

ISOLATION, CHARACTERIZATION AND EXPRESSION OF
cDNAs ENCODING HUMAN AND MARMOSET
CYTOCHROMES P450's

by

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ABSTRACT

The expression of individual cytochromes P450 (P450s) has become a valuable tool for studying the structure- function relationship of these proteins and their metabolic capacities. A full-length cDNA encoding human CYP2A6 was expressed both in the baculovirus/Sf9 insect cell system and as a fusion protein with the maltose binding protein (MBP) in *Escherichia coli* (*E. coli*) cells. The expressed proteins were detected by SDS-PAGE and western blotting. MBP-CYP2A6 fusion protein was located in the cell membrane fraction of *E. coli* cells. In Sf9 insect cells transfected with a recombinant baculovirus, CYP2A6 was located in the microsomal membranes.

MBP-CYP2A6 fusion protein expressed in *E. coli* was functionally inactive towards the CYP2A6 substrate, coumarin. Although the degradation of MBP-CYP2A6 was not detected by western blot analysis, spectral analysis showed a strong P420 component suggesting misfolding of the polypeptide due to the interaction with the MBP domain. Attempts to purify MBP-CYP2A6 from *E. coli* were not successful. Baculovirus expressed CYP2A6 was found to be enzymatically active towards the metabolism of coumarin but not testosterone. Endogenous NADPH-cytochrome P450 reductase in Sf9 cells did interact with the expressed CYP2A6. However, the amounts of this protein were not sufficient for the amount of CYP2A6 expressed and catalytic studies required the addition of exogenous NADPH-cytochrome P450 reductase to obtain maximum CYP2A6 activity. CYP2A6 was purified from Sf9 cells by affinity chromatography.

A cDNA encoding a CYP2A was isolated from marmoset liver total RNA by reverse transcription and PCR. When compared to the sequence of CYP2A6, marmoset CYP2A cDNA contained a deletion of a nucleotide C after the initiation codon which changed the reading frame. Although not useful for heterologous studies, the cDNA was used as a probe for northern blot analysis of marmoset liver total RNA isolated from the livers of untreated or phenobarbital treated animals. CYP2A mRNA was induced 20-fold on treatment of marmoset with phenobarbital.

DEDICATION

To

My mum and my brother Mahesh

ACKNOWLEDGMENTS

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LIST of ABBREVIATIONS

A ₂₆₀	absorbance at 260 nm
A	adenine
ATP	adenine triphosphate
bp	base pair
BSA	bovine serum albumin
C	cytosine
cDNA	complementary deoxyribonucleic acid
Ci	Curie
cpm	counts per minute
dATP	deoxy adenosine triphosphate
dCTP	deoxycytidine triphosphate
ddATP	dideoxyadenosine triphosphate
ddCTP	dideoxycytidine triphosphate
ddGTP	dideoxyguanosine triphosphate
ddTTP	dideoxythymidine triphosphate
DEAE	diethylaminoethyl
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
G	guanine

Ig	immunoglobulin
IPTG	isopropyl-1-thio- β -D-galactoside
Kb	kilobase
MBP	maltose-binding protein
mRNA	messenger ribonucleic acid
NAD	nicotinamide adenine dinucleotide
OD ₂₆₀	optical density at 260
oligo	oligonucleotide
ORF	open reading frame
ori	origin of replication
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMSF	phenylmethylsulfonyl fluoride
RNA	ribonucleic acid
RNase	ribonuclease
SDS	sodium dodecyl sulfate
T	thymine
TBE	Tris/borate buffer
TBS	Tris-buffered saline
TE	Tris/EDTA buffer
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
TLC	thin-layer chromatography
Tris	tris (hydroxymethyl) aminomethane
UV	ultraviolet
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactoside

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Chapter 1

Introduction

1.1 General characteristics of cytochromes P450

Cytochrome P450 (P450) was identified in mammalian liver microsomes as a reduced pigment that had an absorption band with a λ_{max} at 450nm after binding to carbon monoxide (Klingenberg 1958; Garfinkel 1958). This pigment was further characterized, by Omura and Sato in 1964, as a P450 haemoprotein. P450s are now known to be a family of proteins whose members are present in biological sources as diverse as microorganisms, plants and animals (Porter and Coon, 1991). In mammals, P450s are present in almost all tissues (reviewed in Omura *et al.*, 1993).

P450s share the following characteristics: i) they contain a noncovalently bound haem; ii) they are intrinsic membrane proteins firmly bound to intracellular membranes and iii) they use reducing equivalents from NADPH (and sometimes NADH) and an atom of oxygen derived from molecular oxygen to oxygenate substrates. The reducing equivalents are transferred to P450 via a second enzyme. P450s can be divided into two major classes based on their intracellular location and the enzyme from which they receive electrons.

The mitochondrial P450s, first found in mitochondria isolated from the adrenal cortex (Harding *et al.*, 1964) are now known to be widely distributed amongst animal organs. All steroidogenic organs and some non steroidogenic organs including liver and kidney contain P450s in their mitochondria. These enzymes are synthesized on membrane-free polyribosomes (Nabi *et al.*, 1983) as a large precursor and are then transported into the mitochondria concomitant with the cleavage and the removal of an NH₂-terminal extra peptide (Kramer *et al.*, 1982). Mitochondrial

P450s are bound to the inner mitochondrial membrane and receive electrons from NADPH via two soluble redox proteins in the matrix, NADPH-adrenodoxin reductase and adrenodoxin (Baron *et al.*, 1972). Several mitochondrial P450s have been isolated from various animal sources, and their enzymatic and molecular properties have been elucidated. These proteins include the cholesterol side chain cleavage enzyme, steroid 11 β -hydroxylase, aldosterone synthase, and sterol 26 hydroxylase. Mitochondrial P450s are involved in the metabolism of steroids and related physiological substrates for example vitamin D₃.

The majority of P450s are of the second class, found primarily in the endoplasmic reticulum membranes. These proteins are synthesized on membrane-bound polyribosomes and inserted directly into the lipid bilayer via the signal sequence recognition system (Bar-Nun *et al.*, 1980; Sabatini *et al.*, 1982). Microsomal P450s receive electrons from NADPH via the flavoprotein, NADPH-cytochrome P450 reductase (Lu *et al.*, 1969) and in some cases, one electron is derived from NADH via cytochrome b₅ (Pompon and Coon, 1984).

Microsomal P450s play a central role in the metabolism of endogenous substances such as steroids and fatty acids (Guengerich *et al.*, 1986), and in the detoxification of foreign substances (xenobiotics) including plant toxins, drugs and environmental pollutants (Guengerich *et al.*, 1986) and in the activation of procarcinogens (Guengerich, 1988). Most P450s, particularly those found in the liver, function to convert hydrophobic substances to more hydrophilic derivatives that can be easily eliminated from the body directly or after conjugation with water soluble agents such as glucuronic acid and glutathione (Jakoby and Ziegler, 1990). The hydrophilic, conjugated material

is then easily passed from the body via urine or bile. This is usually a detoxification process, but in some instances, foreign substances are converted to products with much greater cytotoxicity, mutagenicity and carcinogenicity (Guengerich, 1988) This property of P450s emphasizes the importance of understanding their multiplicity, substrate specificities, and regulation.

1.2 Multiplicity of P450s and reactions catalysed by these proteins

The vast array of foreign substances to which organisms are exposed makes it impractical to have one enzyme for each compound, or even each class of compounds. Thus, while most cellular functions tend to be very specific, metabolism of foreign substances requires enzymes with diverse substrate specificity. Much of this role is assumed by P450s.

Partial purification of a form of P450 from phenobarbital treated rabbit liver microsomes was first reported by Lu and Coon (1968). Over the next 20 years it became evident through protein purification experiments that multiple forms of P450 exist in mammals and other species. Studies using systems of purified P450s reconstituted with lipid and NADPH-cytochrome P450 reductase (for example, Haugen and Coon, 1976; Coon *et al.*, 1975) revealed that individual forms of P450 can exhibit either highly specific or less specific overlapping substrate positional or stereospecificities (reviewed by Guengerich *et al.*, 1986).

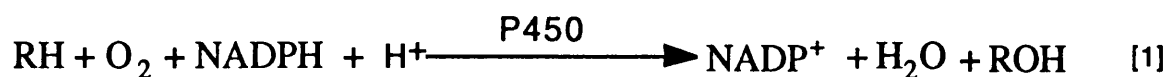
Most of the P450 mediated reactions begin with transfer of electrons from NAD(P)H to either NADPH-cytochrome P450 reductase in the microsomal system or a ferredoxin reductase and a nonhaem iron protein in the mitochondrial and bacterial systems, and then to P450. This leads to the

reductive activation of molecular oxygen followed by the insertion of one oxygen atom into the substrate. Reactions that have been demonstrated to be catalysed by P450s include hydroxylation, epoxidation, peroxygenation, deamination, desulfuration, dehalogenation and reduction (Nebert and Gonzalez, 1987). Examples of P450 mediated reactions are shown in figure 1.1.

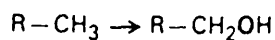
1.2.1 Mechanism of P450 catalysed reactions

The active site of P450 contains iron protoporphyrin IX bound in part by hydrophobic forces. The fifth ligand is a thiolate anion provided by a cysteine residue, a feature that contributes to the unusual spectral and catalytic properties of P450s, and the sixth coordinate position may be occupied by an exchangeable water molecule. Upon reduction of iron, molecular oxygen can be bound in the sixth position (Poulos *et al.*, 1987). Stoichiometric studies on monooxygenase reactions catalysed by P450 indicated the consumption of one molecule each of NADPH and oxygen to introduce one oxygen atom to the substrate molecule, indicating the supply of two electrons from NADPH to one P450 molecule during one cycle of reaction (Cooper *et al.*, 1977).

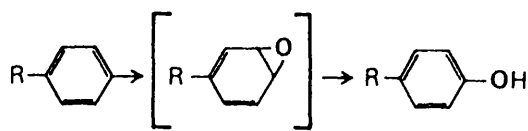
Stoichiometry of the P450 catalyzed hydroxylation reaction:



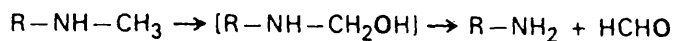
RH represents the substrate.



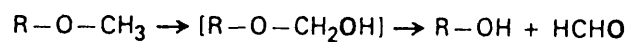
ALIPHATIC OXIDATION



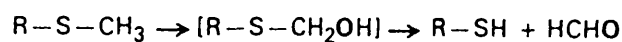
AROMATIC HYDROXYLATION



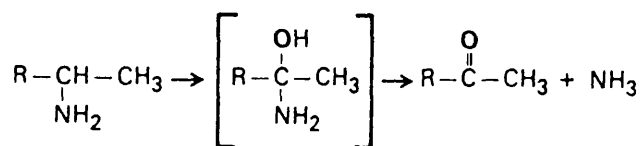
N-DEALKYLATION



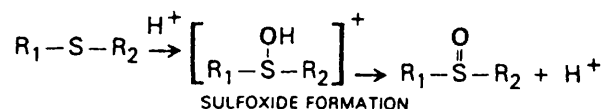
O-DEALKYLATION



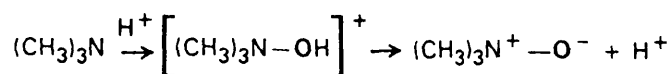
S-DEALKYLATION



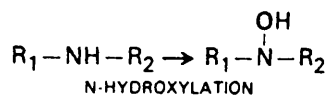
OXIDATIVE DEAMINATION



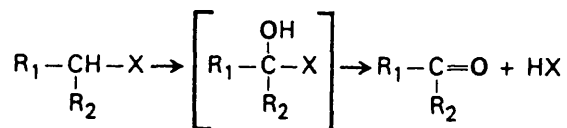
SULFOXIDE FORMATION



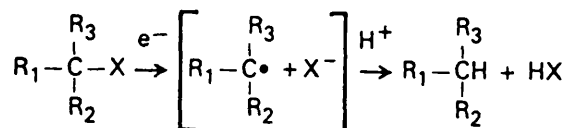
N-OXIDATION



N-HYDROXYLATION



OXIDATIVE DEHALOGENATION



REDUCTIVE DEHALOGENATION

Figure 1.1 Examples of reactions catalyzed by cytochromes P450
(Reproduced from Nebert and Gonzalez; 1987)

A cyclic mechanism for P450 catalysed monooxygenation was postulated in accordance with the known stoichiometry of the hydroxylation reaction (equation 1) and on the basis of observations of substrate induced spectral changes and spectral detection of the oxygenated form of P450 (Estabrook *et al.*, 1971). Further insight into the mechanism has also been gained from studies of the crystallized form of P450_{cam} from *Pseudomonas putida* (Poulos *et al.*, 1992). A schematic diagram of the overall P450 reaction cycle is shown in figure 1.2.

The first step in the reaction cycle is the binding of substrate to the active site of P450 resulting in the conversion of low- spin ferric haem in the enzyme to a high spin-ferric state. This facilitates the uptake of one reducing equivalent from the electron transfer system (in microsomes, this electron is transferred by NADPH-cytochrome P450 reductase from NADPH) to form a substrate- bound ferrous form (step 2).

Substrates that undergo reduction rather than oxygenation such as epoxides, N-oxides, nitro and azo compounds and lipid hydroperoxides, accept two electrons in stepwise manner as shown to give RH(H)₂. To initiate the oxidative reactions the ferrous enzyme reacts with molecular oxygen to form a ternary complex of oxygen , substrate and ferrous P450 commonly referred to as Oxy-P450. Introduction of a second reducing equivalent into the Oxy-P450 (step 4) is mandatory for the oxygenation reaction to occur. In microsomes, the second reducing equivalent is provided by NADPH cytochrome P450 reductase or in some cases, cytochrome b₅ (as an additional electron donor) (Pompon and Coon, 1984). The next step (5) is not well understood but involves splitting of the O-O bond with the uptake of two protons and the generation of “activated oxygen” and the release of water.

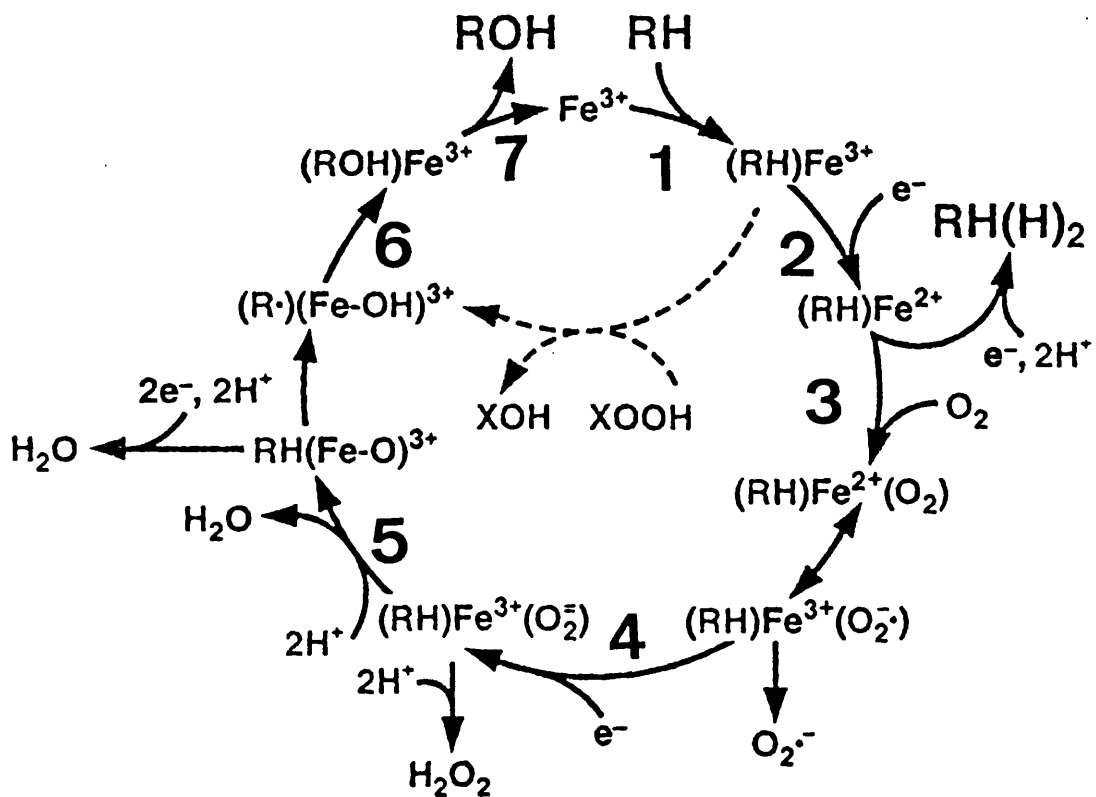


Figure 1.2 Mechanism of P450 catalyzed reactions

Fe represents the heme iron atom at the active site, RH the substrate, $RH(H)_2$ a reduction product, ROH a monooxygenation product and XOOH a peroxy compound that can serve as an alternative oxygen donor (Reproduced from Porter and Coon, 1991)

Oxygen insertion into the substrate (step 6) is believed to involve hydrogen abstraction from the substrate and the recombination of the resulting transient hydroxyl and carbon radicals to give the product (Groves *et al.*, 1978). Dissociation of the product, ROH, then restores the P450 to the ferric state (step 7).

Also indicated, in figure 1.2, is the way in which a peroxy compound may substitute for molecular oxygen and reducing equivalents in what is termed as the peroxide shunt. Homolytic cleavage is envisioned with the formation of an iron-bound hydroxyl radical capable of hydrogen abstraction from the substrate (White and Coon, 1980).

Much remains to be learned about the factors controlling regio and stereo specificity in P450 catalysed reactions. Heterologous cDNA based expression of large amounts of individual P450s will greatly aid such studies. Some of the systems that have been used for the heterologous expression of P450s are described in section 3 of this thesis.

1.2.2 Nomenclature

The confusing nature of P450 nomenclature has been one of the hallmarks of P450 research. Most laboratories involved in the purification of P450s developed their own system of nomenclature, often based on enzyme characteristics, which included typical P450 inducing agents or substrates for the purified enzyme or the sequential order in which a particular research group had purified a particular series of P450s. For example, CYP1A1 has been designated P-450-C, P450 β NF-B, P-448₂, P-450MC-1, P-450 isozyme 6 etc (Gonzalez, 1989). Such nomenclature systems proved to be inadequate

as a result of the ever increasing number of P450s to be isolated and the broad and overlapping substrate specificity of these enzymes. Characterization based on full length amino acid sequences was impeded by technical difficulties and the required labour intensive procedures inherent in this biochemical approach.

A major breakthrough in the structural analysis of P450s came with the application of recombinant DNA technology (Fujii-Kuriyama *et al.*, 1982) which subsequently resulted in the isolation and sequence determination of many individual P450 forms. Sequence analysis has provided the basis for a unified nomenclature system (Nebert *et al.*, 1991).

In the P450 gene superfamily, families and subfamilies have been defined on the basis of amino acid sequence similarity. A P450 protein sequence from one gene family is defined as usually having less than or equal to 40% amino acid identity to a P450 protein from any other family. Within a single family, the P450 protein sequences are > than 40% identical. Sequences of P450 proteins within the same subfamily are > 55% identical within the same species.

Recommendations for naming a P450 gene or cDNA include the root symbol *CYP* (*cyp* for the mouse) denoting P450, an Arabic number designating the P450 family, a letter indicating the subfamily and an Arabic number representing the individual gene. With the mouse genes or cDNAs, the final number is generally preceded by a hyphen. P (p in mouse) after a gene number is used to denote a pseudogene. The same nomenclature is recommended for the corresponding gene product (enzyme). For example, *CYP1A1* (*cyp1a-1*, in mouse) for the gene and cDNA, and CYP1A1 for the mRNA and protein in all species including mouse.

One of the problems with the P450 nomenclature system is in distinguishing allelic variants of one protein from closely related P450 forms. For example, rodent P450 genes can differ by only a few bases. Allelic variants can be distinguished by genetic crosses in rodents when the enzymes can be resolved by two dimensional electrophoresis (Rampersaud and Walz, 1987). Allelic variants can also be distinguished by direct analysis of genomic DNA using the polymerase chain reaction (PCR) or Southern blotting following genetic crosses of rodents and by linkage studies using large pedigrees in humans.

Another problem faced by the P450 nomenclature system is a reasonable method to determine orthologous P450s. Genes or proteins in two or more species are said to be orthologous if both are believed to have evolved from a single ancestral gene present at the time of divergence of the species. This is problematic when multiple forms of P450s exist in certain subfamilies. For example rats, mice, rabbits and humans contain many P450s in the CYP2C subfamily, however, no two P450s between these different P450s display the same catalytic activities. In addition, a single CYP2C P450 in rat does not have significantly higher sequence similarity with a single P450 in the same subfamily in humans than with the other P450s in the human CYP2C subfamily. The same applies when comparisons are made between other species. Thus P450 forms in the CYP2C family are given individual form designations. In contrast, in the CYP2E subfamily, most species except rabbits contain a single P450 form designated CYP2E1 that has qualitatively similar catalytic activities across species (Gonzalez, 1989). A recent update of the P450 nomenclature, and genes isolated, has been published by Nelson *et al.*, 1993.

1.2.3 Evolution of P450s

Based on the existence of two distinct classes of P450s, the possible pathways of evolution giving rise to mammalian P450s can be hypothesized. The mitochondrial P450s may have arisen from bacterial P450s such as P450_{cam} (CYP101) from *Pseudomonas putida* and other prokaryotes that use an iron sulphur protein and flavoprotein as electron donors. Microsomal P450s might have evolved from the mitochondrial forms by transfer of genetic material from the mitochondrial genome to the nucleus. Alternatively, P450 and NADPH cytochrome P450 reductase may have arisen from a fusion protein such as that found in *Bacillus megaterium* followed by the separation of the two domains. In fact, the mammalian CYP3 gene family and NADPH-cytochrome P450 reductase are both found on human chromosome 7 (Yamano *et al.* , 1989).

Much of the insight into the evolution of P450s has come from a correlation of catalytic activities of the P450s with the information obtained from the examination of phylogenetic trees of the P450s. Construction of the phylogenetic tree is based on amino acid sequence relatedness of different P450 forms. The percentage differences calculated after pairwise sequence comparisons are converted to evolutionary distance in units defined as accepted point mutations (Nelson and Strobel, 1987). Evolutionary distance (d) is a mathematical approximation of the number of mutations per 100 amino acids that have occurred since a divergence event. The tree (figure 1.3) is constructed using the unweighted-pair-group method of analysis (UPGMA). Proper interpretation of data in a phylogenetic tree requires calibration of d values with real time. This is generally achieved by using divergence times from early in evolution (for example, bird-mammalian split at 300 million years

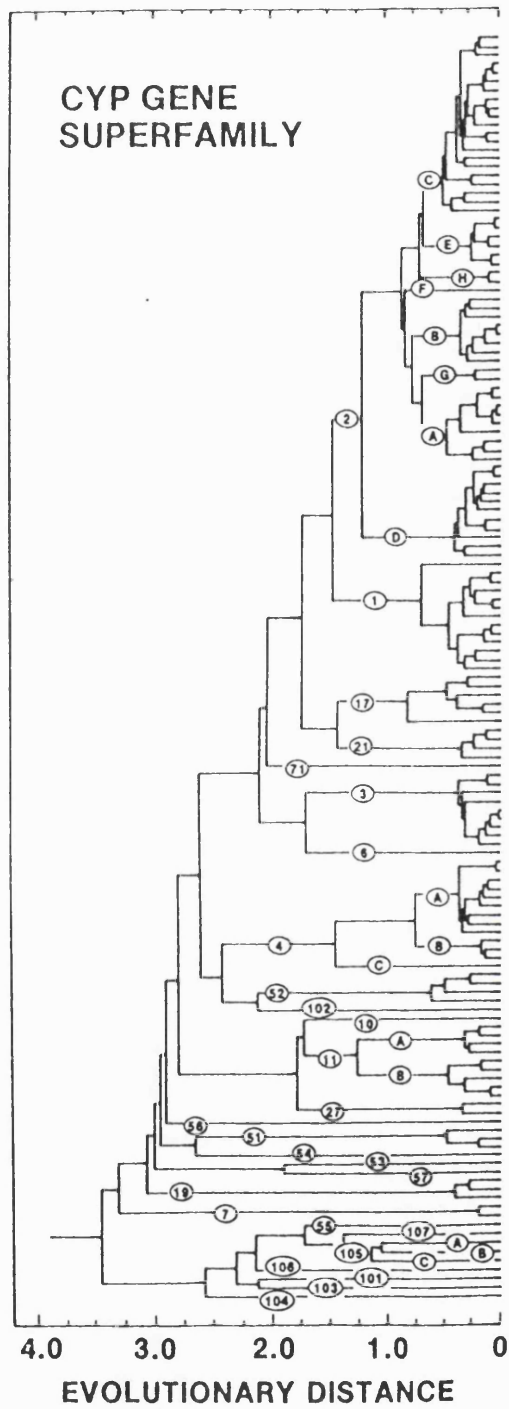


Figure 1.3 Phylogenetic tree of P450 gene superfamily

147 protein sequences (derived from cDNA sequences) have been compared (Reproduced from Nebert *et al.*, 1991)

ago) and more recent divergence times (for example, mammalian radiation at about 80 million years ago and rat-mouse divergence at 17 million years ago).

From an analysis of the P450 phylogenetic tree (figure 1.3.), it appears that the oldest mammalian P450s closely related to the bacteria and yeast P450s, are the cholesterol 7 α -hydroxylase (CYP7) and the P450 aromatase (CYP19) involved in bile acid and oestrogen synthesis respectively.

Two other "old" P450s are the cholesterol 27-hydroxylase (CYP27) and the cholesterol side chain cleavage enzyme (CYP11A1). These are related to P450s that metabolize fatty acids such as the lauric acid hydroxylases of the CYP4 family. Cholesterol and fatty acid metabolizing P450s are thought to have been involved in the maintenance of membrane integrity of early eukaryotes.(Gonzalez, 1989). A later evolutionary event, about 900 million years ago, was the formation of the drug metabolising P450s. These diverged into major drug and carcinogen metabolising enzymes of the CYP1, CYP2 and CYP3 families. More recently, between 400 and 600 million years ago, a tremendous expansion in the *CYP2* gene family has occurred. The reason for the increase in the number of P450 genes during the past several million years may be related to environmental factors including dietary components and toxins.

An interesting feature of P450 gene evolution is the occurrence of multiple genes, within a particular subfamily, that in many cases are present in some species but not in others. These are thought to have arisen through the process of gene duplication and the fixation of duplicated genes through natural selection. For example, rabbits, rats and humans are known to have diverged about 75 million years ago. The CYP2E subfamily in rats and

humans contains a single gene, whereas two highly similar genes (*CYP2E1* and *CYP2E2*) are present in rabbits (Gonzalez, 1989). On the basis of amino acid sequence similarity of the two rabbit proteins, it is predicted that a gene duplication occurred in this species about 10 million years ago, long after the rabbit-rat-human speciation.

The net result of recent gene duplications and gene conversion events is that individual species contain their own unique P450 genes. A scheme for the formation of P450 gene families, subfamilies and gene conversion is shown in figure 1.4. Gene duplication, conversion or gene loss probably account for some of the major interspecies differences in drug and carcinogen metabolism that have been observed over the years. Although humans have more recently been exposed to a vast number of man made chemicals, it is apparent, given an average unit evolutionary period for P450s of 4, that human P450s cannot evolve rapidly enough to meet this challenge. This may be an important factor underlying the increase in chemically induced diseases such as cancer.

1.3 Regulation, tissue specific expression and catalytic activities of cytochromes P450.

221 P450 genes and 12 putative pseudogenes have been characterized (Nelson *et al.*, 1993). These genes have been described in eleven prokaryotes and thirty one eukaryotes (including eleven mammalian and three plant species). Of the thirty six gene families described, twelve families exist in all mammals examined.

The mammalian P450 families can be functionally subdivided into two major classes; those involved in the synthesis of steroids and bile acids and

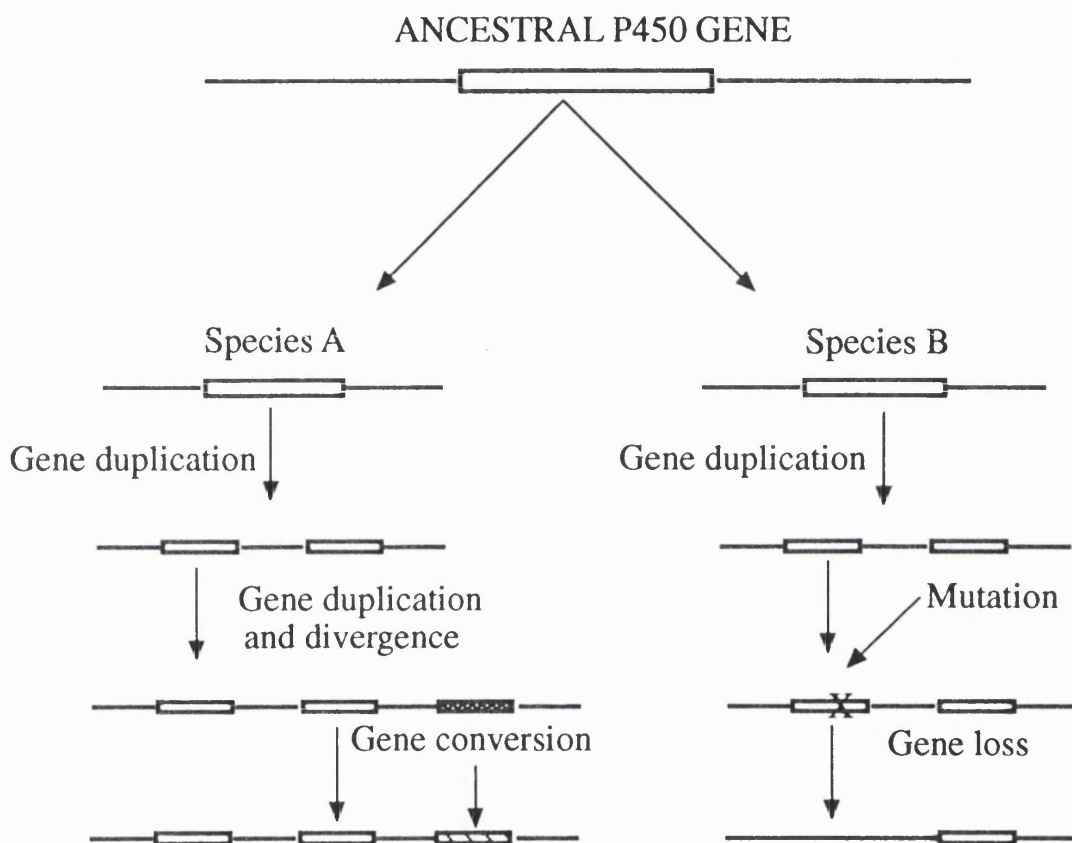


Figure 1.4 Evolution of P450 gene superfamily

A hypothetical scheme is shown in which two species, A and B evolve P450 genes. Gene duplications occur independently in both species. A second gene duplication has occurred in species A while a mutation (X) has eliminated a gene in species B. Within the species A family, a gene has diverged from the other two due to changes in sequence and natural selection. This gene represented as a striped rectangle, was then involved in a gene conversion with the neighbouring gene. After a period of time, species A and B have evolved their own distinct set of P450 genes.

those that primarily metabolize xenobiotics.

1.3.1 P450s Involved in the metabolism of xenobiotics

CYP1 family

The CYP1 family comprises of two genes (*CYP1A1* and *CYP1A2*) in every mammalian species so far examined. In humans *CYP1A* genes are located on chromosome 15 (Jaiswal *et al.*, 1987). *CYP1A1* is associated with high aromatic hydrocarbon hydroxylase activity and its expression is dependent on the presence of inducers such as benzo (a) pyrene and can be demonstrated in almost all tissues of an animal. The induction of the *CYP1A1* gene is mediated through a cytosolic receptor called the Arylhydrocarbon hydroxylase (Ah) receptor, that dimerises with the protein Arnt (Ah receptor nuclear translocator), to initiate transcription of this gene by binding to xenobiotic responsive elements (XREs) located in the 5' flanking sequences of this gene (Gonzalez *et al.*, 1993).

Expression of the *CYP1A1* gene has been correlated with development of polycyclic aromatic hydrocarbon- associated cancers in rodents (Nebert, 1989). High and low-inducibility phenotypes have been found in humans (Petersen *et al.*, 1991). A restriction fragment length polymorphism near the *CYP1A1* gene was found to be associated with increased lung cancer risk in Japanese smokers (Nakachi *et al.*, 1993), however, the same association was not found in a cohort study of Norwegian lung cancer patients (Tefre *et al.*, 1991). The biochemical basis of lung cancer association with *CYP1A1* is not known.

Human *CYP1A1* cDNA has been isolated from a human liver cDNA library (Quattrochi *et al.*, 1986) and its expression in COS cells produced an

enzyme capable of N-hydroxylating acetylaminofluorine, hydroxylating benzo(a)pyrene, and activating the food derived promutagen 2-amino-1-methyl-6-phenylimidazo [4,5-b] (PhIP) (McManus *et al.*, 1990).

CYP1A2 is constitutively expressed in the liver and is responsible for the activation of numerous promutagens and procarcinogens including aflatoxin B1, heterocyclic arylamine promutagens and the drugs phenacetin and caffeine (Guengerich and Shimada 1991). Gene regulation studies demonstrated that a heterologous *CYP1A2* promoter is activated by 3-methylcholanthrene in human hepatoma HepG2 cells and a region of DNA that supports Ah receptor binding and promoter specific induction has been identified (Quattrochi *et al.*, 1994).

A human *CYP1A2* cDNA has been cloned (Jaiswal *et al.*, 1987) and its expression in B-lymphoblastoid cells (Crespi *et al.*, 1990) produced an enzyme capable of metabolically activating aflatoxin B1. The activity was higher than other human P450s known to metabolize this compound. *CYP1A2* therefore may be the principal enzyme functional *in vivo* under typical human exposure concentrations of aflatoxin B1.

CYP2 family

CYP2 is the largest of the 12 mammalian P450 families and is divided into 10 subfamilies.

CYP2A subfamily

The *CYP2A* subfamily has been extensively studied in rodents. Rats and mice possess three *CYP2A* (*CYP2A1*, *CYP2A2* and *CYP2A3*) and two *CYP2A* (*CYP2A4* and *CYP2A5*) subfamily members respectively (Nelson *et*

al., 1993). CYP2A1 is highly specific for testosterone 7 α -hydroxylase activity. In contrast, CYP2A2 exhibits a high testosterone 15 α -hydroxylase activity. CYP2A1 and CYP2A2 are liver specific, but they are regulated differently. CYP2A1 production is increased in young male and female rats but the expression of this gene is suppressed in males at the onset of puberty. In contrast, CYP2A2 is never expressed in females and the gene is activated only when males reach puberty (Matsunaga *et al.*, 1988). CYP2A3 is constitutively expressed in the rat lung and absent from the liver. The mRNAs encoding this protein are induced 3 fold by treatment of rats with 3-methylcholanthrene (Kimura *et al.*, 1989 a).

The two P450s in the mouse CYP2A subfamily bear a high sequence similarity with the rat CYP2A3 have been characterized (Lindberg and Negishi, 1989). CYP2A4 carries out testosterone 15 α -hydroxylation whereas CYP2A5-Coh^h and CYP2A5-Coh^l are allelic variants having high and low coumarin 7-hydroxylase activities respectively and differing at only a single nucleotide associated with the codon 117, Ala117 in CYP2A5-Coh^l and Val117 in CYP2A5-Coh^h (Lindberg and Negishi; 1989). The expression of the *Cyp2a-5* gene is induced by phenobarbital (Aida and Negishi; 1991) whereas *CYP2A3* gene expression is induced by 3-methylcholanthrene (Kimura *et al.*, 1989 a) suggesting that the regulatory elements controlling the *CYP2A* genes have evolved differently in rats and mice. In humans, two distinct cDNAs have been isolated, one of which (*CYP2A6*) encodes a P450 capable of hydroxylating coumarin (Yamano *et al.*, 1990). A second cDNA (*CYP2A7*) also isolated from a human liver library (Yamano *et al.*, 1990) encoded a protein that contained a complete reading frame and was 94% similar in amino acid

sequence to CYP2A6. Interestingly, CYP2A7 did not incorporate haem when the cDNA was expressed using a vaccinia virus based system (Yamano *et al.*, 1990). It is possible that CYP2A7 contains an amino acid substitution that renders it unstable. The most conspicuous feature of CYP2A6 is the extremely large inter-individual variations observed at the protein, mRNA and enzyme activity levels (Yamano *et al.*, 1990, Palmer *et al.*, 1990, Rounio *et al.*, 1988). Large interspecies variation in COH activity has also been observed (Pelkonen *et al.*, 1985).

Depending on the human livers analysed, 40 to 100 fold differences in COH activity were detected. Palmer *et al.* 1990 found that the expression of CYP2A6 mRNA varied 1000-fold between the individual samples they analysed. In 10% to 20% of livers with high COH activity, CYP2A6 represents only about 1% of total P450 indicating that CYP2A6 is a low abundance P450 (Yun *et al.*, 1991). In a number of human livers, only trace amounts of CYP2A6 protein and associated enzyme activities were observed suggesting that the protein may be inducible by some dietary components or drugs. Several compounds have been reported to increase COH activity in mouse liver after administration *in vivo* (Hahnemann *et al.*, 1992). Examples include phenobarbital, pyrazole and cobalt. As yet, inducibility of CYP2A6 by similar compounds has not been demonstrated in humans. It is possible that the *CYP2A6* gene is polymorphically expressed in humans and that the mutant alleles in the population cause the marked variation in expression similar to the findings with the *CYP2D6* allele and the debrisoquine/sparteine polymorphism (Kimura *et al.*, 1989 b). This idea is supported by the presence of *CYP2A6v*, a variant cDNA that was isolated from a human liver cDNA library. The protein expressed from this cDNA did not show COH activity

(Yamano *et al.*, 1990). As yet, it is not established whether this variant is common within the human population and whether it accounts for the lack of CYP2A6 protein activity observed in some human liver samples.

Coumarin has been used as an *in vivo* metabolic probe to test for the presence of CYP2A6 polymorphism in humans (Cholerton *et al.*, 1992). These studies have presented evidence for a wide inter-individual variation in 7-hydroxylation of coumarin. In the light of the possibility that a CYP2A6 polymorphism exists in humans, it will be interesting to determine whether the expression of CYP2A6 is associated with an increased risk for chemically induced cancer since CYP2A6 is capable of metabolically activating aflatoxin B₁, N-nitrosodiethylamine (Crespi *et al.*, 1991 a), and 1,3-butadiene (Duescher and Elfarra; 1994). It would be interesting to determine whether CYP2A6 is expressed in human lung, the site of tobacco smoke and air pollution associated with carcinogen exposure. As yet, human *CYP2A* genes have not been isolated, but a cluster is known to exist on human chromosome 19 (Phillips *et al.*, 1985).

CYP2B subfamily

The cDNAs for *CYP2B1* and *CYP2B2* in rats were the first P450 cDNAs to be isolated and completely sequenced (Fujii-Kuriyama *et al.*, 1982). *CYP2B1* and *CYP2B2* proteins exhibit 97% amino acid sequence similarity. These enzymes have similar broad and overlapping substrate specificities but *CYP2B1* has two to ten fold higher activity (depending on the substrate) than does *CYP2B2* when these enzymes are purified and analysed for activity in a reconstituted system (reviewed by Gonzalez, 1984). A third cDNA in the rat *CYP2B* subfamily designated *CYP2B3* has also been isolated and shown to

have 77% amino acid sequence identity to CYP2B1 and CYP2B2. However, the genes that encode CYP2B1, 2B2, and 2B3 are differentially regulated. CYP2B1 is absent or expressed in a very low amount in the liver but is highly inducible by phenobarbital treatment. CYP2B2 is constitutively expressed in the liver and induced by phenobarbital. CYP2B3 is constitutively expressed and not inducible by phenobarbital. Furthermore CYP2B1 is constitutively expressed and not inducible by phenobarbital in lung and testis while CYP2B2 is absent from these tissues regardless of treatment (reviewed by Gonzalez, 1989). In the small intestine, CYP2B1 is constitutively expressed and inducible by various agents including phenobarbital (Traber *et al.*, 1988).

These observations suggest that the genes encoding CYP2B subfamily members contain tissue specific enhancer and inducer control elements. To date, no receptor for phenobarbital has been identified. Recently, two cis-acting elements located between -199 and -183 and -72 and -31 of a *CYP2B2* gene have been identified that bind nuclear proteins that are either more abundant or activated in response to phenobarbital treatment (Shephard *et al.*, 1994). It is known that induction of *CYP2B1/2* genes by phenobarbital occurs through transcriptional activation (Waxman and Azaroff 1992).

Progress has been made in defining the molecular mechanism of phenobarbital induction of P450s in bacteria. A region of DNA upstream of the bacterial P450 gene *CYP102* was found to confer transcriptional activation in the presence of phenobarbital. This activation appears to be due to the release of a repressor protein designated Bm3R1 that binds to a palindromic operator sequence located immediately upstream of the *CYP102* gene (Shaw and Fulco 1992). It is not clear whether phenobarbital acts directly or indirectly to release the repressor from the operator.

Two CYP2B cDNAs, *CYP2B6* and *CYP2B7* have been isolated from a human liver cDNA library. *CYP2B6* cDNA was expressed using the vaccinia virus based system and the protein was able to catalyze the O- deethylation of 7-ethoxycoumarin. The *CYP2B7* cDNA deduced amino acid sequence displayed 93% identity to that of *CYP2B6* and contained a premature termination codon due to a C to T transition (Yamano *et al.*, 1989 b). A large degree of inter individual variation in the expression of CYP2B protein and mRNA has been observed in human liver specimens (Miles *et al.*, 1989, Yamano *et al.* ;1989 b). However, no evidence was presented to suggest the presence of a known P450 inducing drug in patients whose liver specimens contained high amounts of CYP2B mRNA or protein.

CYP2C subfamily

The CYP2C subfamily is generally thought to represent a class of constitutively expressed genes. A total of 9 rabbit and 8 rat *CYP2C* genes have been identified to date (Nelson *et al.*, 1993). In rats these enzymes are noted for their sex specific and developmentally regulated expression (reviewed by Gonzalez, 1989). In rabbits, none of the CYP2C enzymes are uniquely expressed in males or females. In humans six CYP2C forms have been identified through cDNA cloning (Nelson *et al.*, 1993). CYP2C8 (Ged *et al.*, 1988) and CYP2C9 (Srivastava *et al.*, 1991) have been purified. Another P450 designated CYP2C10 (Ged *et al.*, 1988) has identical cDNA deduced amino acid sequence to CYP2C9 apart from an abrupt sequence change in the 3' non-coding region of the cDNA. Could *CYP2C10* be a cloning artefact? The confirmation of the existence of *CYP2C10* can be achieved by PCR analysis and sequencing of genomic DNA.

A member of the CYP2C subfamily is believed to encode a P450 responsible for the human S-mephenytoin polymorphism, which effects 2% to 5% of Caucasians and up to 27% of Japanese who lack S-mephenytoin 4-hydroxylase activity (Wilkinson *et al.*, 1989). A *CYP2C9* cDNA isolated from the Japanese population has been expressed in yeast (Yasumori *et al.*, 1989) and the expressed protein shows stereo selective metabolism of mephenytoin but at low turnover rates. Proteins expressed from *CYP2C9* cDNAs other than that isolated from a Japanese liver cDNA library did not show S-mephenytoin 4-hydroxylase activity, but readily metabolized tolbutamide (Relling *et al.*, 1990).

Additionally, three cDNAs *CYP2C17*, *CYP2C18* and *CYP2C19* have been isolated from a human liver cDNA library and their expression in COS cells (Romkes *et al.*, 1991) produced enzymes capable of metabolizing racemic mephenytoin with *CYP2C18* displaying high activities as compared to *CYP2C9* and *CYP2C19*. These observations suggest that *CYP2C18* might be a candidate for the P450 responsible for the mephenytoin polymorphism.

CYP2C9 is also the major human hepatic P450 involved in the metabolism of the therapeutically active S-isomer of the anticoagulant drug, warfarin (Rettie *et al.*, 1992). cDNA expressed *CYP2C9* catalyses 7-hydroxylation of S-warfarin and the 7-hydroxy metabolite is the major metabolite found in humans after administration of the drug. Thus the cause of large patient variability in warfarin effectiveness may be due to interindividual variation in *CYP2C9* expression. The locus of *CYP2C* genes has been mapped to human chromosome 10 (Shephard *et al.*, 1989).

CYP2D subfamily

Mice and rats contain five *CYP2D* genes that are expressed in the liver and kidney. Three *CYP2D* genes are present in humans and are expressed in the liver, kidney and the intestine (Nelson *et al.*, 1993). The rat and human enzymes carry out the oxidation of the drugs debrisoquine, and bufuralol. In humans, *CYP2D6* is responsible for the debrisoquine/sparteine polymorphism (Gonzalez and Meyer, 1991). About 7.5% of European and north American Caucasians possess two copies of the mutant *CYP2D6* alleles and are thus unable to metabolize debrisoquine and over 20 other drugs. The three major mutant *CYP2D6* alleles have been sequenced including one locus in which the *CYP2D6* gene is deleted (Hanioka *et al.*, 1990, Gaediak *et al.*, 1991). The majority of deficient metabolisers can be identified through PCR genotyping (Broly *et al.*, 1991, Daly *et al.*, 1991,).

None of the mouse *CYP2D* genes encode a P450 able to metabolize debrisoquine and other *CYP2D6* substrates demonstrating a species difference in the *CYP2D* subfamily. Heterologous cDNA expression studies have demonstrated that *CYP2D6* is capable of metabolically activating 4-(methylnitrosamino)-1-(3-pyridyl)-1-butone (NNK), a tobacco smoke nitrosamine carcinogen (Crespi *et al.*, 1991 a). It has been observed that the tobacco smoke associated lung cancer risk is less in smokers who lack a functional *CYP2D6* gene (Caporaso *et al.*, 1991). A deficient metabolizer genotype has also been shown to be predominant in patients with Parkinsons disease suggesting that *CYP2D6* expression offers some protection against possible neurotoxins (Smith *et al.*, 1992). The locus of human *CYP2D* genes has been mapped to chromosome 22 and consists of *CYP2D6*, *CYP2D7P*, and *CYP2D8P* (Kimura *et al.*, 1989 b). One common allelic locus consists of

four genes and includes a duplication of *CYP2D7P* (Heim and Meyer, 1992).

CYP2E subfamily

CYP2E1 is among the best conserved P450 forms in the CYP2 family. A single *CYP2E* gene exists in humans, rats and mice and there are two very similar genes in rabbits. The catalytic activities of CYP2E1 across species are quite similar, suggesting that this enzyme might play a role in the metabolism of some physiologically important compounds. In this respect, CYP2E1 was shown to play a role in the metabolism of ketone bodies in the propandiol pathway of glyconeogenesis (Koop and Casazza, 1985). CYP2E1 is a major xenobiotic metabolising enzyme capable of metabolically activating numerous low molecular weight solvents and carcinogens (Guengerich *et al.*, 1991 a). At least 15 promutagens and procarcinogens have been identified as being mainly activated by CYP2E1 (Guengerich and Shimada, 1991). This P450 is constitutively expressed in human liver and is probably influenced by inducers many of which are CYP2E1 substrates, such as ethanol (Koop and Tierney, 1990). CYP2E1 is probably expressed in extrahepatic epithelial tissues, especially after induction, for example CYP2E1 expression has been detected in lymphocytes from uncontrolled diabetics (Song *et al.*, 1990).

Human CYP2E1 has been purified and was found to carry out the metabolic activation of N-nitrosodimethylamine, similar to the rodent enzyme (Wrighton *et al.*, 1987). These studies were confirmed by using B-lymphoblastoid cell lines transfected with a human *CYP2E1* cDNA (Crespi *et al.*, 1990 b). These cell lines are very useful in determining the mutagenicity of nitrosamines which are relatively inert in the commonly used Ames test. The CYP2E1 protein is posttranscriptionally regulated in rodents through substrate

induced protein stabilization (Gonzalez *et al.*, 1991). Administration of ethanol, acetone and other substrates causes an increase in CYP2E1 protein without affecting its mRNA. This increase is due to a decrease in the degradation of the protein (Song *et al.*; 1989). It is believed that substrates block site specific phosphorylation of the protein, a posttranscriptional modification that may serve as a trigger for CYP2E1 degradation (Eliasson *et al.*, 1990).

Both cDNA and genomic clones encoding human CYP2E1 have been isolated and completely sequenced (Umeno *et al.*, 1988). The human *CYP2E1* gene has been mapped to chromosome 10 and shares many features with its rat counterpart. The 5' flanking sequence of both the rat and human genes contain a binding sequence for the liver specific transcription factor HNF-1 (Gonzalez *et al.*, 1991). This factor controls the expression of the *CYP2E1* gene and triggers its transcription in rats within a few hours after birth.

CYP2F subfamily

A *CYP2F1* cDNA has been isolated from a human lung cDNA library (Nhamburo *et al.*, 1990). The corresponding protein has however not been purified from human lung but a mouse counterpart has been isolated from lung tissue (Ritter *et al.*, 1991). The mouse enzyme activates pneumotoxin naphthylamine and was purified on this basis. Little is known about the catalytic activities of human CYP2F1 except for its involvement in activation of lung toxin, 3-methylindole (Thornton-Manning *et al.*, 1991).

CYP2G, CYP2H, CYP2I and CYP2J subfamilies

cDNAs for the P450 members of the above subfamilies have been

isolated from various species but as yet these P450 forms have not been characterized fully at the DNA or protein level (Nelson *et al.*, 1993).

CYP3 family

The CYP3A subfamily of P450s are the most abundantly expressed in human liver (Shimada and Guengerich, 1989). CYP3A3 and CYP3A4 are very similar having only 11 amino acid substitutions and no known differences, in catalytic activities. A third CYP3A form designated CYP3A5 is expressed in only 10% to 20% of human livers analysed (Aoyama *et al.*, 1989) and exhibits similar catalytic activities to CYP3A3 and CYP3A4 except for some differences in positional specificities on certain substrates such as cyclosporin. A P450 designated CYP3A7, that is absent in adults, has been purified (Wrighton and Vanden branden; 1989) and its cDNA cloned (Komori *et al* ; 1989) from foetal liver sources. CYP3A7 is the major P450 expressed in human foetal liver (Kamatani *et al.*, 1991). In contrast, rodents do not express foetal liver CYP3A P450s. The role of CYP3A7 in human foetal liver is not clear.

All CYP3A P450s are able to metabolize numerous drugs for example, erythromycin, cyclosporin, warfarin and lidocaine and can also metabolically activate aflatoxin B1 and other procarcinogens (Guengerich and Shimada, 1991).

Expression of CYP3A P450s is highly variable in human liver specimens, with some livers having up to 60% of their total P450 as CYP3A4 while others contain less than 10%. This finding has important implications for the metabolism of drugs that serve as a substrates for these enzymes. *In vivo* analysis of levels of these enzymes can be accomplished using an

erythromycin breath test in which $^{14}\text{CO}_2$ is monitored as a result of demethylation reaction mediated by CYP3A enzymes (Watkins *et al.*, 1989). The *CYP3A* locus has been mapped to human chromosome 7 (Brooks *et al.*, 1988).

CYP4 family

P450s in the CYP4 family are involved in the metabolism of fatty acids and are present in both liver and extrahepatic tissues such as the kidney, lung, brain and prostate. cDNAs encoding members of CYP4 family have been isolated from rabbits, rats, mice, human and guinea pig (Nelson *et al.*, 1993).

Structurally diverse xenobiotics including hypolipidaemic drugs such as clofibrate and chlorinated phenoxy acid herbicides induce rat CYP4A1 (reviewed by Gonzalez, 1989). CYP4A1 is characterized by a narrow substrate specificity for oxidation of terminal (ω) carbon of fatty acids such as lauric and arachidonic acids. Clofibrate administration to rats results in a rapid transcriptional activation of CYP4A1 and genes coding for peroxisomal enzymes and recent evidence suggests that these effects may be receptor mediated (Issemann and Green, 1990). Interest in this phenomena results from the knowledge that induction of hepatic CYP4A1 is related to hepatic peroxisome proliferation and hepatocellular carcinoma in rodents (Lock *et al.*, 1989).

1.3.2 Steroidogenic and cholesterol metabolising P450s

The steroidogenic and cholesterol metabolising P450s in families CYP7, CYP19, CYP21 and CYP27 contain only one member. CYP11 has two

subfamilies, CYP11A and CYP11B comprising of 1 and 2 forms respectively. All of these enzymes are well conserved in mammals with respect to their catalytic activities and display rigid substrate and product specificities. This conservation is due to their critical role in steroid and bile acid synthesis (Guengerich *et al.*, 1986).

The *CYP11* gene family codes for two mitochondrial P450s namely steroid 11 β -hydroxylase (CYP11B1) and cholesterol side chain cleavage enzyme (CYP11A1). Conversion of cholesterol to pregnenolone is the first rate limiting step in the synthesis of steroid hormones and involves three consecutive monooxygenation steps all of which are catalysed by CYP11A1. CYP11A1 is expressed in adrenal cortex, ovary, testes, and placenta. CYP11B1 is expressed only in the adrenal cortex and mediates 11 β -hydroxylation of 11-deoxycortisol to cortisol as well as the synthesis of aldosterone (the key mineralocorticoid required for salt retention) from 11-deoxycorticosterone.

The intermediate steps leading from the conversion of pregnenolone to 11-deoxycorticosterone occur in the endoplasmic reticulum and are mediated by CYP17 and CYP21. CYP17 is found in all steroidogenic tissues and is a critical determinant in the synthesis of mineralocorticoids and glucocorticoids. CYP17 also functions as 17, 20-lyase and this activity distinguishes between synthesis of glucocorticoids and the C19 precursors of androgens. Aromatisation of androgens into oestrogens is catalyzed by CYP19. CYP21 catalyses the hydroxylation of progesterone and 17-hydroxyprogesterone at carbon 21 to yield 11-deoxycorticosterone and 11-deoxycortisol respectively. The deficiency of this 21-hydroxylase which occurs in about 1 in every 7000 persons results in adrenal hyperplasia, a common inborn error of metabolism

(New, 1988).

The pathway leading to synthesis of bile acids involves several P450s including cholesterol 7 α -hydroxylase (CYP7 family) which catalyses the first and rate limiting step, a 27-hydroxylation (CYP27 family) that initiates side chain oxidation and a 12 α -hydroxylation that leads to cholic acid formation. P450 dependent cholesterol metabolism also includes the 25-hydroxylation of vitamin D precursors, a step required for the activation of vitamin D₃ (Okuda et al., 1992).

1.4 Tools for predicting P450-mediated metabolism of xenobiotics

The level of expression of some metabolic enzymes including P450s in individuals is known to vary within a population. Such variations may be genetic or affected by environmental factors (Vesell, 1991). Consequently, there is considerable individuality in our capacity to activate and/or deactivate xenobiotics. An understanding of the role played by specific xenobiotic metabolizing enzymes in the *in vivo* biotransformation of compounds will therefore enable us to establish which of these proteins are important in specific pathways of drug metabolism and our susceptibility to environmental toxins and carcinogens.

In recent years, recombinant DNA technology has provided us with the tools to express and study individual xenobiotic metabolizing enzymes, via their cDNAs, in heterologous systems such as bacteria, yeast and mammalian cells. These systems provide several advantages :

1) Expression of a protein allows one to study its function (for example

catalytic activity) under conditions devoid of interference from activities of other enzymes. This is important for the discrimination of the activities of closely related proteins;

2) A good expression system is an invaluable tool for studying structure and function relationships through approaches such as site directed mutagenesis;

3) Expression systems provide a source of large quantities of protein for biophysical investigations. For example NMR and crystallization and X-ray diffraction;

4) In cases where a protein is expressed at very low levels in the tissue concerned, reasonable amounts can be obtained, through cDNA based expression, to carry out biochemical studies and

5) It is possible to study protein-protein interactions in a multicomponent enzyme system through simultaneous expression of individual components in a heterologous cell.

Cloning of genes and heterologous expression

To initiate any expression studies, the cDNA clone must be available. One approach to obtain a cDNA clone (and the one used in this thesis) is by reverse transcription of mRNA and amplification of the cDNA by PCR. This approach requires gene specific primers which can be designed to incorporate restriction endonuclease sites to aid cloning into the appropriate expression vector. The use of a suitable source of mRNA is important and it is essential to start from an appropriate organ and in the case of P450s, from an animal preferably treated with an appropriate inducer. In this way, the gene of interest is expressed or even overexpressed. However, the authenticity of the PCR product is not guaranteed and requires confirmation by DNA

sequencing.

The delivery of the recombinant expression vector to its host cell is usually achieved by conventional techniques. For eukaryotic cells, techniques used include transfection by the DEAE-dextran method (Zuber *et al.*, 1986), by calcium phosphate-coprecipitation (Doehmer and Oesch, 1991) or by retroviral infection (Battula, 1989). For *E.coli*, simple transformation procedures can be used (Hanahan, 1983). The cells containing a cDNA are usually selected by making use of bacterial genes preferably those encoding antibiotic resistance (Doehmer *et al.*, 1988). The selected cellular clones are then amplified in a culture to check for the presence of the cDNA by Southern blotting, for expression of the cDNA by northern blotting and for translation of the mRNA by western blotting. Finally, the enzymatic activity can be probed using P450 specific substrates, for example, coumarin in the case of CYP2A6. Once established, the system can be used to study P450 related metabolism problems in toxicology, pharmacology and immunology. Figure 1.5 shows a scheme for cloning specific DNA sequences and their subsequent expression in a heterologous cell.

1.4.2 Expression systems

No single optimal host cell system is available for heterologous expression of P450s. An expression system is dependent on the effectiveness of the expression vector and the usefulness of the host cell. Criteria for choice of the host cell for heterologous expression of a protein include, stable or transient expression, growth conditions, expression capacity, morphological appearance of the cell, chromosomal makeup, karyotype stability and if

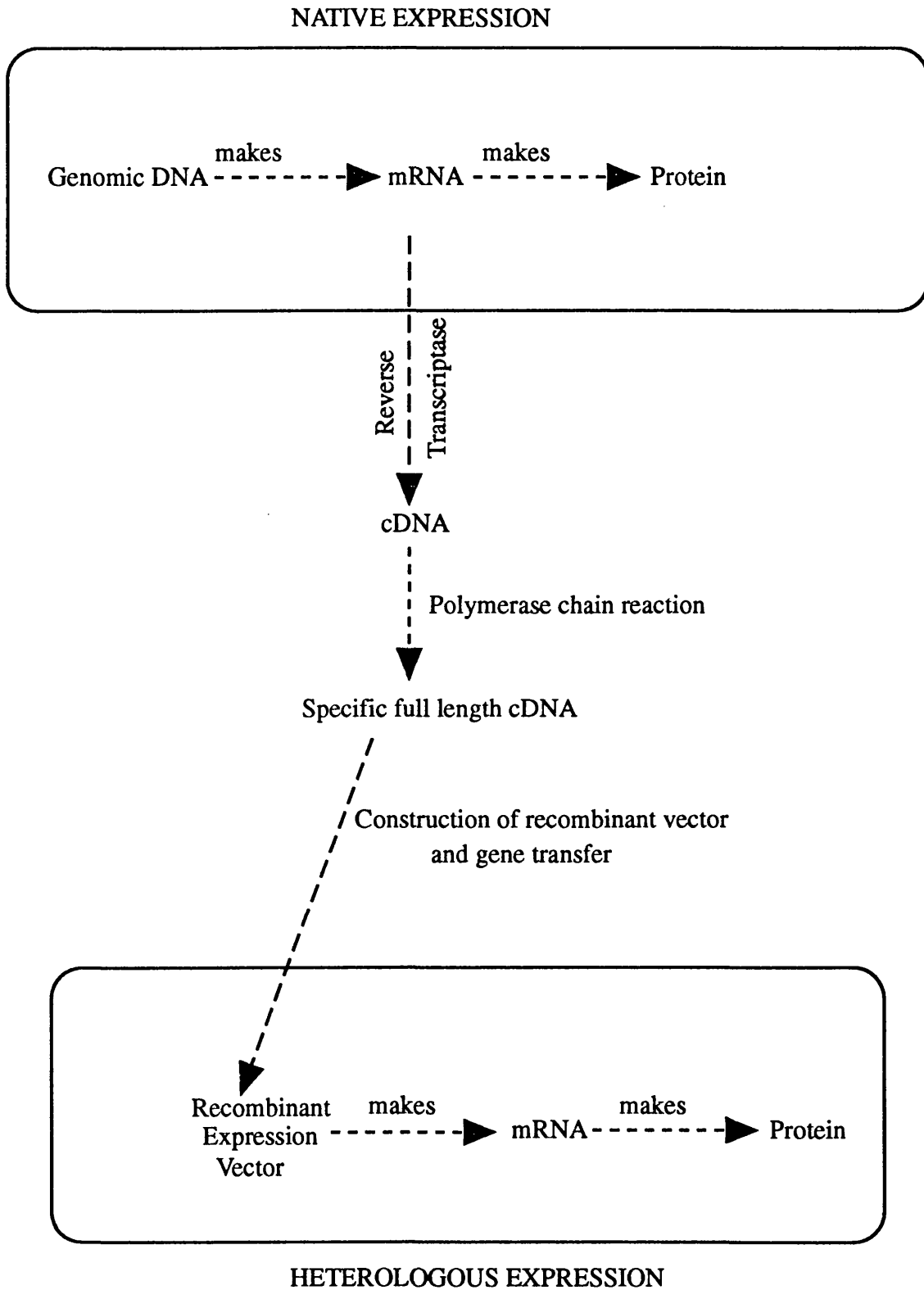


Figure 1.5 Cloning of specific DNA sequences and their subsequent expression in heterologous cell.

possible provision by the host cell to test for certain biological end points for example, genotoxicity or neoplastic transformation.

1.4.2.1 *E. coli*

E. coli is a suitable host for the production of large quantities of foreign P450s. This provides a good source of P450 for spectroscopic studies, for example for the production of reduced, CO complexed Soret bands at 450nm and spin-type absorption spectra, production of antibodies and attempts to crystallize proteins for X-ray structure analysis. Limitations for expression of eukaryotic proteins in *E. coli* include; different patterns of codon usage between eukaryotic and prokaryotic cells which can prevent expression of a desired protein even when placing a cDNA under the control of a very strong prokaryotic promoter. In this case, the cDNA sequence may be partially or wholly redesigned to optimize codon usage for *E. coli* (without necessarily altering the amino acid sequence of the final protein. Efficient expression of functionally active bovine P450 CYP17 in *E. coli* has been achieved by adjusting the P450 cDNA sequence to the codon demands in *E. coli* (Barnes *et al.*, 1991). Additionally, the exogenous addition of NADPH-cytochrome P450 reductase was not required to produce functional P450, CYP17. However, foreign proteins expressed in *E. coli* are prone to proteolysis and overexpression results in the formation of inclusion bodies for large proteins. This necessitates the optimization of expression conditions for each protein and use of protease inhibitors during the preparation of cellular fractions or use of protease deficient *E. coli* strains.

1.4.2.2 Yeast

Yeast is somewhere between *E. coli* and cultivated mammalian cells in terms of a host cell system for heterologous expression of P450s. Yeast has a fairly well known genetics, is easy to transform yielding stable transformants and yeast culture is cheap and can be adapted to large scale culture (Guengerich *et al.*, 1991 b). It is generally possible to measure P450 spectra directly in cells or microsomes prepared from whole cell preparations. When compared to bacteria, yeast cells have the advantage that they are eukaryotic with all the cellular structures present for example, nucleus, endoplasmic reticulum and plasma membrane comparable but not identical to that of mammalian cells. Generally there is less trouble with proteolysis and the formation of inclusion bodies. Yeast are also capable of some post translational modifications and of undergoing homologous recombination which may be used in the production of chimaeric mutants and screening them for activity. Moreover, yeast possesses its own NADPH-cytochrome P450 reductase which can interact with expressed P450s leading to functional monooxygenase complexes within the microsomal membranes. However, the levels of expression of a particular protein are not as high as can be potentially obtained in bacteria. Moreover, provision of haem may be limiting and endogenous P450s may complicate interpretation of spectra and catalytic data in some strains (Ching *et al.*, 1991).

Due to the ease of genetic manipulation, yeast cells have been the preferred expression system, in conjunction with site directed mutagenesis for structure/function analysis of sequences in the protein. In this way, various domains in the amino acid sequence of some P450s have been identified. For

example, the amino acids surrounding the haem binding cysteine (Shimizu *et al.*, 1988), or the amino acids in the N-terminal part of a P450 responsible for anchoring the P450 in the microsomal membrane (Yabusaki *et al.*, 1988) and the identification of the substrate binding domain of CYP1A1 (Sakaki *et al.*, 1987).

Several P450s of different species have been expressed in yeast cells for example, rat CYP1A2 (Shimizu *et al.*, 1986) and human CYP3A4 (Renaud *et al.*, 1990). The yield of P450 from heterologously expressed cDNA in yeast was found to be in the range of 0.2 to 0.5 mg/l of culture and all of the cDNA encoded P450s were found to be active towards the substrates tested.

1.4.2.3 Mammalian cells

Two basic mammalian systems are available for expression work, stable and transient. With stable cell expression systems, the known characteristics of a cell line can be used to advantage. Mutagenicity and toxicity (to the cells) can be investigated in a manner precluded with the use of prokaryotes or yeast by their dissimilarities to mammalian cells. Disadvantages include, the need for complex cell culture facilities and the relatively low levels of expression. Large scale work is tedious and expensive, furthermore, it is important to establish how stable the expression is in such long term cultures. Transient expression systems have the advantage that higher levels of expression can generally be achieved than in stable cell lines and there is a ready availability of vectors and host cells. Again cell culture facilities are required and cells must be transfected each time they are to be used and the level of expression must be checked each time. A variety of expression vectors and mammalian host cells have been applied for the

heterologous cDNA based expression of P450s from different species.

The following include the examples of mammalian expression systems used for expression of these proteins:

The Monkey Kidney COS cell transient expression vector system

The basis of the COS cell expression system is the Simian Virus 40 (SV40). Monkey kidney COS cells were developed that contain a portion of the SV40 genome encoding a T-antigen required for viral replication (Gluzman-Y, 1981). The cDNA is expressed under the control of a strong promoter on a plasmid containing the SV40 origin of replication. This allows the propagation of multiple DNA copies of the plasmid in T-antigen containing COS cells, resulting in the overproduction of the expressed protein from its cDNA. The first P450 cDNA expressed in this system was the bovine adrenal steroid 17α -hydroxylase (CYP17A1) (Zuber *et al.*, 1986). These studies established that CYP17A1 catalyses both, 17α -hydroxylase activity and $17,20$ lyase activity. Although expression of foreign cDNAs is readily achieved using the COS cell system, the level of enzyme production is in practice very low requiring highly sensitive assays for enzyme activity studies. Additionally, the plasmid used for expression is limited to the use in COS cells.

Vaccinia virus vector mediated expression

A Vaccinia virus based vector allows the expression of high amounts of enzymes in a wide variety of mammalian cells (Smith *et al.*, 1984). Twelve

different full length cDNAs encoding human P450s have been prepared and expressed in vaccinia virus (see for example, Aoyama *et al.*, 1990). These recombinant vaccinia vectors have been used to introduce into the human hepatoma-derived cell line HepG2 cDNAs encoding eleven human P450s including CYP2A6, CYP3A4, CYP2C8, and CYP2E1. This permitted comparative studies on steroid hormone hydroxylase specificities to be carried out (Waxman *et al.*, 1991). The HepG2 cell line is well suited to such P450 studies as it has good amounts of endogenous human NADPH-cytochrome P450 reductase.

The COS cells and vaccinia virus based expression systems transiently express P450 cDNAs since the vectors used are multicopy and lytic in nature. Several cell lines have been developed that stably express P450 cDNAs. Examples include, the V79 Chinese hamster cell line, Chinese hamster ovary (CHO) cell line, a mouse NIH3T3 cell line and the human B-lymphoblastoid cell line AHH1.

The V79 Chinese hamster cell line

Stable expression of P450s has been achieved in V79 Chinese hamster cells by using a vector that stably integrates into the chromosomal DNA of these cells (Doehmer and Oesch; 1991). The expression of the cDNA is under the control of the SV40 early promoter. However, this cell line has been repeatedly observed to be deficient in P450 mediated metabolic activation of xenobiotics and hence requires to be combined with a metabolically active component for example liver homogenate or primary liver cells. To date, no hamster P450 has been found to be expressed in this cell line. Hence the genetically modified V79 cell lines are defined for the P450

encoded by the transfected cDNA. This cell line, when transfected with sequences encoding CYP2B1, CYP1A1 and CYP1A2, has been successfully used for metabolic studies on steroids (Waxman *et al.*, 1989), diagnostic drugs for example caffeine (Fuhr *et al.*, 1992) and mutagenicity studies on polycyclic aromatic hydrocarbons (Dogra *et al.*, 1990).

The CHO cell line

CHO cell lines deficient or proficient for DNA repair have been genetically engineered for the expression of mouse CYP1A2 (Thompson *et al.*, 1991) and CYP1A1 (Trinidad *et al.*, 1991). Such engineered cell lines are useful in mammalian genotoxicity studies for the assessment of the molecular mechanisms involved in DNA damage and repair.

The mouse NIH3T3 cell line

Recombinant retroviral vectors have been developed for the stable expression of mouse CYP1A2 in mouse NIH3T3 cells (Battula, 1989). These cells are useful for studies on metabolically activated procarcinogens as their growth is density dependent (contact inhibition) and thus suitable for neoplastic transformation studies.

The Human B-lymphoblastoid cell line AHH1

The choice of the human AHH1 cell line is based on the assumption that these human cells, expressing human P450s, would be a better model for human susceptibility to the toxic and mutagenic effects of xenobiotics than are non-human cell systems. This system comprises expression vectors containing sequences from the origin of replication of the Epstein-Barr virus

which allow it to replicate autonomously in Epstein-Barr virus transformed lymphoblastoid cells (Sugden *et al.*, 1985). This cell line is suitable for mutagenicity studies (Crespi and Thilly 1984) and has also been genetically engineered for stable expression of several human P450s including CYP1A1, CYP1A2 and CYP2A2 (Davies *et al.*, 1989, Crespi *et al.*, 1990 a) and the AHH1 derived cell line MCL-5 expressing simultaneously five different P450s (Crespi *et al.*, 1991). All AHH1 derived cell lines have been validated in enzyme assays using various test substrates.

The baculovirus/insect cell expression system

The baculovirus system uses insect cells for transient expression. The system is not trivial to use but can produce extremely high amounts of expressed proteins, comparable to some of the better expression vectors in bacteria and yeast. This system has been used for the expression of P450s (Assefa *et al.*, 1989; Ohta *et al.*, 1991) and was used to express full length human *CYP2A6* cDNA in the present study and is fully described in chapters 2 and 3.

The aims of the work described in this thesis were to isolate, characterize, and express cDNAs encoding human and marmoset monkey P450s of the CYP2A subfamily.

Chapter 2

Materials and Methods

2.1 Bacterial growth media

2.1.1 Liquid media

Water : Autoclaved deionized water was used in all experiments, unless mentioned otherwise.

Bactotryptone, Bactoyeast extract and Bactoagar were from Difco.

LB Medium (Luria-Bertani Medium): Bactotryptone (10 g), Bactoyeast extract (5 g), and NaCl (5 g) were dissolved in water, the pH adjusted to 7.5, the volume made up to 1 litre and autoclaved for 20 minutes at 15 lb/sq.in. on liquid cycle.

SOB Medium: Bactotryptone (20 g), Bactoyeast extract (5 g), NaCl (0.5 g) and KCl (0.19 g) were dissolved in water, the pH adjusted to 7.0 with 5N NaOH and the volume was made up to 1 litre. This was autoclaved for 20 minutes at 15 lb/sq.in. on liquid cycle.

SOB/Mg²⁺ : A 2 M stock of Mg²⁺ (1 M MgCl₂, 1 M MgSO₄) was made in deionized water and sterilized by filtration through a 0.45 µm filter (Millipore). This was added to autoclaved SOB medium just before use, to a final concentration of 20 mM Mg²⁺.

SOC Medium: A 2 M stock of glucose was made in deionized water and sterilized by filtration through 0.45 µm filter (Millipore). This was added to autoclaved SOB medium just before use, to a final concentration of 20 mM.

M9 Minimal medium: Na₂HPO₄ (6 g), KH₂PO₄ (3 g), NaCl (0.5 g), and NH₄Cl (1 g) were dissolved in water and the volume made up to 1 litre. After autoclaving for 15 minutes at 15 lb/sq.in. on liquid cycle, the following components (each sterilized separately) were added: 1 M MgSO₄ (2 ml), 20% glucose (10 ml) and 1 M CaCl₂ (0.1 ml).

2.1.2 Media containing agar

Liquid media were prepared according to the recipes given above and just before autoclaving the appropriate amount of bacto-agar was added: for plates, 15 g/l and for top agar, 7 g/l.

2.1.3 Antibiotics

Before addition of antibiotic media were cooled to 50°C.

Ampicillin: a stock solution of the sodium salt of ampicillin (Beecham) was made up to 50 mg/ml in distilled water, sterilized by filtration through a 0.45 µm nitrocellulose filter (Millipore) and stored in aliquots at -20°C. Ampicillin was added to appropriate sterile media at a concentration of 100 µg/ml. Plates containing ampicillin were stored at 4°C and used within 1-2 weeks. Liquid media containing ampicillin were used immediately.

2.2 Bacterial strains

Strains of *Escherichia coli* (*E. coli*) used in these studies were:

JM109: recA1, endA1, gyrA96, thi, hsd1r17, supE44, rel1A1, Δ(lac-proAB), {F', traD36, proAB, lacI^qZM15}.

GM2163: F⁻, ara⁻14, leuB6, tonA31, lacY1, tsx⁻78, supE44, galK2, galT22, hisG4, rpsL136, xyl⁻5, mtl⁻1, thi⁻1, dam⁻13, Tn9, (Cr^r)^{*}, dcm⁻6, hsdR2, mcrA⁻, mcrB⁻.

The bacteria were streaked on an M9 agar plate and incubated at 37°C for 1-2 days. 5 ml of LB medium was inoculated with a single bacterial colony and incubated with shaking at 37°C overnight. 0.5 ml aliquots from the culture were diluted 1:1 with 30% glycerol/LB medium, snap-frozen in liquid nitrogen and stored at -70°C to form a stock. The plates were sealed and stored at 4°C

for up to 4 weeks.

2.3 Isolation of plasmid DNA

2.3.1 Growth and harvesting of bacteria on a large scale

5 ml of LB medium containing the appropriate antibiotic was inoculated with 10 μ l of a bacterial glycerol stock and incubated overnight in a shaker-incubator. 1 ml of this starter culture was used to inoculate 100 ml of LB medium containing the appropriate antibiotic. The culture was incubated overnight at 37°C in a 250 ml flask with shaking. The bacterial cells were harvested by centrifugation at 4000 g for 10 minutes at 4°C. The pellet was washed on ice by resuspending it in 15 ml of ice-cold STE [10 mM Tris-HCl (pH 8.0)/100 mM NaCl/1 mM EDTA]. The suspension was then centrifuged at 4000 g for 10 minutes at 4°C

2.3.2 Isolation of plasmid DNA on a large scale

Plasmid DNA was isolated as described by Birnboim and Doly (1979) as modified by Ish-Horowicz and Burke (1981)

Solutions

Solution I: 50 mM glucose/25 mM Tris-HCl(pH8.0)/10 mM EDTA. This solution was autoclaved for 20 minutes at 10 lb/sq. in. and stored at 4°C.

Solution II: 0.2 N NaOH/1% SDS. This was made using stock solutions (10 N NaOH and 10% (w/v) SDS) diluted in water just before use.

Solution III: Potassium acetate(pH 4.8) was made by mixing 60 ml 5 M potassium acetate, 11.5 ml glacial acetic acid and 28.5 ml of water. This solution was autoclaved for 20 minutes at 10 lb/sq. in. and stored at 4°C.

The washed bacterial pellet was resuspended in 4 ml of cold solution I, left at room temperature for 5 minutes and then transferred to a 50 ml capped polypropylene centrifuge tube. 8 ml of freshly prepared solution II was added, the tube capped and the contents mixed by inverting the tube several times. The tube was left on ice for 10 minutes and then 4 ml of solution III was added and the contents were mixed by inverting the tube several times. The tube was incubated on ice for 10 minutes and then centrifuged at 10000 g for 10 minutes at 4°C. The supernatant was transferred to a 30 ml corex tube and 0.6 volumes of isopropanol was added. The contents of the tube were mixed and incubated at room temperature for 15 minutes. The tube was then centrifuged at 9000 g for 30 minutes at room temperature. The pellet was washed in 70% (v/v) ethanol, dried and dissolved in 1.25 ml of TE [10 mM Tris-HCl (pH8.0)/1 mM EDTA]. This solution was transferred to a sterile microfuge tube and 39 µl of 5 M NaCl and 25 µl of 10 mg/ml RNase A was added. This mixture was incubated at 37°C for 90 minutes. The sample was first extracted with an equal volume of buffered phenol (phenol was buffered once with 1 M Tris-HCl(pH 8.0)/1 mM EDTA and twice with 100 mM Tris-HCl (pH 8.0)/1 mM EDTA), then with an equal volume of buffered phenol/chloroform/isoamyl alcohol (25:24:1) and finally with an equal volume of chloroform/isoamyl alcohol (24:1). Thorough vortexing was carried out (1 minute) at each stage of extraction. The phases were separated by centrifugation in a microfuge for 2 minutes. Two volumes of absolute ethanol were added to the final aqueous phase and mixed by vortexing. The tubes were stored on dry ice for 20 minutes and the plasmid DNA was pelleted by centrifugation in a microfuge for 15 minutes at room temperature. The pellet was washed in 70% ethanol, dried and resuspended in 100 µl of TE. The DNA solution was heated at 65°C for 5

minutes to destroy endogenous nucleases and stored at -20°C.

2.3.3 Small scale plasmid preparation

5 ml of LB medium containing the appropriate antibiotic was inoculated with 5 µl of bacterial glycerol stock and incubated with shaking at 37°C overnight. Stock solutions were as described in section 2.3.2. 1.5 ml of the overnight culture was transferred to a sterile microfuge tube and centrifuged for 2 minutes at room temperature in a microfuge. The supernatant was removed and the dry pellet was resuspended in 100 µl of ice-cold solution I. The tube was stored at room temperature for 5 minutes, then 200 µl of freshly prepared solution II was added. The tube contents were mixed by inverting the tube several times and the sample was placed on ice for 5 minutes. 150 µl of ice-cold solution III was then added, the tube vortexed in an inverted position for 10 seconds and placed on ice for 5 minutes. The tube was then centrifuged for 5 minutes in a microfuge. The supernatant was extracted with an equal volume of buffered phenol/chloroform/isoamyl alcohol (25:24:1) with thorough vortexing. The tube was centrifuged for 5 minutes in a microfuge at room temperature. The supernatant was transferred to a new microfuge tube and mixed with two volumes of ethanol. The tube was then centrifuged for 5 minutes in a microfuge and the pellet was washed with 70% ethanol and vacuum dried. The pellet was finally dissolved in 20 µl of TE and heated at 65°C to destroy endogenous nucleases. The DNA was stored at -20°C.

2.4 Restriction endonuclease digestion

Enzymes were obtained from Pharmacia. The reaction conditions were as described by the manufacturer. Digests were carried out using the buffer

supplied with the enzymes. The volume of digest varied according to the amount of DNA and the concentration of the restriction endonuclease. In general, digestion contained final concentrations of 10-100 µg/ml DNA, 1 mM dithiothreitol/10 µg/ml RNase A/1x buffer (see above) and 2-10 units of restriction endonuclease per µg of DNA. Samples were usually incubated in a water bath at 37°C for 2-16 hours unless otherwise indicated for a particular restriction enzyme.

2.5 Electrophoresis of DNA

2.5.1 Stock solutions

10 x TBE: 108 g Tris base, 55 g Boric acid, and 9.3 g EDTA made up to 1 litre with distilled water and autoclaved for 20 minutes.

Ethidium Bromide: Made up as 10 mg/ml stock solution and stored at 4°C

10 x Loading buffer: 0.025% bromophenol blue and 20% glycerol.

2.5.2 Agarose gels

Horizontal agarose gels containing ethidium bromide were used for electrophoresis of DNA. The percentage of agarose used depended on the size of DNA to be analysed and the resolution required. Agarose was dissolved by heating in 1 x TBE, cooled to approximately 50°C and made up to a final concentration of 0.5 µg/ml with respect to ethidium bromide. The solution was poured into a template containing a comb to form wells. The gel was allowed to set for 45-60 minutes at room temperature or at 4°C if the low melting point agarose was used. One-tenth volume of 10 x loading buffer was added to DNA samples before loading onto gels. 1 Kb ladder (GIBCO-BRL) resuspended in 1 x loading buffer was used as a DNA molecular weight

marker. Electrophoresis was carried out in 1 x TBE at 10 V/cm for approximately 1.5 hours. DNA was visualised by photography under ultra-violet (UV) light using Polaroid 55 film in a Polaroid land camera (f 4.5 for 50 seconds) fitted with a red filter. The sizes of DNA fragments were determined by using a standard curve plotted from the DNA molecular weight markers.

2.6 Amplification of CYP2A sequences

Total RNA isolated from human and marmoset liver samples was first reverse transcribed and then amplified by the Polymerase Chain Reaction (PCR) using oligoprimers based on sequences located at the ends of the coding region of human CYP2A6 (Yamano et al, 1990).

Primer sequences are presented in the 5' to 3' direction

Forward primer: GCT CAG TCT AGA ACC ATG GTG GCC TCA GGG ATG.

Reverse primer: CGA GTC TCT AGA TCA GCG GGG CAG GAA GCT CA

2.6.1 Reverse transcription

Total RNA (15 µg) was incubated in a volume of 17 µl at 65°C for 10 minutes with 50 ng of reverse oligonucleotide primer at 65°C for 10 minutes. The sample was overlaid with 50 µl of light paraffin oil. After incubation the mixture was snap-chilled on ice and made up to 50 µl with a pre-mix that contained 10 µl of 5 x Moloney Murine Leukemia Virus Reverse Transcriptase(M-MLVRT) buffer (final concentration of 50 mM Tris-HCl(pH 8.3), 75 mM KCl, 3 mM MgCl₂, and 10 mM DTT), 100 µg/ml nuclease free BSA, 500 µM each of dCTP, dGTP, dTTP, and dATP, 50 units of RNase inhibitor (Pharmacia), and 200 units of M-MLVRT (GIBCO-BRL). This mixture was incubated at 37°C for 90 minutes, followed by incubation at 65°C for 10 minutes.

To the Reverse Transcription product, two volumes of ethanol were added and the contents of the tube mixed. The tube was left on dry-ice for 30 minutes and then centrifuged in a microfuge for 10 minutes at room temperature. The supernatant was removed and the pellet was washed with 70% ethanol, vacuum dried and dissolved in 50 µl of sterile water.

2.6.2 Polymerase chain reaction (PCR)

The PCR was performed in 100 µl of a mixture containing 200 µM each of dATP, dGTP, dCTP and dTTP, 10 µl of 10X reaction buffer (200 mM Tris-HCl(pH 8.2), 100 mM KCl, 60 mM (NH₄)₂SO₄, 20 mM MgCl₂, 1% (v/v) Triton X-100 and 100 ng/µl nuclease free BSA), 10 µl of reverse transcription product, 500 ng each of reverse and forward oligonucleotide primers, 10% (v/v) DMSO, 2% (v/v) glycerol and 4 units of Pfu (*Pyrococcus furiosus*) DNA polymerase (Stratagene). The reaction mix was overlaid with 100 µl of light paraffin oil. PCR was performed in a programmable thermal cycler (Hybaid). Reaction conditions consisted of initial denaturation at 95°C for 5 minutes, then 5 cycles of 90°C for 1 minute, 25°C for 5 minutes, and 75°C for 7 minutes, then 30 cycles of 90°C for 1 minute, 38°C for 3 minutes and 75°C for 7 minutes, with a final polymerisation step of 75°C for 15 minutes. 10 µl of the PCR product was electrophoresed on a 0.8% agarose gel containing 0.5 µg/ml ethidium bromide. The DNA was visualised as in section 2.4.2.

2.7 Preparation of full-length cDNA Insert for subcloning into plasmid vector pUC19

2.7.1 Restriction endonuclease digest and gel electrophoresis

To the PCR product, two volumes of ethanol were added and the contents of the tube mixed. The tube was left on dry-ice for 30 minutes and then centrifuged in a microfuge for 10 minutes at room temperature. The supernatant was removed and the pellet was washed with 70% ethanol, vacuum dried and dissolved in sterile water. The DNA was then digested with Xba I as described in section 2.4. The digest was then electrophoresed on a horizontal agarose gel containing ethidium bromide (section 2.5.2). The gel was visualised under UV light for location and excision of the required band.

2.7.2 Isolation of DNA fragments from agarose gels

2.7.2.1 Elution of DNA from DEAE-cellulose membranes

(Schleicher and Schuell NA-45) was prepared by first soaking in 10 mM EDTA (pH 8.0) for 5 minutes and then in 0.5 N NaOH for a further 5 minutes and finally washed six times in sterile water. Using a scalpel blade, an incision was made in the gel directly in front of the leading edge of the DNA band of interest. DEAE-cellulose membrane was cut to a size slightly deeper (1 mm) than the gel and the same width as the incision. The membrane was inserted into the slit in the gel and the gel electrophoresed at 200 V to run the DNA fragment on to the membrane. The progress of electrophoresis was followed with a hand held UV lamp. The piece of membrane bound with the DNA fragment of interest was rinsed in 50 mM Tris-HCl (pH 8.0)/150 mM NaCl/10 mM EDTA (pH 8.0), then placed in a microfuge tube containing 500 μ l of 50 mM Tris-HCl (pH 8.0)/150 mM NaCl/10 mM EDTA (pH 8.0).

The tube was incubated at 65°C for 1-2 hours to elute the DNA from the membrane. The liquid was then placed in a fresh tube and extracted with an equal volume of buffered phenol/chloroform/isoamyl alcohol (25:24:1) and the organic phase was back extracted with 10 µl of TE. The aqueous layers were combined and mixed with one volume of 3 M sodium acetate and four volumes of absolute ethanol. The contents of the tube were placed on dry-ice for 20 minutes and then centrifuged for 15 minutes at room temperature in a microfuge. The pellet was washed with 70% ethanol, dried briefly and resuspended in 20 µl of TE.

2.7.2.2 Recovery of DNA from agarose gels using liquid nitrogen

DNA fragments were separated by electrophoresis through low-melting temperature agarose gels containing 0.5 µg/ml ethidium bromide. The DNA band of interest was cut out of the gel as small as possible using a scalpel blade. The gel piece was placed in a 0.5 ml eppendorf tube previously punctured centrally at the bottom and plugged with siliconized glass wool. The capped tube was then incubated in liquid nitrogen for 5 minutes and placed in a 1.5 ml Eppendorf tube. The tube was then centrifuged for 5 minutes in a microfuge to collect the aqueous solution containing the DNA in a second tube. DNA was extracted with an equal volume of buffered phenol/chloroform/isoamyl alcohol (25:24:1) and the organic phase was back extracted with 10 µl of TE. The aqueous layers were combined and mixed with one volume of 3 M sodium acetate and four volumes of absolute ethanol. The contents of the tube were placed on dry-ice for 20 minutes and then centrifuged for 15 minutes at room temperature in a microfuge. The pellet was washed with 70% ethanol, dried briefly and resuspended in 20 µl of TE.

2.7.3 Quantitation of DNA fragment isolated

A sample of DNA fragment isolated by one of the methods described above was loaded onto an agarose gel with a known amount of DNA molecular weight markers in an adjacent well. Comparison of the intensity of the bands under UV light gave a good indication of the amount of DNA in the sample isolated from the gel. Concentration of DNA was also measured spectrophotometrically by measuring the A_{260} of the sample.

2.8 Subcloning of cDNAs into the plasmid vector pUC19

2.8.1 Ligation of cDNAs into the plasmid vector pUC19

Plasmid DNA was digested with Xba I restriction endonuclease as described in section 2.4. A Sample of the digest was run on an agarose gel (section 2.5.2) to confirm that the sample had digested to completion. The rest of the digest was made up to a volume of 50 μ l and then extracted once with an equal volume of buffered phenol/chloroform/isoamyl alcohol (25:24:1) and the DNA precipitated with 2 volumes of absolute ethanol on dry-ice for 20 minutes. The DNA was pelleted by centrifugation for 15 minutes in a microfuge at room temperature. The pellet was washed in 70% ethanol, vacuum dried and resuspended in sterile water.

200 ng of cDNA fragment isolated as described in section 2.7.2 was mixed with 20 ng of digested plasmid vector in a volume of 20 μ l containing 66 mM Tris-HCl (pH 7.5)/ 6 mM MgCl/ 1 mM ATP and 10 units of T4 DNA ligase.

The mixture was incubated at room temperature for 3-16 hours.

2.9 Standard transformation of *E. coli* JM109.

2.9.1 Solutions

STB (Standard Transformation Buffer): KCl (7.46 g), MnCl₂·4H₂O (8.91 g), CaCl₂·2H₂O (1.47 g) and 100 ml glycerol were dissolved in distilled water. 10 ml of a 1 M (MES) (2-[N-morpholino]ethanesulfonic acid), pH 6.3 stock was added and the volume was made up to 1 litre. The final pH was 6.1. The solution was filtered through a 0.45 µm filter and stored at 4°C.

5-Bromo-4-Chloro-3-Indolyl-β-D-Galactoside (X-gal) from Sigma: was made up as a 20 mg/ml stock solution in dimethylformamide and stored in aliquots at -20°C.

isopropyl-1-thio-β-D-galactoside (IPTG) from Sigma: was made up as a 0.5 M solution in water, filtered through 0.45 µm filter (Millipore) and stored in aliquots at -20°C.

DnD solution: 1.53 g dithiothreitol, 9 ml DMSO, 100 µl 1 M potassium acetate (pH 7.5) were dissolved in water and the volume made up to 10 ml. The solution was filtered through a 0.45 µm filter and stored in aliquots at -20°C.

Competent cells were prepared by the method of Hanahan (1983).

A single JM109 colony from an M9 plate was inoculated into 5 ml of SOB medium and incubated overnight with shaking at 37°C. 1 ml of this starter culture was used to inoculate 100 ml of SOB medium in a 1 litre flask containing MgSO₄ at a final concentration of 20 mM. Cells were grown at 37°C with gentle shaking until an optical density of 0.45-0.55 OD units/ ml at a wavelength of 550 nm was reached (2-3 hours). The culture was transferred to sterile, disposable 50 ml polypropylene Falcon tubes and chilled on ice for 10

minutes. Cells were recovered by centrifugation at 900 g for 15 minutes at 4°C. The pellet was then resuspended by gentle pipetting in a volume of STB that was 1/3rd of the original culture volume. The cells were centrifuged at 900 g for 15 minutes at 4°C and the pellet was resuspended in a volume of STB that was 1/12.5th of the original culture volume. At this stage the cells were prepared for either immediate use (i) or for storage as a frozen stock of competent cells to be used at later date (ii).

(i) For preparation of fresh competent cells, 140 µl of DnD solution was added to the suspension, mixed by swirling gently and stored on ice for 15 minutes. This step was repeated once and 200 µl aliquots of the suspension was added to chilled polypropylene tubes (Falcon, 17 mmX100 mm) and stored on ice. The cells are now ready for addition of DNA.

(ii) For preparation of a frozen stock of competent cells, 140 µl of DMSO was added to the cells suspended in STB, mixed by swirling gently and stored on ice for 15 minutes. This step was repeated once more and 200 µl aliquots of suspended cells were transferred to chilled microfuge tubes. The tightly closed tubes were then snap-frozen by immersing in liquid nitrogen and stored at -70°C. When needed, competent cells from -70°C were thawed and stored on ice for 10 minutes.

Cells were then transferred to chilled polypropylene tubes (Falcon) and stored on ice. The cells are now ready for addition of DNA.

Ligation mixture (to the maximum of 5% total volume of cells) was then added to the competent cells. As a control, 1 ng (10 µl) of uncut plasmid vector was added to an aliquot of competent cells. The contents of each tube were mixed by swirling gently and stored on ice for 30 minutes. Following this, the cells were heat shocked by incubation in a 42°C water bath for exactly 90 seconds

and immediately chilled on ice for 1-2 minutes. To each tube, 800 μ l of SOC medium were added and the tubes were incubated at 37°C for 60 minutes with gentle shaking.

2.9.2 Plating out of pUC19 transformants

40 μ l each of 20 mg/ml X-gal and 0.5 M IPTG were spread onto LB-agar plates containing 100 μ g/ml ampicillin using a sterile glass spreader. The plates were incubated at 37°C until all the fluid had disappeared. 200 μ l of ligation mix/ competent cells was then spread onto the plates using a sterile glass spreader. For the uncut vector, 10 μ l and 100 μ l aliquots of ligation mix/competent cells diluted in SOB medium were taken and spread on LB-agar plates containing ampicillin, X-gal and IPTG as above. The plates were inverted and incubated overnight at 37°C.

2.9.3 Preparation of recombinant plasmid DNA

Single transformed colonies were picked using sterile toothpicks into 5 ml SOB medium containing 100 μ g/ml ampicillin and incubated at 37°C overnight with shaking. 1.5 ml of this culture was transferred to a microfuge tube and centrifuged for 2 minutes at room temperature in a microfuge. A small scale plasmid preparation was carried out on the pellet (section 2.3.3).

This plasmid DNA was digested with the appropriate restriction endonucleases and electrophoresed on an agarose gel for analysis of the insert contained within a plasmid vector. 0.5 ml aliquots from the remaining culture were diluted 1:1 with 30% (v/v) glycerol/LB, snap-frozen and stored at -70°C to form a stock.

2.10 DNA sequence determination

The sequence of cloned DNA fragments was determined by the dideoxynucleotide chain termination method (Sanger et al; 1975) using double stranded recombinant pUC19 plasmid DNA and a T7 sequencing kit from Pharmacia.

2.10.1 Isolation of plasmid DNA (Mierendorf and Pfeffer, 1987)

Small scale plasmid preparation was carried out as described in section 2.3.3 up to the addition of solution III and removal of precipitated cell debris and chromosomal DNA. To the supernatant, RNaseA was added to the final concentration of 20 µg/ml and the tube was incubated at 37°C for 30 minutes. The mixture was then extracted with an equal volume of buffered phenol/chloroform/isoamyl alcohol (25:24:1) with thorough vortexing. To the final aqueous phase, 2.5 volumes of ethanol were added and mixed by vortexing. The plasmid DNA was pelleted by centrifugation in a microfuge for 15 minutes at room temperature. The supernatant was removed and the pellet was washed with 70% ethanol, vacuum dried and resuspended in a mixture containing 16 µl of water, 4 µl 4 M NaCl and 20 µl of 13% (w/v) polyethyleneglycol (PEG 8000). The tube was incubated on ice for 20 minutes and then centrifuged in a microfuge for 10 minutes at room temperature. The resulting pellet was washed with 70% ethanol, vacuum dried and resuspended in 20 µl of sterile water.

2.10.2 Denaturation of the template DNA

To a sterile 1.5 ml microfuge tube, 10 μ l (2 μ g) of plasmid DNA prepared as above was mixed with 2 μ l of 2 N NaOH and the tube was incubated at room temperature for 5 minutes. Following this 110 μ l of ice cold absolute ethanol and 10 μ l of ammonium acetate (pH 7.5) was added and the tube was incubated in dry ice for 20 minutes. The tube was then centrifuged for 10 minutes at room temperature in a microfuge, and the pellet was washed with 70% ethanol, vacuum dried and resuspended in 10 μ l of sterile water.

2.10.3 Annealing of primer to template DNA

10 μ l (1.5-2 μ g) of denatured recombinant pUC19 plasmid DNA (section 2.10.2) was mixed with 2 μ l of annealing buffer (1 M Tris-HCl (pH 7.6), 100 mM MgCl₂ and 100 mM DTT), 2 μ l (10 pmol) of appropriate primer in 1.5 ml sterile microfuge tube and the mixture was incubated at 37°C for 20 minutes. The tube was left at room temperature for at least 10 minutes and then briefly centrifuged in a microfuge. If not used immediately, the annealed primer/template DNA was stored at -20°C.

2.10.4 Sequencing reactions

Stock solutions

Deaza Labelling mix-dATP: 1.37 μ M each dCTP, c7dGTP, and dTTP, 333.3 mM NaCl.

ddA Termination mix ('A' mix): 95 μ M each c7dATP and c7dGTP, 840 μ M dCTP and dTTP, 2 μ M ddATP, 40 mM Tris-HCl(pH 7.6) and 50 mM NaCl.

ddC Termination mix ('C' mix): 95 μ M each c7dATP, c7dGTP and dCTP, 840 μ M dTTP, 10 μ M ddCTP, 40 mM Tris-HCl(pH 7.6) and 50 mM NaCl.

ddG Termination mix ('G' mix): 95 μM each c7dATP and c7dGTP, 840 μM each dCTP and dTTP, 4 μM ddGTP, 40 mM Tris-HCl(pH 7.6) and 50 mM NaCl.

ddT Termination mix ('T' mix): 95 μM each c7dATP, c7dGTP and dTTP, 840 μM dCTP, 6 μM ddTTP, 40 mM Tris-HCl (pH 7.6) and 50 mM NaCl.

T7 DNA polymerase: Diluted to 1.5 units/ μl in 20 mM Tris-HCl (pH 7.5)/ 5% glycerol/0.1 mg/ml BSA/ 5 mM dithiothreitol just before use.

Stop solution: 0.3% each Bromophenol Blue and Xylene Cyanol FF, 97.5% deionised formamide and 10 mM EDTA (pH 7.5).

2.10.5 Labelling reaction

To the tube containing annealed template and primer, 3 μl of deaza labelling mix-dATP, 10 μCi [α - ^{35}S]-dATP (600Ci/mmol) and 2 μl of diluted T7 DNA polymerase was added. The components were mixed by gentle pipetting and incubated at room temperature for 5 minutes. Meanwhile to the four microfuge tubes labelled T, C, G, A, 2.5 μl of the 'T' mix, 'C' mix, 'G' mix and 'A' mix were added to the corresponding tube and pre-warmed at 37°C for 1-5 minutes.

2.10.6 Termination reactions

When the labelling reaction was complete, a 4.5 μl aliquot of the reaction was transferred to each of the pre-warmed sequencing mixes. The tubes were incubated at 37°C for 5 minutes. After the incubation, 5 μl of stop solution was added to each tube and the tubes were centrifuged briefly to collect the contents at the bottom of the tube. A 3 μl aliquot of each stopped reaction was transferred to a separate microfuge tube and boiled for 3 minutes and immediately loaded into the appropriate wells of the sequencing gel. The

remaining material was stored at -20°C for up to one week.

2.10.7 Gel electrophoresis of sequencing reactions

Sequencing gels were prepared using stock solutions obtained from National Diagnostics.

Stock Solutions

Sequagel Concentrate: Contained the equivalent of 237.5 g of acrylamide, 12.5 g of methylene bisacrylamide and 500 g of urea(8.5 M) per litre of solution.

Sequagel Diluent: Contained 500 g of urea(0.5 M) per litre of solution.

Sequagel Buffer: Contained 50% urea (8.5 M) in 1.0 M Tris-Borate/20 mM EDTA buffer (pH 8.3).

8% polyacrylamide/8.3 M urea gels (100 ml) were made up by mixing 32 ml of Sequagel Concentrate, 58 ml of Sequagel Diluent, and 10 ml of Sequagel buffer. Freshly made ammonium persulphate (0.8 ml of a 10% (w/v) solution) and 40 µl of TEMED were added; the solution swirled to mix and the gel poured immediately into a sequencing cassette that lay at an angle of 45°. Sample application combs were clamped into position with the teeth outermost to form the top of the gel. The gel was allowed to polymerise for at least 1 hour and was used within 24 hours. The combs were removed and re-inserted so that the teeth just touched the surface of the gel to form wells. The gel was placed into the electrophoresis apparatus and pre-run at 1200-1500 V for 30-60 minutes. The electrophoresis buffer was 1 x TBE. Following this, the power supply was switched off and the wells were washed with electrophoresis buffer. Aliquots of denatured sequencing reactions (section

2.10.6) were loaded into adjacent wells and electrophoresed at 30 mA (approximately 1500 V) for times between 1.5 to 12 hours. Gels were transferred to Whatman 3 MM paper, covered with Saran wrap and dried at 80°C for 40 minutes under vacuum. The Saran wrap was removed and the gel was exposed to Fuji RX X-ray film overnight at room temperature.

2.11 [α -³²P] Radioactive labelling of DNA

2.11.1 Random primer labelling

Recombinant pUC19 plasmid DNA with the insert of interest was prepared as described in section 2.10.1. Prior to labelling, the DNA sample was boiled for 3 minutes to denature the DNA and snap-chilled on ice. The random primer labelling was carried out as described by Feinberg and Vogelstein (1984).

Solutions

Solution O: 1.25 M Tris-HCl (pH 8.0)/ 0.125 M MgCl₂.

Solution A: 1 ml solution O/18 μ l 2-mercaptoethanol/ 5 μ l dATP/ 5 μ l dTTP/ 5 μ l dGTP (each deoxynucleoside triphosphate previously dissolved in 3 mM Tris-HCl/0.2 mM EDTA pH 7.0 at a concentration of 0.1 M).

Solution B: 2 M HEPES (pH 6.6 with 4 M NaOH).

Solution C: Hexadeoxyribonucleotides evenly suspended at 90 O.D units/ml in 3 mM Tris-HCl/0.2 mM EDTA pH 7.0.

Oligo Labelling Buffer (OLB): Solutions A:B:C were mixed in a ratio of 100:250:150. OLB was stored at -20°C.

The labelling reaction was carried out at room temperature by the addition of the following reagents in the order: (i) water (to a final volume of 50 μ l), (ii) 10

μl OLB, (iii) 2 μl (10mg/ml) BSA, (iv) DNA (up to 32.5 μl), (v) 5 μl of [α - ^{32}P]dCTP (3000 Ci/mmol) and (vi) 5 units of Klenow fragment of Escherichia Coli DNA polymerase I. The labelling reaction was left at room temperature for 3-16 hours. The efficiency of incorporation of radioactivity was determined using Whatman DE81 paper.

1 μl of the reaction mixture was spotted in duplicate onto 1 cm square pieces of Whatman DE81 paper. One piece was used to determine total counts and the second piece was used to determine incorporated counts. For assessing incorporated counts, the filter paper was washed 6 times for 5 minutes each in 0.5 M disodium hydrogen orthophosphate, twice for 1 minute each in distilled water, and once for 1 minute in ethanol. Both filters were dried under a heat lamp and radioactivity on the filters was determined by liquid scintillation spectrophotometry (Phillips model 4700 scintillation counter) in 4 ml aquasol (New England Nuclear). Incorporation of radioactivity was generally in the range of 50%-80%. DNA samples were labelled to a specific activity of 2×10^7 to 1×10^8 cpm/ μg .

2.12 Northern blotting

Northern blotting was carried out as described by Fournay *et al.* (1988)

2.12.1 Solutions

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

Electrophoresis sample buffer: 0.75 ml deionised formamide, 0.15 ml 10 x MOPS/EDTA buffer, 0.24 ml formaldehyde, 0.1 ml RNase-free water, 0.1 ml

glycerol, 0.08 ml 10% (w/v) bromophenol blue. This buffer was stored at -20°C in small aliquots.

Electrophoresis buffer: 1 x MOPS/EDTA buffer

Other solutions required: 37% formaldehyde, 20 x SSC [3 M NaCl/0.3 M sodium citrate (pH 7.0)], 1 mg/ml ethidium bromide in deionized RNase-free water.

2.12.2 Gel preparation

1.0-1.5 g of agarose was dissolved by melting in 10 ml of 10 x MOPS/EDTA and 87 ml of RNase-free water in an RNase free flask. In a fume hood, the agarose solution was allowed to cool to 50°C and 5.1 ml of 37% formaldehyde was then added and the solution mixed gently. The gel was poured into an RNase-free gel tray and allowed to set for at least 1 hour before use. The gel was placed in an electrophoresis chamber and sample wells were flushed by pipetting electrophoresis buffer in and out prior to loading the samples.

2.12.3 Sample preparation and gel electrophoresis

25 µl of electrophoresis sample buffer was added to 30 µg of total RNA in an RNase free microfuge tube and incubated at 65°C for 15 minutes and chilled on ice. Following this, 1 µl of ethidium bromide solution (1 mg/ml) was added to the samples and mixed thoroughly. Samples were then loaded on the gel and electrophoresed at 50-60 V for 3-4 hours. RNA was visualised by photography under UV light using Polaroid 55 film in a Polaroid land camera (f 4.5 for 5-10 seconds) fitted with a yellow filter.

2.12.4 RNA transfer preparation

RNA was transferred to a nylon membrane (Hybond-N, Amersham International) by capillary action. A glass plate forming a platform was covered with 2 Whatman 3MM paper 'wicks' and placed so that the 'wicks' were in a reservoir of 20 x SSC. The gel was placed face downwards on top of the saturated 'wick'. A piece of membrane cut to the same size as the gel was soaked in 20 x SSC and placed on top of the gel. Two sheets of Whatman 3MM paper cut to the same size as the gel were soaked in 20 x SSC and placed on top of the membrane. 10 sheets of Quick draw blotting paper (Sigma) were placed onto Whatman 3MM paper on top of the membrane to draw the transfer buffer up through the gel and the membrane. Finally, a second glass plate and a 500 g weight were placed on top of the Quick draw blotting paper stack. The gel was blotted for 4-16 hours. After the transfer was complete, the membrane was wrapped in Saran wrap and the RNA was fixed to the membrane under UV light for 4 minutes.

2.12.5 Northern blot hybridization procedure for [α -³²P]-labelled probe

Solutions

Deionised formamide (Sigma)

Denatured Salmon Sperm DNA: This was dissolved in water to a final concentration of 10 mg/ml and then sheared through a 25 gauge needle. The DNA was then denatured by boiling for 10 minutes and stored at -20°C. Prior to use, the DNA was boiled for 5 minutes and snap-chilled on ice.

50 x Denhardt's solution with BSA: ficoll (5 g), polyvinylpyrrolidone (5 g), and

BSA (5 g) (pentax fraction V, Miles scientific) were dissolved in deionized water and the volume made up to 500 ml. The solution was filter sterilized through 0.45 μm nitrocellulose filter (Millipore) and stored in aliquots at -20°C .
20 x SSPE: 3.6 M NaCl/ 0.2 M NaH_2PO_4 / 0.02 M EDTA (pH 7.4). This solution was autoclaved and stored at room temperature.

2.12.5.1 Pre-hybridization of the filter

Pre-hybridization buffer: 50% formamide/ 5 x Denhardt's with BSA/ 5 x SSPE/ 0.1% SDS/200 $\mu\text{g}/\text{ml}$ salmon sperm DNA.

Prehybridisation: The filter was placed, RNA side up on a nylon gauze cut to a size slightly bigger than the filter, in a roller bottle containing pre-hybridization buffer (100 $\mu\text{l}/\text{cm}^2$ filter). Pre-hybridization was carried out at 42°C for 4-16 hours.

2.12.5.2 Hybridization of the filter

Hybridization buffer: 50% formamide/ 2 x Denhardts with BSA/5 x SSPE/0.1% SDS/100 $\mu\text{g}/\text{ml}$ salmon sperm DNA/9% (w/v) dextran sulphate.

The pre-hybridization buffer was removed from the roller bottle and replaced with hybridization buffer (50 $\mu\text{l}/\text{cm}^2$ filter) and incubated at 42°C for 1 hour.

Addition of the probe: pUC19 recombinant plasmid labelled with [α - ^{32}P]-dCTP (section 2.11) was boiled for 3 minutes, chilled on ice and added at an equivalent of 8 ng/ml of hybridization buffer. Hybridization was carried out at 42°C for 16-20 hours.

2.12.5.3 Filter washes

To remove unbound [α -³²P] labelled probe, the filter was washed twice with 2 x SSPE/0.1% SDS for 15 minutes each at room temperature, twice with 2 x SSPE/0.1% SDS for 15 minutes each at 50°C and twice with 0.1 x SSPE/0.1% SDS for 15 minutes each at 50°C. The filter was blotted dry with tissue paper, covered in Saran wrap and autoradiographed at -70°C using Fuji RX-X-ray film. If necessary the filter was washed again for 15 minutes in 0.1 x SSPE/0.1% SDS at temperatures ranging from 55°C-65°C and the blot was autoradiographed as above. For complete removal of the probe, the filter was incubated in boiling 0.1% SDS with gentle shaking until the solution reached room temperature. The filter was exposed to Fuji RX X-ray film at -70°C overnight to ensure that all the probe had been removed.

2.15 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS/PAGE)

(Laemmli, 1979)

Stock solutions

30% (w/v) Acrylamide/ 0.8% (w/v) N,N'-methylenebisacrylamide: As supplied by National Diagnostics (U.K)

pH 8.8 buffer: 1.5 M Tris-HCL (pH 8.8)/0.4% (w/v) SDS. Stored at room temperature.

pH 6.8 buffer: 0.5 M Tris-HCL (pH 6.8)/0.4% (w/v) SDS. Stored at room temperature.

TEMED: Stored at 4°C

Ammonium persulphate: 3% (w/v) solution in sterile water. This was prepared immediately before use.

Loading buffer (2 x): 0.125 M Tris-HCL (pH 6.8)/ 4% (w/v) SDS/ 20% (v/v) glycerol/ 10% (v/v) 2-mercaptoethanol. Stored at -20°C.

Electrophoresis buffer: 0.025 M Tris-base/ 0.192 M glycine/ 0.1% (w/v) SDS. This was made fresh on the day.

Staining solution: 0.2% (w/v) Kenacid Blue in 45:45:10 (v:v:v) mixture of water, methanol and acetic acid.

Destaining solution: 6:3:1 mixture of water, methanol and acetic acid.

Gel preparation:

(i) 7.5% separating gel: Stock acrylamide (10 ml), pH 8.8 buffer (10 ml), and water (17 ml) were mixed by stirring. Ammonium persulphate (3 ml) and TEMED (40 µl) were added, the solution was quickly mixed and the gel was poured. The gel was immediately overlaid with 0.1% (w/v) SDS and left to polymerise overnight.

(ii) Stacking gel: The overlay solution from the separating gel was removed and the comb was inserted. Stock acrylamide (2 ml), pH 6.8 buffer (5 ml), and water (10 ml) were mixed by stirring. Ammonium persulphate (3 ml) and TEMED (40 µl) were added, the solution was quickly mixed and the gel was poured. The gel was immediately overlaid with 0.1% (w/v) SDS and left to polymerise for at least one hour.

Preparation of samples for electrophoresis:

10 µl of prestained SDS molecular weight markers (Sigma) and 20-50 µl (15-20 µg) of protein samples suspended in 1 x loading buffer were boiled for 5

minutes in microfuge tubes and centrifuged briefly in a microfuge at room temperature prior to loading the gel.

Electrophoresis of samples:

The gel assembly was placed into the electrophoresis chamber containing electrophoresis buffer. The comb was removed and the samples were loaded into the wells and electrophoresed at 15 mA until the tracking dye entered the separating gel. The current was then increased to 30 mA until the tracking dye had almost reached the bottom of the gel.

Analysis of SDS-Polyacrylamide gels:

Protein bands were stained for 2-16 hours in staining solution which was then replaced with destaining solution. When the gel was almost completely destained, it was transferred to a 7% (w/v) acetic acid solution for storage.

2.16 Western blotting

Solutions:

Transfer buffer: 0.025 M Tris-base/ 0.192 M glycine/ 20% (v/v) methanol.
Prepared fresh.

Tris Buffered Saline (TBS): 20 mM Tris-base/ 500 mM NaCl, pH 7.5. Stored at room temperature.

Tween-20 Tris Buffered Saline (TTBS): TBS with final concentration of 0.05% (v/v) of Tween-20.

Antibody buffer: 1% (w/v) of gelatin dissolved in TTBS.

Blocking solution: 3% (w/v) of gelatin dissolved in TTBS.

Other reagents: Primary antibodies (i) Anti-MBP serum: Rabbit serum prepared using purified MBP (NEB). Serum was diluted 1:10000 in antibody buffer.

(ii) Anti-CYP2A7 antibody: Sheep serum prepared using purified CYP2A7 protein. Serum was diluted at 1:4000 in antibody buffer.

Secondary antibodies: (i) Goat-Anti rabbit IgG (whole molecule) HRP conjugate (Biorad). Antibody was diluted at 1:30000 in TBS.

(ii) Donkey-Anti sheep IgG (whole molecule) Alkaline phosphatase conjugate (Sigma immunochemicals). Antibody was diluted at 1:30000 in TBS prior to use.

HRP colour development solution:

(a) 60 mg of HRP colour development reagent was dissolved in 20 ml of methanol immediately before use.

(b) Immediately prior to use, 60 μ l of ice-cold 30% (v/v) hydrogen peroxide was added to 100 ml of TBS and mixed with (a) at room temperature and used immediately.

Alkaline Phosphatase colour development solution:

33 μ l of 50 mg/ml NBT (nitroblue tetrazolium) substrate and 33 μ l of 50 mg/ml BCIP (5-Bromo-4-Chloro-3-Indolylphosphate) substrate were mixed with 5 ml of alkaline phosphatase buffer (100 mM Tris-HCl (pH 9.5)/ 100 mM NaCl/ 5 mM MgCl₂) and the solution was used immediately.

Stop Solution: 20 mM Tris-HCl (pH 8.0)/ 5 mM EDTA. Stored at room temperature.

Electrophoretic blotting procedure:

Proteins were first separated by electrophoresis through a SDS/polyacrylamide gel as described in section 2.15.

Four pieces of Whatman 3MM paper and a nitrocellulose membrane were cut to the same size as the gel and pre-wetted in transfer buffer. Two pieces of pre-wetted Whatman 3MM paper were placed on top of the scotch brite pad pre-saturated in transfer buffer in an opened hinged holder. The electrophoresed gel was placed on top of the Whatman 3MM paper. Pre-wetted nitrocellulose membrane was placed on top of the gel and care was taken to remove the air bubbles. A second set of pre-wetted 3MM Whatman paper was placed on top of the nitrocellulose membrane and the sandwich was completed by placing a second scotch brite pad pre-saturated in transfer buffer on top of the 3MM Whatman paper. The hinged holder was closed and placed in the transfer chamber filled with transfer buffer with the nitrocellulose membrane facing the anode. Electrophoretic transfer was carried out at 100 mA overnight.

Immunological detection of proteins on nitrocellulose:

After the electrophoretic transfer, the membrane was washed briefly in TBS and incubated at room temperature in a blocking solution for 1 hour with gentle shaking. Following this, the membrane was incubated in a primary antibody solution for two hours with gentle shaking. The membrane was then washed once in distilled water for 5 minutes and twice for 10 minutes each in TTBS and incubated in secondary antibody solution for 1 hour. Finally the membrane was washed twice for 10 minutes each in TTBS and once for 5 minutes in distilled water.

Colour reaction

When using Horseradish-peroxidase-conjugated IgG preparations, the membrane was soaked in HRP colour development solution and the reaction was terminated after 5-10 minutes by washing with water. The membrane was dried between filter paper and stored protected from light.

When using alkaline phosphatase conjugated IgG preparations the membrane was soaked in alkaline phosphatase colour development solution and the reaction was terminated after 5-10 minutes by replacing the colour development solution with stop solution. The membrane was dried between filter paper and stored protected from light.

2.17 Expression of human CYP2A6 in *E. coli*

2.17.1 Construction of the fusion plasmid

E. coli GM2163 cells were transformed (as in section 2.9) with pUC19 plasmid DNA containing a full length human CYP2A6 cDNA inserted at an Xba I site. A small scale plasmid preparation was carried out from the transformants (as in section 2.3.3) and the plasmid DNA was digested with Xba I. The Xba I fragment was isolated from the agarose gel (as in section 2.7.2) and ligated (as in section 2.8.1) into pMAL-C or pMAL-P plasmid expression vectors (New England Biolab (NEB)) which had been digested with Xba I.

E. coli JM109 cells were transformed with the ligation mixture (as in section 2.9) and plated onto LB/agar/ampicilin plates. Following overnight incubation at 37°C, the transformants were plated onto LB/agar/ampicilin/IPTG/X-gal indicator plates. The plates were incubated at 37°C overnight. Small scale plasmid preparation was carried out from candidate transformants and the

presence and orientation of the insert was confirmed by restriction mapping.

2.17.2 Bacterial expression and cellular fractionation

An overnight culture of *E. coli* JM109 cells transformed with a fusion plasmid was grown at 37°C in LB medium containing 100 µg/ml ampicillin. A 10 ml aliquot was used to inoculate 1 liter of SOC medium containing 100 µg/ml ampicillin. The cells were grown at 30°C with gentle shaking to an OD₅₅₀ = 0.45-0.55. 1 ml aliquot of the culture (uninduced cells) was centrifuged in a microfuge at room temperature and the pellet was resuspended in 50 µl of 1X SDS/PAGE sample buffer and stored at -20°C. To the remaining culture, IPTG was added to a final concentration of 0.3 mM.

The cells were grown for 72 hours at 30°C with gentle shaking. 1 ml aliquot of the culture (induced cells) was centrifuged in a microfuge at room temperature and the pellet was resuspended in 50 µl of 1 x SDS/PAGE sample buffer and stored at -20°C. The cells were chilled on ice for 1 hour, harvested by centrifugation at 3000 g for 10 minutes at 4°C and then the pelleted cells were washed by resuspending in 50 ml of column buffer and centrifuged at 4000 g for 20 minutes at 4°C. The supernatant was removed and the pellet was drained and resuspended in 50 ml of column buffer (10 mM sodium phosphate/ 0.5 M NaCl/ 1 mM sodium azide/ 10 mM 2-mercaptoethanol, pH 7.0) containing PMSF (0.4 mM) and stored at -20°C overnight. For the preparation of membranes, cells were thawed in cold water and subjected to one cycle of disruption with a cooled French pressure cell. The disrupted cells were centrifuged at 3000 g for 20 minutes at 4°C to remove unbroken cells. The supernatant was centrifuged at 100000 g for 60 minutes at 4°C. 100 µl of

the supernatant (cytosolic fraction) was resuspended in 100 μ l of 2 x SDS/PAGE sample buffer and stored at -20°C.

The pellet was resuspended using a Dounce homogenizer in a 5 ml volume of column buffer containing 0.4 mM PMSF and 10% (v/v) glycerol. 100 μ l of this sample (membrane fraction) was resuspended in 100 μ l of 2 x SDS/PAGE sample buffer and stored at -20°C. Membrane bound proteins were solubilized on ice by dropwise addition of deoxycholate (Sigma) and lubrol (Sigma) to the membrane fraction at final concentrations of 0.4% (v/v) and 0.2% (v/v) respectively. The sample was left on ice with gentle stirring for 30 minutes and then centrifuged at 100000 g for 2 hours at 4°C. 100 μ l of the supernatant (soluble fraction) was resuspended in 100 μ l of 2 x SDS/PAGE sample buffer and stored at -20°C. The soluble fraction was stored at -70°C. The expression of MBP-CYP2A6 fusion protein was analysed by SDS/PAGE and Western blotting (sections 2.15 and 2.16). JM109 cells harboring pMAL-p without the insert and induced with IPTG were used as controls.

2.17.3 Spectral analysis and coumarin 7-hydroxylase (COH) activity of MBP-CYP2A6 fusion protein

P450 spectral analysis was carried out as described by Omura and Sato (1964).

Solubilized membrane samples (1 ml) from *E. coli* expressing MBP-CYP2A6 fusion protein was placed in each of two cuvettes. The contents of both cuvettes were reduced by addition of few crystals of sodium dithionite and the baseline recorded between 400nm and 500nm. The sample cuvette was then bubbled with CO and rescanned as above. Measurement of COH activity of MBP-CYP2A6 fusion protein was carried out as described in section 2.18.7.1

(ii) except the microsomes from Sf9 cells infected with the recombinant baculovirus were replaced with membranes from E. coli cells expressing MBP-CYP2A6 fusion protein.

2.17.4 Purification of MBP-CYP2A6 fusion protein by affinity chromatography using cross linked amylose

2.17.4.1 Analysis of Affinity of MBP-CYP2A6 fusion protein for amylose resin)

Amylose resin (NEB) was rehydrated by mixing 1 g of resin and 50 ml of column buffer. The mixture was incubated at room temperature for 30 minutes and sodium azide was added to a final concentration of 1 mM. The rehydrated resin was stored at 4°C.

50 µl of proteins solubilised from bacterial membranes was mixed with 50 µl of a slurry of amylose resin and incubated on ice for 15 minutes. The sample was centrifuged for 1 minute in a microfuge at room temperature and 50 µl of the supernatant was resuspended in 50 µl of 2 x SDS/PAGE sample buffer and stored at -20°C. The pellet was washed with 1 ml of column buffer and centrifuged for 1 minute in a microfuge at room temperature. The pellet was then resuspended in 50 µl of 1X SDS/PAGE sample buffer and stored at -20°C. The binding of MBP-CYP2A6 fusion protein to amylose resin was analysed by western blotting (section 2.16.)

2.17.4.2 Analysis of the effect of detergent on affinity of MBP-CYP2A6 fusion protein to amylose resin

50 µl samples of proteins solubilised from bacterial membranes were diluted at 1:10 and 1:100 in column buffer and to one undiluted sample SDS was added to a final concentration of 0.1% (w/v). Each sample was mixed with 50 µl slurry of amylose resin and incubated on ice for 15 minutes. The samples were then centrifuged for 1 minute in a microfuge at room temperature and the pellets were each resuspended in 50 µl of 1 x SDS/PAGE sample buffer and stored at -20°C.

The binding of MBP-CYP2A6 fusion protein to amylose resin was analysed by western blotting (section 2.16). Purified MBP-Paramyosin fusion protein was used as a positive control for binding of MBP to amylose resin.

2.18 Baculovirus mediated expression of human CYP2A6

2.18.1 Construction of recombinant transfer plasmid

The Nco I-EcoR I fragment of the recombinant pUC19 plasmid(section 2.8) containing the entire coding region for human CYP2A6 was ligated into the NcoI-EcoRI sites downstream of the polyhedrin promoter in pAcC5 baculovirus transfer vector. The ligation product was then used to transform *E.Coli* JM109 cells as described previously. The transformants were plated onto LB/Agar plates containing 200 µg/ml ampicillin and incubated at 37°C overnight. A small scale plasmid preparation was carried out from the transformants and the presence and the orientation of the insert was confirmed by restriction enzyme mapping.

2.18.2 Maintenance and culture of insect cells

Sf9 cells were cultured at 27°C in TNM-FH medium in the presence of the antibiotics, gentamycine, 50 mg/ml (GIBCO-BRL) and fungizone, 2.5 mg/ml (GIBCO-BRL). TNM-FH medium is composed of Grace's insect cell culture medium (GIBCO-BRL) supplemented with 10% foetal calf serum (GIBCO-BRL), 3.3 g/l yeastolate (TC-yeastolate, Difco) and 3.3 g/l lactalbumin hydrolysate (TC-lactalbumin, Difco). The pH of the medium was adjusted to 6.2 with sodium phosphate buffer and the medium was filter sterilized and stored at 4°C. Sf9 cells are routinely grown at 27°C. However, they tolerate temperatures from 25°C to 28°C well and because they have no requirement for CO₂, they may be cultured successfully on the lab bench providing the room temperature does not fluctuate excessively.

2.18.2.1 Passage of cells

Sf9 cell cultures require the use of plasticware that has been treated to permit cell attachment. Plasticware from Falcon or Corning was used.

Approximately 2×10^6 cells were used to inoculate 10 ml of TNM-FH medium in 75 cm² tissue culture flasks. The flasks were incubated in an incubator at 27°C. The TNM-FH medium was renewed after 3 days. Cells attained confluence after 5 to 7 days ($\sim 2 \times 10^7$ cells/flask). The cells were then scraped from the flask and resuspended in 100 ml of fresh TNM-FH medium prewarmed to 27°C in 1 litre Erlenmeyer flask (starting density of 2×10^5 cells/ml). 10 ml of resuspended cells were seeded into 75cm² flask as above. The remainder of the cells were grown as a suspension culture at 27°C in a rotating shaker (50 RPM) for use such as the production of recombinant proteins or for operations to select the recombinant viruses. The cells were

pelleted (500 g for 5 minutes) when they reached the density of $\sim 1 \times 10^6$ cells/ml (three to four days after inoculation) and resuspended in 100 ml of fresh TNM-FH medium. Viability of cells was measured by trypan blue staining. 0.1 volumes of trypan blue solution (0.4% (w/v) trypan blue in PBS) was added to a small aliquot of cells. The cells were immediately examined on a haemocytometer under a light microscope. Cells that take up trypan blue are dead cells. The total number of cells were counted. A healthy culture of cells was taken to be > 97% viable. Generally, growth of the cells reaches a plateau when cell density reaches 6×10^6 cell/ml.

2.18.2.2 Long term cell storage

Cells from an exponentially growing culture (1×10^6 cell/ml, viability ~98%) were centrifuged and resuspended at 2×10^6 cells/ml in ice-cold TNM-FH medium containing 10% DMSO. Aliquots of cells were dispensed into screw-top cryostat freezing vials and placed for 2 hours at -20°C and then overnight at -70°C . The frozen cells were then transferred to the liquid nitrogen freezer for long term storage.

2.18.3 Homologous recombination

500 ng of AcMNPV linear DNA (Baculogold Bsu 36l linearised AcMNPV DNA from A.M.S. Biotechnology, U.K) and 5 μg of the recombinant transfer vector were diluted in 1 ml of Grace's medium, serum-free (GIBCO-BRL). 50 μl of lipofectin (GIBCO-BRL) were diluted in 1 ml of the same medium, in a 5 ml polystyrene tube. The DNA solution was then mixed with liposomes, and the liposome/DNA complex was allowed to form for 20 minutes at room temperature. After careful washing of the Sf9 layer (2×10^6 cells in a 60 mm

diameter petri dish) with serum-free Grace's medium, the cells were overlaid with the DNA/liposome complex and co-transfection was allowed to proceed for 4 hours. The lipofection medium was then replaced with TNM-FH medium, the dishes wrapped with parafilm to prevent desiccation and incubated for 4 days at 27°C. After 48 hours post infection, it is possible to observe some virogenic trauma in the cells. At the end of the incubation period, supernatant (containing the virus) was collected and stored at 4°C.

2.18.4 Identification and purification of the recombinant virus

Recombinant virus can be purified from co-transfection stocks by plaque purification, end-point dilution or by the combination of both. Recombinants may be identified during this purification procedure by visual screening, DNA hybridization, PCR amplification or immunoblotting. The recombinant transfer plasmid and the parent virus used in this study allow the visual screening of the recombinant virus. This method is based on the fact that viruses that lack a polyhedrin gene (*polh*) are incapable of forming occlusion bodies and, therefore, form plaques that are visually distinguishable from Wt (*occ*⁺) virus plaques. This screen has the advantage of being based on the loss of a gene, therefore, it only identifies double recombinants.

60 mm diameter tissue culture plates were seeded with 4×10^6 cells (at exponential growth phase) in a final volume of 4 ml of TNM-FH medium per plate. The plates were rocked gently immediately after adding the cells to ensure an even monolayer. The plates were incubated at 27°C for 1 hour to allow the cells to attach. Ten-fold serial dilutions of the supernatant from a co-transfection experiment were made in Grace's medium (10^{-5} to 10^{-8}). Tissue culture medium was removed from the cell monolayers and the cells were

infected with 500 μ l of each virus dilution in duplicate. For the controls, 500 μ l of Grace's medium was added to 2 plates (non-infected cells) and 500 μ l of Wt (occ⁺) virus diluted in Grace's medium was added to two additional plates (cells infected with wild type virus). Plates were incubated for 90 minutes at room temperature with gentle rocking. Meanwhile, 4% agarose (Sea Plaque) was prepared in MilliQ water and autoclaved. The melted agarose was cooled to 60°C and diluted to 0.5% in TNM-FH medium pre-warmed to 60°C. The virus inoculum was removed from the cells after 90 minutes and 4 ml of agarose overlay was added to each plate (agarose was cooled to 38°C prior to overlaying the cells). The plates were left at room temperature for 90 minutes until the agarose had hardened. The plates were then wrapped with parafilm and incubated at 27°C for four days.

Occlusion phenotype was determined using a dissecting microscope with a platform, a blackbackground and a strong light source at an acute angle to the platform.

The plates were inverted and scanned. Occ⁺ plaques appear as yellowish-white shiny cells against a background of light grey uninfected cells. The bright shiny appearance is due to the high refractivity of occlusion bodies. Occ⁻ plaques are much less refractive than occ⁺ plaques. Because cells become rounded during infection, occ⁻ plaques are more refractive and appear whiter than surrounding uninfected cells. Plaques displaying the appropriate phenotype were circled and examined using an inverted phase-contrast tissue culture microscope. For occ⁺ plaques, many occlusion bodies are visible in the nuclei of the infected cells. Occ⁻ plaques lack occlusion bodies. The tip of a sterilized Pasteur pipette was placed directly onto the plaque and

gentle suction was applied until a small plug of agarose was drawn into the pipette. The agarose plug was placed in 1 ml of TNM-FH medium and vortexed to release the virus particles from the plug. The 1 ml stock was used to plaque purify the virus at least once more. The virus stock was stored at 4°C.

2.18.5 Amplification of virus stocks

The isolation of viral plaques were diluted in 1 ml of TNM-FH medium and aliquots of 50 µl were used to amplify the viral titre in 96 wells titration plate, each well containing 2×10^5 fresh cells in 150 µl of fresh TNM-FH medium. The amplified passage 1 viral supernatant (400 µl) was directly used to produce high titre passage 2 virus by infecting 3×10^6 cells in 60 mm tissue culture plates. Passage 2 viral supernatant was collected and centrifuged to remove the debris. The amplified stocks of passage 2 virus were titrated using the TCID-50 method before their use for larger scale infections. The virus stocks were stored at 4°C. Passage 2 infected cells were scraped, pelleted and resuspended in 400 µl of lysis buffer (50 mM HEPES pH 7.4/ 0.25 M succrose/0.4 mM PMSF/0.2% Triton-X 100). This procedure resulted in solubilization of cell membranes and extraction of the nuclei. 50 µl aliquots were mixed with an equal volume of 2 x SDS/PAGE loading buffer. Samples were stored at -20°C for later analysis by SDS/PAGE and immunoblotting along with the lysates of uninfected cells and wild type AcMNPV infected cells.

2.18.6 Preparation of Sf9 cellular extracts

The passage 2 viral stocks were used to infect the cells from a 100 ml suspension culture (density 2×10^6 cells/ml, at exponential growth phase,

cultivated in a 1 litre Erlenmeyer flask at 25°C on a rotating shaker, 60 RPM). Cells were pelleted at 500 g for 6 minutes and resuspended in 10 ml of TNM-FH medium in 50 ml sterile tubes (Falcon). Passage 2 virus was added in a volume of 1 ml at MOI of 5 and the cells were incubated at 25°C for 60-90 minutes with gentle agitation to prevent sedimentation. The cells were then re-seeded in a flask containing 100 ml of fresh TNM-FH medium. The cells were harvested by centrifugation 60 hours post infection and resuspended in 10 ml of isotonic buffer (50 mM HEPES, pH 7.4/0.25 M sucrose/0.4 mM PMSF). The resuspended cells were lysed by sonication (3 bursts of 20 seconds at 0°C). The cell homogenates were centrifuged at 12000 g for 15 minutes, at 4°C. The nuclear-mitochondrial pellet (P1) was resuspended in 2 ml of storage buffer (isotonic buffer containing 20% (v/v) glycerol). The microsomal fraction (P2) was then pelleted from the post-mitochondrial supernatant (S1) by ultracentrifugation (100000 g for 90 minutes at 4°C) and resuspended in 4 ml of storage buffer. Glycerol was added (20% (v/v) final concentration) to soluble cytosolic fraction (S2). All fractions were stored at -70°C. 20 µl aliquots of each fraction were analysed by SDS/PAGE and western blotting. Aliquots of total cell lysates from cells infected with Wt virus and uninfected cells were used as controls. Protein assay was carried out using the Biorad protein assay kit (Bio-Rad) as instructed by the manufacturer.

2.18.7 Enzymatic activity of baculovirus expressed CYP2A6

2.18.7.1 Coumarin 7-hydroxylase (COH) activity of the expressed CYP2A6

(i) COH activity in Sf9 cells infected with recombinant baculovirus

60 mm diameter tissue culture plates were seeded with 2×10^6 cells in a final volume of 4 ml of TNM-FH medium per plate, supplemented with 5-ALA and Iron citrate each at a final concentration of 100 μ M. The plates were incubated at 27°C for 24 hours and then the cells were infected with recombinant baculovirus expressing CYP2A6 at MOI of 5. Cells infected with wild type baculovirus (AcMNPV) were used as controls. 24 hours post infection, coumarin solution (5 mM in methanol) was added to the culture medium at a final concentration of 50 μ M. 48 hours post infection, the culture medium was collected and analysed for the formation of 7-hydroxycoumarin.

Measurement of formation of 7-hydroxycoumarin

To the culture medium, an equal volume of 6% (w/v) trichloroacetic acid (TCA) was added and the mixture was incubated on ice for 10 minutes. The precipitated proteins were removed by centrifugation and 0.5 ml of the supernatant was then added to 3 ml of 0.8 M Tris-HCl/0.8 M glycine, pH 9.0. The formation of 7-hydroxycoumarin from coumarin was measured fluorimetrically in 1cm quartz cuvettes at $\lambda_{\text{excitation}} = 376$ using a Perkin Elmer LS 50B fluorescence spectrometer.

(ii) COH assay using microsomal fraction of Sf9 cells infected with recombinant baculovirus

Sf9 cellular extracts were prepared as described in section 2.18.6 except that the cell culture medium (TNM-FH) was supplemented with 5-ALA and Iron citrate each at a final concentration of 100 μ M.

COH assay conditions

COH activity was measured in a reaction mixture (1.0 ml) containing the following components added in the order:

Microsomes: 1 mg of protein

Purified rat NADPH cytochrome P450 reductase: 1.25 units

HEPES: 50 mM, pH 7.4

MgCl₂: 1.5 mM

Water: to make up the volume to 1 ml

Sodium deoxycholate: 0.01% (w/v)

NADPH: 500 μ M

Glucose-6-phosphate-dehydrogenase (G6PDH): 1 unit

Glucose-6-phosphate (G6P): 2 mM

Coumarin: 50 μ M

The tubes were incubated at 37°C for 3 minutes and the reaction was initiated by addition of coumarin. 100 μ l samples were taken at 10 minute intervals and added to an equal volume of 6% (w/v) TCA. The precipitated protein was removed by centrifugation and the supernatant was added to 2 ml of 0.8 M Tris-HCl/0.8 M glycine, pH 9.0.

The amount of 7-hydroxycoumarin formed from coumarin was measured fluorimetrically in 1cm quartz cuvettes at $\lambda_{\text{excitation}} = 376$ using a Perkin Elmer

LS 50B fluorescence spectrometer and compared with 7-hydroxycoumarin standards made up in the same reaction/stop mixture but with no added protein. Microsomal membranes from Sf9 cells infected with wild type baculovirus (AcMNPV) were used as a control for this experiment.

(iii) Effect of adding exogenous reductase on COH activity

COH assay was carried out as described in (ii). 3 separate reactions were carried out where in reaction 1, no reductase was added, in 2, purified rat reductase was added, and in 3, microsomes prepared from Sf9 cells infected with recombinant baculovirus expressing human reductase was added.

Composition of reaction mixtures (total volume of 1 ml) is shown in table1

	No reductase	Rat reductase	Human reductase
Microsomes from Sf9 cells infected with recombinant virus (mg of protein)	1.08	1.08	1.08
NADPH- cytochrome P450 reductase ($\mu\text{mol/Cyc/min/ml}$)	0	1.258	1.338
Hepes pH 7.4 (mM)	50	50	50
MgCl ₂ (mM)	1.5	1.5	1.5
Sodium deoxycholate(%)	0.01	0.01	0.01
Coumarin (μM)	50	50	50
NADPH (μM)	500	500	500
G6PDH (U/ml)	2	2	2
G6P (mM)	2	2	2

Table 1 Composition of reaction mixtures for the experiment on effect of adding exogenous NADPH-cytochrome P450 reductase on COH activity of baculovirus expressed CYP2A6.

2.18.7.2 Analysis of testosterone hydroxylase activity of the expressed CYP2A6

[¹⁴C₄] Testosterone (100 μM, 370 Mbq/mmol) from Amersham international was incubated for 1 hour in 1 ml of reaction mixture containing 50 mM HEPES, pH 7.4, 1.5 mM MgCl₂, microsomes from Sf9 cells infected with recombinant baculovirus (1 mg protein), 500 μM NADPH, 1 unit G6PD, 2 mM G6P and 1.5 units purified rat reductase. The reaction was stopped by adding 1 ml of methanol and the tubes were incubated on ice for 10 minutes. The protein precipitate was removed by centrifugation (12000 g, 10 minutes). The unchanged testosterone and the products of the reaction were extracted using C18 solid phase disposable columns (1 ml Analytichem Bond Elut, Varian). Purified rat CYP2B1 and microsomes prepared from Sf9 cells infected with wild type baculovirus (AcMNPV) were used as controls.

Samples were subjected to TLC as described by Waxman (1993). Briefly samples were applied as an individual spot at the origin, drawn 2 cm up from the bottom of the TLC plate (precoated plate on aluminium backing, 20X20 cm and 0.2 mm thick, E. Merck. F-254 supplied by E M Science, Gibbstown, N J). A short wavelength UV lamp was used to confirm that the amount of material applied to the plate is sufficient to enable UV detection. The TLC plate was developed in a glass tank pre-equilibrated for 30 minutes with 100 ml of developing solvent (chloroform/ ethylacetate/ ethanol, 4/1/0.7) containing 1 sheet of Whatman 3MM paper. Development was halted when the solvent front reached 3-4 cm from the top. The plate was air dried and exposed to X-ray film for four days at room temperature.

2.19 Purification of cytochrome P450 by chromatography on p-chloramphetamine-coupled Sepharose.

(Sundin et al; 1987)

Preparation of chloramphetamine column

Affi-gel 10 (Bio-Rad) has a capacity of 15 μ moles/ml. A ten fold excess of chloramphetamine (150 μ moles, 770 mg) from Sigma was dissolved in 25 ml of 0.1 M NaHCO₃. The pH of the solution was measured as 8.5.

25 ml of affigel suspension in a vial was shaken and poured into a Buchner funnel lined with 3 MM Whatman paper and connected to a vacuum. The solvent from the gel was drained and the gel was washed with 3 bed volumes (75 ml) of ice cold deionized water within 20 minutes to obtain optimal ligand coupling. The gel was then washed out into a bottle containing chloramphetamine solution and put on a roller at 4°C for four hours. The suspension was then packed in a 1.5 x 5 cm column in a Luer-Lock syringe and washed with water to remove excess chloramphetamine. The column was stored at 4°C in water containing 0.2% (w/v) sodium azide.

Prior to use the column was washed with four cycles of Tris buffer, pH 8.0, and sodium acetate buffer, pH 4.5, both containing 100 mM NaCl. The column was then equilibrated in a starting buffer (5 mM sodium phosphate, pH 7.5, 20% (v/v) glycerol, 0.125% (w/v) sodium cholate, and 0.05% (w/v) Emulgen 911(Sigma)).

Purification procedure

The 100000 x g microsomal pellet from Sf9 cells expressing CYP2A6 or a membrane pellet from *E. coli* expressing MBP-CYP2A6 fusion protein was

suspended in solubilization buffer (5 mM sodium phosphate, pH 7.5, 20% (v/v) glycerol, 0.5% (w/v) sodium cholate, 0.2% (w/v) Emulgen 911 and 50 μ M PMSF). The sample was left for 30 minutes at 4°C with gentle stirring and was then diluted to one-fourth with 5 mM sodium phosphate buffer, pH 7.5, containing 20% (v/v) glycerol, giving the same concentration of sodium cholate and Emulgen 911 as in the starting buffer.

The sample was applied to the column at a flow rate of 0.5-1.0 ml/minute. The column was then washed with 5 volumes of starting buffer containing 100 mM NaCl. Cytochrome P450 was then eluted with solubilization buffer. Fractions from each step of purification were analysed by SDS/PAGE and western blotting. The gels were silver stained using a kit from Bio-Rad as described by the manufacturer.

The P450 containing fractions were concentrated using Centriprep-30 centrifugal concentrators (Amicon). Concentration of protein was measured using the Bio-Rad protein assay kit as described by the manufacturer.

Chapter 3

Results and Discussion

The cDNA-based expression of cytochromes P450 in heterologous cells provides an important tool for the production of specific proteins for catalytic studies. Such cell systems will help to define enzymes involved in the metabolism of a particular drug and whether the drug is a potential inhibitor of enzyme activity. An increased knowledge of the structural classes of drugs metabolized by specific human cytochromes P450 may result in drug regulatory agencies requiring such information for each drug that is to be marketed. Another advantage of producing large amounts of a single cytochrome P450 via its cDNA is that the protein can be purified and used to raise antibodies.

If large amounts of protein are required the bacterial expression system may be best suited to this task. In contrast, functional studies require a host cell evolutionary closer to man which is easily cultured in large amounts. Several eukaryotic cDNA based expression systems have been devised. Yeast (Ching *et al.*, 1991), COS cells (Zuber *et al.*, 1986), and insect cells (Assefa *et al.*, 1989) have proved successful for the expression of foreign proteins. Each approach requires a cloning strategy that will tailor and package the cDNA in the vectors needed for that particular cell system. In this study, a strategy was designed to synthesize cDNAs from total RNA by the method of reverse transcription and PCR. The key to this approach is to tailor the cDNA to have suitable restriction sites flanking the initiator (ATG) and the translation stop codon. The coding region can be amplified and subsequently cloned into vectors for expression in bacteria, yeast, insect or mammalian systems. The 5' and 3' flanking non-coding regions of the cDNA are hence not included in the cDNA to be expressed. However, a potential problem with this approach is that the fidelity of the PCR product is not guaranteed. Restriction

mapping and sequence analysis are required to confirm the authenticity of cDNAs cloned in this way.

Such a strategy was used in this study for the isolation of human and marmoset cytochromes P450 of the CYP2A subfamily. The amplified products were cloned in bacteria and characterized. Human CYP2A6 was expressed via its cDNA in bacterial cells and using the baculovirus/insect cell system. The results of these experiments are described in this chapter.

3.1 Amplification of a Human CYP2A6 sequence

Total RNA (15 µg) isolated from a human liver sample known to express CYP2A6, was first reverse transcribed and then amplified by PCR using oligoprimers based on the sequences located at the ends of the coding region of human *CYP2A6* (Yamano *et al.*, 1990). The sequence of these primers is shown in Figure 3.1. A DNA fragment of approximately 1.5 Kb was generated by the PCR. This was the expected size for the coding region of human *CYP2A6* (Figure 3.1).

3.2 Characterization of human CYP2A6 cDNA

The amplified 1.5 Kb DNA fragment was isolated from a low melting point agarose gel and cloned into pUC19 plasmid vector at a unique XbaI site in the polylinker. The pUC19 human CYP2A6 construct was introduced into *E. coli* JM109 cells by transformation. Recombinant plasmid DNA isolated from positive transformants was used to confirm that the amplified product was indeed human *CYP2A6* by restriction endonuclease digestion and sequencing.

3.2.1 Restriction endonuclease analysis of the human *CYP2A6* cDNA clone

pUC19 human *CYP2A6* recombinant plasmid DNA was digested with

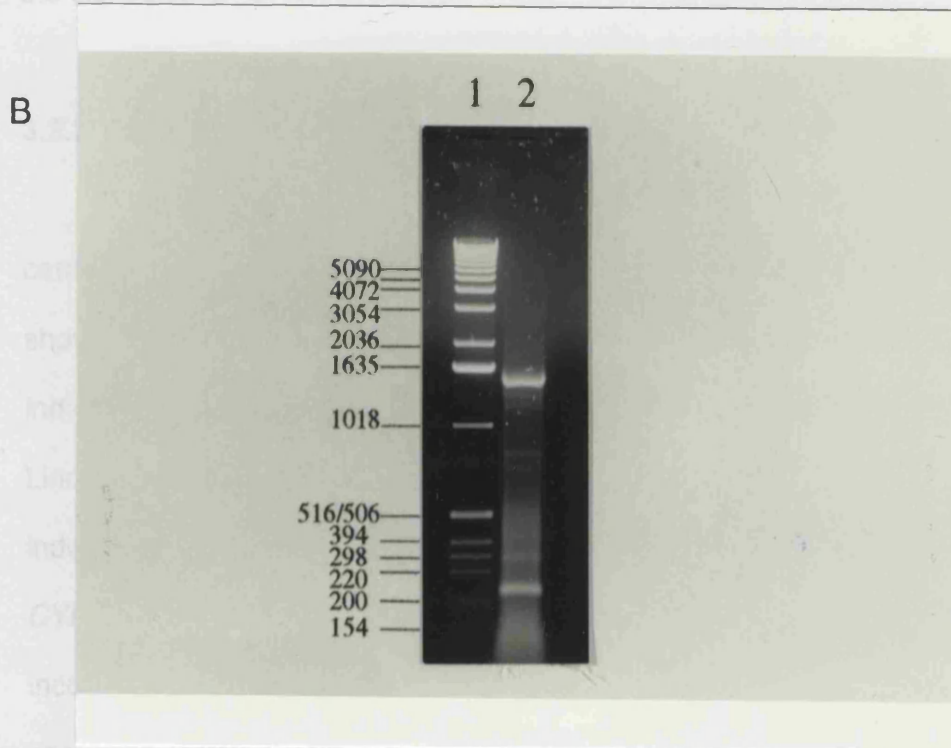
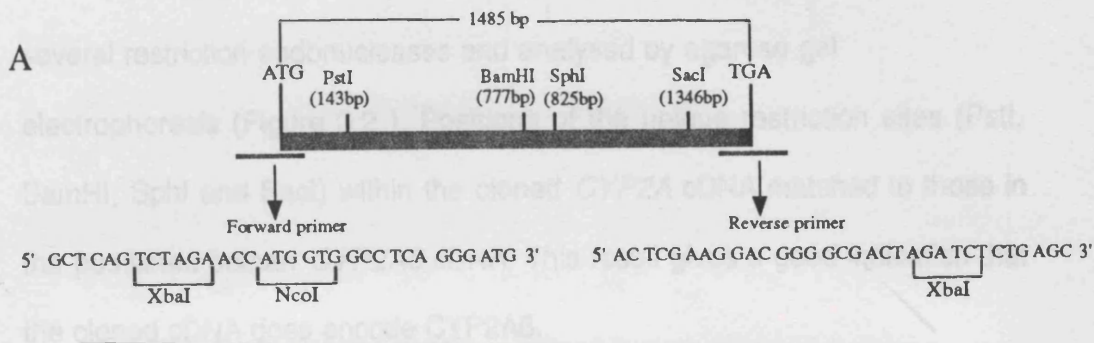


Figure 3.1 Amplification of the human *CYP2A6* sequence from total human RNA.

A: Schematic diagram showing the location of the reverse and forward oligoprimers with incorporated restriction sites. Unique restriction sites within the coding region of human *CYP2A6* as predicted from the published *CYP2A6* sequence are also indicated.

B: Analysis of the PCR product by agarose gel electrophoresis. One fifth of the PCR product (track 2) was electrophoresed through 0.8% agarose gel containing 0.5 μ g/ml ethidium bromide. Track 1 shows DNA molecular size markers (BRL 1Kb ladder). DNA was visualised and photographed under UV light.

3.2.1 Restriction endonuclease analysis of the human CYP2A cDNA clone

pUC19 humanCYP2A recombinant plasmid DNA was digested with several restriction endonucleases and analysed by agarose gel electrophoresis (Figure 3.2). Positions of the unique restriction sites (PstI, BamHI, SphI and SacI) within the cloned *CYP2A* cDNA matched to those in the published human *CYP2A6* cDNA. This result gives a good indication that the cloned cDNA does encode CYP2A6.

3.2.2 Validation of the cloned CYP2A cDNA by sequencing

Final characterization of the recombinant human CYP2A plasmid was carried out by DNA sequence determination. The sequencing strategy is shown in Figure 3.3A. Regions of the *CYP2A* sequence determined are indicated in Figure 3.3B. Further sequence determination by Susannah Lindey (of our laboratory) confirmed that cloned human *CYP2A* cDNA was indeed identical in sequence to the coding region of the published human *CYP2A6* sequence except for the XbaI and NcoI restriction sites which were incorporated into the PCR primers. This result confirms that the *CYP2A* sequence isolated from human total RNA represents the coding region of human *CYP2A6* and that no errors were introduced into the amplified *CYP2A6* during the PCR reaction.

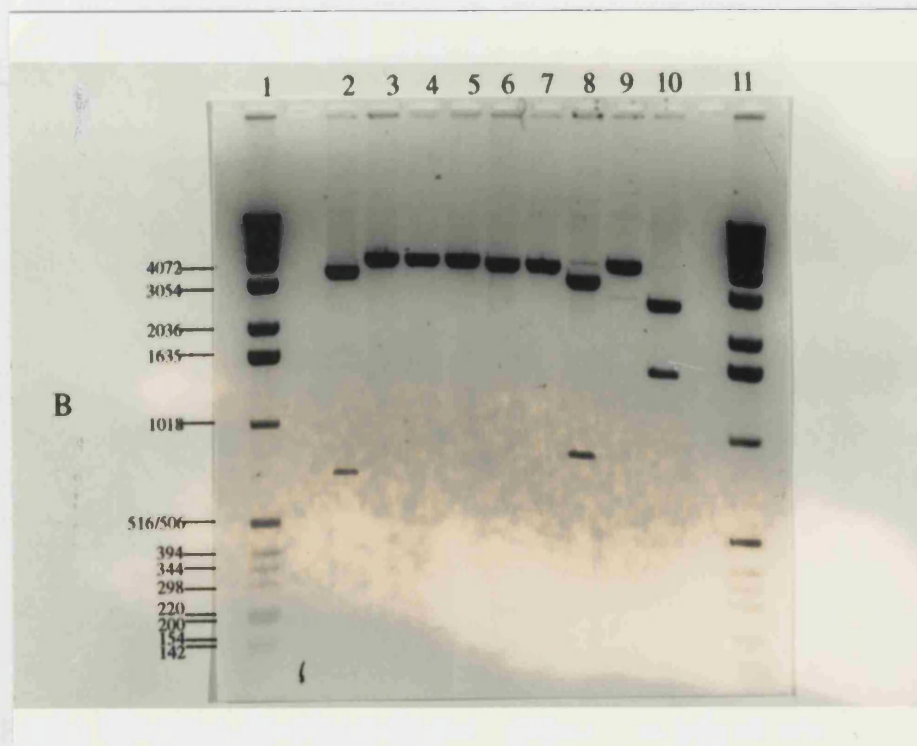
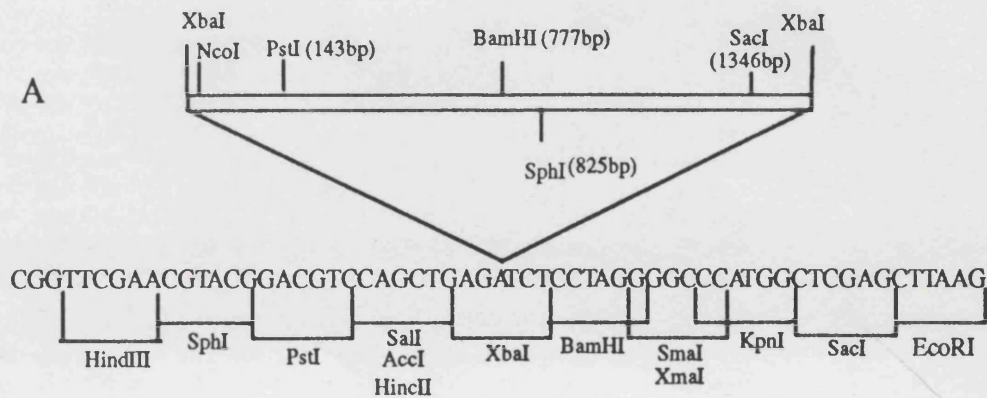


Figure 3.2 Analysis of the human CYP2A cDNA clone by restriction endonuclease digestion.

A: Schematic diagram showing restriction map of the human CYP2A cDNA clone and the polylinker sequence of pUC19.

B: pUC19-human CYP2A recombinant plasmid DNA was digested with BamHI (track 2), EcoRI (track 3), HindIII (track 4), NcoI (track 5), PstI (track 6), SacI (track 7), SphI (track 8), XbaI (track 9), and XbaI/EcoRI (track 10). Tracks 1 and 11 show DNA molecular size markers (1Kb ladder from BRL). The digests were electrophoresed through a 1% agarose gel containing 0.5 μ g/ml ethidium bromide. DNA was visualised and photographed under UV light.

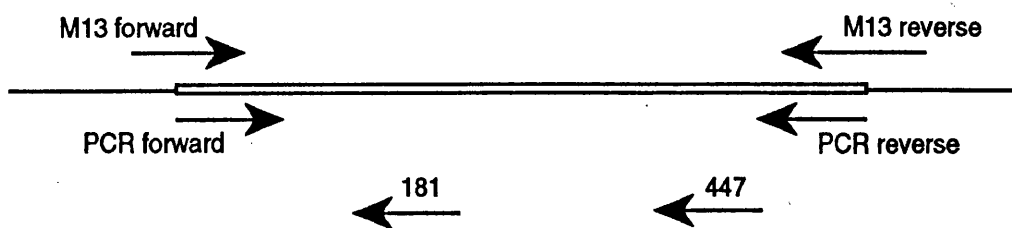


Figure 3.3A Sequencing strategy for human *CYP2A* cDNA clone. Primers used and their direction are indicated. Additionally *CYP2A* cDNA fragments were subcloned into pUC19 and sequenced using the same primers.

3.3 Isolation and characterization of the murine CYP2A

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ATG CTG GCC TCA GGG ATG CTT CTG GTG GCC TTG CTG GTC TGC CTG ACT
ATG CTG 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
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 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
CTG AAC ACA GAG CAG ATG TAC AAC TCC CTC ATG AAG ATC AGT GA
TAT GGC CCC GTG TTC ACC AAT 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
TC TTC AAA
GGC TAT GGC GTG GTA TTC AGC AAC GGG GAG CGC GCC AAG CAG CTC CGG
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
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
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 CT
CGG GGC ACT GGC GGC GCC AAT ATC GAT CCC ACC TTC TTC CT
ACA GTC TCC AAT GTC ATC AGC TCC AAT 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 CAT CCC AAT TCC CCA CGG GAC TTC AAT GAC TCC TTT CTC
ATC CGC ATG CAG GAG GAG AAG AAC CCC AAC ACG GAG TTC TAC TTG
TC 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 AAT GGG GGC ACC GAG
AAA AAC CTG GTG ATG ACC ACG TTG AAC CTC TTC AAT GGG GGC ACC GAG
ACC GTC AGC ACC ACC CTG CGC TAT GGC TTC TTG CTG CTC ATG AAG CAC
ACC GTC AGC ACC ACC CTG CGC TAT GGC TTC TTG CTG CTC ATG AAG CAC
CCA GAG GTG GAG GCC AAG GTC CAT GAG GAG AAT GAC AGA GTG ATC GGC
CCA GAG GT
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
AG CAC TTC
CTG AAT GAG AAG GGG CAG TTT AAG AAG AGT GAT GCT TTT GTG CCC TTT
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
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
CTC TTT CTC TTC TTC ACC ACC GTC ATG CAG AAC TTC CGC CTC AAG TCC
TCC CAG TCA CCT AAG GAC AAT GAC GTG TCC CCC AAA CAC GTG GGC TTT
TCC CAG TCA CCT AAG GAC AAT GAC GTG TCC CCC AAA CAC GTG GGC TTT
GCC ACG ATC CCA CGA AAC TAC ACC ATG AGC TTC CTG CCC CGC TGA
GCC ACG ATC CCA CGA AAC TAC ACC ATG AGC TTC CTG CCC CGC TGA

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Figure 3.3B Human CYP2A cDNA sequence (blue) compared to the published human CYP2A6 cDNA sequence (black). Codons shown on positions 117 and 209 are for valine (GTA) and phenylalanine (TTC) respectively (see page 146). The blue sequence represents the DNA sequence determined in this study. The rest of the sequence was determined by Susanah Lindey in our laboratory.

3.3 Isolation and characterization of the marmoset CYP2A sequence.

An attempt was made to amplify a *CYP2A* from total liver RNA isolated from phenobarbital treated marmosets. Total RNA (15 µg) isolated from phenobarbital treated marmoset liver samples was first reverse transcribed and then amplified by PCR using oligoprimers based on the sequences located at the ends of the coding region of human *CYP2A6* (Yamano *et al.*, 1990). The sequence of these primers is shown in Figure 3.1. A DNA fragment of approximately 1.5 Kb was generated by the PCR (Figure 3.4). This was similar in size to the coding region of human *CYP2A6* (Figure 3.1). The PCR product was cloned into the pUC19 plasmid and analysed by restriction endonuclease digestion and sequencing. Analysis of the restriction map shown in Figure 3.5 revealed that the cloned insert is larger than the PCR product. Subsequent DNA sequence analysis showed that extra 400 bases had been ligated to the original PCR product at the 3' end. In addition, sequence analysis of the 5' end of the marmoset *CYP2A* shows that the reading frame has been altered due to the deletion of a C after the initiation codon (Figure 3.6). This is probably due to the 3' to 5' exonuclease (proofreading) ability of Pfu DNA polymerase. It was decided not to continue with the isolation of marmoset *CYP2A* cDNA clones.

However, the clone was used as a probe to analyze the expression of *CYP2A* mRNA in the livers of untreated and phenobarbital-treated marmosets by northern blotting (Figure 3.7). A mRNA species of approximately 1.9 Kb was detected in both samples and was inducible 20 fold by phenobarbital. In a similar experiment, a mRNA species of 1.9 Kb has been identified by northern blot analysis of total RNA from marmoset liver using a human *CYP2A*

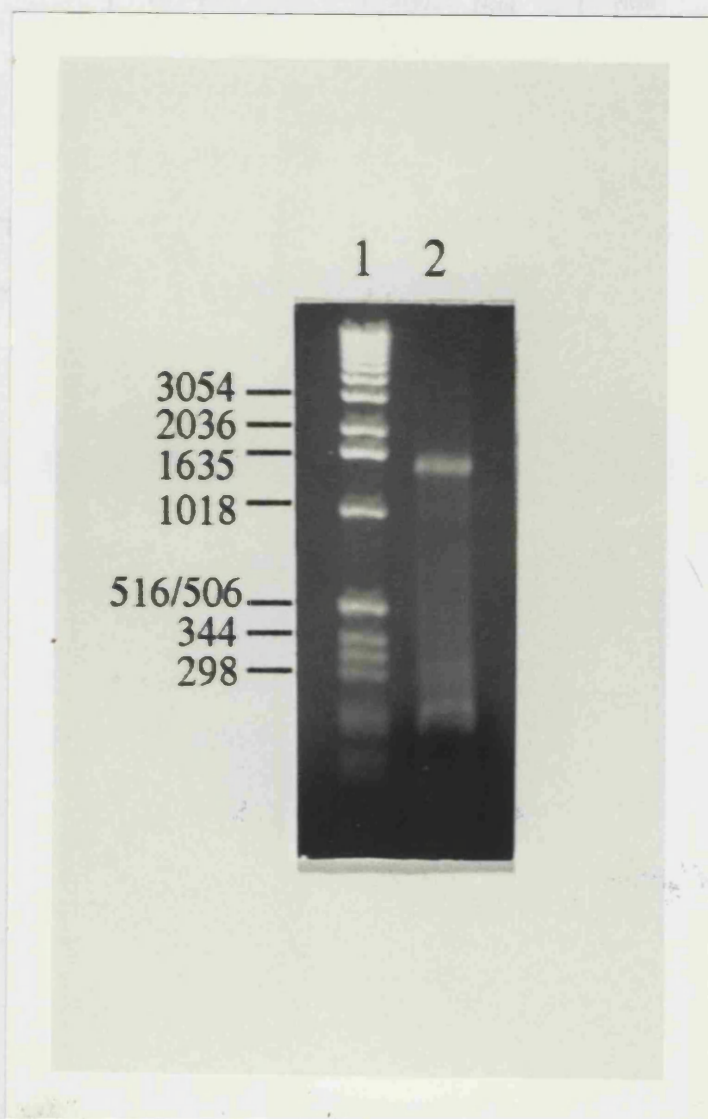
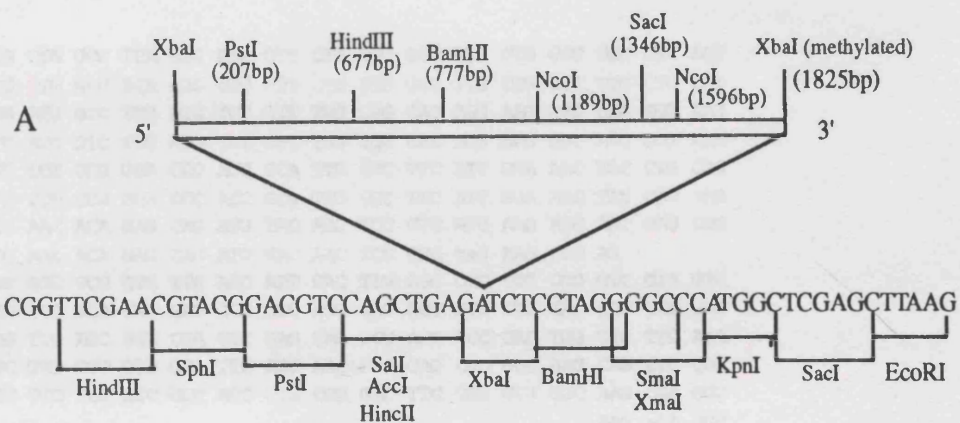


Figure 3.4 Amplification of the marmoset *CYP2A* sequence from total RNA isolated from liver of marmoset treated with phenobarbital.

One fifth of the PCR product (track 2) was electrophoresed through 0.8% agarose gel containing 0.5 $\mu\text{g/ml}$ ethidium bromide. Track 1 shows DNA molecular size markers (BRL 1Kb ladder). DNA was visualised and photographed under UV light.



B

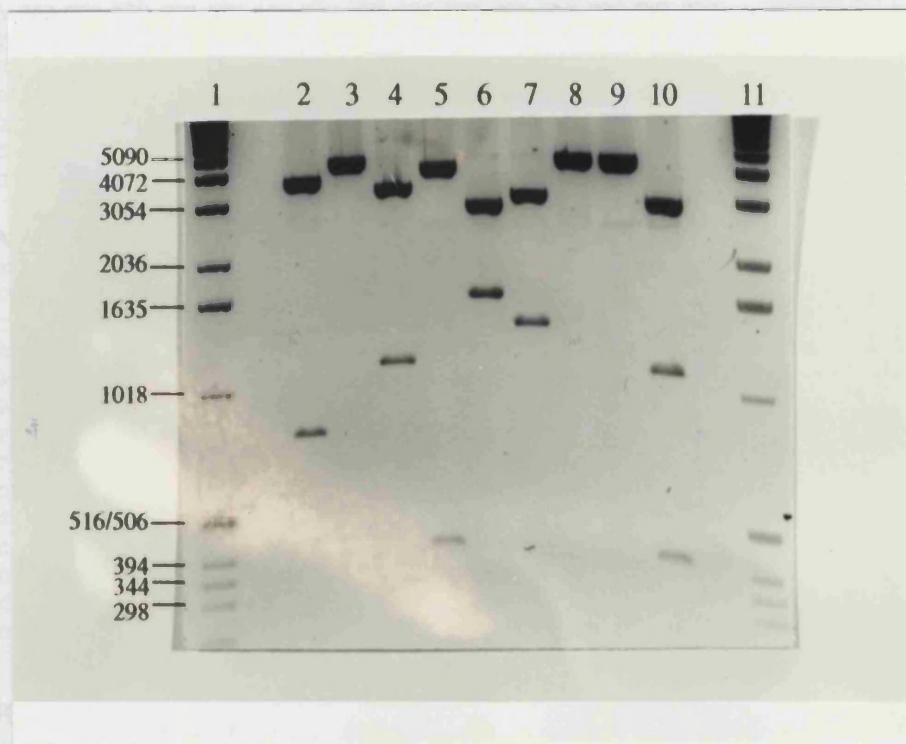


Figure 3.5 Analysis of the marmoset CYP2A cDNA clone by restriction endonuclease digestion.

A : Schematic diagram showing restriction map of the marmoset CYP2A cDNA clone and the polylinker sequence of pUC19.

B : pUC19-marmoset CYP2A recombinant plasmid DNA was digested with BamHI (track 2), EcoRI (track 3), HindIII (track 4), PstI (track 5), XbaI/EcoRI (track 6), SacI (track 7), SphI (track 8), XbaI (track 9), and XbaI/NcoI (track 10). Tracks 1 and 11 show DNA molecular size markers (1Kb ladder from BRL). The digests were electrophoresed through a 1% agarose gel containing 0.5µg/ml ethidium bromide. DNA was visualised and photographed under UV light.

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ATG CTG GCC TCA GGG ATG CTT CTG GTG GCC TTG CTG GTC TGC CTG ACT
ATG XTG GCC TCA GGG CTG 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
GTG ATG GTC TTG ATG TCT GTT TGG agG CAG AGG AAG AGC AAG GGG AAG
CTG COT COG GGA CCC ACC CCA TTG CCC TTC AIT GGA AAC TAC CTG CAG
CTG COT COG GGA CCC ACC CCG TTG CCC TTC AIT GGA AAC TAt CTG CAG
CTG AAC ACA GAG CAG ATG TAC AAC TCC CTC ATG AAG ATC AGT GAG CGC
CTG AAt ACA GAG CAG ATG TAC AAC TCC CTC taG AAG taC AG
TAT GGC CCC GTG TTC ACC AIT 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
AAG aGA GGC
ATC GAG GAG CGC ATC CAG GAG GAG GCG GGC TTC CTC ATC GAC GCC CTC
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CGG GGC ACT GGC GGC GCC AAT ATC GAT CCC ACC TTC TTC CTG AGC CGC
CGG GGC ACg cGC GGC GCC AAT ATC GAT CCC ACC TTC TTC C
ACA GTC TCC AAT GTC ATC AGC TCC AIT 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 AIT GAC TCC TTT CTC
ATC CGC ATG CAG GAG GAG GAG AAG AAC CCC AAC ACG GAG TTC TAC TTG
CGC ATt CAG GAG GAG GAG AAG AAC CCC cAC ACG GAG TTC TAC TTG
AAA AAC CTG GTG ATG ACC ACG TTG AAC CTC TTC AIT GGG GGC ACC GAG
AAg AAC CTG GTG CTG ACC ACG CTG AAC CTC TTC tTT GcG GGC Act GAG
ACC GTC AGC ACC ACC CTG CGC TAT GGC TTC TTG CTG CTC ATG AAG CAC
ACC GTC AGC ACg ACC CTG CGC atT GGC TT
CCA GAG GTG GAG GCC AAG GTC CAT GAG GAG AIT 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 AAT 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 AIT GAC GTG TCC CCC AAA CAC GTG GGC TTT
GCC ACG ATC CCA CGA AAC TAC ACC ATG AGC TTC CTG CCC CGC TGA

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Figure 3.6 Marmoset CYP2A cDNA sequence (blue) compared to the published human CYP2A6 cDNA sequence (black). X indicates the deleted nucleotide C after the initiation codon ATG. The sequence matching to the reverse PCR primer sequence is shown in red. The blue sequence represents the DNA sequence determined in this study. The rest of the sequence was determined by Susanah Lindey in our laboratory.

cDNA clones as a probe (Shephard et al., 1987).

3.4 Expression of Human CYP2A6 in E. Coli

The basic approach used to express all foreign genes in *E. coli* is to insert the insert of a gene into a plasmid expression vector. This vector generally contains the following elements: 1) sequences encoding a selectable marker that allows maintenance of the vector in the cell; 2) a

controllable transcription start site (lac) which, under inducing conditions can control the amount of mRNA from the cloned gene; 3) transfer origin, such as an appropriately positioned ribosome binding site (RBS) or ATG, and 4) a multiple cloning site to simplify the insertion of the gene to be expressed in the correct orientation within the vector. Once constructed, the recombinant plasmid containing the gene to be expressed is introduced into the *E. coli* strain by transformation.

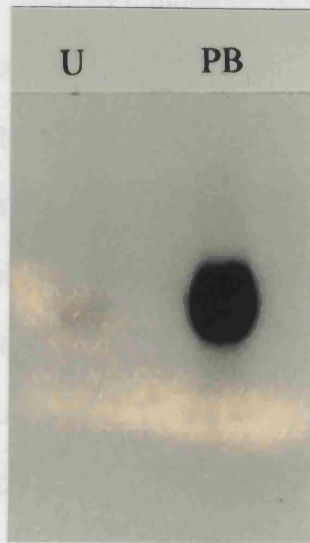


Figure 3.7 Northern blot analysis of marmoset liver RNA

Total RNA (30µg) from the livers of untreated (U) or phenobarbital treated (PB) marmosets was electrophoresed and transferred to a nylon membrane (section 2.12). The membrane was incubated with ³²P-labelled pUC19marmoset CYP2A recombinant plasmid, washed and exposed to X-ray film at -70°C overnight. The film was developed and photographed.

the natural protein; however, sometimes a different region of the protein may be desired. For instance, the N-terminus of some P450s is hydrophobic and thought to be involved in membrane anchoring; so deletion of this sequence might be used to produce a soluble protein (Jansen et al., 1991). In other cases modifying part of the cDNA sequence, for example, by alteration of the 5' sequence upstream of the start codon can improve ribosome

cDNA clone as a probe (Shephard *et al.*, 1987).

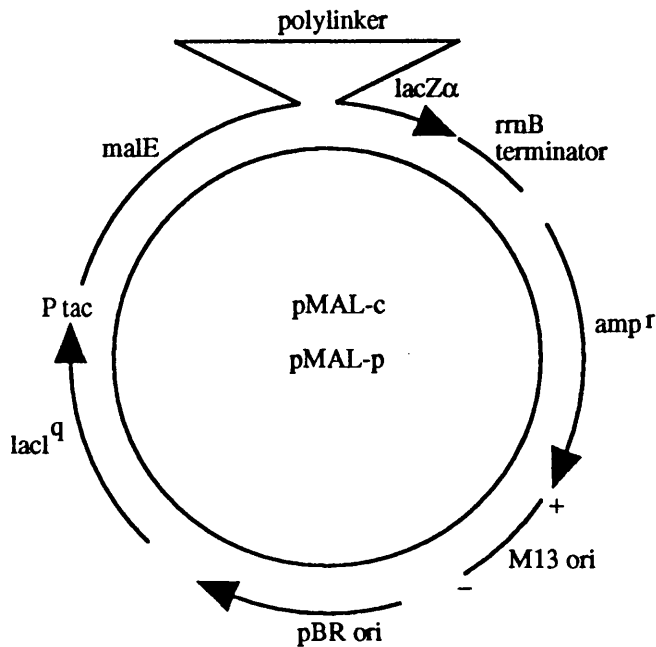
3.4 Expression of human CYP2A6 in *E. coli*

The basic approach used to express all foreign genes in *E. coli* begins with the insertion of a gene into a plasmid expression vector. This vector generally contains the following elements: 1) sequences encoding a selectable marker that assures maintenance of the vector in the cell, 2) a controllable transcriptional promoter (eg. lac or tac) which, under inducing conditions can control the production of large amounts of mRNA from the cloned gene, 3) translational control sequences, such as an appropriately positioned ribosome-binding site and an initiator ATG, and 4) a multiple cloning site to simplify the insertion of the gene in the correct orientation within the vector. Once constructed, the expression vector containing the gene to be expressed is introduced into an appropriate *E. coli* strain by transformation. Although insertion of the gene of interest into an expression vector followed by transformation in *E. coli* is common to all bacterial systems, the final application of the expressed protein usually dictates the expression strategy to be used.

In general, expression of the full length normal protein is the safest way to ensure that the recombinant gene product matches as closely as possible the natural protein; however, sometimes a shortened version of the protein may be desired. For instance, the N-terminus of most P450s is hydrophobic and thought to be involved in membrane anchoring, so deletion of this sequence might be used to produce a soluble protein (Larson *et al.*, 1991). In other cases modifying part of the cDNA sequence, for example, by alteration of the 5' sequences upstream of the start codon can improve ribosome

binding (Barnes *et al.*, 1991). Tagging the desired cDNA onto that of a more easily expressed bacterial protein may both enhance expression levels and facilitate purification of the expressed protein. The latter is the strategy used in this investigation to express CYP2A6 in *E. coli* cells. The maltose-binding protein vectors (MBP) were chosen because it was reported that these were very good for the expression of foreign proteins and that the expressed protein can be easily purified by affinity chromatography. The maltose-binding protein (MBP) vectors (pMAL vectors) allow the expression and purification of a protein encoded by a cloned gene by fusing it to MBP encoded by the *malE* gene of *E. coli* (Figure 3.8).

The gene of interest is subcloned into one of the MBP vectors, pMAL-c or pMAL-p so that the coding sequence of interest is fused in-frame to the 3' end of the *malE* gene. The vector pMAL-c has an exact deletion of the *malE* signal sequence which leads to cytoplasmic expression of the fusion protein. Fusion plasmids made with this vector usually give a higher yield of fusion protein than those made with pMAL-p. The vector pMAL-p contains the normal signal sequence of the *malE* protein which potentially directs the fusion protein through the cytoplasmic membrane to the periplasm. This promotes proper folding and disulphide bond formation for some proteins and allows purification from the periplasm (Hsiung and Becker, 1988; Lauritzen *et al.*, 1991). The MBP vectors also include a sequence that encodes the four-amino-acid recognition site for the specific protease, factor Xa. The site is placed so that it can be used to separate the protein of interest from MBP after affinity purification. Factor Xa cuts after arginine in the sequence Ile-Glu-Gly-Arg (Figure 3.9)



polylinker :

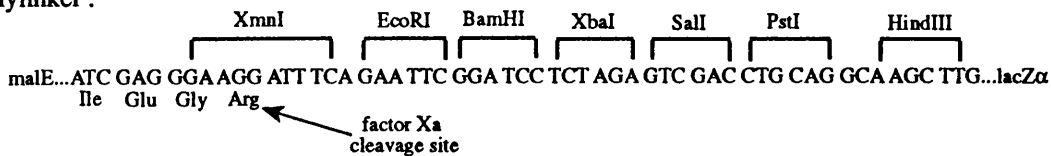


Figure 3.8 pMal Vectors.

The vectors contain the inducible Ptac promoter positioned to transcribe a malE-lacZ α gene fusion. The lacI gene encodes the lac repressor, which turns off transcription from Ptac until IPTG is added. The polylinker provides several restriction endonuclease sites to insert the gene of interest, so it is fused to the malE gene. The rrnB terminators prevent transcription from Ptac from interfering with plasmid replication or maintenance functions. pMAL-c (6646 base pairs) has an exact deletion of the malE signal sequence. pMAL-p (6721 base pairs) includes the malE signal sequence. Arrows indicate the direction of transcription. Restriction sites indicated are unique.

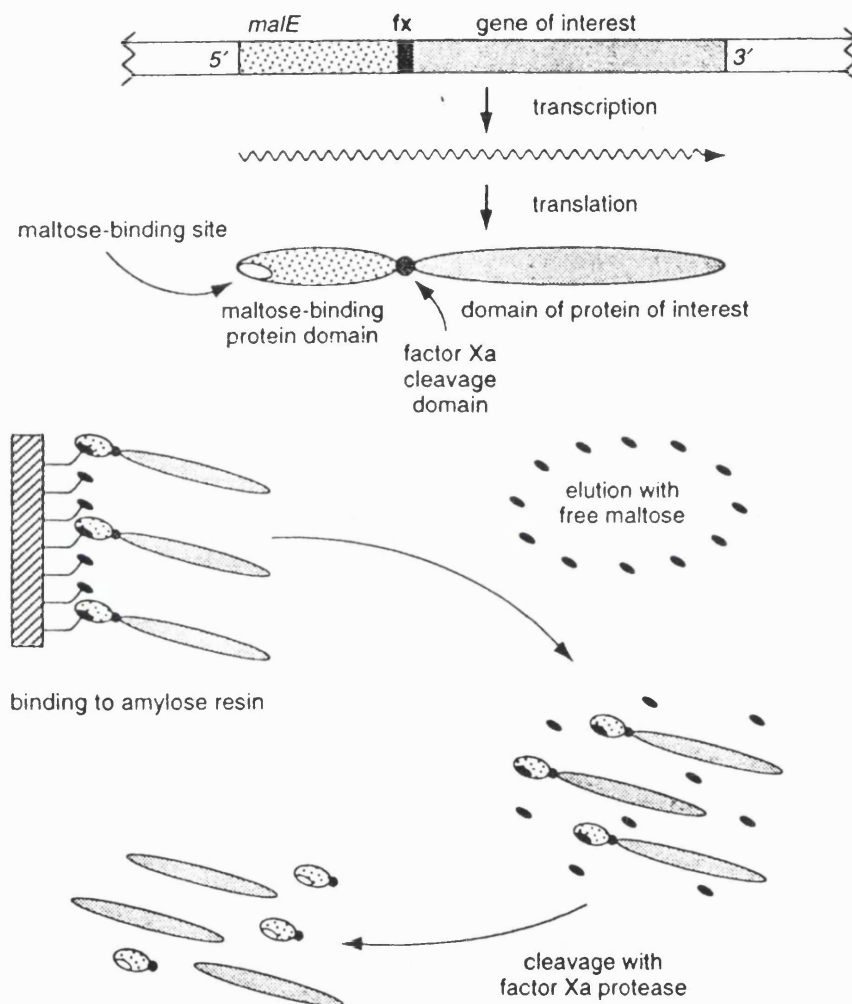


Figure 3.9 Schematic representation of expression and purification using the MBP vectors.

'fx' denotes the sequence coding for recognition site of factor Xa. The gene of interest is cloned 3' to the *malE* gene and expressed, cells are lysed, and the extracts are poured over a column of amylose resin. The MBP fusion protein binds to the column and the remaining proteins in the cell extract are washed through the column. The fusion protein is eluted with free maltose and then cleaved with factor Xa to separate MBP from the protein of interest. (Kellerman and Ferenci, 1982)

3.4.1 Construction of pMAL-c-CYP2A6 fusion plasmid

As seen from the sequence of the reverse oligoprimers (Figure 3.1), the XbaI site overlaps a site for dam methylation. This site is not cleaved efficiently in DNA isolated from dam⁺ *E. coli* (Figure 3.2, track 9). This problem was solved by introducing pUC19CYP2A6 recombinant plasmid DNA into dam⁻ *E. coli* (GM2163). The XbaI fragment was excised from the pUC19CYP2A6 construct and ligated into pMAL-c which had been digested with XbaI. *E. coli* JM109 cells were transformed with the ligation mixture and plated onto LB/Agar/Ampicillin plates. Following overnight incubation at 37°C, the transformants were plated onto LB/Agar/Ampicillin/IPTG/Xgal indicator plates. Very few (10-20) transformants were observed on some indicator plates whereas no transformants were present on other plates. Miniprep DNA from positive transformants was analysed for the presence of the CYP2A6 insert by restriction endonuclease digestion (Figure 3.10). The results show that various fragments were generated following digestion of fusion plasmids with XbaI/HindIII none of which matched the size of the original insert. These results indicate plasmid loss and instability suggesting that high level expression of CYP2A6 is toxic to *E. coli*. Although repeated several times, it was never possible to recover the CYP2A6 pMAL-c fusion plasmid from transformed *E. coli* cells.

3.4.2. Construction of pMAL-p-CYP2A6 fusion plasmid

Switching to an expression system using a pMAL-p vector could alleviate the toxicity problem encountered with pMAL-c. Fusion plasmids made with pMAL-p give a lower yield of fusion protein (5% to 10% of total

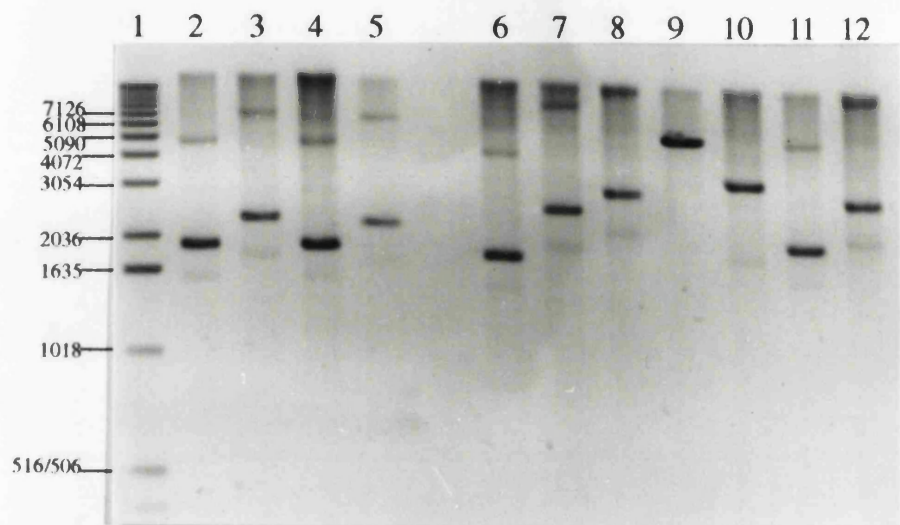


Figure 3.10 Restriction endonuclease digestion of plasmid DNA from putative positive transformants following transformation of *E. coli* JM109 cells with pMAL-c+CYP2A6 ligation mixture. Plasmid DNA from each transformant was digested with XbaI/HindIII and analysed by electrophoresis through a 0.8% agarose gel containing 0.5µg/ml ethidium bromide (tracks 2 to 12, excluding track 9). Track 9 shows XbaI/HindIII digest of plasmid DNA isolated from JM109 cells harboring pMAL-c. Track 1 shows molecular size marker. DNA was visualized and photographed under UV light.

cellular protein compared to 20% to 40% of total cellular protein with pMAL-c). Transformation of *E. Coli* JM109 cells using pMAL-p-CYP2A6 fusion plasmid resulted in a higher number of transformants as compared to transformation with the pMAL-c-CYP2A6 fusion plasmid. Restriction endonuclease digestion with XbaI/HindIII of miniprep plasmid DNA from positive transformants showed the presence of the full length *CYP2A6* cDNA insert. A single clone with the *CYP2A6* insert in the correct orientation in the pMAL-p-CYP2A6 fusion plasmid (Figure 3.11) was used for subsequent expression studies.

3.4.3 Expression of fusion protein using pMAL-p-CYP2A6 fusion plasmid

JM109 cells (100 ml culture) containing the pMAL-p-CYP2A6 fusion plasmid were grown at 37°C to exponential growth phase. IPTG (0.3 mM) was then added to induce expression of the fusion protein mRNA. The cells were grown overnight and harvested by centrifugation. A sample of total cell lysate was analysed by western blotting using an antibody against baboon CYP2A7. Initial results showed very faint protein bands on the western blot which ran between the size of the full length MBP-CYP2A6 fusion protein (M_r of 91000) and MBP (M_r of 42000). Possible explanations for these truncated proteins is the proteolysis of the fusion protein by *E. coli* proteases or (rarely) premature translation termination of the fusion. Fusion proteins expressed in *E. coli* have been shown to be degraded by *E. coli* proteases during the harvesting of the cells and preparation of crude extracts (Grodberg and Dunn, 1988; Sugimura and Higashi, 1988). Addition of protease inhibitors such as PMSF could help avoid this problem.

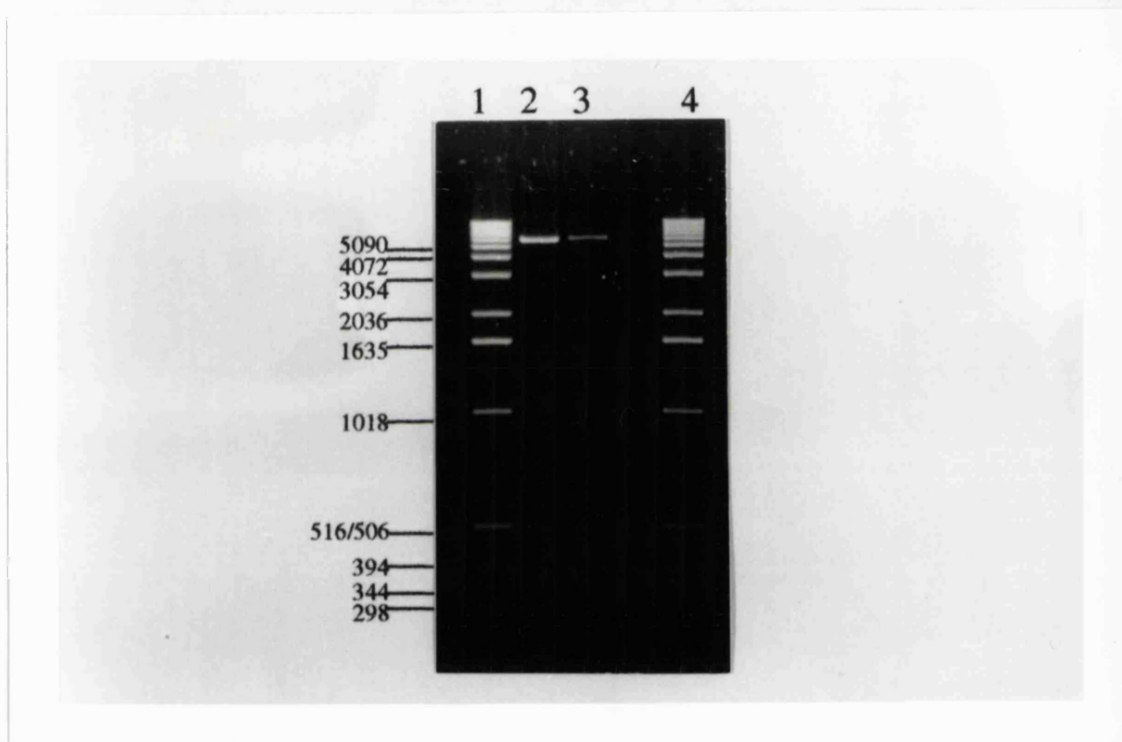


Figure 3.11 Restriction endonuclease digestion of pMAL-p-CYP2A6 fusion plasmid. pMAL-p plasmid DNA (track 2) and pMAL-p-CYP2A6 plasmid DNA (track 3) were digested with XbaI/HindIII and analysed by electrophoresis through a 0.8% agarose gel containing 0.5 μ g/ml ethidium bromide. Tracks 1 and 4 show DNA molecular size standards (BRL 1 Kb ladder). The DNA was visualised and photographed under UV light.

Additionally, lowering the temperature of expression can lead to a higher proportion of soluble full-length fusion protein (Tagaki *et al*, 1988; Schein 1989). The temperature for expression was therefore lowered to 30°C and JM109 cells bearing the pMAL-p-CYP2A6 fusion plasmid were cultured for 40 hours post induction with 0.3 mM IPTG. A 1 ml sample of the culture was taken at ten hour intervals (following the addition of IPTG) and the cells were pelleted by centrifugation. The cell pellets were resuspended in 1 x SDS/PAGE sample buffer containing PMSF at a final concentration of 1 mM. These samples were then analysed by western blotting using antibodies against baboon CYP2A7. Lowering of the temperature for growth and use of the protease inhibitor, PMSF resulted in the recovery of a full length MBP-CYP2A6 fusion (Figure 3.12).

3.4.4 Cellular localization of MBP-CYP2A6 fusion protein

Having established conditions for the expression of full length CYP2A6 an analysis was undertaken to find the cell fraction containing the expressed protein. Cytosolic and membrane fractions of *E. coli* JM109 cells expressing MBP-CYP2A6 fusion protein were prepared (section 2.17.2) and analysed by SDS/PAGE (Figure 3.13) and western blotting using antibodies against purified MBP (Figure 3.14). Results show that the fusion protein was localised in the membrane fraction of cells treated with IPTG. However, soluble protein is required for affinity purification. For this purpose, the membrane bound protein was solubilised using deoxycholate and lubrol at final concentrations of 0.4%(v/v) and 0.2% (v/v) respectively. The soluble fraction was separated by centrifugation and the fusion protein was identified by western blotting using antibodies against purified MBP (Figure 3.14, track 7).

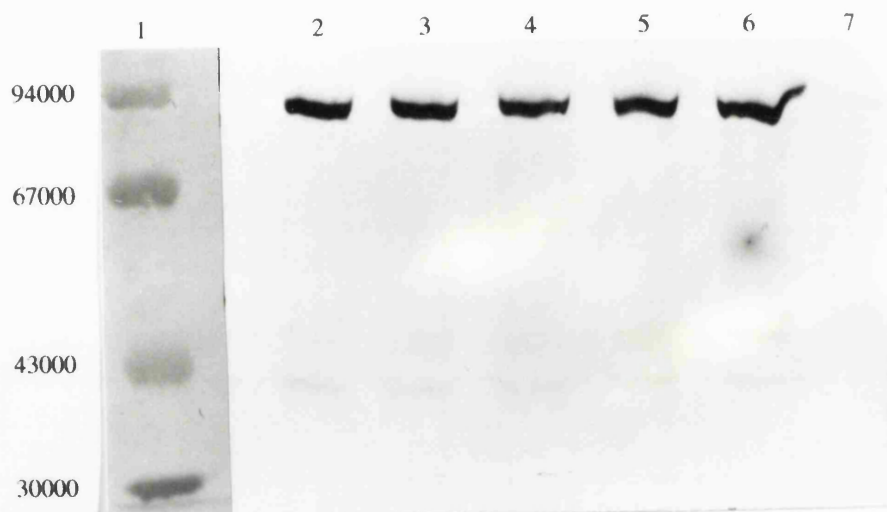


Figure 3.12 Western immunoblot analysis of *E. coli* expressed MBP-CYP2A6 fusion protein.

E. coli cells that had been transformed with pMAL-p-CYP2A6 fusion plasmid were subjected to electrophoresis on SDS- containing polyacrylamide gel and western blotting. The blot was developed by using sheep antibody against purified baboon CYP2A7 and alkaline phosphatase conjugated donkey anti-sheep IgG. Track 1 shows relative molecular weight standards.

Total cell lysates from uninduced cells (track 7) or IPTG treated cells harvested at 10 hour intervals (tracks 3 to 5).

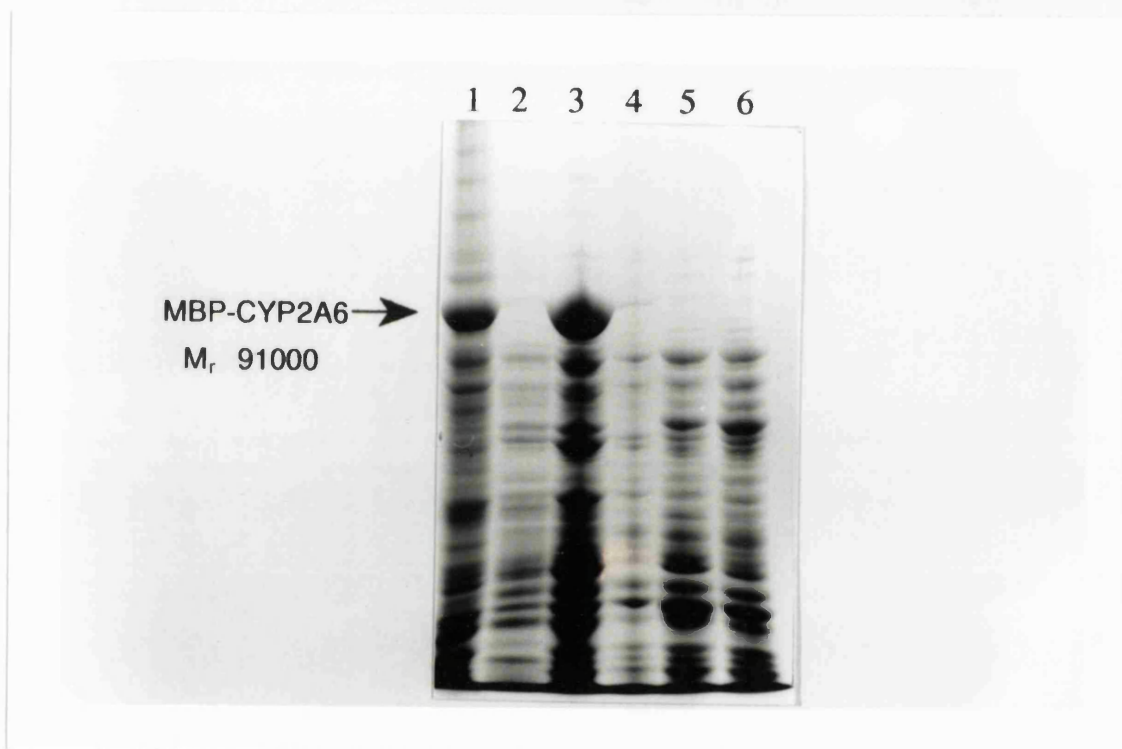


Figure 3.13 Cellular localization of MBP-CYP2A6 fusion protein (SDS/PAGE analysis)

Tracks 1, 2, 3 and 4 respectively contain membrane fraction, cytosolic fraction and total cell lysate from IPTG treated and uninduced *E. coli* cells harboring pMAL-p-CYP2A6 fusion plasmid. Tracks 5 and 6 respectively contain total cell lysate from IPTG treated and uninduced *E. coli* cells harboring pMAL-p plasmid. Samples were loaded onto SDS containing polyacrylamide gel and subjected to electrophoresis. The gel was stained with coomassie blue.

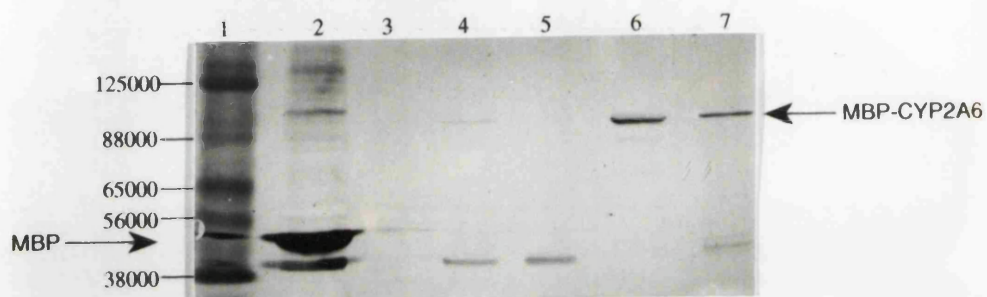


Figure 3.14 Cellular localization of MBP-CYP2A6 fusion protein
(immunoblot analysis)

Track 2 shows total cell lysate from IPTG treated *E. coli* cells harbouring the pMAL-p plasmid vector. Tracks 3 and 4 respectively show total cell lysate from uninduced and IPTG treated *E. coli* cells harbouring pMAL-p-CYP2A6 fusion plasmid. Tracks 5, 6 and 7 respectively show cytosolic fraction, membrane fraction and solubilised membranes from IPTG treated *E. coli* cells harboring pMAL-p-CYP2A6 fusion plasmid. Track 1 shows relative molecular weight standards. Samples were loaded onto SDS containing polyacrylamide gel and subjected to electrophoresis and western blotting. The blot was developed using rabbit antibody against purified MBP and HRP conjugated goat anti-rabbit IgG.

3.4.5 Spectral analysis of MBP-CYP2A6 fusion protein

The bacterial pellet from cells transformed with the pMAL-p-CYP2A6 construct were bright red in colour. This could have been due to the increased expression of the haemoprotein CYP2A6. To determine whether the fusion protein had indeed bound haem a CO-reduced difference spectrum of MBP-CYP2A6 protein was recorded as described in section 2.17.3

The results of the spectral analysis show a strong P420 component (Figure 3.15). From the western blot analysis of solubilized membranes (Figure 3.14, track 7), degradation of the MBP-CYP2A6 fusion protein is not observed. Thus the lack of a peak at 450nm in the spectrum is more likely to be due to improper folding of the expressed polypeptide possibly due to the presence of the MBP sequence.

The primary aim of this part of the investigation was not to produce a functional CYP2A6 but rather to express sufficient protein to allow antibodies to be raised against purified CYP2A6. Therefore the recovery of an expressed protein with a CO-difference spectral peak at 420nm was not of major concern. Additionally, when membranes from *E. coli* JM109 cells expressing MBP-CYP2A6 fusion protein were reconstituted in the assay for coumarin hydroxylase activity, there was no formation of 7-hydroxycoumarin from the CYP2A6 substrate, coumarin. This indicates that MBP-CYP2A6 fusion protein is enzymatically inactive.

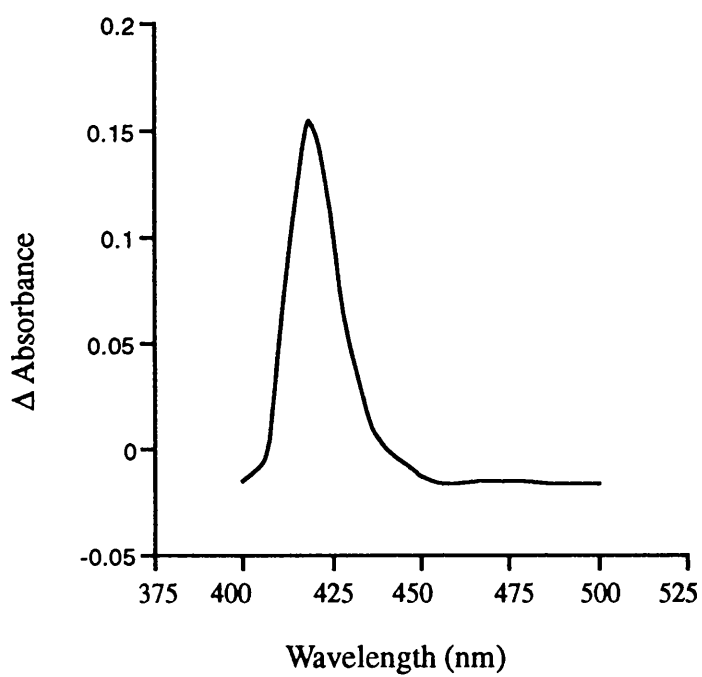


Figure 3.15 CO-Reduced difference spectrum of E. Coli expressed MBP-CYP2A6 fusion protein.

3.5 Purification of MBP-CYP2A6 fusion protein

3.5.1 Affinity of MBP-CYP2A6 fusion protein to amylose resin

Binding of MBP-CYP2A6 fusion protein to amylose resin was analysed by mixing a sample of protein (50 µg) solubilised from bacterial membranes and 50 µl of a slurry of amylose resin (section 2.17.4). The supernatant containing unbound proteins and the amylose resin fraction were analysed by western blotting using antibodies against purified MBP. Results indicate that, under the conditions suggested by the manufacturer, the solubilised MBP-CYP2A6 fusion protein does not bind to amylose resin (Figure 3.16). This could be due to a low intrinsic affinity of the fusion protein for the amylose resin or the presence of some intrinsic factor in the extract that interferes with binding. A low intrinsic affinity could be caused by an interaction between CYP2A6 and MBP that either blocks or distorts the maltose binding site of the MBP part of the fusion protein. Non ionic detergents present in the solubilized membrane fraction may also interfere with binding of the protein to the resin. Therefore the effect of the presence of detergents on the affinity of the MBP-CYP2A6 fusion protein for the amylose resin was analyzed.

50 µg of proteins solubilised from bacterial membranes were diluted at 1:10 and 1:100 in column buffer. To one undiluted sample, SDS was added to a final concentration of 0.1% (w/v) (section 2.17.4). SDS was added in anticipation that it would relax the fusion protein and unblock the maltose binding site. As a control, purified MBP-paramyosin fusion protein (0.5 µg) was resuspended in column buffer containing deoxycholate and lubrol at a final concentration of 0.4% (v/v) and 0.2% (v/v) respectively. Each sample was mixed with a 50 µl slurry of amylose resin (section 2.17.4) The supernatants containing unbound proteins and the amylose resin fractions were analysed

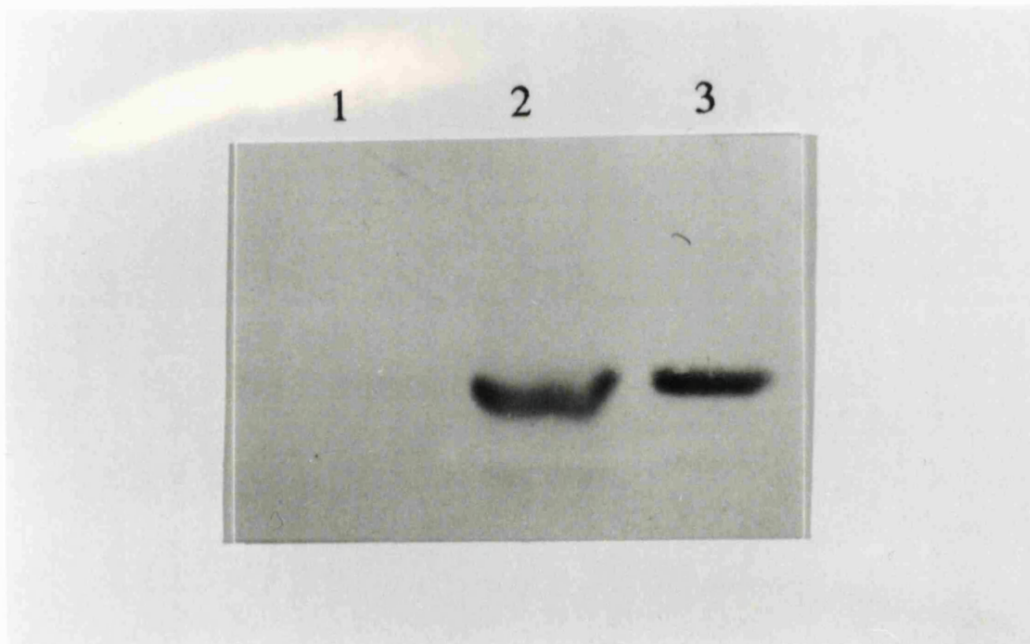


Figure 3.16 Affinity of MBP-CYP2A6 fusion protein to amylose resin. Amylose resin bound fraction (track 1), the supernatant containing unbound protein (track 2), and proteins solubilised from bacterial membranes (track 3) were loaded onto a SDS containing polyacrylamide gel and subjected to electrophoresis and western blotting. The blot was developed using rabbit antibody against purified MBP and HRP conjugated goat anti- rabbit IgG.

by western blotting using antibodies against purified MBP (Figure 3.17)

The results (Figure 3.17) show that, diluting the protein samples to counteract the effect of detergents or addition of SDS did not improve the binding of the MBP-CYP2A6 fusion protein to amylose resin. Additionally, even in the presence of detergents, MBP-paramyosin fusion protein did bind to amylose resin. These results suggest that the lack of affinity of the MBP-CYP2A6 fusion protein for the amylose resin may be due to an interaction between CYP2A6 and MBP that either blocks or distorts the maltose binding site.

An alternative purification strategy was then used in an attempt to purify the fusion protein. This involved the use of p-chloramphenicol-coupled Sepharose which has been used successfully to purify brain P450s (Sundin *et al.*, 1987).

3.5.2 Purification of MBP-CYP2A6 fusion protein by Chromatography on p-chloramphenicol-coupled Sepharose

Membranes from *E. coli* expressing the MBP-CYP2A6 fusion protein were solubilized and subjected to purification as described in section 2.19. Fractions from each stage of the purification were analysed by SDS/PAGE (Figure 3.18) and western blotting using an antibody against baboon CYP2A7 (Figure 3.19). The results indicate that only partial purification of the fusion protein was achieved on the affinity column (Figure 3.18). Western blot analysis of fractions from each stage of the purification show that the binding of MBP-CYP2A6 fusion protein to the column is weak as the bulk of the fusion protein is

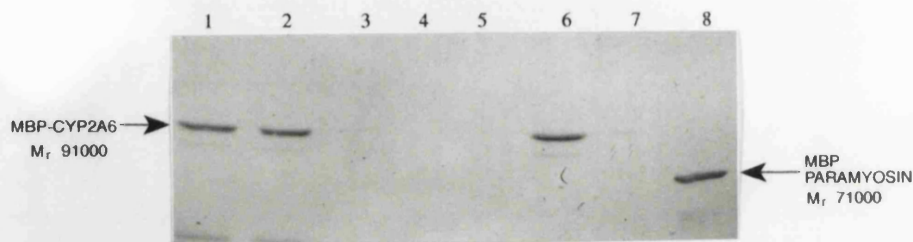


Figure 3.17 Analysis of the effect of detergent concentration on the affinity of MBP-CYP2A6 fusion protein for amylose resin. Tracks 1 and 2 show supernatants (unbound protein) from samples diluted at 1:10 and 1:100 respectively. Tracks 3 and 4 show the amylose resin fraction from samples diluted at 1:10 and 1:100 respectively. Track 5 shows the amylose resin fractions from a sample of solubilized microsomal membranes containing 0.1% (w/v) SDS. Track 6 shows the supernatant from a sample containing 0.1% (w/v) SDS. Track 7 shows supernatant from a sample containing purified MBP-paramyosin fusion protein. Track 8 shows amylose resin fraction from the sample containing purified MBP-paramyosin fusion protein. Samples were loaded onto a SDS containing polyacrylamide gel and subjected to electrophoresis and western blotting. The blot was developed using rabbit antibody against purified MBP and HRP conjugated goat anti- rabbit IgG.

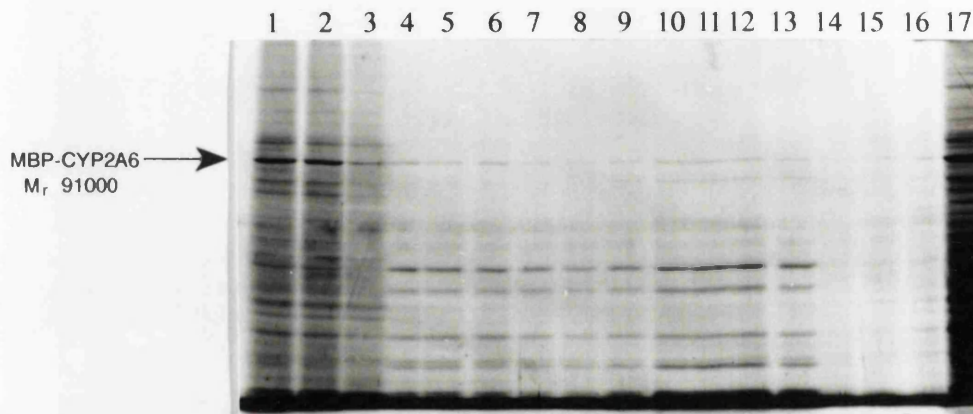


Figure 3.18 Purification of MBP-CYP2A6 fusion protein by chromatography on *p*-chloramphenicol-coupled Sepharose (analysis by SDS/PAGE). Tracks 1, 2 and 3 show the flow through fractions following the application of solubilized membranes onto the column. Tracks 4 to 8 show the fractions collected during the washing of the column. Tracks 9 to 16 show the fractions collected during the elution of proteins from the column. Track 17 shows the sample from solubilized membranes prior to purification. Samples (20 μ l) were subjected to electrophoresis through SDS containing polyacrylamide gel. The gel was silver stained using the Bio-Rad silver staining kit.

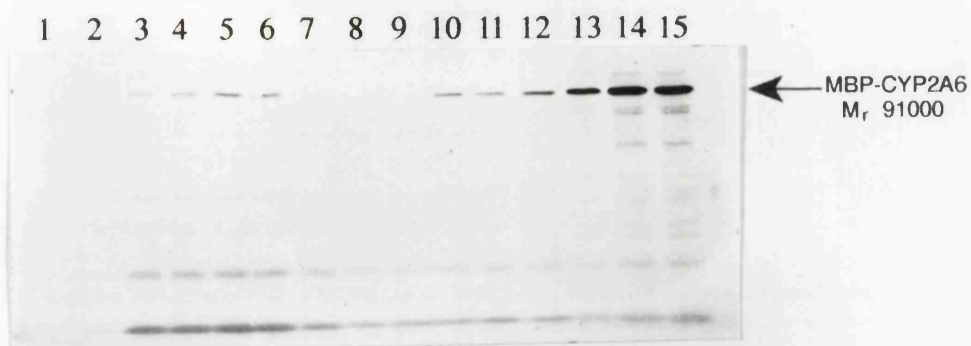


Figure 3.19 Purification of MBP-CYP2A6 fusion protein by chromatography on p-chloramphetamine-coupled Sepharose (analysis by western blotting) Tracks 1 to 6 show the fractions collected during the elution of proteins from the column. Tracks 7 to 10 show the fractions collected during the washing of the column. Tracks 11 to 14 show the flow through fractions following the application of solubilized membranes to the column. Track 15 shows the sample from solubilized membranes prior to purification. Samples (20 μ l) were electrophoresed through a SDS containing polyacrylamide gel and subjected to western blotting. The blot was developed by using sheep antibody against purified baboon CYP2A7 and alkaline phosphatase conjugated donkey anti-sheep IgG.

seen in the flow through fraction (Figure 3.19, tracks 11-14). Although the binding of the fusion protein to the affinity column is weak the elution fractions are “enriched” for this protein. Additionally, it is observed that the fusion protein is partially degraded during the purification process. Further purification of the fusion protein could possibly be achieved using an alternative method such as DE52 chromatography. Because of lack of time no other purification strategies were tried in an attempt to purify MBP-CYP2A6 fusion protein.

Although the expression of MBP-CYP2A6 fusion protein in bacteria was successful it was not possible to purify the protein in sufficient amounts to permit factor Xa cleavage of MBP from CYP2A6. An alternative expression system was then adopted. It was hoped that the use of the baculovirus/insect cell system would permit the expression of functional CYP2A6 and produce sufficient protein for purification.

3.6 Baculovirus mediated expression of human CYP2A6

Baculoviruses have become a popular system for overproducing recombinant proteins in eukaryotic cells (O’ Reilly *et al.*, 1992). Several factors have contributed to this popularity. Unlike bacterial expression systems, the baculovirus-based system is a eukaryotic expression system and thus uses many of the protein modification, processing and transport systems present in higher eukaryotic cells. In addition, the baculovirus expression system uses a helper- independent virus that can be propagated to high titres in insect cells adapted for growth in suspension cultures at 27°C without the need for CO₂, making it possible to obtain large amounts of recombinant proteins (50-500

mg/l). The majority of the overproduced protein remains soluble in insect cells in contrast to the insoluble proteins often obtained from bacteria. Furthermore the viral genome is large (130 Kb) and thus can accommodate large segments of foreign DNA. Additionally, baculoviruses are non infectious to vertebrates, and their promoters have been shown to be inactive in mammalian cells (Carbonell and Miller, 1987), which gives them a possible advantage over other systems when expressing oncogenes or potentially toxic proteins.

The most widely used baculovirus system utilizes *Spodoptera fugiperda* (Sf9) cells derived from fall armyworm ovaries and a lytic virus known as *Autographa Californica nuclear polyhedrosis virus* (AcMNPV) from the family *Baculoviridae*.

The baculo portion of the name refers to the rod-shaped capsids of the virus particles. Within the capsid, the double stranded, covalently closed circular DNA is condensed into a nucleoprotein structure known as the core. The capsid plus the core are collectively referred to as the nucleocapsid. Nucleocapsids are made in the nucleus of infected cells and acquire a membrane (i.e. are enveloped) either within the nucleus where they are produced or by budding through the plasma membrane of the infected cell. The plasma membrane budded form of the virus is referred to as non-occluded virus. Virions that obtain their envelopes within the nucleus are usually occluded within the crystalline protein matrix known as polyhedrin. The occluded virus particle together with the polyhedrin are referred to as occluded virus. In this study, some unique features of the viral life cycle have provided an important aid for construction, isolation and propagation of the recombinant virus.

For replication in cell culture, the infection cycle can be considered to occur in three basic phases: early, late and very late. Briefly, these three phases correspond biologically to: 1) reprogramming the cell for virus replication, 2) producing budded virus and 3) producing occluded virus.

During the early phase of replication, 0 to 6 hours post infection, the budded virus enters the cell by adsorptive endocytosis. Nucleocapsids migrate through the cytoplasm to the nucleus where the core is released. Viral RNA can be detected 30 minutes post infection. Cytoskeletal rearrangements and enlargement of the nucleus occur within the cells during the first 6 hours of infection. During the late phase of infection which extends from 6 hours post infection to approximately 20 to 24 hours post infection, there is extensive viral DNA replication and late gene expression resulting in accumulation of viral proteins in the nucleus. At this stage, an electron dense structure known as virogenic stroma forms accompanied with the formation of nucleocapsids. Virogenic stroma is a good indicator of the state of infection of the cells and is clearly visible 20 hours post infection under a phase contrast microscope.

During the late phase, progeny nucleocapsids leave the nucleus and move to the cytoplasmic membrane where they are enveloped through budding. Production of budded virus occurs exponentially from approximately 12 hours post infection to 20 hours post infection, decreasing in rate thereafter.

During the very late phase, 20 to 24 hours post infection, nucleocapsids interact with membrane envelope segments within the nucleus and become enveloped. The enveloped virions are then embedded within the polyhedrin matrix of an occlusion body. At 24 hours post infection, occlusion bodies can be seen within the nucleus of the infected cells. At 48 hours post infection, the nucleus of the infected cells becomes virtually filled with occlusion bodies.

The crystalline nature of the polyhedra gives a highly refractive appearance to groups of infected cells or plaques. Synthesis of polyhedrin and a p10 protein which forms the mass of fibrous material mainly in the nucleus continues following 24 hours post infection. The replication cycle of the virus is terminated following the lysis of the cell and release of occlusion bodies (approximately 70 hours post infection). At this time, polyhedrin constitutes 50% of the total cellular protein.

The baculovirus expression system takes advantage of several facts about polyhedrin protein: 1) that it is expressed to very high levels in infected cells where it constitutes more than half of the total cellular protein late in the infection cycle, 2) that it is nonessential for infection or replication of the virus, meaning that the recombinant virus does not require any helper function, and 3) that the viruses lacking the polyhedrin gene have a distinct plaque morphology from viruses containing the gene. Recombinant baculovirus are generated by replacing the polyhedrin gene with a foreign gene through homologous recombination.

3.6.1 Replacement of the polyhedrin gene through homologous recombination

Manipulation of the baculovirus genome at the molecular level is challenging, as they comprise some 130 Kb of DNA, too large to be amenable to conventional plasmid cloning techniques. The traditional solution to this problem has been to introduce foreign genes, as a cassette also comprising a suitable promoter and termination sequences, by homologous recombination. This is accomplished by flanking the cassette, in a plasmid vector, by viral DNA sequences which flank the point at which it is to be inserted. Viral DNA

and this plasmid “ transfer vector” are cotransfected into insect cells, and the cassette will recombine into the wild type baculovirus genome. However the efficiency of this technique is less than 1%.

In 1990, Paul Kitts and coworkers observed that it is possible to introduce foreign genes into the baculovirus genome if it is in a linear form. They derivatized wild-type baculovirus DNA by introducing a unique restriction site (Bsu 361) at the polyhedrin locus. Linearizing the baculovirus genome using this restriction site reduced the infectivity of the viral DNA on transfection into insect cells, however, co-transfection with a transfer vector driving recombination into the polyhedrin locus produced the higher proportion of recombinant viruses. Between 10% to 25% of the progeny viruses from such cotransfection are recombinant due to the reduced background of wild type viruses. The subsequent development of this system effectively combined it with both a visual (lacZ-based) and a replication based strategy (Kitts and Possee, 1993). As shown in Figure 3.20 , recombination occurs between a conventional transfer vector and a derivative of the wild type baculovirus genome, containing lacZ at the polyhedrin locus and two further Bsu361 sites (at ORF 603 and ORF 1629) in the polyhedrin flanking sequences. Bsu361 digestion of this baculovirus DNA not only linearizes the molecule, but also removes two genomic fragments, thus disrupting the open reading frame ORF 1629. This gene is essential for baculovirus replication, so, with the two Bsu361 fragments removed from the genome, competent viruses will only be reconstituted by recombination with the transfer vector, whereby an intact ORF1629 will be restored to the genome. This strategy yields recombinant viruses at a frequency of 85-99%. The linear virus genome system depends on the ability to perform plaque assays and to produce good

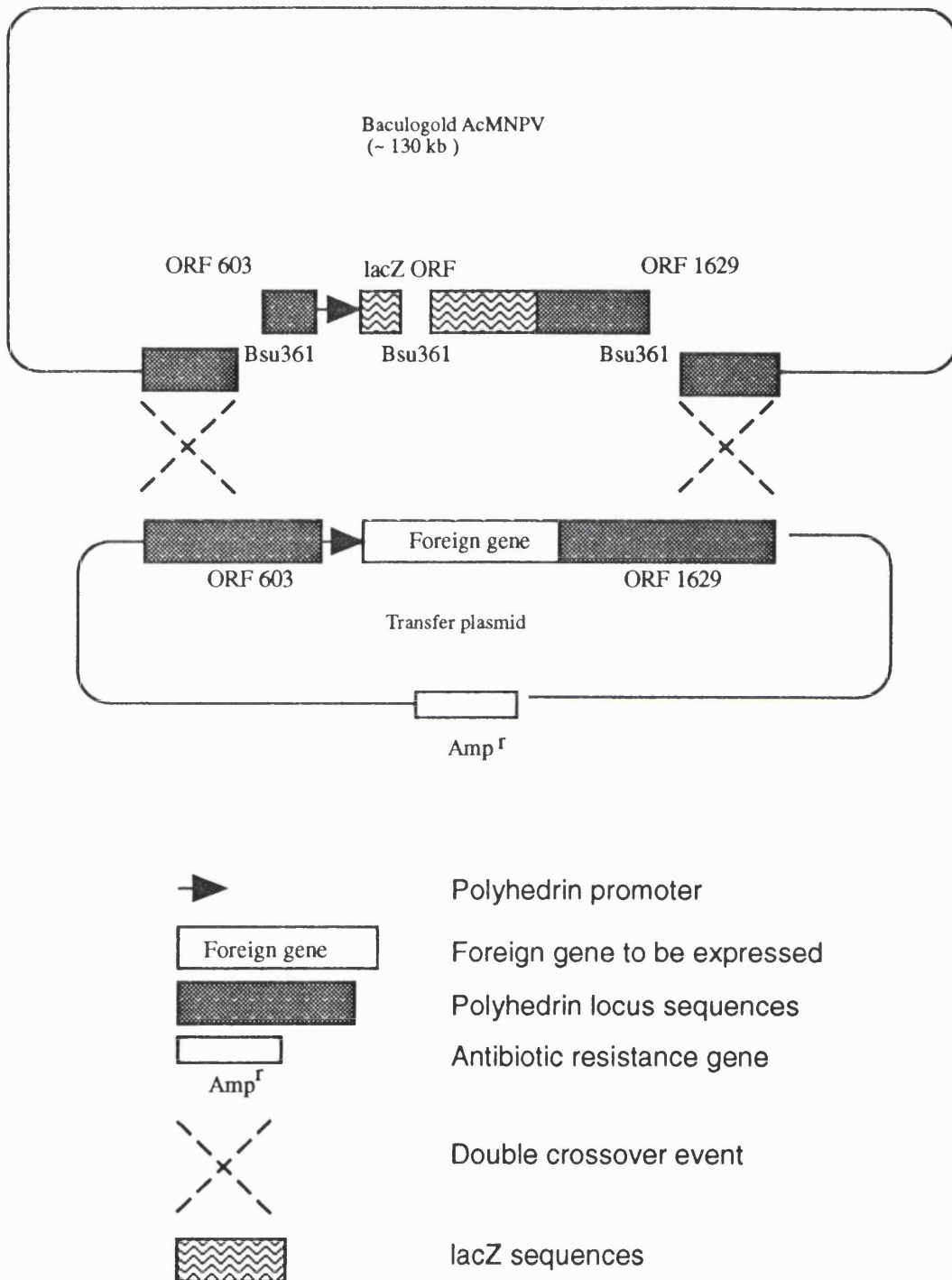


Figure 3.20 Replacement of polyhedrin gene through homologous recombination.

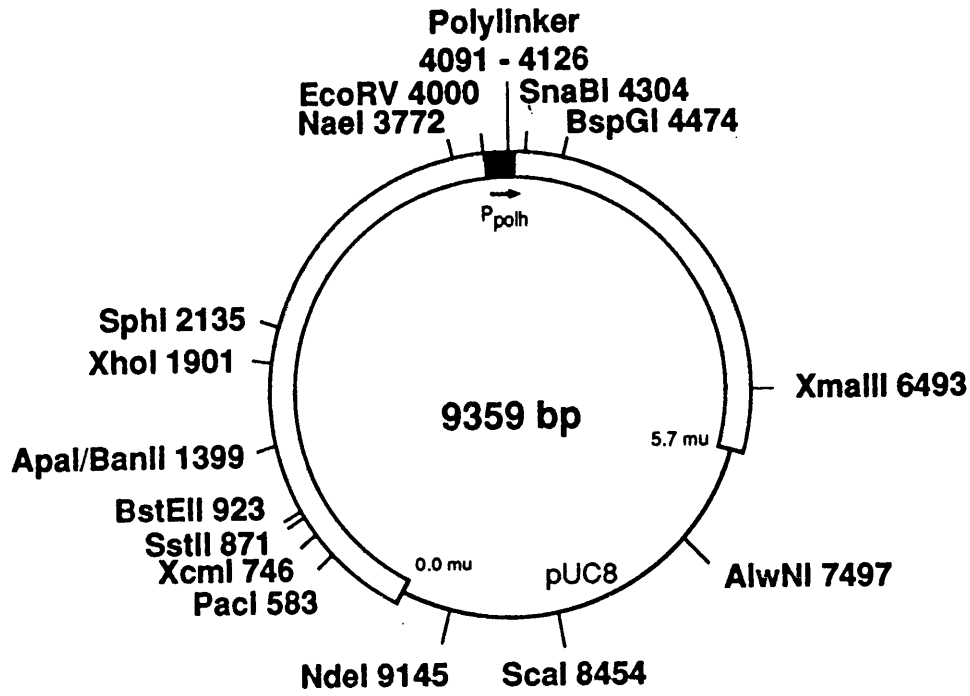
quality Bsu361 digested DNA. Ready to use linear virus DNA is available commercially .

3.6.2 Isolation of recombinant viruses and expression of CYP2A6

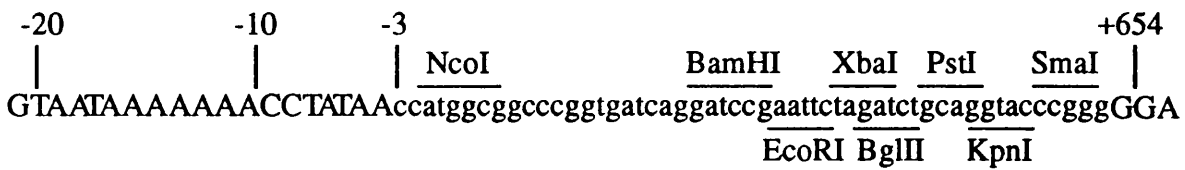
cDNA encoding full length CYP2A6 was inserted at the Nco1/EcoR1 sites downstream of the polyhedrin promoter in the baculovirus transfer vector, pAcC5 (Figure 3.21) Recombinant baculovirus were generated by co-lipofection of Sf9 cells with recombinant transfer vector and linearized AcMNPV DNA as described in section 2.18.3. Lysates of cells infected during the second passaging of each recombinant virus contained a polypeptide of the expected molecular weight of 49000 detected by western blotting using antibodies raised against CYP2A7 isolated from baboon liver. No signal was obtained with the lysates of non-infected cells or cells infected with wild type baculovirus (Figure 3.22).

3.6.3 Sub-cellular localization of expressed CYP2A6

Analysis of subcellular fractions of the insect (Sf9) cells by SDS/PAGE (Figure 3.23) and western blotting (Figure 3.24) revealed that the expressed CYP2A6 was mainly located in the microsomal fraction.



Polylinker



Plasmid	pAcC5
Promoter	P _{polh}
Parent virus	wt AcMNPV
Plaque phenotype	Occ -
Related plasmids	pAcC4
Selection in <i>E.coli</i>	Amp ^r
Source	O'Reilly et al (1992)

Figure 3.21 Baculovirus transfer vector pAcC5

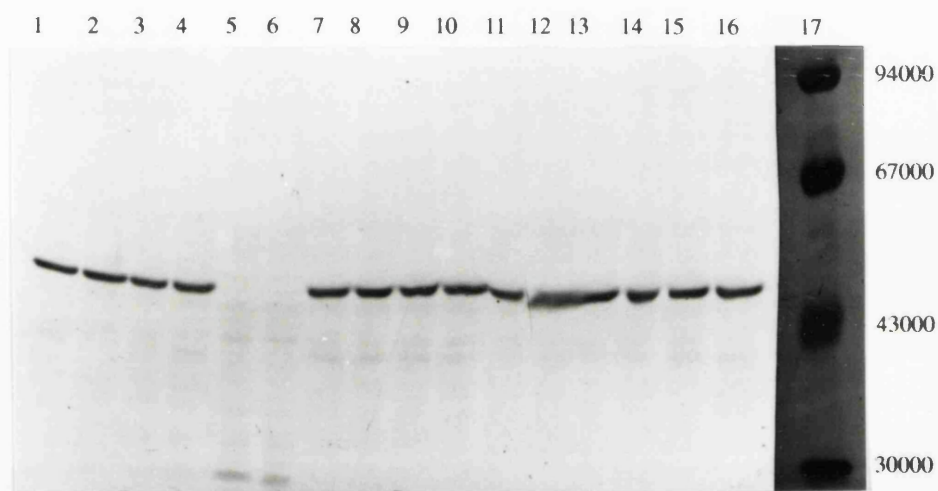


Figure 3.22 Immunodetection of CYP2A6.

Total cell lysates from Sf9 cells infected during the second passaging of each recombinant virus isolate (tracks 1 to 16, except tracks 5 and 6) and from uninfected cells (track 5) and from cells infected with wild type baculovirus (track 6) were subjected to SDS containing polyacrylamide gel electrophoresis and western blotting. The blot was developed by using sheep antibody against purified baboon CYP2A7 and alkaline phosphatase conjugated donkey anti-sheep IgG.

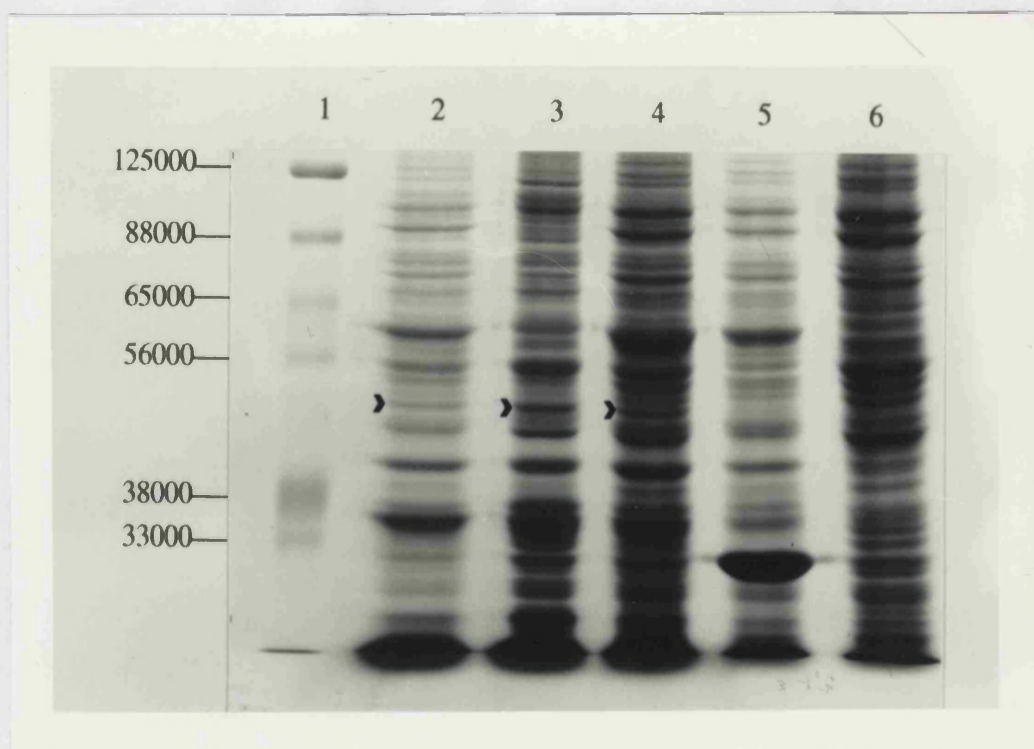


Figure 3.23 Analysis by SDS/polyacrylamide gel electrophoresis of lysates and subcellular fractions of Sf9 cells. Nuclear-mitochondrial fraction (track 2), microsomal fraction (track 3), and total cell lysate (track 4) of cells infected with recombinant baculovirus. Total cell lysates of cells infected with wild type baculovirus (track 5) or of uninfected cells (track 6).Track 1 shows relative molecular weight standards. Samples were subjected to electrophoresis through a SDS containing polyacrylamide gel followed by Coomassie staining. Arrow head indicates the position of the expressed CYP2A6.

3.2.7 Enzymatic activity of baculovirus-expressed CYP2A6

The metabolism of coumarin in man results in the formation of 7-hydroxycoumarin as a major metabolite (Eggen *et al.*, 1990). Coumarin hydroxylase activity is exceptionally high in human liver microsomes compared with the activities observed in the livers of different animal species (Perkunan *et al.*, 1995). With the aid of the antigenic mouse enzyme, it has been shown that this reaction is catalysed mainly by a P450 isoform

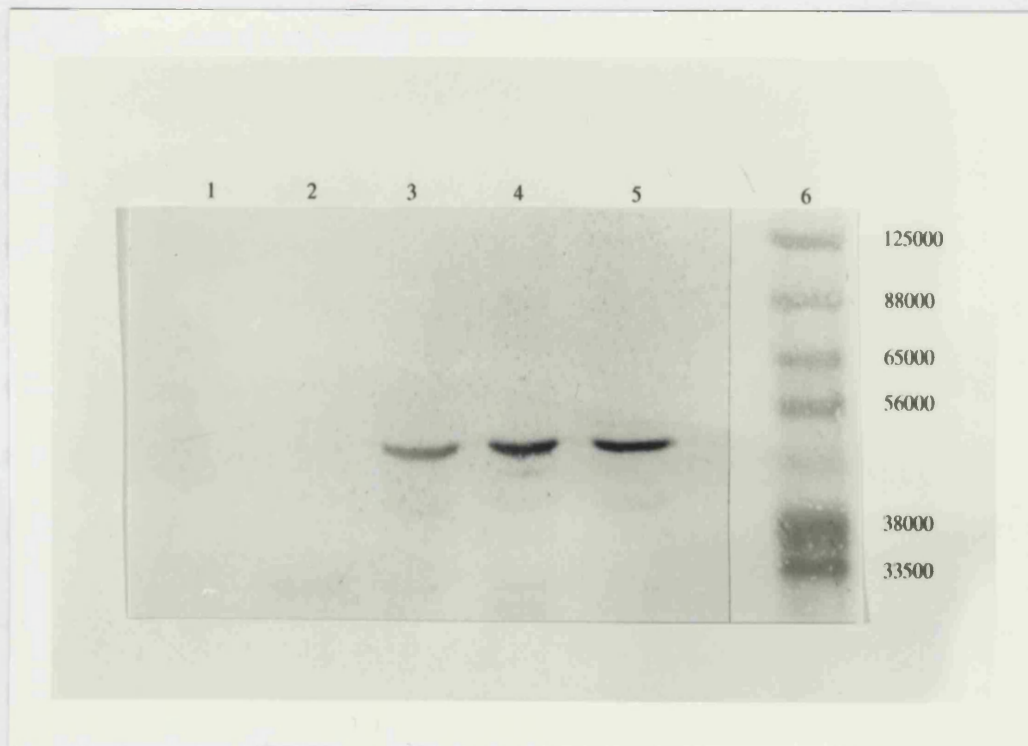


Figure 3.24 Western blot analysis of lysates and subcellular fractions of Sf9 cells.

Total lysates of cells infected with wild type baculovirus (track 1) or uninfected cells (track 2). Nuclear- mitochondrial fraction (track 3), microsomal fraction (track 4), and total cell lysate (track 5) of cells infected with recombinant baculovirus. Track 6 shows relative molecular weight standards. The blot was developed by using sheep antibody against purified baboon CYP2A7 and alkaline phosphatase conjugated donkey anti-sheep IgG.

3.7 Enzymatic activity of baculovirus-expressed CYP2A6

The metabolism of coumarin in man results in the formation of 7-hydroxycoumarin as a major metabolite (Egan *et al.*, 1990). Coumarin hydroxylase activity is exceptionally high in human liver microsomes compared with the activities observed in the livers of different animal species (Pelkonen *et al.*, 1985). With the aid of the antibody to the mouse enzyme, it has been shown that this reaction is catalysed mainly by a P450 isoform belonging to the CYP2A subfamily (Raunio *et al.*, 1988). This was later confirmed when purified human CYP2A6 was shown to catalyse coumarin 7-hydroxylation (Yun *et al.*, 1991). *CYP2A6* cDNA clones isolated from human liver cDNA libraries have been shown to encode for P450s with high coumarin hydroxylase activity when expressed using *Vaccinia virus*/human hepatoma (Hep G2) cell expression system (Yamano *et al.*, 1990). In mouse, Negishi *et al.* (1989) have characterized three P450s in the CYP2A subfamily. Mouse CYP2A4-15 α carries out testosterone 15 α -hydroxylation where as CYP2A5-coh^h and CYP2A5-coh^l are allelic variants having high and low coumarin 7-hydroxylase activities respectively, and differing at only a single nucleotide associated with the codon Ala117 in CYP2A5-coh^l and Val117 in CYP2A5-coh^h. Substitution of Phe at position 209 to Leu, out of a possible eleven differences between CYP2A4 and CYP2A5, conferred the testosterone 15 α -hydroxylase activity. The nucleotide sequence of the *CYP2A6* cDNA isolated in this study shows the codon for Val at position 117 and Phe at position 209 (Figure 3.3B) The expressed protein was expected to have coumarin 7-hydroxylase activity and no testosterone 15 α -hydroxylase activity. The interest

of this study was to analyze whether the baculovirus expressed CYP2A6 was enzymatically active in the metabolism of coumarin and testosterone.

Several reports concerning the expression of haemoproteins such as prostaglandin endoperoxide synthase-1 (Shimokawa and Smith, 1992), myeloperoxidase (Taylor *et al.*, 1992) and CYP2A1 (Asseffa *et al.*, 1989) using baculovirus expression system, agree with the observation that the enzymatic activity observed is less in comparison to the amount of apoprotein expressed. Asseffa *et al.* (1989) observed that there was a 6-fold increase in testosterone hydroxylase activity of expressed CYP2A1 on addition of hemin to the insect cell culture medium.

A series of experiments carried out by Phillippe Clair (Queen Mary and Westfield College, London) to analyze the incorporation of haem into expressed cytochromes P450 showed that addition of hemin (6 μM) to the culture medium during the course of infection with the virus allowed the formation of cytochrome P450 holoenzyme. However, the spectra obtained showed a strong P420 component which increased with hemin concentration in a non-saturable manner as a result of non specific adsorption of hemin to cellular components. To optimize haem incorporation into P450, 24 hours before infection, the culture medium was supplemented with 5-Aminolevulinate (5-ALA) and a source of Fe^{3+} (e.g Iron citrate, Iron dextran and Iron EDTA). This study showed that (i) lack of spectrally detectable P450 was due to a deficiency of the medium rather than to the aggregation or misfolding of the expressed polypeptides, (ii) the limiting factor is protoporphyrin synthesis rather than Iron, (iii) 5-ALA is not toxic to Sf9 cells up to 1mM, whereas Iron toxicity strongly depends on the chelator used and (iv) a concentration of 100 μM for both 5-ALA and Iron citrate supports the same

amount of formation of holo P450 as obtained with hemin but without the appearance of the strong P420 component. These optimised media conditions were used in the following studies when analyzing the enzymatic activities of the baculovirus expressed CYP2A6.

3.7.1 Coumarin 7-hydroxylase activity of the expressed CYP2A6

i) Coumarin 7-hydroxylase activity in Sf9 cells infected with recombinant baculovirus expressing CYP2A6

Sf9 cells were incubated for 24 hours at 27°C with 5-ALA and iron citrate each at final concentration of 100 µM. Cells were then infected with recombinant baculovirus and 24 hours post infection, coumarin solution was added to the culture medium at a final concentration of 50 µM. 48 hours post infection, the culture medium was collected and analysed for the formation of 7-hydroxycoumarin as described in the methods section. Under the same conditions Sf9 cells infected with wild type baculovirus (AcMNPV) were used as control. Formation of 7-hydroxycoumarin was only detected in the supernatant from Sf9 cells infected with recombinant baculovirus (Figure 3.25). This result shows that, the baculovirus expressed CYP2A6 is enzymatically active as a coumarin hydroxylase.

ii) Coumarin 7-hydroxylase activity in the microsomal fraction of Sf9 cells infected with recombinant baculovirus expressing CYP2A6.

Coumarin 7-hydroxylase activity of recombinant CYP2A6 was analysed in a reconstituted system using microsomes from Sf9 cells infected with recombinant baculovirus expressing CYP2A6. Conditions of incubation and measurement of 7-hydroxycoumarin were as described in section 2.18.7.1 (ii).

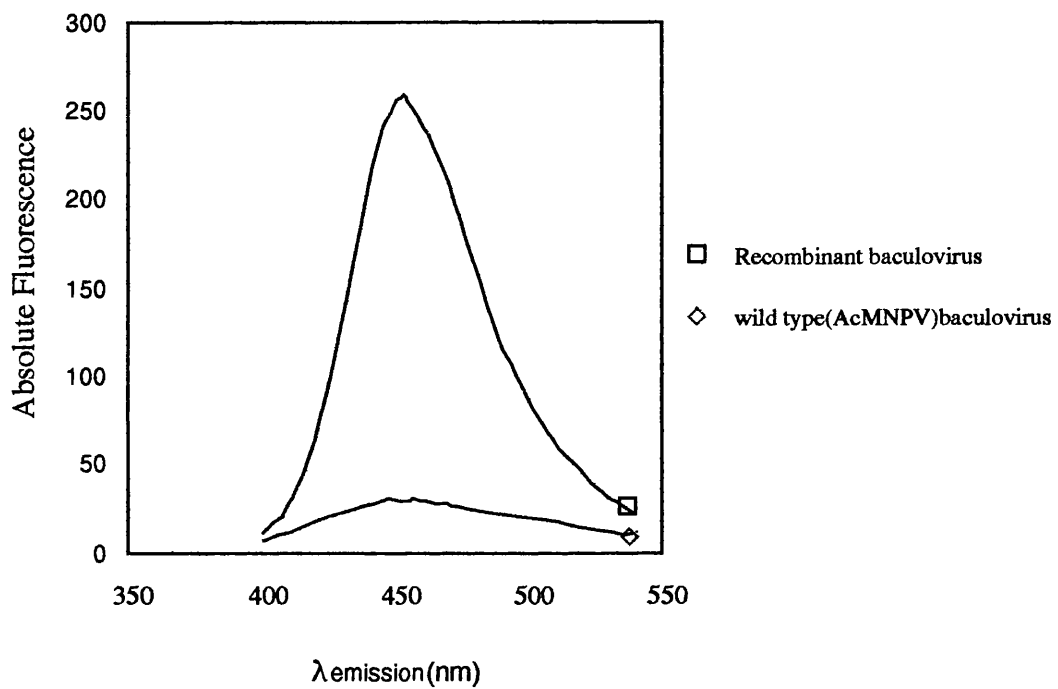


Figure 3.25 Formation of 7-hydroxycoumarin from coumarin in a culture medium of Sf9 cells infected with recombinant baculovirus expressing CYP2A6 or wild type baculovirus (AcMNPV).

In a parallel experiment, microsomes from Sf9 cells infected with wild type baculovirus (AcMNPV) were used as control. Results indicate that the progression of the reaction catalysed by microsomes from Sf9 cells infected with recombinant baculovirus is not linear (Figure 3.26). Coumarin was used in excess in the reaction mixture and thus, the concentration of the substrate could not account for the non-linearity of the reaction. A series of experiments were set up to determine if the amount of insect NADPH-cytochrome P450 reductase present in the microsomal membranes was limiting in the enzyme reaction.

iii) The effect of adding exogenous NADPH-cytochrome P450 reductase on coumarin hydroxylase activity in microsomes from Sf9 cells infected with recombinant baculovirus.

The effect of adding exogenous NADPH-cytochrome P450 reductase on coumarin 7-hydroxylase activity was analysed in a reconstituted system using microsomes from Sf9 cells infected with recombinant baculovirus expressing CYP2A6 and purified rat NADPH-cytochrome P450 reductase or microsomes from Sf9 cells infected with recombinant baculovirus expressing full length human NADPH-cytochrome P450 reductase. Conditions of incubation and measurement of 7-hydroxycoumarin were as described in section 2.18.7.1 (iii).

7-hydroxycoumarin standards (0-4 μ M range) made up under the same reaction conditions gave a linear graph with the regression coefficient of 7.73×10^7 units of fluorescence/M and a correlation coefficient of 0.99. Initial rates of reaction for each of the above curves were calculated from the region of the curve between 0 and 20 minutes and expressed in pmoles/min/mg of

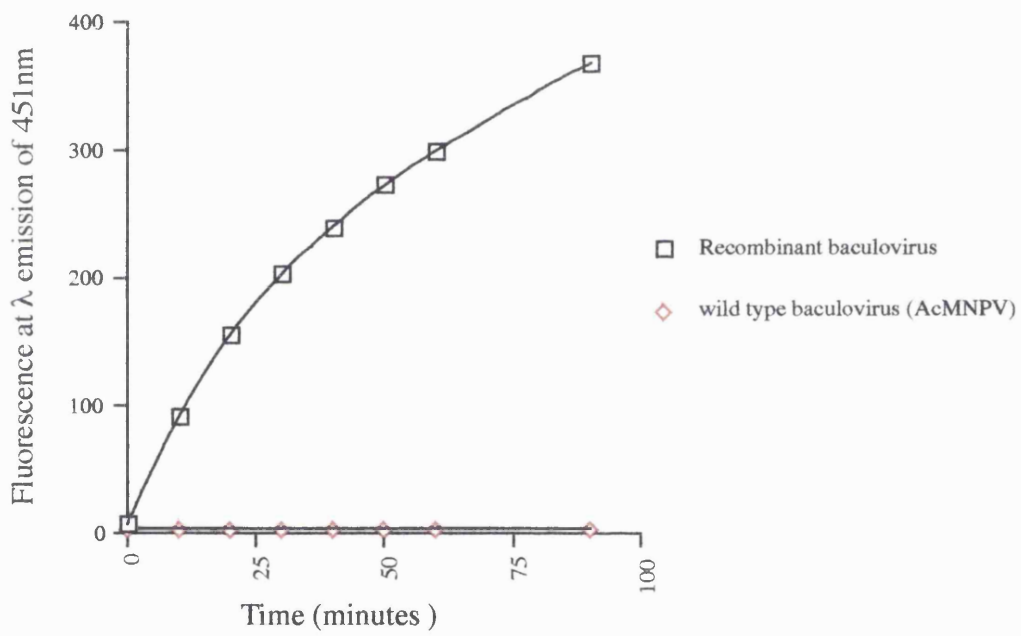


Figure 3.26 Coumarin 7-hydroxylase activity in the microsomes from Sf9 cells infected with recombinant baculovirus or wild type baculovirus(AcMNPV)

microsomal protein. Values obtained were 63.1, 86.4, and 132.5 for reactions with no added NADPH-cytochrome P450 reductase, rat NADPH-cytochrome P450 reductase and Human NADPH-cytochrome P450 reductase respectively (Figure 3.27).

The results of this experiment indicate the presence of endogenous reductase in microsomes from Sf9 cells, capable of interacting with the expressed CYP2A6 in the formation of 7-hydroxycoumarin. However, formation of 7-hydroxycoumarin in the reaction with no added NADPH-cytochrome P450 reductase virtually stops after 60 minutes, whereas in the reactions to which exogenous NADPH-cytochrome P450 reductase was added, the formation of 7-hydroxycoumarin continues. Possible explanation for this result could be that the activity of the endogenous reductase is not sufficient to cope with the amount of CYP2A6 expressed and this is indicated from the observation that the initial rate of reaction with no added reductase is two-fold less than that in the reaction where exogenous rat reductase was added. Subtraction of the graph obtained from the reaction where no NADPH-cytochrome P450 reductase was added from the graph obtained from the reaction where microsomes containing human NADPH-cytochrome P450 reductase were added shows that the rate of formation of 7-hydroxycoumarin catalysed by coupling of CYP2A6 and human NADPH-cytochrome P450 reductase is constant (Figure 3.28).

3.7.2 Analysis of metabolism of testosterone using microsomes from Sf9 cells infected with recombinant baculovirus expressing CYP2A6

Metabolism of testosterone was analysed in a reconstituted system

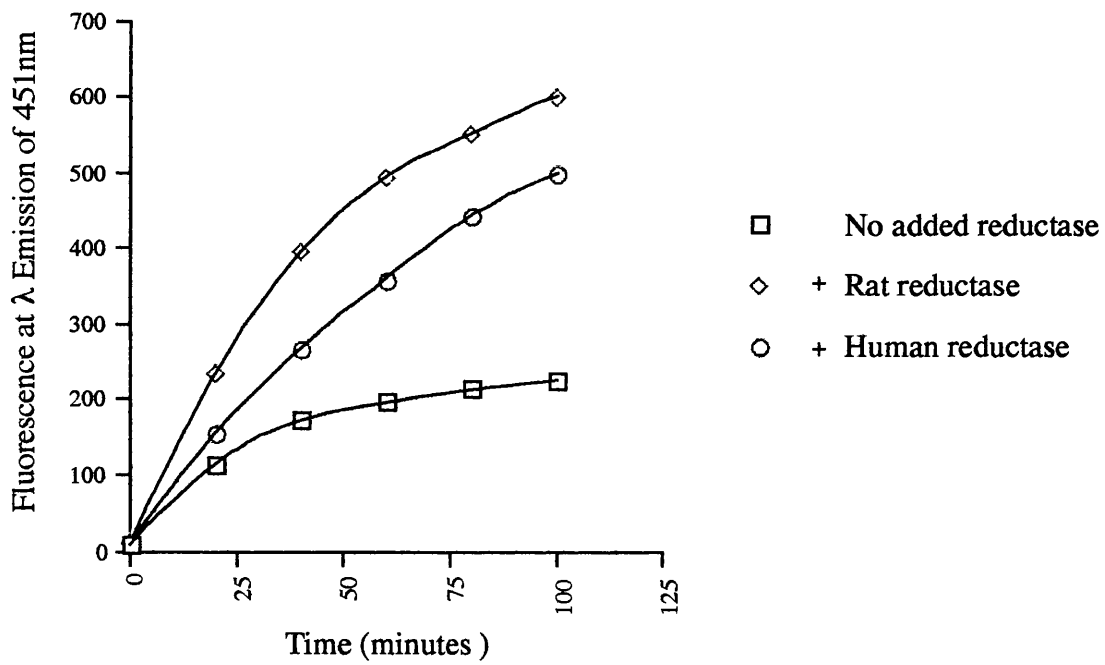


Figure 3.27 Effect of adding exogenous NADPH-cytochrome P450 reductase on coumarin hydroxylase activity in microsomes from Sf9 cells infected with recombinant baculovirus.

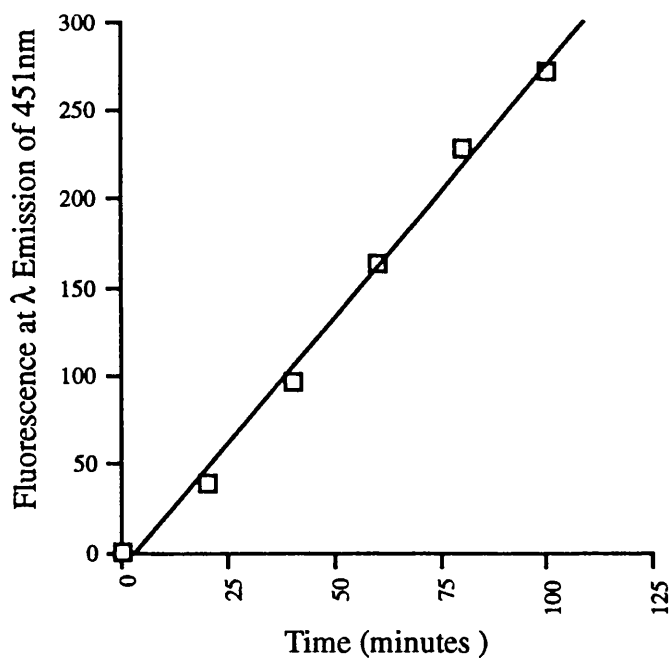


Figure 3.28 Effect of subtracting COH activity with no added NADPH-cytochrome P450 reductase from the COH activity obtained in the presence of added human NADPH-cytochrome P450 reductase.

using microsomes from Sf9 cells infected with recombinant baculovirus expressing CYP2A6. Conditions of incubation, extraction of the products and TLC analysis were carried out as described in section 2.18.7.2. Purified rat CYP2B1 and microsomes prepared from Sf9 cells infected with wild type baculovirus (AcMNPV) were used as controls. Metabolites were identified by comparison with mobilities of monohydroxytestosterones relative to testosterone on a TLC plate using solvent BB (Waxman, 1991)

In Figure 3.29, compound T is the unchanged testosterone. 16α -hydroxytestosterone (16α) and 16β -hydroxytestosterone (16β) are the major products of testosterone hydroxylation produced by purified rat CYP2B1 (used as a positive control for reaction conditions and identification of testosterone metabolites in this experiment). The minor compounds, a, b and c appear to be produced to some extent in every reaction and may be due to endogenous hydroxylation activities of Sf9 cells, the regenerating system or from non-enzymatic degradation. Compounds d and e were not identified. Expected position for 15α -hydroxytestosterone (15α) is indicated. Results indicate that the baculovirus expressed CYP2A6 is devoid of 15α -testosterone hydroxylase activity.

Having established that the expressed CYP2A6 exhibited the expected catalytic activities the next step was to try to purify the protein. The bacterial expressed MBP-CYP2A6 fusion protein had a low affinity for p-chloramphenicol-coupled Sepharose. However, this column has been used fairly successfully in our laboratory to purify human CYP3A4 expressed in baculovirus. It was therefore decided to use this affinity resin in an attempt to purify the baculovirus expressed CYP2A6.

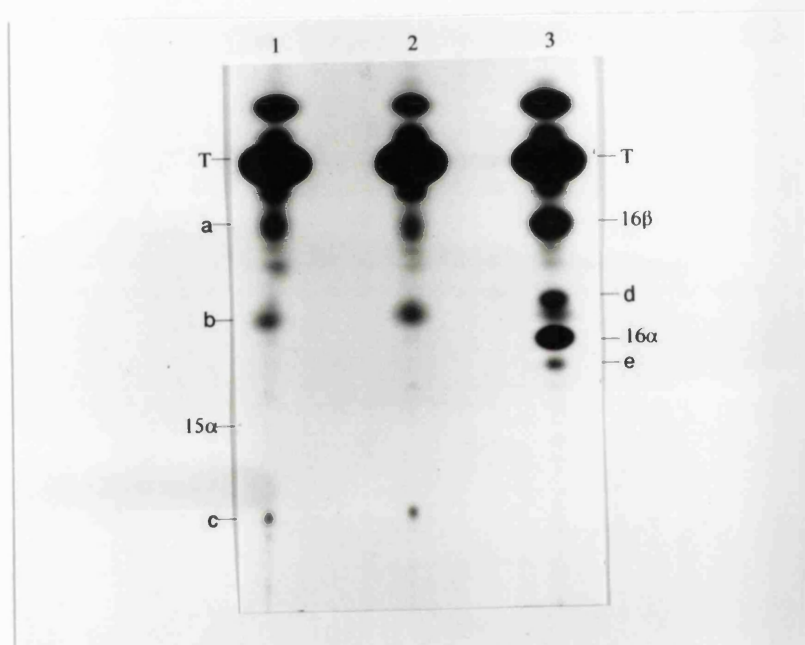


Figure 3.29 Analysis of testosterone hydroxylase activity of baculovirus expressed CYP2A6.

The photograph shows TLC separation of testosterone and its metabolites generated by reactions containing microsomes prepared from Sf9 cells infected with wild type baculovirus (track 1), microsomes prepared from Sf9 cells infected with recombinant baculovirus (track 2), and purified rat CYP2B1(track 3).

3.8 Purification of baculovirus expressed CYP2A6 by chromatography on p-chloramphenicol-coupled Sepharose

Microsomes from Sf9 cells infected with recombinant baculovirus expressing CYP2A6 were solubilized and used to purify CYP2A6 as described in section 2.19. Fractions from each stage of the purification were analysed by SDS/PAGE (Figure 3.30) and western blotting using a CYP2A7 baboon antibody (Figure 3.31). Although a lot CYP2A6 is present in the flow through fractions (Figure 3.31) suggesting that the binding of CYP2A6 to the column was weak, purification of CYP2A6 in low yield was achieved using this approach. CYP2A6 containing fractions were concentrated using Amicon centrprep centrifugal concentrators and a sample from the concentrated fraction was checked by western blotting (Figure 3.32). The amount of CYP2A6 obtained during this purification was 1.23 mg from initial amount of protein (50 mg) loaded onto the column. Purified CYP2A6 was successfully used in our laboratory by Susannah Lindey to generate antibodies in mice against human CYP2A6.



Figure 3.30 Purification of baculovirus expressed CYP2A6 by chromatography on p-chloramphetamine- coupled Sepharose (analysis by SDS/PAGE). Tracks 2, 3, 4, and 5 show the flow through fractions following the application of solubilized membranes onto the column. Tracks 6 to 9 show the fractions collected during the washing of the column. Tracks 10 to 17 show the fractions collected during the elution of proteins from the column. Track 1 shows the sample from solubilized membranes prior to purification. Track 18 shows relative molecular weight standards. Samples were subjected to electrophoresis through an SDS containing polyacrylamide gel. The gel was silver stained using the Bio-Rad silver staining kit.

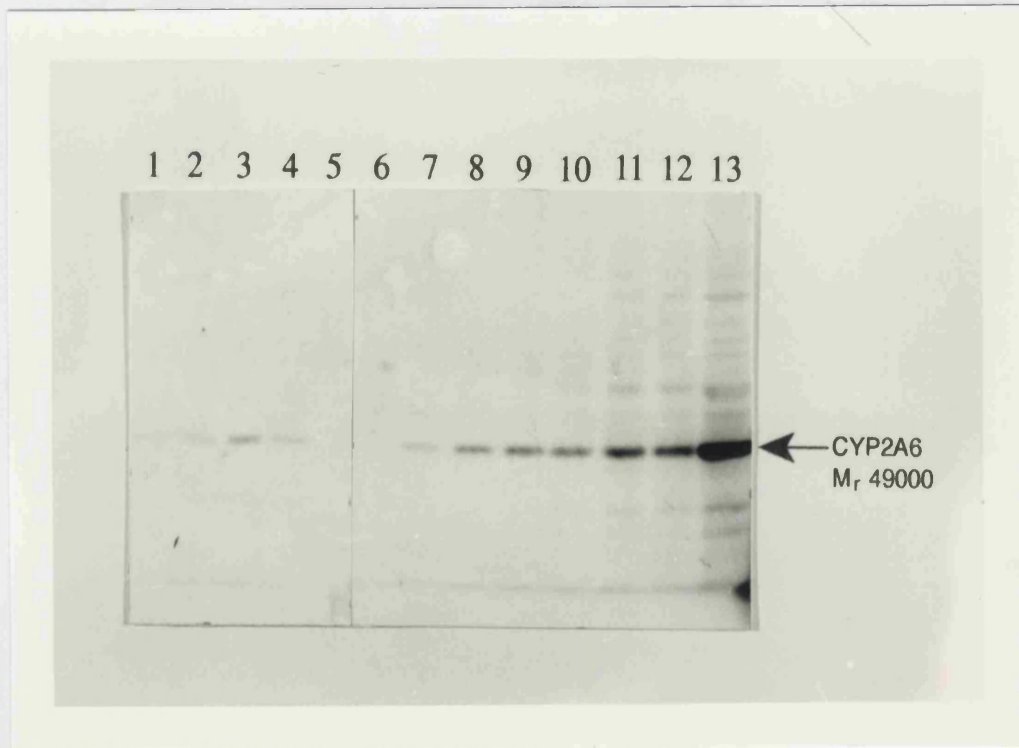


Figure 3.31 Purification of baculovirus expressed CYP2A6 by chromatography on p-chloramphetamine- coupled Sepharose (analysis by western blotting).

Tracks 1 to 4 show the fractions collected during the elution of proteins from the column. Tracks 5 to 9 show the fractions collected during the washing of the column. Tracks 10 to 12 show the flow through fractions following the application of solubilized membranes to the column. Track 13 shows the sample from solubilized microsomes prior to purification. Samples were electrophoresed through an SDS containing polyacrylamide gel and subjected to western blotting. The blot was developed by using sheep antibody against purified baboon CYP2A7 and alkaline phosphatase conjugated donkey anti-sheep IgG.

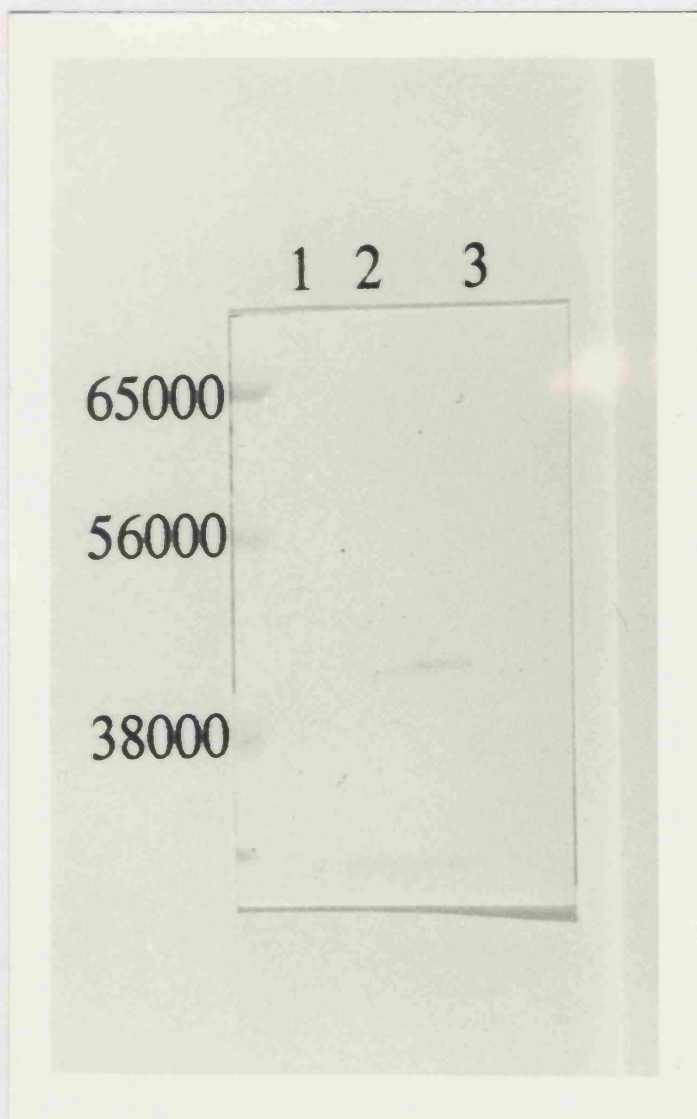


Figure 3.32 Western blot analysis of baculovirus expressed CYP2A6 purified by chromatography on p-chloramphenicol coupled Sepharose and concentrated using the Amicon centriprep-30 concentrator. Sample from diluent (track 2) and concentrate (track 3) were electrophoresed through SDS containing polyacrylamide gel and subjected to western blotting. The blot was developed by using sheep antibody against purified baboon CYP2A7 and alkaline phosphatase conjugated donkey anti-sheep IgG. Track 1 shows relative molecular weight standards.

Chapter 4

Conclusions

The expression of a protein, via its cDNA, in an heterologous cell system is not of course an exact representation of the in vivo situation but such systems can provide useful information about the protein under investigation. It is necessary to have clear goals, for the use of the expressed protein, prior to selecting and using artificial protein expression systems. As a prelude to expression studies, a cDNA which defines the amino acid sequence of the enzyme under study is required. The method of reverse transcription of mRNA and subsequent amplification of the corresponding cDNA by PCR has proved to be convenient in isolating cDNAs encoding individual P450 forms (human CYP2A6 and a marmoset CYP2A subfamily member). However, the authenticity of the PCR product, as in the case of marmoset CYP2A cDNA, is not guaranteed and confirmation by restriction endonuclease analysis and DNA sequencing is necessary.

With the aim of generating antibodies to CYP2A6, an E. coli based expression system using pMAL plasmid expression vectors seemed the attractive choice for producing relatively large amounts of CYP2A6. Expression of CYP2A6 as a fusion protein with MBP was achieved in E. Coli JM109 cells through optimization of E. coli culture conditions, although proteolysis and possibly toxicity due to overexpression of P450 proved to be a problem initially. However, this expression system proved to have many problems and it was not useful in obtaining a pure expressed CYP2A6 as described by the manufacturer.

With the aim of producing functional CYP2A6, the baculovirus/insect cell system seemed attractive, as it offered the potential for producing large amounts of protein in a favourable eukaryotic membrane context, which allows the correct folding of the endoplasmic reticulum proteins. Additionally

Sf9 cells contain endogenous NADPH-cytochrome P450 reductase which makes expressed P450 directly active in Sf9 cells microsomes. Expression and purification of functional CYP2A6 was achieved in Sf9 cells using recombinant baculovirus. However, the amount of haem synthesized in Sf9 cells is not sufficient and the addition of an exogenous source of haem (hemin or precursor of haem) is required. Additionally, the amount of endogenous NADPH-cytochrome P450 reductase was not sufficient for the amount of CYP2A6 expressed and catalytic studies required the addition of exogenous NADPH-cytochrome P450 reductase to the reaction mixture. These results suggest that the baculovirus/insect cell expression system can be used as a convenient tool for the study of the early steps of drug metabolism. In this context, this system can be used to determine whether a particular enzyme has the ability to catalyse a particular reaction. Consequently, it is possible to determine if a single enzyme can catalyse two reactions thought to be linked. Additionally, some insight about the contribution of individual isoenzymes to the metabolism of a drug can be obtained. It is possible also to analyze the question as to whether a particular enzyme can form toxic products of the drug. Such systems can also be used to determine whether a particular compound inhibits the activity of the expressed protein.

It must be stressed that the results obtained with in vitro systems, especially artificial expression ones, must be used with caution. It is necessary to evaluate the amount of the enzyme present in the tissue under investigation and to consider the relevance of an overexpressed system. It is difficult to calculate a specific enzyme activity and relate this to the tissue which is to be modelled. In the normal cellular environment, there is a competition for cofactors, accessory enzymes, etc. and this may not be the case in the

expression system. Physicochemical parameters, for example substrate solubility, may differ in the native and the model systems. Additionally, for development of cellular systems where toxicity or mutagenicity studies are considered, other drug activating and detoxicating enzymes might be limited or absent. However, expression systems are an excellent source of individual enzymes required for structure /function studies especially those involving site directed mutagenesis.

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