electrophoresed through a 1.5% agarose gel along with 2 μg of 100 bp molecular weight standard DNA ladder (Pharmacia Biotech). The gel was photographed under U.V. light. The following samples are shown: 100 bp ladder (lanes 1 and 20); 1 μl and 1.5 μl of control ScFv [sample 1] (lanes 2 and 3); 2.5 μl and 5 μl of ScFv marker (lanes 4 and 5); 1 μl and 1.5 μl of control ScFv [sample 2] (lanes 6 and 7); 1 μl and 1.5 μl of CYP2A6 [sample 1] (lanes 8 and 9); 2.5 μl and 5 μl of ScFv marker (lanes 10 and 11); 1 μl and 1.5 μl of CYP2A6 [sample 2] (lanes 12 and 13); 1 μl and 1.5 μl of CYP3A4 ScFv [sample 1] (lanes 14 and 15); 2.5 μl and 5 μl of ScFv marker (lanes 16 and 17) and 1 μl and 1.5 μl of CYP3A4 ScFv [sample 2] (lanes 18 and 19).

| Sample | Estimated concentration, ng/µl |
|---|---|
| Control ScFv (sample 1) | 2.1 |
| Control ScFv (sample 2) | 14.6 |
| | |
| CYP2A6 ScFv (sample 1) | 25.0 |
| CYP2A6 ScFv (sample 2) | 16.7 |
| | |
| CYP3A4 ScFv (sample 1) | 37.5 |
| CYP3A4 ScFv (sample 2) | 16.7 |

Table 5.2: Estimated concentrations of the six ScFv DNA products.

The two control ScFv samples were pooled and used for the restriction digests along with CYP2A6 ScFv (sample 1) and CYP3A4 ScFv (sample 1).

### 5.3.7. *Sfi* I AND *Not* I RESTRICTION DIGESTS OF ScFv FRAGMENTS

Each ScFv product (120-300 ng) was sequentially digested with *Sfi* I and *Not* I to generate cohesive ends for ligation into the cloning vector (section 5.4.4) as described in section 2.2.34.9. Pharmacia Biotech suggest spun-column purification of the restricted ScFv DNA to remove any small *Sfi* I or *Not* I fragments. However, this was not carried out because of the previously encountered problems with the spun columns. The restricted ScFv fragments were instead phenol/chloroform extracted and ethanol precipitated, as described in section 2.2.34.9., in preparation for ligation into the vector pCANTAB 5 E (section 5.4.4).

## 5.4. EXPRESSION OF ScFv ANTIBODIES IN BACTERIA

The expression module is designed to clone an antibody ScFv fragment, produced in the mouse ScFv module, into the phagemid vector pCANTAB 5 E. The flexible nature of the phagemid vector (described in section 5.4.1.) allows the production of both phage-displayed and soluble antibodies in different strains of *E. coli* (reviewed in: Marks *et al.*, 1991 and Plückthun, 1991). Additional technical information was provided in the protocol leaflets supplied with the module.

### 5.4.1. THE PHAGEMID pCANTAB 5 E

A phagemid vector combines the properties of a phage and plasmid vector. pCANTAB 5 E possesses an M13 and a plasmid origin of replication (figure 5.16). The phagemid can be propagated as either a plasmid or packaged as a recombinant M13 phage with the aid of a helper phage as the phagemid lacks the phage protein genes necessary to produce an entire phage.

pCANTAB 5 E is constructed such that the ScFv fragment is cloned into *Sfi* I and *Not* I restriction sites that lie between the leader sequence and the main part of the M13 gene 3. This gene encodes the phage adsorption protein; this protein mediates phage attachment to the F pilus of a male *E. coli.* A viable M13 phage usually expresses either three or five copies of the gene 3-encoded adsorption protein (g3p) on its tip. The expressed ScFv-g3p fusion protein retains the functions of both of the parent proteins. The g3p leader sequence directs the transport of the protein to the inner membrane/periplasm of *E. coli* where the main part of the g3p protein attaches the fusion protein to the tip of an assembling phage. Mature phage display one copy of the ScFv-g3p fusion protein and either two or four copies of the native g3p protein.

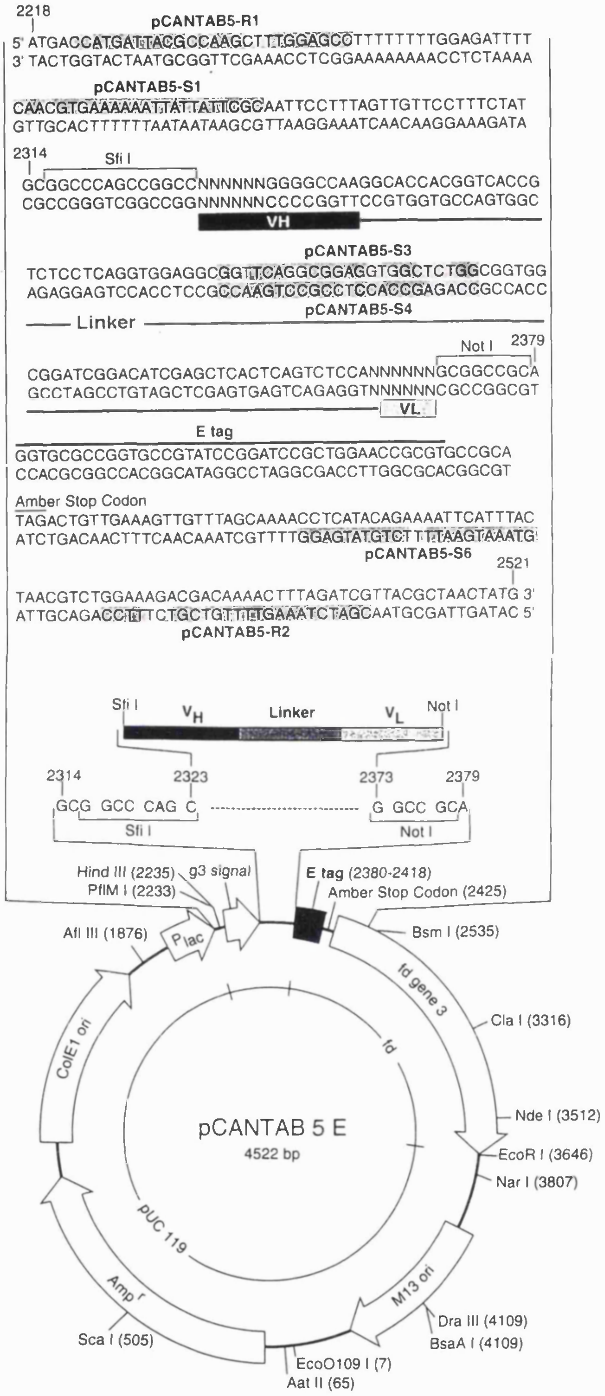

2218
pCANTAB5-R1
5' ATGACCATGATTACGCCAAGCTTTGGAGCCTTTTTTTTGGAGATTTT
3' TACTGGTACTAATGCGGTTCGAAACCTCGGAAAAAAAACCTCTAAAA
pCANTAB5-S1
CAACGTGAAAAAATTATTATTCGCAATTCCTTTAGTTGTTCCTTTCTAT
GTTGCACTTTTTTAATAATAAGCGTTAAGGAAATCAACAAGGAAAGATA
2314
Sfi I
GCGGCCCAGCCGGCCNNNNNNGGGGCCAAGGCACCACGGTCACCG
CGCCGGGTCGGCCGGNNNNNNCCCCGGTTCCGTGGTGCCAGTGGC
VH
pCANTAB5-S3
TCTCCTCAGGTGGAGGCGGTTCAGGCGGAGGTGGCTCTGGCGGTGG
AGAGGAGTCCACCTCCGCCAAGTCCGCCTCCACCGAGACCGCCACC
pCANTAB5-S4
Linker
Not I
2379
CGGATCGGACATCGAGCTCACTCAGTCTCCANNNNNNGCGGCCGCA
GCCTAGCCTGTAGCTCGAGTGAGTCAGAGGTNNNNNNCGCCGGCGT
VL
E tag
GGTGCGCCGGTGCCGTATCCGGATCCGCTGGAACCGCGTGCCGCA
CCACGCGGCCACGGCATAGGCCTAGGCGACCTTGGCGCACGGCGT
Amber Stop Codon
TAGACTGTTGAAAGTTGTTTAGCAAAACCTCATACAGAAAATTCATTTAC
ATCTGACAACTTTCAACAAATCGTTTTGGAGTATGTCTTTTAAGTAAATG
pCANTAB5-S6
2521
TAACGTCTGGAAAGACGACAAAACTTTAGATCGTTACGCTAACTATG 3'
ATTGCAGACCTTTCTGCTGTTTTGAAATCTAGCAATGCGATTGATAC 5'
pCANTAB5-R2
Sfi I
V$_H$
Linker
V$_L$
Not I
2314
2323
2373
2379
GCG GCC CAG C
G GCC GCA
Sfi I
Not I
Hind III (2235)
g3 signal
PflM I (2233)
E tag (2380-2418)
Amber Stop Codon (2425)
Afl III (1876)
Plac
Bsm I (2535)
fd gene 3
Cla I (3316)
fd
ColE1 ori
pCANTAB 5 E
4522 bp
Nde I (3512)
EcoR I (3646)
Nar I (3807)
pUC 119
M13 ori
Amp r
Dra III (4109)
BsaA I (4109)
Sca I (505)
EcoO109 I (7)
Aat II (65)

Figure 5.16: Map of phagemid pCANTAB 5 E showing the control regions and genealogy of the vector (reproduced from the protocol booklet supplied with the expression module).

pCANTAB 5 E also contains a sequence encoding a thirteen amino acid peptide E-tag. This E-tag is followed by an amber translational stop codon (UAG) at the junction between the cloned ScFv fragment and the sequence encoding the g3p protein. When a *supE* strain of *E. coli,* such as TG1, is transformed with the recombinant vector glutamine is inserted at the UAG codon and a ScFv-g3p fusion protein is expressed. In non-suppressor strains, such as HB2151, the stop codon is recognised and protein synthesis is halted at the end of the E-tag. An ScFv protein with a thirteen amino acid E-tag at the C-terminus is expressed.

pCANTAB 5 E also carries an ampicillin resistance gene to enable the selection of *E. coli* transformed with the recombinant ScFv-pCANTAB 5 E vector.

The expression of the ScFv-g3p gene is under control of the *lac* promoter located in the phagemid vector. The *lac* promoter is regulated by the *lac* repressor which is encoded by the $lacI^q$ gene in the *E. coli* genome of the strains TG1 and HB2151. Vast quantities of expressed proteins destined for export, such as ScFv-g3p and ScFv, are toxic to *E. coli.* Therefore the expression of these proteins must be tightly controlled. Repression of the *lac* promoter by the *lac* repressor is rather leaky. Further repression is provided by growing *E. coli* transformed with the recombinant ScFv-pCANTAB 5 E construct in media containing 2% glucose. The expression of the recombinant antibody is controlled through catabolite repression by the presence of glucose in the media. If expression of the antibodies is required, the transformed *E. coli* are grown in the absence of glucose. IPTG is added to the media when large amounts of soluble antibodies are required. IPTG binds the *lac* repressor and prevents it binding to the *lac* promoter. All transformed *E. coli* cells are grown at the sub-optimal temperature of 30°C to slow down the expression of the toxic proteins which are lethal to the host cells when present in vast quantities.

### 5.4.2. PHAGE-DISPLAYED ScFv ANTIBODIES

This thesis is concerned with the production of soluble ScFv antibodies therefore the theory behind the phage-displayed antibodies will be mentioned only briefly. pCANTAB 5 E is a phagemid vector which is deficient in the machinery required for complete viral assembly and replication. The necessary components need to be supplied by a helper phage if phage-displayed antibodies are required. *E. coli* TG1 are transformed with ScFv-pCANTAB 5 E recombinant phagemid; the amber stop codon is suppressed and a ScFv-g3p fusion protein is produced. The suppression of the amber codon is approximately 20% efficient, therefore a small amount of soluble antibodies are also produced.

The transformed *E. coli* are infected with a helper phage, M13KO7 to "rescue" the phagemid (Vieira and Messing, 1987). The majority of the mature phage produced contain phagemid DNA which express usually only one ScFv-g3p protein at their tips. The helper phage contains a wild-type g3p gene which competes with the ScFv-g3p gene in the phagemid for display on the surface of the mature phage.

### 5.4.3. SOLUBLE ScFv ANTIBODIES

The protocol supplied with the expression module suggests that phage-displayed antibodies are generated en route to the production of soluble antibodies. Phage-displayed antibodies that recognise the antigen of interest are selected by panning against the antigen. Phage displaying the antigen-binding recombinant antibodies can be used to reinfect *E. coli* TG1 cells for another round of panning; they can also be assayed for antigen-binding in an ELISA (reagents for this assay are provided with the detection module). Phage antibodies that show a positive signal in an ELISA are used to infect *E. coli* HB2151 cells for the production of soluble antibodies.

The panning process is arduous and has many problems. The antigen may not bind well to the support used for panning, several rounds of panning are required to enrich for antigen-binding phage-displayed antibodies and high affinity antibodies may be difficult to remove from the support. There is also the added problem that some cloned ScFv fragments may contain in frame UAG stop codons that are suppressed in the *E. coli* strain TG1 but are recognised in HB2151; this prevents the expression of a functional ScFv.

As soluble antibodies were required for use as immunological tools, I decided to adapt the protocol and transform the ScFv-pCANTAB 5 E recombinant phagemids directly into the *E. coli* strain HB2151. The amber stop codon is recognised in the non-suppressor strain HB2151 and only soluble ScFv antibodies are produced. The soluble antibodies have a thirteen amino acid E-tag that is used to aid the detection of these antibodies.

### 5.4.4. LIGATION OF ScFv FRAGMENTS TO pCANTAB 5 E

60 ng of *Sfi* I and *Not* I restricted control ScFv fragment, 100 ng of *Sfi* I and *Not* I restricted CYP2A6 ScFv DNA or 150 ng of *Sfi* I and *Not* I restricted CYP3A4 ScFv DNA were ligated into 200 ng of the phagemid vector, pCANTAB 5 E, in separate reactions as described in section 2.2.34.20. pCANTAB 5 E has incompatible sticky ends and should therefore not be able to selfligate. A vector background ligation reaction was set up to test the efficiency of the original restriction endonuclease digests used to produce the ready-to-ligate phagemid by Pharmacia Biotech. A control ligation reaction was set up using 150 ng of a control ScFv insert with 50 ng of pCANTAB 5 E. The ligation reactions were carried out in a volume of 10 μl, this was used instead of the 50 μl reaction volume suggested in the protocol provided with the kit. The smaller volume of the ligation reaction should increase the frequency of successful ligation

events. The protocol suggested an incubation of 1 hour at 16$^{o}$C; the incubation time was increased to 16 hours to hopefully increase the amount of recombinant phagemid.

The ligated constructs were used to transform *E. coli* HB2151 by electroporation. Electroporation results in a far higher transformation efficiency than any chemical method. The recombinant ScFv-pCANTAB 5 E phagemids and the two control reaction samples were phenol/chloroform and chloroform extracted as described in section 2.2.34.10. This was necessary as the DNA must be as pure and free of salt as possible before electroporation.

### 5.4.5. TRANSFORMATION OF *E. COLI* BY ELECTROPORATION WITH ScFv-pCANTAB 5 E CONSTRUCTS

The recombinant ScFv-pCANTAB 5 E phagemids and the two control ligation samples were separately introduced into *E. coli* HB2151 by electroporation as described in section 2.2.34.13. Negative and positive experimental controls were set up using cells only and 5 ng of the plasmid pBluescript (Gibco BRL), respectively. The transformation cultures were plated on SOBAG (ligation and experimental controls) and SOBAG-N (control ScFv-pCANTAB 5 E, CYP2A6 ScFv-pCANTAB 5 E and CYP3A4 ScFv-pCANTAB 5E) agar plates as described in section 2.2.34.13. SOBAG and SOBAG-N agar contain 2% glucose, which represses the production of the ScFv antibodies, and ampicillin which selects for cells containing the recombinant phagemids. SOBAG-N agar contains 100 μg/ml nalidixic acid. *E. coli* HB2151 possess a gene that confers resistance to nalidixic acid on the strain, whereas *E. coli* TG1 do not carry this gene and are therefore sensitive to the antibiotic. SOBAG-N agar was used to plate the three potential ScFv cDNA libraries as a precaution in case the stock of HB2151 cells, supplied with the kit, was contaminated with TG1 cells. The choice of agar medium ensured soluble expression of all the ScFvs.

The transformation experiments were a success; the expected results were obtained for the ligation and transformation experimental controls. The vector background control ligation reaction (pCANTAB 5 E only) produced three white colonies on transformation of the *E. coli* cells. This showed that the *Sfi* I and *Not* I restriction of the phagemid vector carried out by Pharmacia Biotech was not 100% efficient. A low percentage of the colonies in the three ScFv cDNA libraries will contain the pCANTAB 5 E vector with no ScFv DNA insert.

The size of each of the three ScFv cDNA libraries were estimated as described in section 2.2.34.14. The estimated size of each library was: 3.27 x $10^4$ colonies for the control ScFv cDNA library; 1.03 x $10^4$ colonies for the CYP2A6 cDNA library and 3.5 x $10^4$ colonies for the CYP3A4 ScFv cDNA library. Using *E. coli* TG1, Pharmacia Biotech have generated library sizes of >5 x $10^6$/μg of recombinant vector DNA. If the ScFv-pCANTAB 5 E ligation reactions were 100% efficient then the maximum amount of recombinant phagemid would be: 60 ng for control ScFv, 100 ng for CYP2A6 ScFv and 150 ng for CYP3A4 ScFv. The library sizes/μg of recombinant vector DNA would be: 5.45 x $10^5$ for control ScFv; 1.03 x $10^5$ for CYP2A6 ScFv and 2.33 x $10^5$ for CYP3A4 ScFv. Phage antibody repertoires ranging from 1 x $10^6$-1 x $10^8$/μg of vector DNA have been achieved (reviewed in Winter and Milstein, 1991); there are no comparable figures for soluble antibody repertoires.

The size of the resulting cDNA library is determined by the quantity and quality of successfully ligated phagemid DNA. The ScFv, *Sfi* I/*Not* I restricted ScFv fragments and ligated ScFv-pCANTAB 5E recombinant phagemids were phenol/chloroform extracted. Approximately 50% of the DNA can be lost during a phenol/chloroform extraction. The resulting DNA should be of good quality, however any minor contaminants will reduce the efficiency of subsequent reactions. The library size could be increased by using a greater amount of ScFv DNA for the restriction and subsequent ligation reactions. However, there is a balance between increasing the amount of ScFv DNA used and achieving a successful *Sfi* I/*Not* I ScFv digestion.

The size of a cDNA library is also determined by the transformation efficiency of the *E. coli*. Phage-displayed antibody libraries are produced by transforming *E. coli* TG1; the soluble antibody libraries were produced by transforming *E. coli* HB2151. It was mentioned in the protocol booklet, that *E. coli* TG1 has a far greater transformation efficiency than *E. coli* HB2151. Control transformation experiments were performed in both strains of *E. coli* using 5 and 50 ng of pBluescript DNA. A similar high number of transformants were obtained for each strain. The reported difference in transformation efficiencies is therefore unlikely to have caused the reduced library sizes. The reason was probably due to a less than ideal amount of pure successfully ligated ScFv-pCANTAB 5 E recombinant phagemid.

The cDNA libraries were stored as described in section 2.2.34.15.; the stored libraries were titered as described in section 2.2.34.15.

Waterhouse *et al.* (1993) have created an antibody repertoire of 1 x $10^{11}$ clones using a technique known as combinatorial infection. *E. coli* are transformed with a repertoire of heavy chains (encoded on plasmids) and are then infected with a repertoire of light chains (encoded on phage). The heavy and light chain genes are recombined *in vivo* onto the same replicon by use of the *lox*-Cre site-specific recombination system of bacteriophage P1. The combinatorial library produces a wide array of phage-displayed Fab antibodies. Such large libraries are not required if the mice are injected with the antigen of interest.

## 5.5. ISOLATION OF FURTHER PURIFIED CYP2A6 AND CYP3A4

Sufficient purified CYP2A6 and CYP3A4 had been produced for injection of the mice. However, more purified antigen was required for the screening of the ScFv antibody library. A different purification strategy was used in an attempt to increase the yield of purified protein.

The recombinant baculovirus containing the cDNA of CYP2A6 was propagated in *Sf9* insect cells. This was carried out in order to increase the titre of the recombinant baculovirus for expression of CYP2A6 in *T ni* insect cells. These experiments were carried out by myself and Dr. Colin Dolphin in the laboratory of Prof. I. R. Phillips at Queen Mary and Westfield College. The *Sf9* cells did not grow well, despite numerous experiments conducted by Dr. Dolphin the problem could not be solved. The recombinant baculovirus was attempted to be amplified and the titre determined in *T ni* cells, but this was also unsuccessful. It was decided not to continue with this area of research due to a lack of time. However, when CYP2A6 is eventually expressed in *T ni* cells, it will be purified and used to screen the library containing ScFv antibodies to CYP2A6.

Microsomes prepared from *T ni* insect cells expressing CYP3A4 (and P450-reductase) were donated by GlaxoWellcome. The concentration of expressed CYP3A4 was 0.67 nmole/ml; approximately 29 nmoles (≈1.48 mg) of CYP3A4. The sample volume was a more manageable amount of 43 ml.

### 5.5.1. PURIFICATION OF CYP3A4 FROM INSECT CELL MICROSOMES BY CHROMATOGRAPHY ON A *N*-OCTYLAMINO-SEPHAROSE COLUMN

The microsomes containing expressed CYP3A4 were solubilised as in section 2.2.22. and CYP3A4 was purified by chromatography on an *n*-octylamino-sepharose column (adapted from Guengerich and Martin, in press) as described in section 2.2.22. The O.D.$_{280}$ and O.D.$_{417}$ were measured for each fraction and on the basis of these readings certain fractions were selected for electrophoresis on 10% SDS-PAGE gels for Coomassie blue staining (figure 5.17) and on 7% SDS-PAGE gels for western blot analysis (figures 5.18 and 5.19).

The western blots were probed with an antisera to rat P450-reductase (1 in 500 dilution) that had been raised in a rabbit (figure 5.18). The blots were developed by using an alkaline phosphatase conjugated goat anti-rabbit IgG (1 in 3,000 dilution). The western blots were reprobed with an antisera to baboon CYP3A (1 in 400 dilution) that had been raised in a sheep (figure 5.19). This antibody had been prepared in the laboratory of Dr. Patrick Maurel (Dalet-Beluche *et al.*, 1992) and a sample was kindly given to our laboratory. The antibody cross-reacts with the CYP3As but is unable to distinguish between the isoforms of the CYP3A subfamily. The blots were developed by using an alkaline phosphatase conjugated donkey anti-sheep IgG (1 in 30,000 dilution).

Figure 5.17 shows that CYP3A4 was substantially enriched by chromatography on an *n*-octylamino-sepharose column in comparison to the sample of solubilised microsomes. A protein band corresponding to CYP3A4 was observed in fractions 12-33 at an apparent molecular weight of 51,000. These results were confirmed by western blot analysis (figure 5.19). CYP3A4 was not detected in samples from either the flow-through or the wash steps (figure 5.17); this was confirmed by western blotting (figure 5.19). The fractions containing CYP3A4 were pooled and concentrated using an Amicon PM30 ultrafiltration cell; the sample was further purified by anion exchange chromatography on a DEAE-sephacel column (section 5.5.2.).

P450-reductase was observed in fractions 15-100 at the apparent molecular weight of 78,000 (figure 5.17). These results were confirmed by western blot analysis (figure 5.18). The breakdown product of P450-reductase (section 1.2.), apparent molecular

**A**

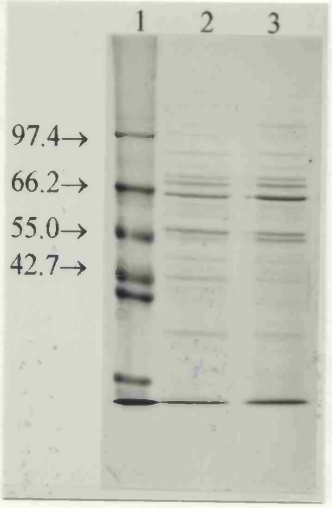


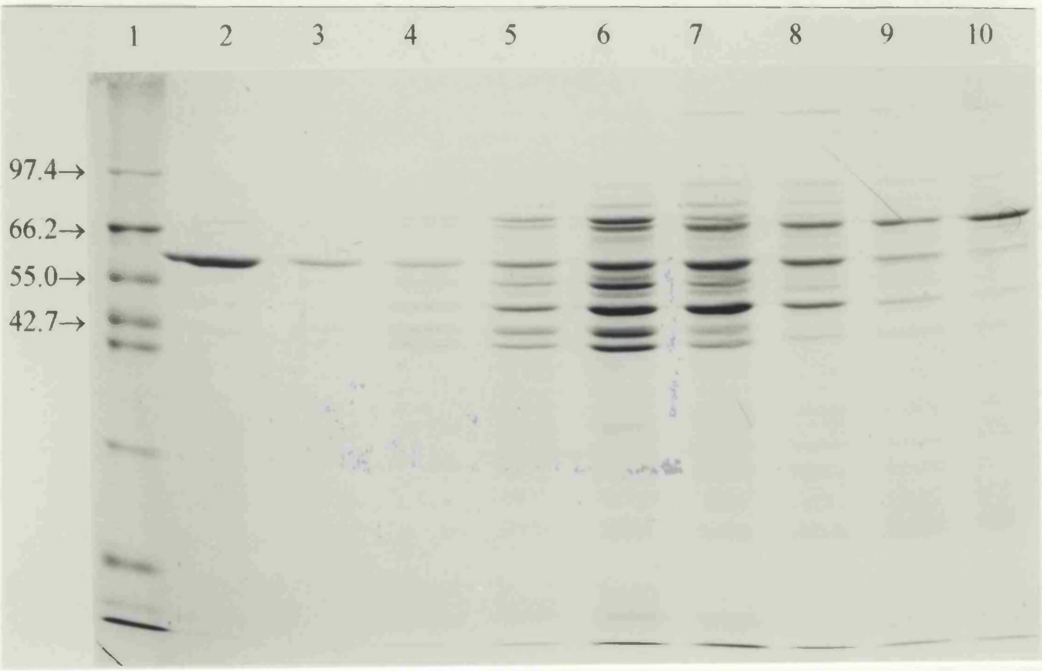
Figure 5.17: Coomassie blue stained SDS-PAGE gels showing fractions collected during the purification of CYP3A4 (and P450-reductase) from insect cell microsomes by chromatography on an *n*-octylamino-sepharose column.

**A**: 50 µl of each sample was electrophoresed through a 10% SDS-PAGE gel; the gel was Coomassie blue stained and photographed with the aid of a light box. The following samples are shown: 2.5 µg of Promega mid-range molecular weight protein standards shown x $10^{-3}$ (lane 1); a sample from a flow-through fraction collected during the loading of the column (lane 2); a sample from a fraction collected during the washing of the column (lane 3).

**B**: As for figure **A** except the following samples are shown: 2.5 µg of Promega mid-range molecular weight protein standards shown x $10^{-3}$ (lane 1); fraction 3 (lane 2); fraction 10 (lane 3); fraction 12 (lane 4); fraction 15 (lane 5); fraction 18 (lane 6); fraction 21 (lane 7); fraction 24 (lane 8); fraction 27 (lane 9) and fraction 30 (lane 10).

**C**: As for figure **A** except the following samples are shown: 2.5 µg of Promega mid-range molecular weight protein standards shown x $10^{-3}$ (lane 1); fraction 33 (lane 2); fraction 40 (lane 3); fraction 45 (lane 4); fraction 50 (lane 5); fraction 60 (lane 6); fraction 70 (lane 7); fraction 80 (lane 8); fraction 90 (lane 9) and fraction 100 (lane 10).

**B**


1
2
3
4
5
6
7
8
9
10
97.4→
66.2→
55.0→
42.7→

**C**

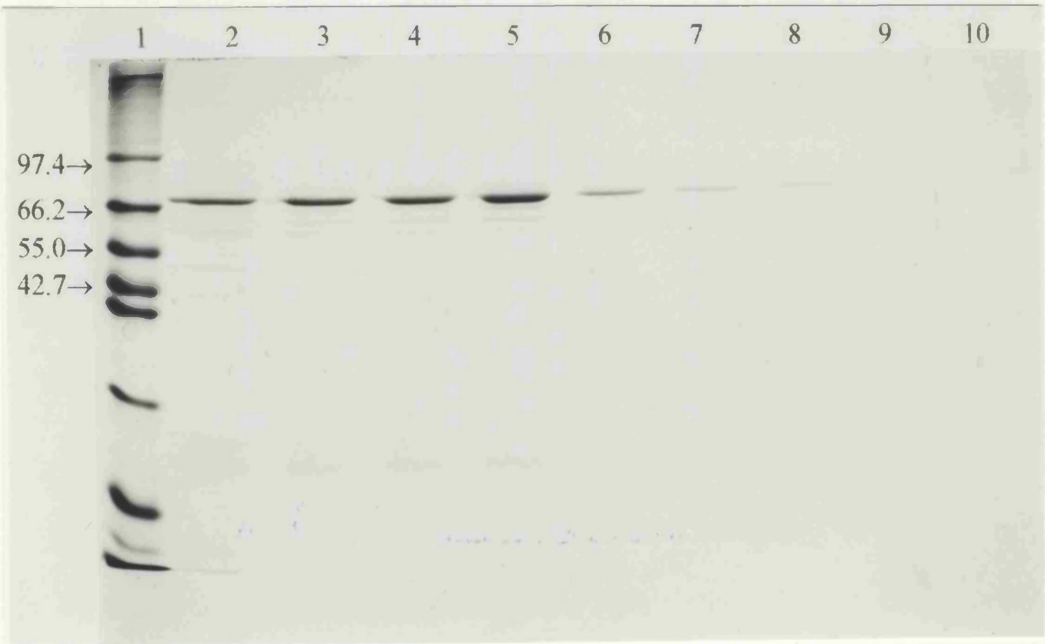
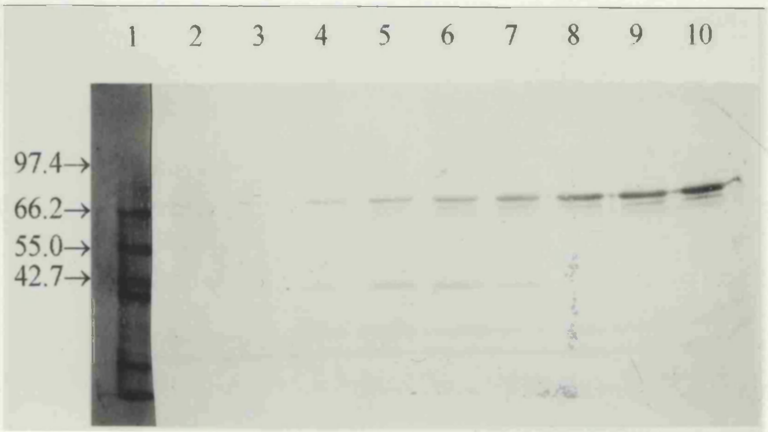

1
2
3
4
5
6
7
8
9
10
97.4→
66.2→
55.0→
42.7→

Figure 5.18: Western blot analysis of fractions collected during the purification of CYP3A4 (and P450-reductase) from insect cell microsomes by chromatography on an *n*-octylamino-sepharose column using a polyclonal antisera to P450-reductase.

**A**: 50 µl of each sample were electrophoresed through a 7% SDS-PAGE gel and transferred to nitrocellulose. The blot was probed with an antisera to rat P450-reductase (1 in 500 dilution) that had been raised in a rabbit. The blot was developed by using an alkaline phosphatase conjugated goat anti-rabbit IgG (1 in 3,000 dilution). The following samples are shown: 2.5 µg of Promega mid-range molecular weight protein standards shown x $10^{-3}$ (lane 1); fraction 3 (lane 2); fraction 10 (lane 3); fraction 12 (lane 4); fraction 15 (lane 5); fraction 18 (lane 6); fraction 21 (lane 7); fraction 24 (lane 8); fraction 27 (lane 9) and fraction 30 (lane 10).

**B**: As for figure **A** except the following samples are shown: 2.5 µg of Promega mid-range molecular weight protein standards shown x $10^{-3}$ (lane 1); fraction 33 (lane 2); fraction 40 (lane 3); fraction 45 (lane 4); fraction 50 (lane 5); fraction 60 (lane 6); fraction 70 (lane 7); fraction 80 (lane 8); fraction 90 (lane 9) and fraction 100 (lane 10).

**A**


1 2 3 4 5 6 7 8 9 10
97.4→
66.2→
55.0→
42.7→

**B**

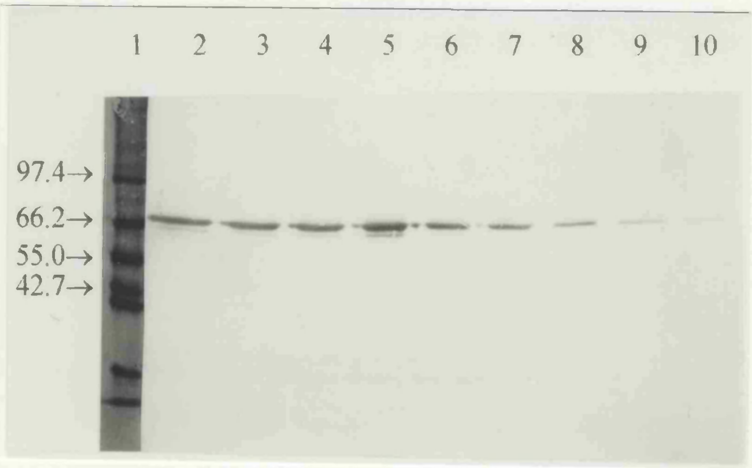
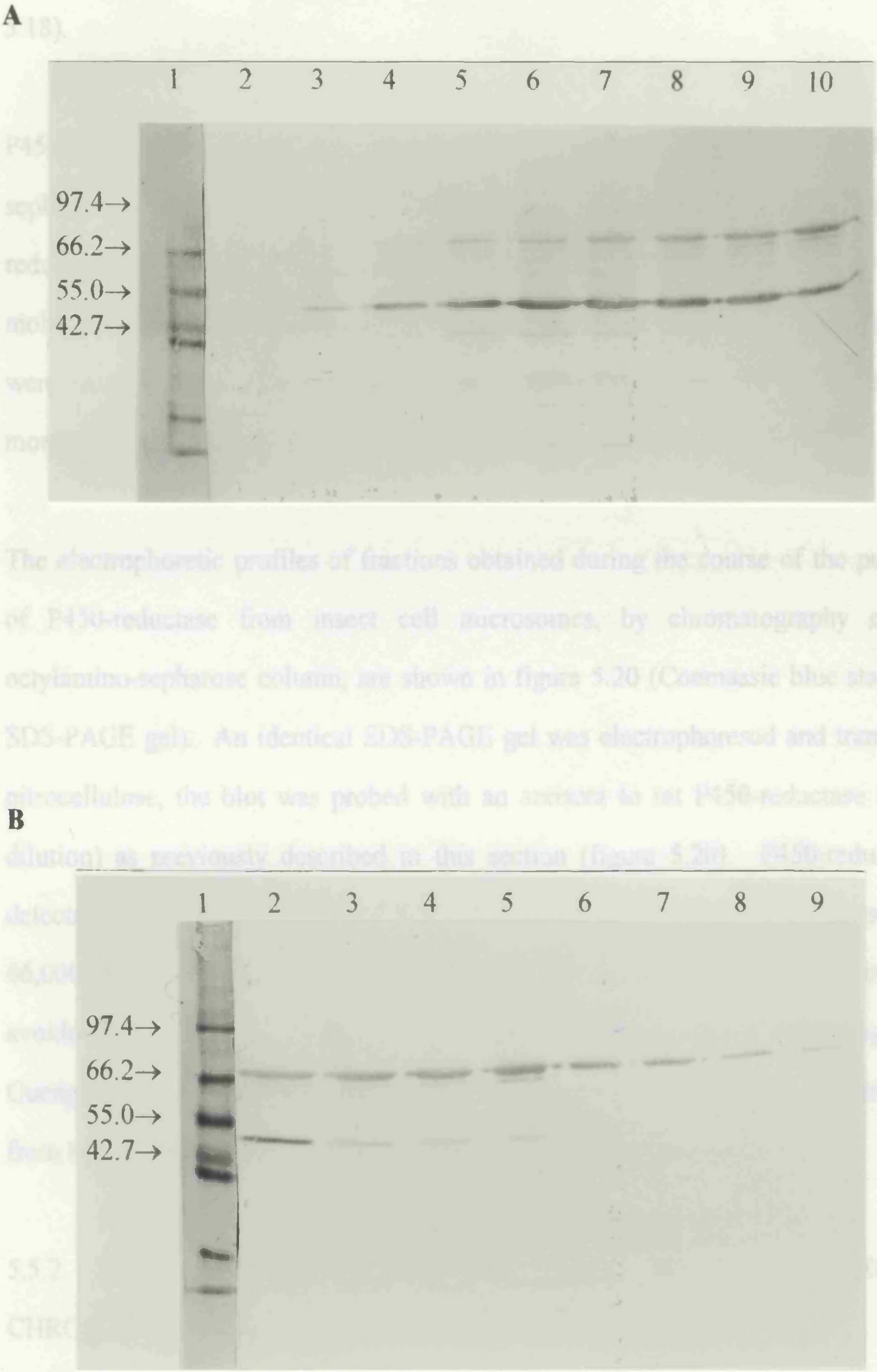

1 2 3 4 5 6 7 8 9 10
97.4→
66.2→
55.0→
42.7→

Figure 5.19: Western blot analysis of fractions collected during the purification of CYP3A4 (and P450-reductase) from insect cell microsomes by chromatography on an *n*-octylamino-sepharose column using a polyclonal antisera to baboon CYP3A.

**A**: The western blot previously analysed with an antisera to P450-reductase, was reprobed with an antisera to baboon CYP3A (1 in 400 dilution) that had been raised in a sheep. The blot was developed by using an alkaline phosphatase conjugated donkey anti-sheep IgG (1 in 30,000 dilution). The following samples are shown: 2.5 μg of Promega mid-range molecular weight protein standards shown x $10^{-3}$ (lane 1); fraction 3 (lane 2); fraction 10 (lane 3); fraction 12 (lane 4); fraction 15 (lane 5); fraction 18 (lane 6); fraction 21 (lane 7); fraction 24 (lane 8); fraction 27 (lane 9) and fraction 30 (lane 10).

**B**: As for figure **A** except the following samples are shown: 2.5 μg of Promega mid-range molecular weight protein standards shown x $10^{-3}$ (lane 1); fraction 33 (lane 2); fraction 40 (lane 3); fraction 45 (lane 4); fraction 50 (lane 5); fraction 60 (lane 6); fraction 70 (lane 7); fraction 80 (lane 8) and fraction 90 (lane 9).


A
1
2
3
4
5
6
7
8
9
10
97.4→
66.2→
55.0→
42.7→
B
1
2
3
4
5
6
7
8
9
97.4→
66.2→
55.0→
42.7→

weight of 66,000, was observed in the flow-through and wash samples (figure 5.17 and 5.18).

P450-reductase was substantially enriched by chromatography on an *n*-octylamino-sepharose column in comparison to the sample of solubilised microsomes. P450-reductase is susceptible to proteolytic cleavage which generates a 66,000 apparent molecular weight breakdown product (section 1.2.). For this reason fractions 45-50 were not concentrated, they were stored at -80°C for further use by our laboratory. The more dilute fractions, 51-70, were concentrated in an Amicon cell.

The electrophoretic profiles of fractions obtained during the course of the purification of P450-reductase from insect cell microsomes, by chromatography on an *n*-octylamino-sepharose column, are shown in figure 5.20 (Coomassie blue stained 10% SDS-PAGE gel). An identical SDS-PAGE gel was electrophoresed and transferred to nitrocellulose, the blot was probed with an antisera to rat P450-reductase (1 in 500 dilution) as previously described in this section (figure 5.20). P450-reductase was detected as a band at an apparent molecular weight of 78,000. A small amount of the 66,000 breakdown product was detected in all the samples (figure 5.20), this could be avoided in the future by the inclusion of PMSF in the solubilisation and storage buffers. Guengerich and Martin (in press) only advise the use of PMSF if the microsomes are from human liver as this organ contains a large amount of proteases.

### 5.5.2. FURTHER PURIFICATION OF CYP3A4 BY ANION EXCHANGE CHROMATOGRAPHY ON A DEAE-SEPHACEL COLUMN

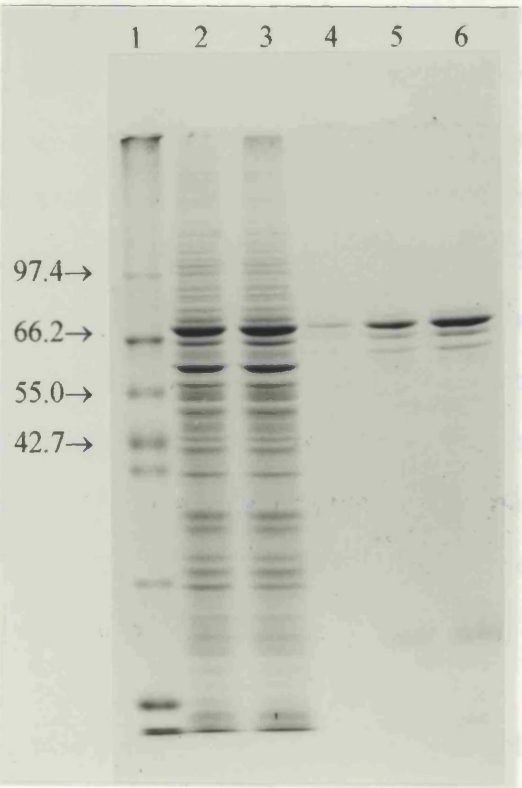
The purified CYP3A4 sample generated in section 5.5.1. was further purified by anion exchange chromatography on a DEAE-sephacel column (adapted from Guengerich and Martin, in press) as described in section 2.2.23. The $O.D._{280}$ and $O.D._{417}$ were measured for each fraction and on the basis of these readings certain fractions were

Figure 5.20: Electrophoretic profiles and western blot analysis of fractions obtained during the course of the purification of P450-reductase from insect cell microsomes, by chromatography on an *n*-octylamino-sepharose column.

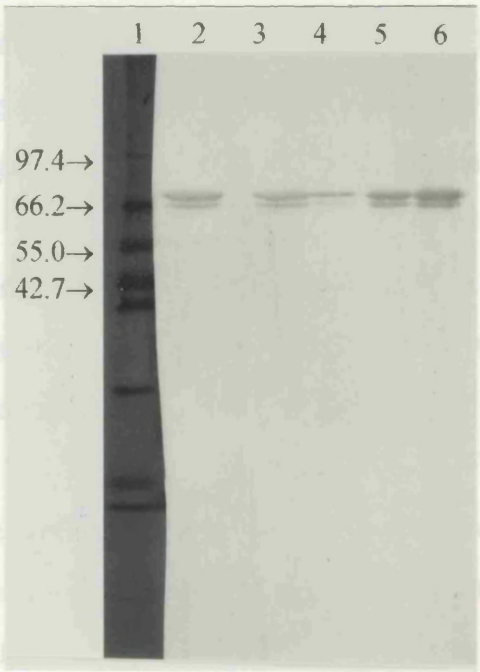
**A**: The samples were loaded on a 10% SDS-PAGE gel. The gel was electrophoresed and stained with Coomassie blue; the gel was photographed with the aid of a light box. The following samples are shown: 2.5 μg of Promega mid-range molecular weight protein standards shown x $10^{-3}$ (lane 1); 20 μg of microsomes containing expressed CYP3A4 and P450-reductase (lane 2); 20 μg of solubilised microsomes (lane 3); 1 μg (lane 4), 5 μg (lane 5) and 10 μg (lane 6) of concentrated P450-reductase.

**B**: The samples were electrophoresed through a 10% SDS-PAGE gel and transferred to nitrocellulose. The blot was probed with an antisera to rat P450-reductase (1 in 500) dilution that had been raised in a rabbit. The blot was developed by using an alkaline phosphatase conjugated goat anti-rabbit IgG (1 in 3,000 dilution). The samples shown are identical to those illustrated in figure **A**.

**A**


1 2 3 4 5 6
97.4→
66.2→
55.0→
42.7→

**B**


1 2 3 4 5 6
97.4→
66.2→
55.0→
42.7→

selected for electrophoresis on a 7% SDS-PAGE gel for Coomassie blue staining (figure 5.21). CYP3A4 was observed as a band at an apparent molecular weight of 51,000 in fractions 6-11; these fractions were collected during the washing of the column (figure 5.21). This indicated that CYP3A4 had bound very weakly to the column. Guengerich and Martin (in press) suggest that if the protein of interest does not bind strongly to the column, the detergent concentrations should be lowered from 0.5% w/v sodium cholate and 0.2% v/v Emulgen 911 to 0.2% w/v sodium cholate and 0.1% v/v Emulgen 911. However, this was unnecessary as most of the contaminating proteins which were present in the sample of CYP3A4 loaded onto the column remained bound to the column. Chen *et al.* (1996) had a similar experience during the purification of human and rat CYP2E1, expressed using baculovirus, from insect cell microsomes using an identical protein purification strategy.

50 µl samples from fractions 5-13 were electrophoresed through a 7% SDS-PAGE gel for Coomassie blue staining (figure 5.22). A duplicate gel was electrophoresed and transferred to nitrocellulose for western blot analysis (figure 5.23). The western blot was probed with an antisera to baboon CYP3A (1 in 400 dilution) that had been raised in a sheep (figure 5.23). The blot was developed by using an alkaline phosphatase conjugated donkey anti-sheep IgG (1 in 30,000 dilution).

Figures 5.22 and 5.23 show that fractions 6, 7 and 8 contain the greatest amount of CYP3A4, fraction 13 contained a small amount of CYP3A4. P450-reductase was detected in fractions 5, 6, 7 and 8 (figure 5.22).

Further purification of these fractions was not carried out as each purification step reduces the amount of sample recovered. Several 100 µgs of CYP3A4 were required for screening the ScFv antibody library; it was thought that too much antigen would be lost by further rounds of purification. Fractions 5-12 were individually concentrated in

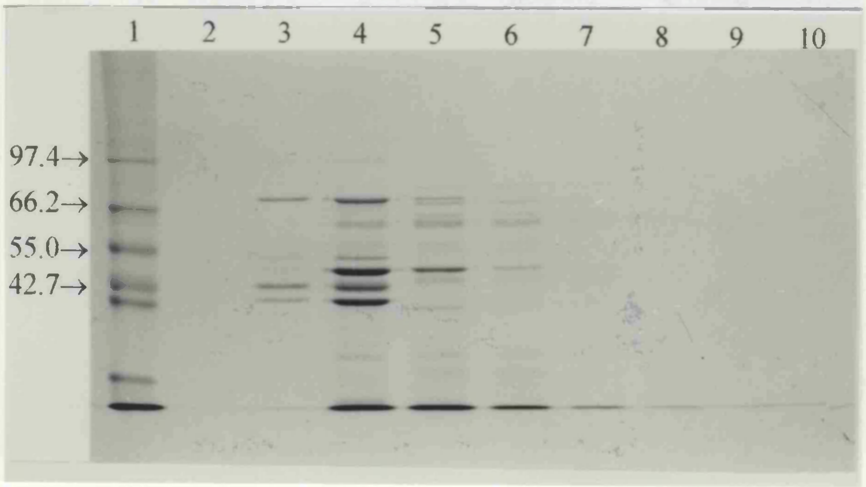


Figure 5.21: Coomassie blue stained SDS-PAGE gel showing fractions collected during the further purification of CYP3A4 by anion-exchange chromatography on a DEAE-sephacel column.

50 µl of each sample was loaded on a 7% SDS-PAGE gel. The gel was electrophoresed and stained with Coomassie blue. The gel was photographed with the aid of a light box. The following samples are shown: 2.5 µg of Promega mid-range molecular weight protein standards shown x $10^{-3}$ (lane 1); fraction 1 (lane 2); fraction 4 (lane 3); fraction 6 (lane 4); fraction 9 (lane 5); fraction 11 (lane 6); fraction 15 (lane 7); fraction 25 (lane 8); fraction 45 (lane 9) and fraction 47 (lane 10).

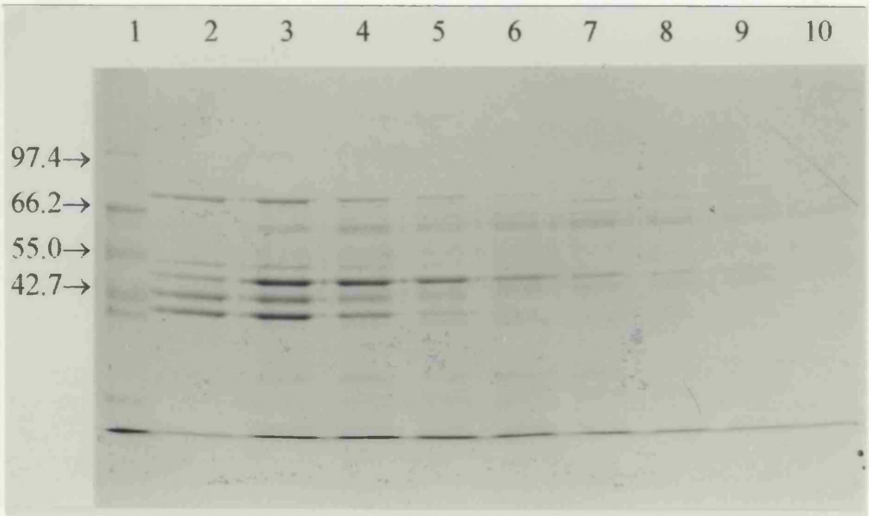


Figure 5.22: Coomassie blue stained SDS-PAGE gel showing fractions 5-13 collected during the further purification of CYP3A4 by anion-exchange chromatography on a DEAE-sephacel column.

50 µl of each sample was loaded on a 7% SDS-PAGE gel. The gel was electrophoresed and stained with Coomassie blue. The gel was photographed with the aid of a light box. The following samples are shown: 2.5 µg of Promega mid-range molecular weight protein standards shown x $10^{-3}$ (lane 1); fraction 5 (lane 2); fraction 6 (lane 3); fraction 7 (lane 4); fraction 8 (lane 5); fraction 9 (lane 6); fraction 10 (lane 7); fraction 11 (lane 8); fraction 12 (lane 9) and fraction 13 (lane 10).

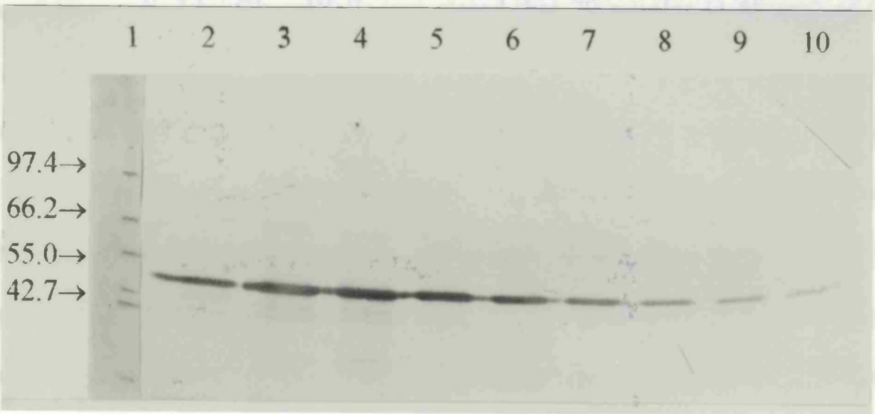


Figure 5.23: Western blot analysis of fractions 5-13 collected during the further purification of CYP3A4 by anion-exchange chromatography on a DEAE-sephacel column using a polyclonal antisera to baboon CYP3A.

50 µl of each sample was electrophoresed through a 7% SDS-PAGE gel and transferred to nitrocellulose. The blot was probed with an antisera to baboon CYP3A (1 in 400 dilution) that had been raised in a sheep. The blot was developed by using an alkaline phosphatase conjugated donkey anti-sheep IgG (1 in 30,000 dilution). The following samples are shown: 2.5 µg of Promega mid-range molecular weight protein standards shown x $10^{-3}$ (lane 1); fraction 5 (lane 2); fraction 6 (lane 3); fraction 7 (lane 4); fraction 8 (lane 5); fraction 9 (lane 6); fraction 10 (lane 7); fraction 11 (lane 8); fraction 12 (lane 9) and fraction 13 (lane 10).

fresh Amicon centricon-30 units. The protein content of each individual fraction was estimated by a Lowry protein determination assay.

The total amount of protein contained in all of the concentrated fractions was approximately 18 mg, of which about 25% was CYP3A4. The data sheet sent with the microsomes supplied by GlaxoWellcome stated that 29 nmoles (1.48 mg) of CYP3A4 were present. This value was obtained from the spectral analysis of CYP3A4 using carbon monoxide as described by Omura and Sato (1964). This analysis only accounts for CYP holoprotein. If the haem group was not present on CYP3A4, this would give an artificially low value for the CYP3A4 content of these microsomes. It has been shown that the addition of hemin or a combination of iron citrate and 5-aminolevulinate to the culture medium of the *T ni* cells during the course of infection with the recombinant baculovirus is critical to producing active expressed CYP holoenzyme (Nanji, PhD. Thesis, 1995).

The electrophoretic profiles of fractions obtained during the course of the purification of CYP3A4 from insect cell microsomes, by chromatography on *n*-octylamino-sepharose and DEAE-sephacel columns, are shown in figure 5.24. An identical SDS-PAGE gel was electrophoresed and the samples were transferred to nitrocellulose. The blot was probed with an antisera to baboon CYP3A (1 in 400 dilution) as previously described in this section (figure 5.24).

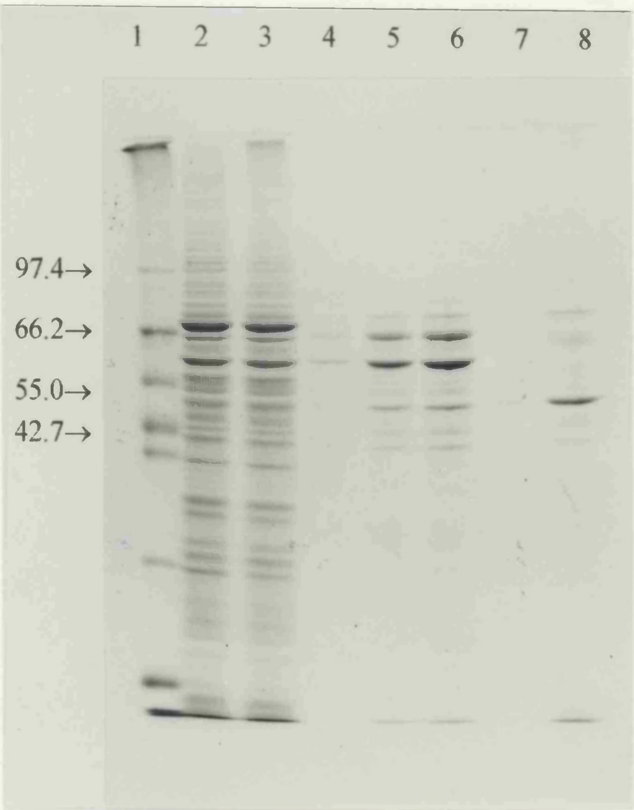
10 μg of each of the eight samples containing CYP3A4 were electrophoresed through a 13% SDS-PAGE gel which was Coomassie blue stained (figure 5.25). Two identical 13% SDS-PAGE gels were electrophoresed and the samples were transferred to nitrocellulose. One blot was probed with an antisera to baboon CYP3A (1 in 400 dilution) as previously described in this section (figure 5.26A). The other blot (figure 5.26B) was probed with the antisera to CYP3A4 (1 in 250 dilution) raised in the balb-c mouse whose sera is shown in well e of figure 5.5A. The reasons for choosing the sera

Figure 5.24: Electrophoretic profiles and western blot analysis of fractions obtained during the course of the purification of CYP3A4 from insect cell microsomes, by chromatography on *n*-octylamino-sepharose and DEAE-sephacel columns.

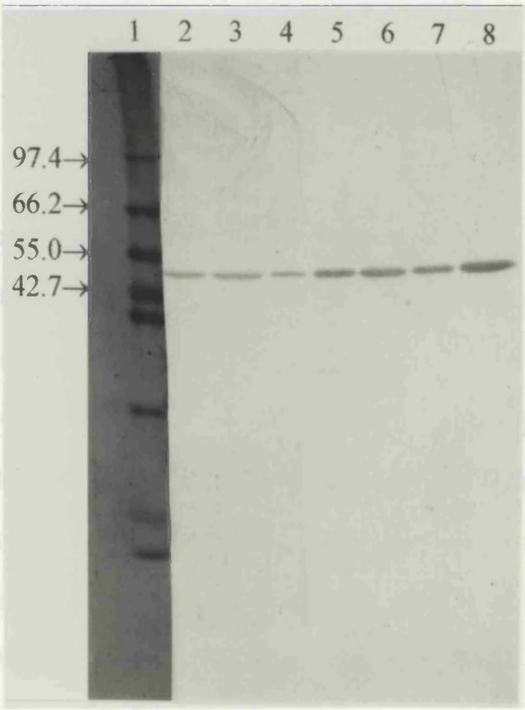
**A**: The samples were loaded on a 10% SDS-PAGE gel. The gel was electrophoresed and stained with Coomassie blue. The gel was photographed with the aid of a light box. The following samples are shown: 2.5 μg of Promega mid-range molecular weight protein standards shown x $10^{-3}$ (lane 1); 20 μg of microsomes containing expressed CYP3A4 and P450-reductase (lane 2); 20 μg of solubilised microsomes (lane 3); 1 μg (lane 4), 5 μg (lane 5) and 10 μg (lane 6) of concentrated CYP3A4 from chromatography on an *n*-octylamino-sepharose column; 1 μg (lane 7) and 5 μg (lane 8) of concentrated CYP3A4 from fraction 8 collected during chromatography on a DEAE-sephacel column.

**B**: The samples were electrophoresed through a 10% SDS-PAGE gel and transferred to nitrocellulose. The blot was probed with an antisera to baboon CYP3A (1 in 400 dilution) that had been raised in a sheep. The blot was developed by using an alkaline phosphatase conjugated donkey anti-sheep IgG (1 in 30,000 dilution). The samples shown are identical to those in figure **A**.

**A**


1 2 3 4 5 6 7 8
97.4→
66.2→
55.0→
42.7→

**B**


1 2 3 4 5 6 7 8
97.4→
66.2→
55.0→
42.7→

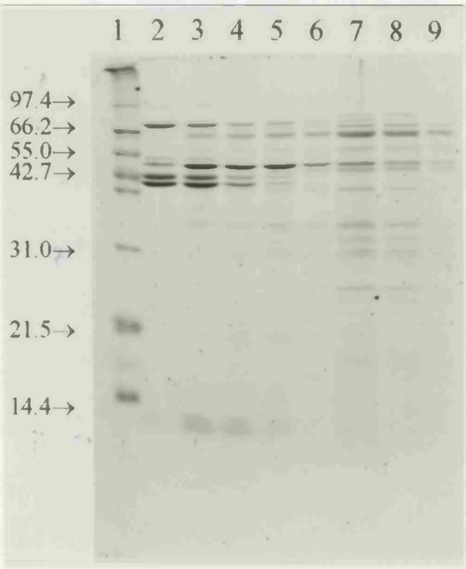


Figure 5.25: Coomassie blue stained SDS-PAGE gel showing 10 µg of the eight purified CYP3A4 preparations obtained from chromatography on *n*-octylamino-sepharose and DEAE-sephacel columns.

The samples were electrophoresed through a 13% SDS-PAGE gel; the gel was stained with Coomassie blue and photographed with the aid of a light box. The following samples are shown: 2.5 µg of Promega mid-range molecular weight protein standards shown x $10^{-3}$ (lane 1); lanes 2-9 contain 10 µg of samples 5-12 of purified CYP3A4 obtained from chromatography on *n*-octylamino-sepharose and DEAE-sephacel columns, respectively.

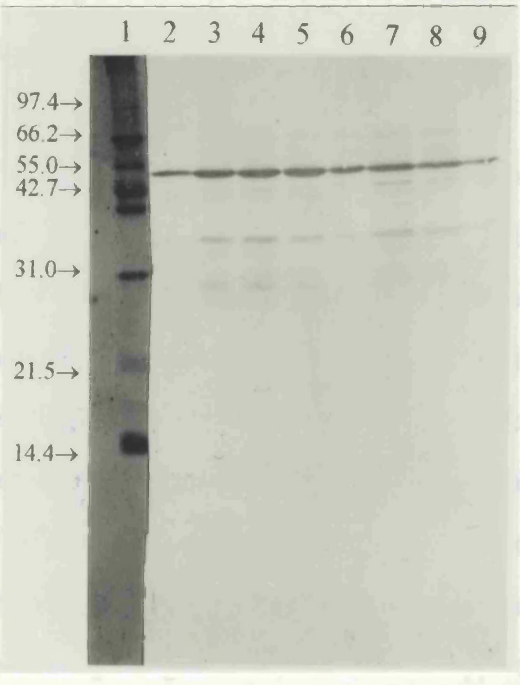
Figure 5.26**A**: Western blot analysis of the eight purified CYP3A4 preparations obtained from chromatography on *n*-octylamino-sepharose and DEAE-sephacel columns using a polyclonal antisera to baboon CYP3A.

The samples were electrophoresed through a 13% SDS-PAGE gel and transferred to nitrocellulose. The blot was probed with an antisera to baboon CYP3A (1 in 400 dilution) which was raised in a sheep. The blot was developed by using an alkaline phosphatase conjugated donkey anti-sheep IgG (1 in 30,000 dilution). The following samples are shown: 2.5 μg of Promega mid-range molecular weight protein standards shown x $10^{-3}$ (lane 1); lanes 2-9 contain 10 μg of samples 5-12 of purified CYP3A4 obtained from chromatography on *n*-octylamino-sepharose and DEAE-sephacel columns, respectively.

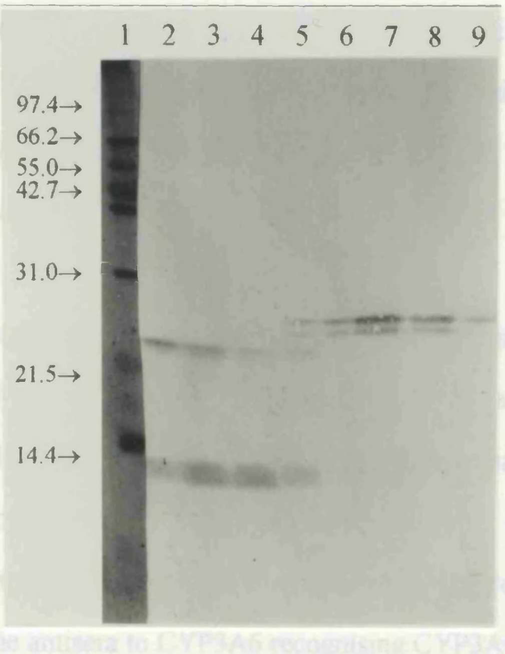
**B**: Western blot analysis of the eight purified CYP3A4 preparations obtained from chromatography on *n*-octylamino-sepharose and DEAE-sephacel columns using mouse antisera raised to CYP3A4.

As for figure **A** except the blot was probed with the mouse polyclonal antisera allegedly raised to CYP3A4 (1 in 250 dilution). The blot was developed by using an alkaline phosphatase conjugated goat anti-mouse IgG (1 in 30,000 dilution). The samples shown are identical to those illustrated in figure **A**.

A


1 2 3 4 5 6 7 8 9
97.4→
66.2→
55.0→
42.7→
31.0→
21.5→
14.4→

B


1 2 3 4 5 6 7 8 9
97.4→
66.2→
55.0→
42.7→
31.0→
21.5→
14.4→

of this mouse for this experiment were previously discussed in section 5.2.3. The blot was developed by using a 1 in 30,000 dilution of an alkaline phosphatase conjugated goat anti-mouse IgG.

Two proteins of similar electrophoretic mobilities were observed as a doublet at an apparent molecular weight of <14,400 in samples 5-8 of the CYP3A4 preparations (figure 5.25). These proteins were recognised by the mouse antisera (figure 5.26B). The mouse antisera also recognised a protein of apparent molecular weight 22,000-23,000 in samples 5-8. This protein was highly immunogenic; a corresponding band of similar electrophoretic mobility could not be detected on the Coomassie blue stained gel (figure 5.25). A protein of slightly higher apparent molecular weight was detected by the mouse antisera in samples 8-11 which was also unable to be seen on the Coomassie blue stained gel (figure 5.25). A band of approximately 28,000 apparent molecular weight was observed in samples 8-12 (figure 5.26B). A corresponding Coomassie blue stained protein was observed in figure 5.25 for samples 10 and 11.

Sample 7 was chosen to screen the ScFv antibody library (section 5.6.) as it contained a large amount of CYP3A4 (figures 5.25 and 5.26A) with the least amount of immunogenic contaminating proteins (figure 5.26B). A small amount of P450-reductase was present in sample 7 (figure 5.25).

The band previously recognised by the mouse antisera at an apparent molecular weight of 50,000-53,000 (figure 5.6) was not observed in any of the samples in figure 5.26B. This implies that the band detected in the purified CYP3A4 sample shown in figure 5.6 did not in fact correspond to CYP3A4 but represented either an insect cell or baculovirus protein. The protein has a similar electrophoretic mobility to CYP3A4 which prevented the antisera to CYP3A6 recognising CYP3A4 in the purified CYP3A4 sample. This protein was removed by chromatography on either the *n*-octylamino-sepharose or the DEAE-sephacel column. Western blot analysis of fractions obtained during the course of the purification of CYP3A4 from insect cell microsomes using the mouse antisera, showed that the protein was removed by chromatography on the *n*-octylamino-sepharose column (figure 5.27).

### 5.5.3. INVESTIGATION OF THE NATURE OF THE IMMUNOGENIC LOW MOLECULAR WEIGHT PROTEINS CONTAMINATING THE PURIFIED CYP3A4 PREPARATION

10 µg of *T ni* insect cell microsomes, 10 µg of microsomes from *T ni* insect cells infected with the wild-type baculovirus AcMNPV, 30 µg of *T ni* insect cell microsomes expressing CYP3A4 and P450-reductase were analysed by western blot analysis using the mouse antisera (figure 5.28) as previously described in this section. The protein of apparent molecular weight of 50,000-53,000 was observed in all three lanes; this protein was derived from the insect cells. The rest of the contaminating proteins were only observed in the insect cell samples that had been infected with baculovirus. An identical western blot was probed with a 1 in 500 dilution of mouse non-immune sera (data not shown). The mouse had not been injected with either CYP2A6 or CYP3A4. No bands were detected; this confirmed that only mice injected with the antigen possessed antibodies that recognised these proteins. An identical blot was also probed with a 1 in 500 dilution of mouse antisera to CYP2A6 (data not shown); bands of similar electrophoretic mobility as those detected in figure 5.28 were observed. This suggests that the immunogenic contaminating proteins were also present in the CYP2A6 preparation used to immunise the mice.

The baculovirus expression system is a useful tool for carrying out mechanistic, biophysical and kinetic studies on expressed enzymes, such as CYPs. However if the expressed proteins are to be purified and used as antigens for the production of antibodies, care must be taken. The expressed proteins must be subject to several rounds of purification in order to remove any traces of insect cell or baculoviral

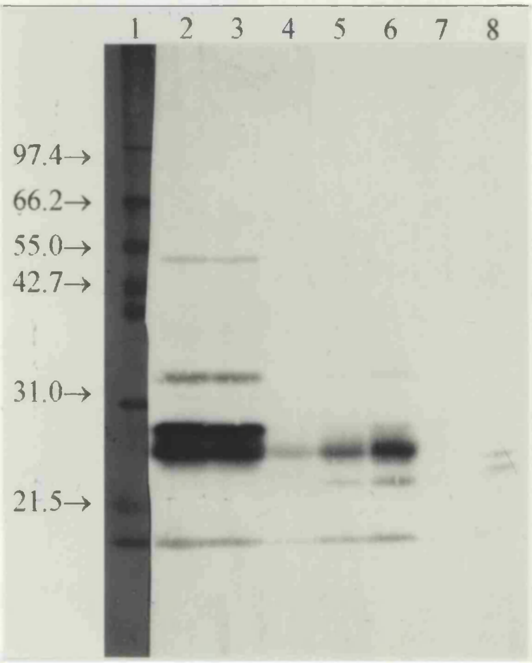


Figure 5.27: Western blot analysis of fractions obtained during the course of the purification of CYP3A4 from insect cell microsomes, by chromatography on *n*-octylamino-sepharose and DEAE-sephacel columns using mouse antisera to CYP3A4. The samples were electrophoresed through a 10% SDS-PAGE gel and transferred to nitrocellulose. The blot was probed with the mouse antisera to CYP3A4 (1 in 250 dilution). The blot was developed by using an alkaline phosphatase conjugated goat anti-mouse IgG (1 in 30,000) dilution. The following samples are shown: 2.5 μg of Promega mid-range molecular weight protein standards shown x $10^{-3}$ (lane 1); 20 μg of microsomes containing expressed CYP3A4 and P450-reductase (lane 2); 20 μg of solubilised microsomes (lane 3); 1 μg (lane 4), 5 μg (lane 5) and 10 μg (lane 6) of concentrated CYP3A4 from chromatography on an *n*-octylamino-sepharose column; 1 μg (lane 7) and 5 μg (lane 8) of concentrated CYP3A4 from fraction 8 collected during chromatography on a DEAE-sephacel column.

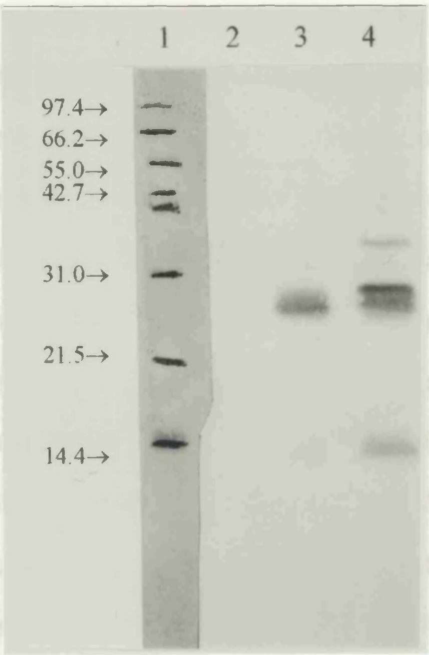


Figure 5.28: Western blot analysis of *T ni* insect cell microsomes, microsomes from *T ni* insect cells infected with the wild-type baculovirus AcMNPV and *T ni* insect cell microsomes expressing CYP3A4 and P450-reductase using the mouse antisera to CYP3A4.

The samples were electrophoresed through a 13% SDS-PAGE gel and transferred to nitrocellulose. The blot was probed with the mouse antisera to CYP3A4 (1 in 250 dilution). The blot was developed by using an alkaline phosphatase conjugated goat anti-mouse IgG (1 in 30,000 dilution). The following samples are shown: 2.5 µg of Promega mid-range molecular weight protein standards shown x $10^{-3}$ (lane 1); 10 µg of *T ni* insect cell microsomes (lane 2); 10 µg of microsomes from *T ni* insect cells infected with the wild-type baculovirus AcMNPV (lane 3) and 30 µg of *T ni* insect cell microsomes expressing CYP3A4 and P450-reductase (lane 4).

proteins. This requires a large amount of insect cell microsomes, expressing the desired protein, to be produced which is very costly. Monoclonal antibodies to CYP2E1 and CYP3A4 have been produced by the traditional hybridoma technology (Gelboin *et al.*, 1996 and Gelboin *et al.*, 1995). Both CYPs were expressed in *Sf9* insect cells using the baculovirus expression system. The CYPs were purified from the insect cell microsomes by hydrophobic interaction and hydroxylapatite chromatography. This combination of chromatography methods may be better for the removal of the contaminating proteins. However, no preliminary data showing western blots of the purified antigen probed with the mice sera was given in the paper. Similar problems may have been encountered but not documented.

The mouse was definitely injected with CYP3A4 as shown by western blot analysis in figure 5.4. It is possible that the mouse produced too low a titre of antibodies to CYP3A4 to enable a signal to be detected on a western blot or that the antibodies produced are unable to recognise CYP3A4 when it is immobilised on nitrocellulose. The former suggestion is a more likely explanation. The low titre of antibodies to CYP3A4 would be cause for concern if the monoclonal antibodies were to produced using the hybridoma technology. As the monoclonal antibodies are to be produced using the recombinant phage antibody system, this was not such a major concern. Nearly every immunoglobulin heavy and light variable ($V_H$ and $V_L$) region genes are amplified from the mice B-lymphocytes and cloned. These are combined at random to create an ScFv library. The original $V_H$ and $V_L$ pairings will be represented in the library along with other random pairings that may also recognise the antigen. Even if the mouse has only produced a few antibodies to CYP3A4, ScFv antibodies should be present, albeit at a low frequency, in the final library.

In conclusion, the assembled ScFv antibody library should contain antibodies to CYP3A4. The purified CYP3A4 preparation used to inject the mouse contained immunogenic contaminating proteins and a small amount of P450-reductase. Sample 7 of purified CYP3A4 will be used to screen the ScFv antibody library. This sample contains CYP3A4, the immunogenic contaminating proteins and a trace amount of P450-reductase. Consequently, a large number of false positive results are expected to be obtained during the screening of the library for antibodies to CYP3A4 (section 5.6.) with the sample of enriched CYP3A4.

A similar situation is expected when the ScFv antibody library to CYP2A6 is eventually screened. The CYP2A6 preparation used to inject the mice also contained the low molecular weight contaminating baculoviral proteins. However, the number of false positive results may be able to be reduced if a better purification strategy can be found.

## 5.6. SCREENING THE ScFv ANTIBODY LIBRARY

The CYP3A4 ScFv antibody library was screened by detection of antigen binding using a two-membrane system (adapted from Skerra *et al.*, 1991 and Dreher *et al.*, 1991) as described in section 2.2.34.16. The technique is based on a colony lift assay using a HRP/Anti-E tag conjugate to detect ScFv antibodies that have bound to the antigen.

One of the six glycerol stocks (a-f) made with *E. coli* HB2151 transformed with CYP3A4 ScFv-pCANTAB 5 E recombinant phagemid DNA (section 2.2.34.13.) was thawed on ice. A master plate was prepared by inoculating a 2 x YT-AG agar plate with the transformed *E. coli*. The expression of the soluble ScFv antibodies are repressed by the inclusion of glucose in the agar medium. The plate was incubated overnight. The next step was proceeded to once well isolated single colonies were detected on the plate. Approximately 500 colonies were expected to be obtained per glycerol stock. Only a third of these were actually observed on the agar plate. This was probably because the freshly transformed bacterial cells had not had enough time to recover from the transformation process before being stored at -80°C. This lead to a higher than normal proportion of unviable bacterial cells being present in the glycerol stock.

A nitrocellulose membrane filter (0.45 μm pore size) was coated with the antigen preparation (50 μg/ml in PBS) and was placed antigen-side up onto the surface of a 2 x YT-AI agar plate. The antigen preparation was enriched CYP3A4 obtained from chromatography on *n*-octylamino-sepharose and DEAE-sephacel columns (sections 5.5.1. and 5.5.2.). It has been shown that the antigen preparation contained low molecular weight baculovirus proteins to which the mice had produced antibodies (section 5.5.3.). Sample 7 of purified CYP3A4 was used to screen the ScFv antibody library as it contained a large amount of CYP3A4, the least amount of immunogenic contaminating proteins and a trace amount of P450-reductase in comparison to the other CYP3A4 fractions (figures 5.25, 5.26A and B). Consequently, a large number of false positive results were expected to be obtained during the screening of the library.

Bacterial colonies were lifted from the surface of the master plate using a fresh piece of nitrocellulose (master filter) with a pore size of 0.22 μm. The master filter was carefully placed colony-side up onto the surface of the antigen-coated nitrocellulose filter on the 2 x YT-AI agar plate (figure 5.29). The 2 x YT-AI agar plate, containing the two filters, was inverted and incubated overnight.

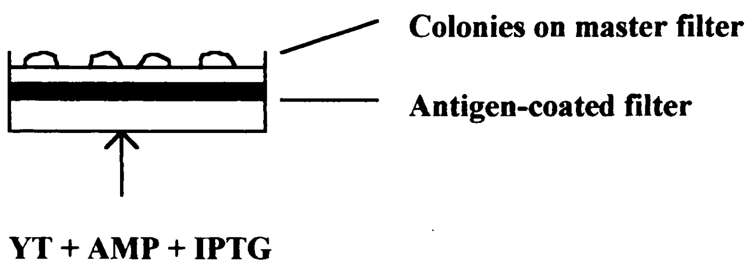


**Figure 5.29**: Schematic diagram showing arrangement of filters in a colony lift assay.

The ScFv antibodies are expressed due to the absence of glucose and inclusion of IPTG in the agar medium. As the antibodies are produced, they diffuse through the master filter and bind to the antigen-coated filter. The pore size of the master filter is large enough to allow the passage of the antibodies but small enough to prevent bacteria diffusing through to the second filter.

The next day, the master filter was removed and stored colony-side up at $4^{O}C$ on a fresh 2 x YT-AG agar plate. The antigen-coated filter was probed with the HRP/Anti-E tag conjugate and developed with the HRP colour substrate, 4-CN, as described in section 2.2.34.16. Colonies of different intensity of colour development were observed on the filter. This could be due to either colonies expressing different amounts of the antibodies or varied antibody affinities for the antigen preparation.

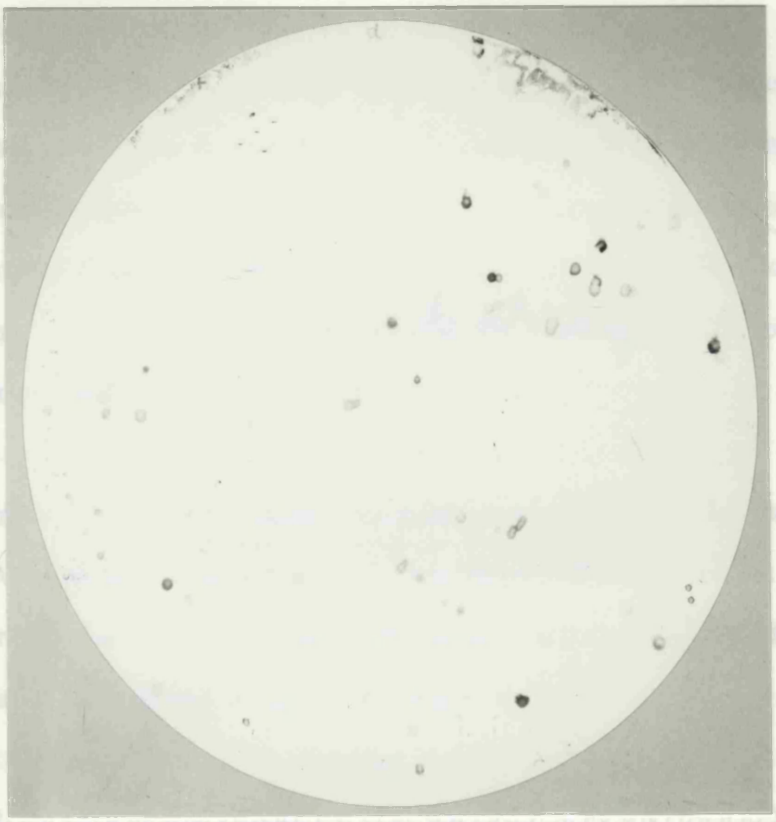
The library screening was repeated four times using the rest of the glycerol stocks for the CYP3A4 ScFv-pCANTAB 5 E cDNA library. Glycerol stocks a and f were combined and plated on one 2 x YT-AG agar plate whereas glycerol stock e was split between two agar plates, approximately 50 µl per plate. This was carried out in order to provide different colony densities for the screening assay. The colour-developed filters obtained from the colony lift assays of glycerol stock d and one of the filters from stock e are shown in figure 5.30. The colonies that were detected to have bound the antigen were observed to be well isolated on the filters.

The master filters were aligned with the antigen-coated filters to identify and recover those colonies that had expressed antigen-binding E-tagged ScFv antibodies. Twelve colonies of different intensity of colour development were selected from a variety of antigen-coated filters. The corresponding bacterial colonies were picked from the appropriate master filters (data not shown). Soluble antibodies were produced from each of these colonies (section 5.7.1.). Due to a lack of time, I was limited in the number of colonies that could be picked and used to produce soluble antibodies.

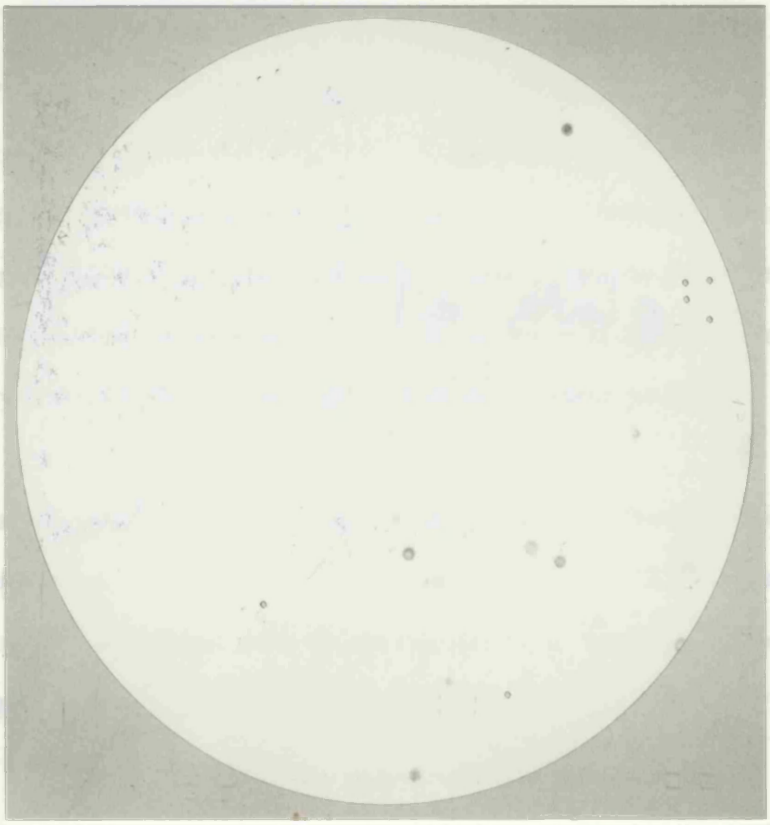
Figure 5.30: Screening the CYP3A4-ScFv antibody library by detection of antigen binding with a two-membrane system.

Bacteria from glycerol stocks d and e of *E. coli* HB2151 transformed with CYP3A4 ScFv-pCANTAB 5 E were screened for expression of antigen-binding ScFv antibodies. The antigen-coated filters were probed with an HRP/Anti-E tag conjugate and colonies were detected with an HRP colour substrate. Figure **A** shows the colour-developed filter from the library screen of glycerol stock d; figure **B** shows one of the colour-developed filters from the library screen of glycerol stock e.

**A**



**B**



Further colonies expressing antigen-binding ScFv antibodies will be isolated, by another member of our laboratory, using the same antigen preparation and the stored library scrapes (section 2.2.34.15.). Several dilutions of the stored library scrapes will need to be plated out in order to achieve the correct colony density. As has been previously mentioned in section 5.1.1., the purified CYP3A4 antigen preparation used to inject the mice contained trace quantities of P450-reductase. If soluble antibodies to CYP3A4 are eventually isolated, then the library may well be rescreened using purified P450-reductase-coated filters.

The CYP2A6 ScFv antibody library was unable to be screened due to a lack of purified CYP2A6 (discussed in section 5.5.1.). This library will be screened in the future by another member of our laboratory. The control (naive) ScFv antibody library was stored at -80°C for future use by our laboratory.

## 5.7. PRODUCTION AND ANALYSIS OF SOLUBLE ScFv ANTIBODIES

### 5.7.1. PRODUCTION OF SOLUBLE ScFv ANTIBODIES

Soluble ScFv antibodies are directed to the bacterial periplasmic space by the g3p leader sequence located at their N-termini. However, soluble antibodies may also be located in the culture supernatant or inside the bacterial cell. The location of the majority of the antibodies is dependent on a particular bacterial clone.

Supernatant, periplasmic and whole cell extracts were produced (section 2.2.34.17.) from eleven out of the twelve colonies (clones 1-8 and 10-12) isolated from the screening of the CYP3A4 ScFv library (section 5.6.). Clone 9 was not used as the overnight culture was accidentally contaminated.

### 5.7.2. WESTERN BLOT ANALYSIS OF THE THREE SOLUBLE ScFv-CONTAINING EXTRACTS

Each of the three extracts, supernatant, periplasmic and whole cell, produced from the eleven selected bacterial clones were analysed by electrophoresis through a 10% SDS-PAGE gel. The samples were transferred to nitrocellulose and were analysed by western blotting as described in section 2.2.34.18. The E-tagged soluble antibodies were detected by using a 1 in 1,000 dilution of HRP/Anti-E tag conjugate; this conjugate was purchased from Pharmacia Biotech. Unfortunately, when the blots were developed with an HRP colour substrate, no bands were detected. Bands of apparent molecular weight of 30,000-32,000 corresponding to the ScFvs were expected to be seen. The absence of any bands may have been due to too low a concentration of ScFvs in the samples. All the samples were concentrated using 20% trichloroacetic acid as described in section 2.2.34.18. The blank membranes were stained with amido black to ensure that the samples had actually been transferred to the nitrocellulose (figure 5.31).

The entire sample (25 µl) of each of the concentrated extracts from the eleven clones were analysed by western blotting as described above. The western blots are shown in figure 5.32. Bands of apparent molecular weight of approximately 32,000 were detected in the following samples: supernatant and periplasmic extract from clone 1, supernatant extract of clone 2, all three extracts of clone 7, supernatant and periplasmic extract of clone 8, supernatant and periplasmic extract of clone 12. Bands of apparent low molecular weight were detected in all three extracts of clone 8. This suggested that the ScFv antibodies were being degraded by bacterial proteases. The apparent low molecular weight bands corresponded to small ScFv peptides containing the E-tag.

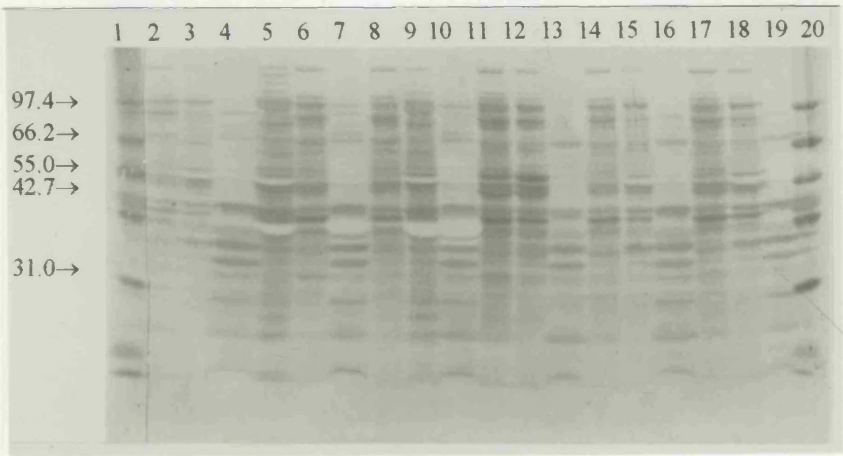
No bands were detected for the extracts derived from clones 3, 4, 5, 6, 10 and 11. This could be due to a variety of reasons:

Figure 5.31: Amido black stained nitrocellulose membranes showing the three soluble ScFv-containing extracts produced from *E. coli* HB2151 clones expressing ScFv antibodies to the CYP3A4 preparation.

**A**: 30 µl of each of the three extracts, supernatant, periplasmic and whole cell, were electrophoresed through a 10% SDS-PAGE gel. The samples were transferred to nitrocellulose and probed with an HRP/Anti-E tag conjugate (1 in 1,000 dilution); as no bands were detected on the addition of an HRP colour substrate the blot was stained with amido black. The following samples are shown: 2.5 µg of Promega mid-range molecular weight protein standards shown x $10^{-3}$ (lanes 1 and 20); supernatant (lane 2), periplasmic (lane 3) and whole cell (lane 4) extracts from *E. coli* HB2151 clone 1; periplasmic (lane 5), supernatant (lane 6), and whole cell (lane 7) extracts from *E. coli* HB2151 clone 2; supernatant (lane 8), periplasmic (lane 9) and whole cell (lane 10) extracts from *E. coli* HB2151 clone 3; supernatant (lane 11), periplasmic (lane 12) and whole cell (lane 13) extracts from *E. coli* HB2151 clone 4; supernatant (lane 14), periplasmic (lane 15) and whole cell (lane 16) extracts from *E. coli* HB2151 clone 5; supernatant (lane 17), periplasmic (lane 18) and whole cell (lane 19) extracts from *E. coli* HB2151 clone 6.

**B**: As for figure **A** except the following samples are shown: 2.5 µg of Promega mid-range molecular weight protein standards shown x $10^{-3}$ (lanes 1 and 17); supernatant (lane 2), periplasmic (lane 3) and whole cell (lane 4) extracts from *E. coli* HB2151 clone 7; supernatant (lane 5), periplasmic (lane 6) and whole cell (lane 7) extracts from *E. coli* HB2151 clone 8; supernatant (lane 8), periplasmic (lane 9) and whole cell (lane 10) extracts from *E. coli* HB2151 clone 10; supernatant (lane 11), periplasmic (lane 12) and whole cell (lane 13) extracts from *E. coli* HB2151 clone 11; supernatant (lane 14), periplasmic (lane 15) and whole cell (lane 16) extracts from *E. coli* HB2151 clone 12.

A


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
97.4→
66.2→
55.0→
42.7→
31.0→

B

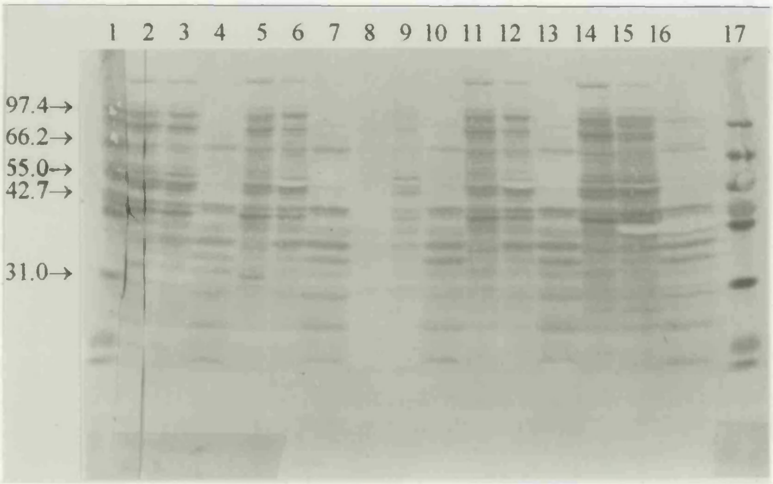
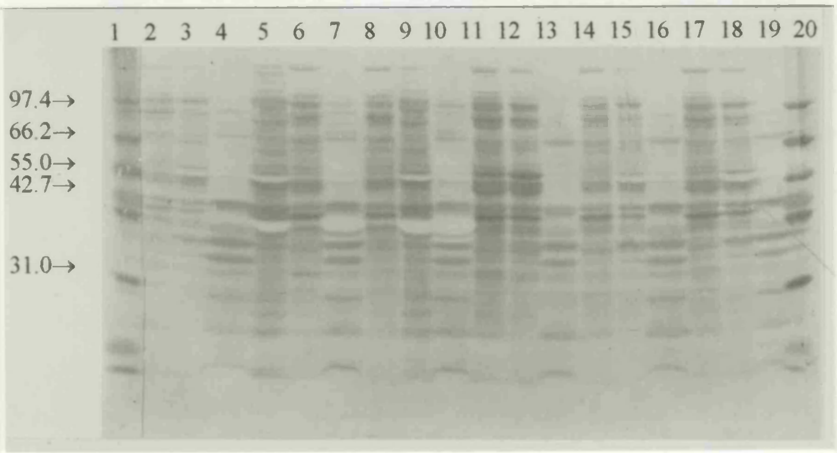

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
97.4→
66.2→
55.0→
42.7→
31.0→

Figure 5.31: Amido black stained nitrocellulose membranes showing the three soluble ScFv-containing extracts produced from *E. coli* HB2151 clones expressing ScFv antibodies to the CYP3A4 preparation.

**A**: 30 µl of each of the three extracts, supernatant, periplasmic and whole cell, were electrophoresed through a 10% SDS-PAGE gel. The samples were transferred to nitrocellulose and probed with an HRP/Anti-E tag conjugate (1 in 1,000 dilution); as no bands were detected on the addition of an HRP colour substrate the blot was stained with amido black. The following samples are shown: 2.5 µg of Promega mid-range molecular weight protein standards shown x $10^{-3}$ (lanes 1 and 20); supernatant (lane 2), periplasmic (lane 3) and whole cell (lane 4) extracts from *E. coli* HB2151 clone 1; periplasmic (lane 5), supernatant (lane 6), and whole cell (lane 7) extracts from *E. coli* HB2151 clone 2; supernatant (lane 8), periplasmic (lane 9) and whole cell (lane 10) extracts from *E. coli* HB2151 clone 3; supernatant (lane 11), periplasmic (lane 12) and whole cell (lane 13) extracts from *E. coli* HB2151 clone 4; supernatant (lane 14), periplasmic (lane 15) and whole cell (lane 16) extracts from *E. coli* HB2151 clone 5; supernatant (lane 17), periplasmic (lane 18) and whole cell (lane 19) extracts from *E. coli* HB2151 clone 6.

**B**: As for figure **A** except the following samples are shown: 2.5 µg of Promega mid-range molecular weight protein standards shown x $10^{-3}$ (lanes 1 and 17); supernatant (lane 2), periplasmic (lane 3) and whole cell (lane 4) extracts from *E. coli* HB2151 clone 7; supernatant (lane 5), periplasmic (lane 6) and whole cell (lane 7) extracts from *E. coli* HB2151 clone 8; supernatant (lane 8), periplasmic (lane 9) and whole cell (lane 10) extracts from *E. coli* HB2151 clone 10; supernatant (lane 11), periplasmic (lane 12) and whole cell (lane 13) extracts from *E. coli* HB2151 clone 11; supernatant (lane 14), periplasmic (lane 15) and whole cell (lane 16) extracts from *E. coli* HB2151 clone 12.

A


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
97.4→
66.2→
55.0→
42.7→
31.0→

B

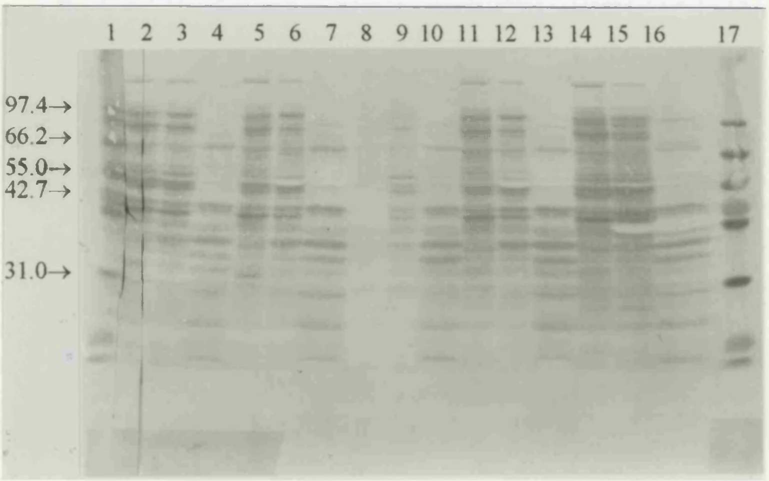
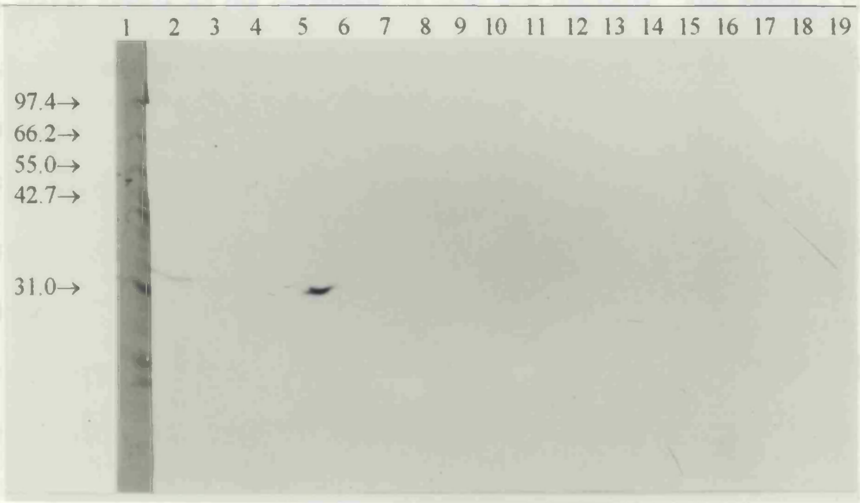

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
97.4→
66.2→
55.0→
42.7→
31.0→

Figure 5.32: Western blot analysis of the three soluble ScFv-containing extracts produced from *E. coli* HB2151 clones expressing ScFv antibodies to the CYP3A4 preparation.

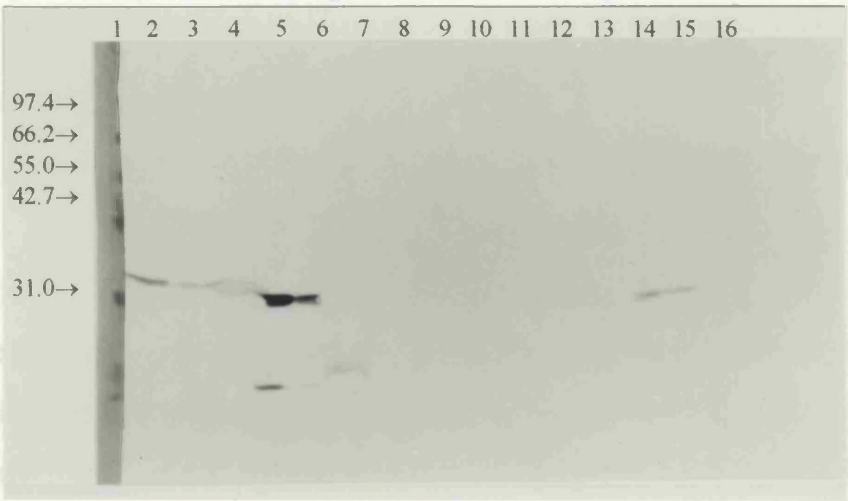
**A**: 30 μl of each of the three extracts, supernatant, periplasmic and whole cell, were electrophoresed through a 10% SDS-PAGE gel. The samples were transferred to nitrocellulose and probed with an HRP/Anti-E tag conjugate (1 in 1,000 dilution); bands were detected by the addition of an HRP colour substrate. The following samples are shown: 2.5 μg of Promega mid-range molecular weight protein standards shown x $10^{-3}$ (lane 1); supernatant (lane 2), periplasmic (lane 3) and whole cell (lane 4) extracts from *E. coli* HB2151 clone 1; periplasmic (lane 5), supernatant (lane 6), and whole cell (lane 7) extracts from *E. coli* HB2151 clone 2; supernatant (lane 8), periplasmic (lane 9) and whole cell (lane 10) extracts from *E. coli* HB2151 clone 3; supernatant (lane 11), periplasmic (lane 12) and whole cell (lane 13) extracts from *E. coli* HB2151 clone 4; supernatant (lane 14), periplasmic (lane 15) and whole cell (lane 16) extracts from *E. coli* HB2151 clone 5; supernatant (lane 17), periplasmic (lane 18) and whole cell (lane 19) extracts from *E. coli* HB2151 clone 6.

**B**: As for figure **A** except the following samples are shown: 2.5 μg of Promega mid-range molecular weight protein standards shown x $10^{-3}$ (lane 1); supernatant (lane 2), periplasmic (lane 3) and whole cell (lane 4) extracts from *E. coli* HB2151 clone 7; supernatant (lane 5), periplasmic (lane 6) and whole cell (lane 7) extracts from *E. coli* HB2151 clone 8; supernatant (lane 8), periplasmic (lane 9) and whole cell (lane 10) extracts from *E. coli* HB2151 clone 10; supernatant (lane 11), periplasmic (lane 12) and whole cell (lane 13) extracts from *E. coli* HB2151 clone 11; supernatant (lane 14), periplasmic (lane 15) and whole cell (lane 16) extracts from *E. coli* HB2151 clone 12.

**A**


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
97.4→
66.2→
55.0→
42.7→
31.0→

**B**


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
97.4→
66.2→
55.0→
42.7→
31.0→

- The clones contained pCANTAB 5 E only; the ScFv DNA was lost due to a rearrangement of vector DNA by the *E. coli.*
- The clones expressed the antibodies at very low amounts. The extracts contained ScFv antibodies but western blot analysis was not sensitive enough to detect such low amounts.
- ScFv antibodies were expressed but were cleaved by bacterial proteases. If the cleavage site was just upstream of the E tag then the cleaved ScFvs would not be able to be detected.
- The colonies picked from the master filter were mistaken for colonies that had given a positive signal on the antigen-coated filter from the screening of the library (section 5.6.). This is the least plausible explanation as great care was taken during the alignment of the master and antigen-coated filters and the subsequent selection of the colonies.

The amount of expression of ScFv antibodies was not related to the intensity of colour development observed on the antigen-coated filters during the colony lift assay (section 5.6.).

### 5.7.3. *Sfi* I AND *Not* I RESTRICTION DIGESTS OF RECOMBINANT PHAGEMID DNA

Phagemid DNA was isolated from the twelve bacterial clones using a protocol for isolation of plasmid DNA (section 2.2.2.1.). The twelve phagemids were digested with *Sfi* I and *Not* I in order to ascertain if they each contained an ScFv DNA insert. A similar protocol to that described in section 2.2.34.9. for the digestion of the ScFv fragments was used. Each digested sample was electrophoresed through a 1.5% agarose gel (figure 5.33). Figure 5.33 shows that the phagemid DNA isolated from clones 1, 2, 3, 5, 6, 7, 8, and 12 contain ScFv fragments of approximately 750 bp. Phagemid DNA isolated from clones 4, 9, 10 and 11 do not contain an insert of ScFv DNA. These

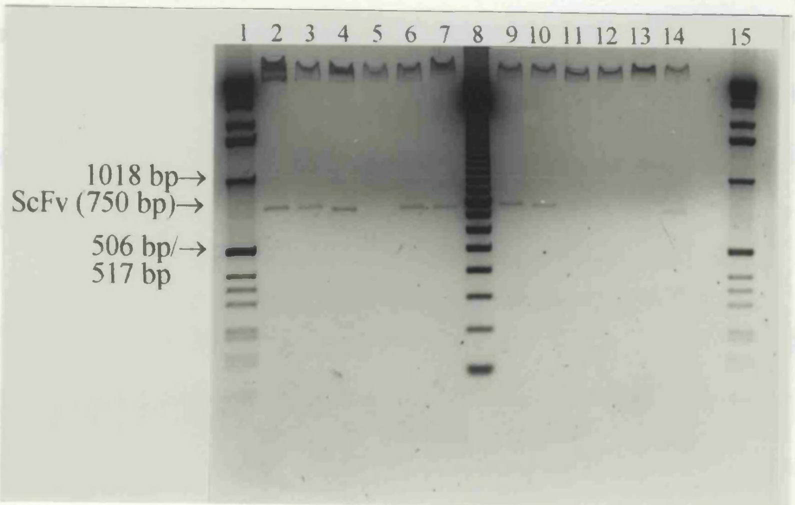


Figure 5.33: *Sfi* I and *Not* I restriction digests of recombinant phagemid DNA.

CYP3A4 ScFv-pCANTAB 5 E recombinant phagemid DNA was isolated from each of the twelve *E. coli* HB2151 clones. Each recombinant phagemid DNA was digested with *Sfi* I and *Not* I and 10 µl of each sample was electrophoresed through a 1.5% agarose gel. The gel was photographed under U.V. light. The following samples are shown: 1 µg of 1 Kb molecular weight standard DNA ladder (Gibco BRL, lanes 1 and 15); *Sfi* I and *Not* I digested recombinant phagemid DNA from clones 1 (lane 2), 2 (lane 3), 4 (lane 5), 5 (lane 6), 6 (lane 7), 7 (lane 9), 8 (lane 10), 9 (lane 11), 10 (lane 12), 11 (lane 13), 12 (lane 14); 2 µg of 100 bp molecular weight standard DNA ladder (lane 8).

phagemids either never contained an ScFv fragment or the ScFv DNA was lost due to a rearrangement of the vector DNA by the *E. coli.*

These observations account for the results obtained in section 5.7.2. Supernatant, periplasmic and whole cell extracts produced from clones 4,10 and 11 did not show any bands for ScFv. The extracts derived from clones 3, 5, and 6 also did not give a positive western blot analysis for ScFv bands. The phagemid DNA isolated from each of these clones does carry an ScFv insert. It can be concluded that these clones either express the soluble ScFv antibodies at very low amounts or the antibodies were undetected due to the cleavage of the E tag by proteases.

### 5.7.4. WESTERN BLOT ANALYSIS OF THE CYP3A4 ANTIGEN PREPARATION USING E-TAGGED ScFv ANTIBODIES

Phagemid DNA isolated from clones 1, 2, 3, 5, 6, 7, 8, and 12 contain ScFv fragments, therefore these clones should express antibodies to the CYP3A4 antigen preparation. As has been previously discussed in section 5.5.3., the CYP3A4 preparation used to screen the CYP3A4 ScFv antibody library contained baculovirus proteins and a trace amount of P450-reductase. These baculovirus proteins and a small amount of P450-reductase were present in the antigen preparation used to inject the mice (sections 5.1.1. and 5.2.3.). Therefore some of the selected clones will produce ScFv antibodies to the baculovirus proteins and others, hopefully, to CYP3A4. As such small amounts of P450-reductase were present in the CYP3A4 sample used to screen the library, it was unlikely that any of the selected antibodies would recognise this protein. In order to determine which ScFv antibody was recognising baculoviral protein or CYP3A4, the following western blot analysis was carried out.

Eight aliquots of 10 μg of the CYP3A4 antigen preparation used to screen the ScFv library (section 5.6.) were electrophoresed through a 13% SDS-PAGE gel. Each sample was transferred to a strip of nitrocelluose. The strips were analysed by western blotting as described in section 2.2.34.19. Each blot was probed with a 1:1 dilution of a soluble ScFv antibody from the supernatant extract in blocking buffer. The E-tagged soluble antibodies were detected by using a 1 in 1,000 dilution of HRP/Anti-E tag conjugate (data not shown).

Unfortunately, when the blots were developed with an HRP colour substrate, no bands were detected. ScFv antibodies have been detected in the supernatant fractions of clones 1, 2, 7, 8, and 12 (section 5.7.2.). The strips were incubated overnight with both the diluted soluble ScFv antibodies and the HRP/Anti-E tag conjugate. However, no bands were detected when the blots were developed.

This suggests that the antibodies did not bind to any of the proteins present in the CYP3A4 preparation. As these clones were selected for the expression of antibodies that bound to the antigen preparation in section 5.6. these results were surprising. Some proteolytic degradation of the antibodies produced from clone 8 were observed in figure 5.32. These samples were taken from freshly produced extracts. It is possible that during the incubation of the blots with the soluble antibodies, the antibodies were completely degraded by bacterial proteases. Another possibility, is that the antibodies were unable to recognise the proteins when they were immobilised on nitrocellulose. A dot blot experiment was performed in order to determine if this was the case.

### 5.7.5. DOT BLOT ANALYSIS OF THE CYP3A4 PREPARATION USING E-TAGGED ScFv ANTIBODIES

10 μg and 20 μg of the CYP3A4 preparation were dotted onto a piece of BA85 nitrocelluose membrane. 5 μl of the soluble E-tagged ScFv antibody (supernatant extract) under test was placed above the other two dots. This was repeated seven times to enable the soluble antibodies (supernatant extract) produced from clones 1, 2, 3, 5, 6,

7, 8, and 12 to be analysed for antigen binding. The pieces of nitrocellulose were analysed for antigen-antibody binding as described for the western blot analysis of antigen using an E-tagged ScFv antibody from the supernatant extract (section 2.2.34.19).

Antigen-antibody binding was not detected for any of the ScFv antibodies. The only dots detected were those of the HRP/Anti-E tag conjugate binding to the soluble ScFv dots from clones 1, 2, 7, 8 and 12. These results correlate with the findings of the western blot analysis of soluble/supernatant ScFv antibodies using the HRP/Anti-E tag conjugate (section 5.7.2.).

The soluble ScFv antibodies were unable to recognise and bind to the proteins in the antigen preparation when the antigen was either immobilised or dotted on nitrocellulose. It may be concluded that this was caused by proteolytic degradation of the antibodies by bacterial proteases during the incubation steps of western blotting. Proteins expressed in *E. coli* have been shown to be degraded by *E. coli* proteases during the harvesting of cells and preparation of crude extracts (Grodberg and Dunn, 1988; Sugimura and Higashi, 1988). This problem could be avoided by adding protease inhibitors, such as PMSF, benzamide or pepstatin A or a combination of all three, to the culture medium during the expression of the antibodies and to all the prepared crude extracts. Protease inhibitors should also be added to the soluble antibody preparation each time it is used for western blotting.

The eight *E. coli* clones shown to harbour phagemid DNA carrying an ScFv insert, will be used to express soluble antibodies in the presence of protease inhibitors by another member of our laboratory.

# *CHAPTER SIX*

# CONCLUSIONS

Rodents are often used for laboratory experiments and drug toxicology trials. There are many examples of species differences in drug metabolism; a rat *in vivo* model may not provide a valid comparison for drug metabolism in man. Three novel marmoset CYP2A cDNA clones were isolated and their nucleotide and protein sequences were compared to a human CYP2A6 cDNA clone. The nucleotide sequences of the marmoset cDNAs were found to be approximately 92% identical to the nucleotide sequence of the cDNA of CYP2A6. The protein sequences of the marmoset CYP2A cDNA clones were approximately 88% identical to the protein sequence of CYP2A6 and CYP2A13, another member of the human CYP2A subfamily. The first twenty amino acids of human and non-human CYP2A sequences were compared; marmoset, monkey and baboon CYP2As were more similar to the human CYP2As than the mouse or rat CYP2As. Non-human primates, such as the marmoset, may provide a better animal model for drug toxicology studies than rodents. Parallel drug toxicology studies in marmosets and rodents must be carried out with a drug that is not subject to polymorphic metabolism and is commonly administered to man. If the pattern of metabolism of this drug in marmosets matches the metabolism of the compound in man more closely than that of rodents, then marmosets should provide a better animal model.

A hepatocyte culture system can be used as an alternative to an *in vivo* animal model. A human hepatocyte culture system was characterized by western blot analysis using different polyclonal antisera raised to several CYPs and P450-reductase. The induction responses of these enzymes to phenobarbital and valproic acid were examined. Two cultures, derived from different individuals, were characterized. Phenobarbital and valproic acid induced the expression of the CYP2As, CYP2B6, CYP2Cs, CYP3As, and P450-reductase after four and seven days of culture (three and six days treatment with the anti-epileptic agents). These results were observed for both the cultures; the average fold inductions of expression of the drug metabolising enzymes showed inter-individual variation. A small amount of expression of hGSTP1-1 was detected in hepatocyte homogenates from day 4 of culture. This indicated that the hepatocytes were slowly dedifferentiating. This culture system is ideal for short-term analysis of expression of drug metabolising enzymes in response to xenobiotics and therapeutic drugs.

The main aim of this thesis was to produce monoclonal antibodies to CYP2A6 and CYP3A4 using an adaptation of the recombinant phage display antibody system (McCafferty *et al.*, 1990). This system generates either monoclonal phage-displayed or monoclonal soluble ScFv antibodies (Marks *et al.*, 1991; reviewed in Winter and Milstein, 1991). CYP2A6 and CYP3A4, expressed in insect cells using the baculovirus expression system, were purified by affinity chromatography on *p*-chloroamphetamine-coupled sepharose (adapted from Sundin *et al.*, 1987). The purified proteins were separately injected into mice. CYP3A4 was also purified from insect cell microsomes by chromatography on *n*-octylamino-sepharose and DEAE-sephacel columns (adapted from Guengerich and Martin, in press). The two different chromatographic strategies significantly enriched for the CYPs however, low molecular weight contaminating baculovirus proteins remained in the purified samples. Western blot analysis with mice sera showed that the mice had preferentially raised antibodies to these proteins. If the baculovirus expression system is to be used for purification of antigens, then there must be several stages of purification to remove these immunogenic proteins. However, this would require a large amount of insect cell microsomes containing the expressed protein. Control (naive) ScFv cDNA, CYP2A6 ScFv cDNA and CYP3A4 ScFv cDNA libraries were successfully constructed; the library sizes ranged from 1 x $10^5$-5 x $10^5$/μg of recombinant vector DNA. The CYP3A4 ScFv library was screened by detection of antigen binding using a two-membrane system (adapted from Skerra *et al.*, 1991 and Dreher *et al.*, 1991). Supernatant, periplasmic and whole cell extracts were produced from selected bacterial clones determined to express antigen-binding ScFv antibodies. The soluble antibodies were expressed at a low amount in many of these clones. This could be due to low mRNA or protein stability or the expressed proteins remain in inclusion bodies. The ScFv antibodies were unable to be used as a primary antibody for western blot analysis of the antigen due to protease degradation. Protease inhibitors must be included in the culture medium used during expression of the soluble antibodies, all the prepared crude extracts and in the final antibody preparation each time it is used for western blotting.

# *CHAPTER SEVEN*

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