Studies on Cytochrome P450 4X1

BY

Mohammed Mashog Al-anizy

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To my Mother and Father

To my Wife and Sons

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Abstract

Antisera raised against recombinant human CYP4Z1 (4Z1), mouse Aryl hydrocarbon receptor (AhR), and *C.elegans* Latrophilin (LpH) proteins were titred over the course of several bleeds. The sensitivity of the antibodies shows an increase over the course of several immunizations, with the minimum amount detected being 1 ng of 4Z1, 0.3 ng of Ah-R, and 0.3 ng of LpH. Terminal bleeds were taken for the AhR and LpH antisera. The AhR antisera detects proteins in rat and mouse liver cytosol consistent with previous reports of the AhR. LpH is predicted to be localized in a membrane compartment on the basis of its primary structure, so sub-cellular fractions of *C.elegans* were isolated and tested, revealing a protein of ~113 kDa in a crude membrane preparation. This protein was not solubilized in < 1% Emulgen 913, but was soluble in 2% dodecylmaltoside.

A fresh 15k supernatant treated with 2% dodecylmaltoside showed a strong band of LpH protein at ~113 kDa. However, 66 kDa band was detected when the samples were stored overnight at –80°C due to presence of a G protein coupled receptor proteolysis site (GPS) between position M536 and C547 of LpH, which is a characteristic feature in the LpH protein family.

In order to study the expression of the CYP 4X1 in mouse tissues, RNase protection assays were performed. Different tissues were assayed at the same time and the same riboprobe was used to hybridise all the samples. The CYP 4\x1 probe was shown to be full length (-ve control). However, the +ve control (RNase positive) shows an absence of signal when hybridised to the yeast tRNA demonstrating the specificity of the signal in the samples. Several RNA

samples were hybridised with mouse cyp4X1 gene probe, such as aorta, brain and heart and liver. The mouse cyp4X1 gene appears to have 12 exon from the genomic sequence and encodes a protein which high identity with the human and rat cyp4X1 gene. The full-length of the probe was 424 b.p and the protected fragments were 177 b.p. The murine cyp4X1 was not expressed in control liver, but is expressed in brain at high level.

Cyp4X1 gene was also investigated in aorta tissue and found to be expressed at low levels. Known inducers of hepatic cytochrome P450 were used (Ciprofibrate, TCDD, PB, and dexamethasone), but had no induction effect in the samples.

Western blotting of brain confirmed that the cyp4X1 protein is expressed in brain, and quantification showed that this is a major cytochrome P450 in brain.

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Abbreviations

4A1	Cytochrome P450 4A1
A	Adenine
a.a	Amin Acid
AA	Arachidonic Acid
Ah-R	Aryl Hydrocarbon Receptor
AMP	Ampicillin
APS	Ammonium Persulphate
b5	Cytochrome b5
BSA	Bovine Serum Albumin
bp	base Pair
BWSV	Black Widow Spider Venom
Cipro	Ciprofibrate
CPR	Cytochrome P450 Reductase
CTP	Cytosine Triphosphate
CYP	Cytochrome P450
C.elegans	Caenorhabditis Elegans
CYP 4Z1	Cytochrome P450 4Z1
DAB	Diaminobenzidine
Dex	Dexamethasone
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid

cDNA	Complementary DNA
DMSO	Dimethyl sulphoxide
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
EET	Epoxyeicosatetraenoic acid
ER	Endoplsmic Reticulum
FAD	Flavin Adenine Dinucleotide
FMN	Flavin Mononucleotide
G	Guanine
GFAP	Glial Fibrillary Acidic Protein
HETE	Hydroxyeicostetraenoic acid
His Tag	Histidine Tag
IPTG	Isopropyl-β-D-thiogalactopyranoside
Kb	Kilobase
kDa	kilo Dalton
LB	Luria-Bertani broth
LpH	Latrophilin
LTB	Leukotriene
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NFT	Neurofilament
OD	Optic Density
P450	Cytochrome P450
PAGE	Polyacrylamide Gel Electrophoresis
PAH	Polycyclic aromatic hydrocarbon
PB	Phenobarbital

PBS	Phosphate Buffered Saline
PVDF	Polyvinylidene Difluoride
PCR	Polymerase Chain Reaction
PG	Prostaglandine
PMSF	Phenylmethylsulfonylfluoride
PPAR	Peroxisome Proliferator
RNA	Ribonucleic acid
mRNA	Messenger RNA
RPA	RNase Protection Assay
SDS	Sodium Dodecyl Sulphate
T	Thymine
TB	Terrific Broth
TBE	Tris-Boric acid-EDTA buffer
TBS	Tris-Buffered Saline
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TEMED	N, N, N`, N`-tetramethylethylenediamine
TTBS	Tween – TBS
UHP	Ultra High Purity
WWW	World Wide Web
4X1	Cytochrome P450 4X1
4Z1	Cytochrome P450 4Z1

Chapter 1 Introduction

1.1 Cytochrome P450

The cytochrome P450 enzymes are a super family of hemoproteins, which contain a thiolate-bound heme protoporphyrin IX prosthetic group and catalyse the oxidation of a large variety of substrates, including endogenous lipids and Xenobiotics. They were described as a microsomal carbon monoxide binding pigment (Omura and Sato 1962). Cytochrome P450 was discovered by Klingenberg and Garfinkle in 1958 as a new kind of pigment in rat liver microsomes (Klingenberg 2003), and it was characterised as a P450 hemoproteins by Omura and Sato (Klingenberg 2003). At first, it was thought that the P450 protein was a single protein. However, it later became apparent that there were several different P450s with differing substrate and physiochemical characteristics (Lewis 2001).

A characteristic of all cytochrome P450 proteins is that in the reduced state they bind carbon monoxide giving a unique absorption maximum at 450 nm (Garfinkel 1958) (Jecoate 1978). The name of cytochrome P450 was derived from this unique absorption and named by Omura and Sato(Omura and Sato 1962; Omura and Sato 1964). This spectral characteristic is due to the presence of the thiolate ligand which not found in most other heme proteins with histidine-derived imidazole ligand (Ortiz de Montellano 1997). For instance, absorption of the ferrous-CO complex in myoglobin occurs at 423 nm and not at 450 nm (Ortiz de Montellano 1997).

The cytochrome P450 enzymes play an important role in the mixed function oxidase (MFO) system which consists of cytochrome P450, NADPH-P450 cytochrome P450 reductase and a lipid component (phosphatidylcholine), in the detoxification of xenobiotic substances in a wide variety of species. In order to distinguish between cytochrome P450s and other redox proteins such as cytochrome (a, b, and c), heme-thiolate enzymes were recently described (Lewis 2001).

The complete reaction can be summarized as follow, where RH is a drug substrate and ROH is the metabolite:

$$NADPH + RH + O_2 + H^+ \xrightarrow{\text{Cytochrome P450}} NADP^+ + H_2O + ROH.$$

Cytochrome P450 is present in most living organisms, both plants and animals (Nelson et al. 1996).It has a molecular weight of approximately 50-60 kDa (Skett 2001)**,** but shows an apparent mobility of 48-55 kDa on SDS-PAGE**.**

Proteins of the cytochrome P450 superfamily occur as soluble or membrane-associated forms. The soluble form is found in bacteria, whereas the membrane-bound form occurs in yeast and higher organisms, particularly in the endoplasmic reticulum and mitochondrial membranes (Lee 2003).

CYP450 proteins usually contain approximately five hundred amino acids with haem as the prosthetic group (Hoch and de Montellano 2001). The haem group in CYP450 is non-covalently bound to an apoprotein (Skett 2001), but recently, it was found that some CYP4 family members are covalently linked to their prosthetic haem group by an ester linkage(Zheng et al. 2003).

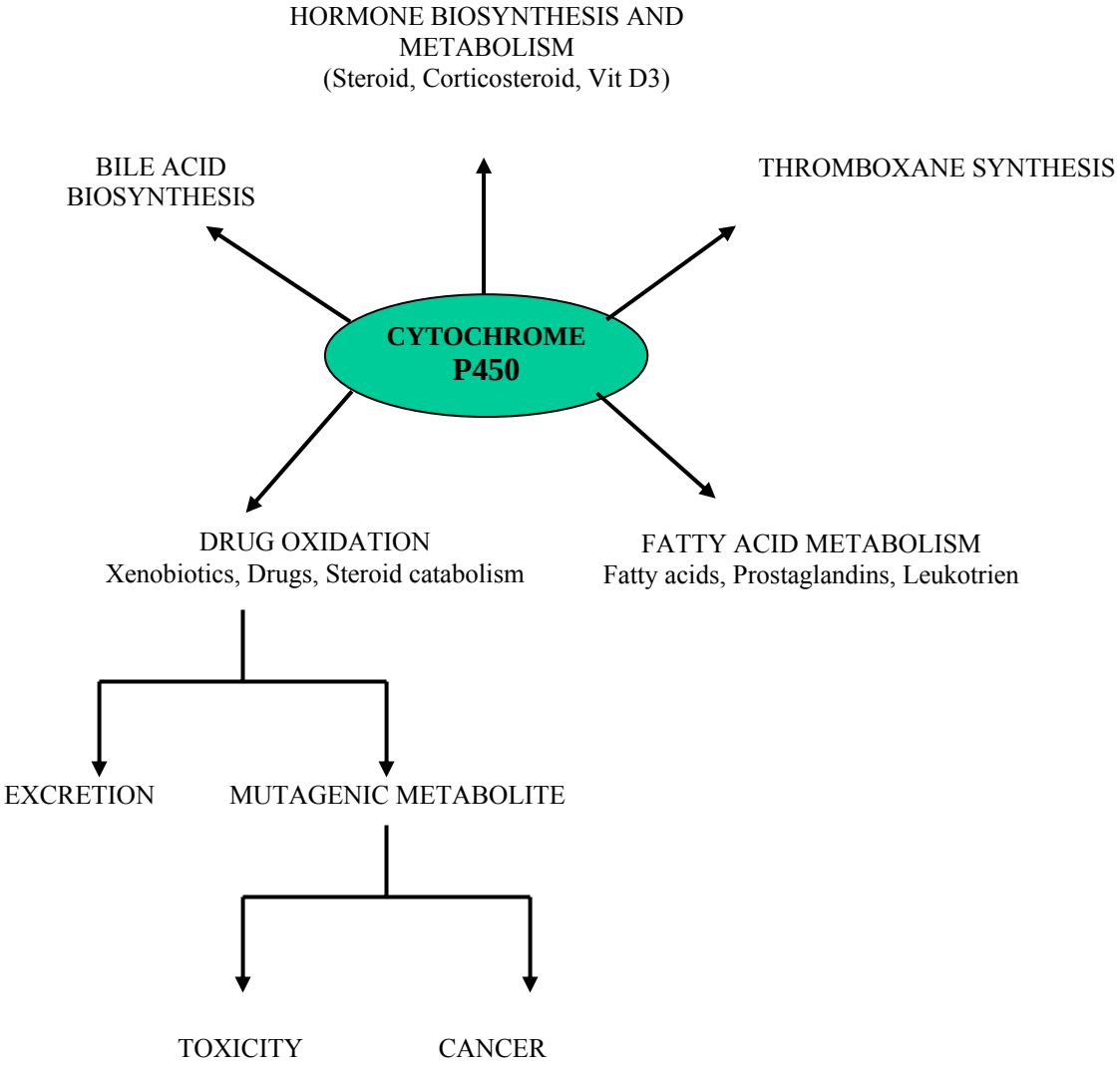
In mammals, it was initially thought that P450 enzymes were found only in the liver and endocrine organs. However, they were subsequently found in the kidney, prostate, breast (Williams et al. 2000), brain, skin and intestine (Gonzalez and Lee 1996) ((Hellmold et al. 1998) ; (Hakkola et al. 1998).

The liver is well-studied because it contains the greatest concentration of cytochrome P450 enzymes, and most of our knowledge regarding drug metabolism was obtained from studying the enzymes in liver.

The typical function of cytochrome P450 is the mono-oxygenation of various substrates by introducing oxygen into a wide variety of substrates and structural classes of compounds (Porter and Coon 1991). A cytochrome P450 catalytic cycle has been proposed (Guengerich 1991) (Guengerich and Johnson 1997) (Guengerich et al. 1998) (Ortiz de Montellano 1997) (Mansuy 1998). Although some parts of this mechanism are poorly understood, it is still accepted by most workers to date. It participates in a wide variety of additional reactions, including epoxidations, dealkylations, deaminations and dehalogenations. The cytochrome P450 system is responsible for biotransformation of drugs, environmental pollutants, pesticides and industrial chemicals (Simpson 1997), Fig 1.1.

CYP450 enzymes can catalyse a vast variety of exogenous and endogenous substrates simultaneously due to its substrate specificity and overlapping reactions. For example, P450 can convert fatty acids to their ω and/or ω-1 hydroxylated form (Gibson et al. 1982) (Bains et al. 1985), or be involved in drug metabolism (by the CYP2D subfamily) (Distlerath et al. 1985)

Fig 1.1 The Functional Roles of Cytochrome P450s. The Figure has been taken from Simpson (Simpson 1997)



1.2 Nomenclature of P450

The nomenclature system introduced originally by Nebert et al (1987) proposes that all members of a family share >40% identity at the amino acid sequence level (e.g. CYP2A6 and CYP2B6), and members of a sub-family share >55% identity (e.g. CYP2A6 and CYP2A7) (Coon et al. 1992) (Nelson et al. 1996). The CYP prefix is used to describe a P450, followed by an Arabic number for the family (e.g. CYP4), a letter for the sub-family and a number for individual gene (e.g. CYP4A1) and italicized names refer to genes (e.g. *CYP4A1*)..

To date, 265 CYP450 Families have been identified, of which 18 families and 43 subfamilies are from mammals (http://drnelson.utmem.edu/famcount.html).

There are now over 400 known members of the cytochrome P450 family in such diverse organisms as mammals, fish, insects, nematodes, plants, yeast, fungi and bacteria (Nelson 1999), but the substrate specificities for only a few of these are known.

1.3 Function of cytochrome P450

Cytochrome P450 plays an important role in the metabolism of endogenous chemicals and the detoxification of natural toxins, drugs and xenobiotics.

The main function of CYP450 is the mono-oxygenase activity whereby it introduces one atom of oxygen into a substrate but there are others functions depending on the substrate such as N-hydroxylation, N-oxidation, oxidative deaminations, S-dealkylations,

O-dealkylations, aliphatic and aromatic hydroxylation (Mansuy 1998). CYP450 enzymes catalyze many steps of sterol biosynthesis, metabolism of fatty acids, sterols, and the vast majority of drugs and xenobiotics. Furthermore, they are responsible for the metabolism of xenobiotics to metabolites responsible for toxic or carcinogenic effects (Josephy 1997). The vast number of catalytic reactions is due to the diversity and overlapping specificities of CYP450 enzymes. For example, CYP1, CYP2 and CYP3 are responsible for metabolism of exogenous compounds, whereas other families such as CYP4 are responsible mainly for endogenous metabolism with some exogenous substrates such as phthalate ester (Lewis 2001).

CYP450s from 13 mammalian gene families are involved in the metabolism of endogenous substrates that are physiologically important in the biosynthesis of thromboxanes (CYP5), prostacyclins (CYP8A), steroid hormones (CYP11, CYP17, CYP19 and CYP21), bile acids (CYP7A, CYP8B and CYP27A) and calcitriol (CYP27A and CYP27B) (Eggertsen et al. 1996) (Shinki et al. 1997) (Shinki et al. 1997) (Hedlund et al. 2001).

1) - Metabolism of exogenous compounds

The role of CYP450 in the oxidation of foreign chemicals is that by converting these hydrophobic chemicals into hydrophilic forms (water soluble) by introducing polar groups, such as hydroxyl, the chemicals become easier to excrete or metabolize by other enzymes (Smith et al. 1998). In humans, three CYP450 families (CYP1, CYP2, and CYP3) are responsible for this phase-I metabolism of foreign compounds and

particularly they have an important role in the metabolism of drugs (Parikh et al. 1997) (Murray 1999). Some CYP450s such as CYP2D6, 2C19 and 3A4 were found to be involved in metabolism of a vast range of medicines (van der Weide and Steijns 1999) (Hasler 1999).

2) - Biosynthesis and biotransformation of endogenous chemicals

CYP450s are involved in catalyzing pathways of endogenous metabolism in both animals and plants (Lewis 2001). CYP51 has been shown to catalyze steroid hormone biosynthesis (Pikuleva 1999). The biosynthesis and metabolism of steroids was found to be a major endogenous function of P450 (Lewis et al. 1998) (Nelson 1999). The catalysis of cholesterol biosynthesis via CYP51 forms the first step in the formation of many steroid hormone derivatives, such as progesterone, pregnenolone, cortisol, testosterone, and estradiol (Takemori and Kominami 1984). Several CYP450s were found to be involved in the production of bile acid from cholesterol (Hasler 1999) (Pikuleva 1999). Some CYP450s are involved in the metabolism of Vitamin D3 to the physiological active form, 1,25-dihydroxyvitamin D3 in the animal tissues (Omura 1999). CYP450 is involved in the synthesis and metabolism of eicosanoids (Omura 1999), and in mammals, the CYP4 family is responsible for metabolizing fatty acids such as lauric acid, palmitic acid, myristic acid and arachidonic acid (Capdevila 1999). Moreover, it was found that some CYP450s metabolize arachidonic acid (Capdevila and Falck 2001) to produce epoxyeicosatrienoic acids (EETs, dihydroxyeicosatetraenoic acids (DiHETEs) and 19 and 20-hydroxyeicosatetrienoic acids (19- and 20-HETE) (Hoagland et al. 2001) (Yue et al. 2004). The arachidonic acid metabolites are

thought to play an important role in the pathogenesis of hypertension (Harder et al. 2000), (Capdevila et al. 2000) (Holla et al. 2001),

(Capdevila and Falck 2001), (Hoagland et al. 2001) and (Llinas et al. 2004).

Recently, it has been reported that non-fatty acid substrates are metabolized by CYP4A (Okita and Okita 2001).

1.4 Redox Partners

Redox partners are essential enzymes required for CYP450 function. Two types of electron transfer protein are involved in the oxidation and reduction of CYP450, cytochrome P450 reductase and cytochrome b5. The presence of cytochrome P450 reductase is essential for cytochrome P450 catalytic reactions (Dierks et al. 1998). Cytochrome P450 reductase is a 68 kDa flavoprotein that has of two flavins prosthetic groups, FMN and FAD, and it is found in the mammalian endoplasmic reticulum (Strobel 1978). FMN and FAD are found at in equal amounts in the cytochrome P450 reductase (Strobel 1978). The pathway of electron transfer is starting from NADPH to FAD to FMN and finally to P450 haem (Shen et al. 1989).

Cytochrome b5 is a heme enzyme present in the endoplasmic reticulum and has a molecular weight of approximately 17 kDa (Schenkman and Jansson 1999). This protein facilitates many reactions including fatty acid desaturation, fatty acid elongation and cytochrome P450 monooxygenation (Schenkman and Jansson 1999). Cytochrome b5 was purified from liver (Strittmatter 1978).

The role of cytochrome b5 in the CYP450 catalytic reaction is not well understood and still controversial. It is known that some P450s can be stimulated by either b5 or by the absence of heme b5 (abo-b5) with a lack of electron transfer, whereas some P450s were reported to mediate the reaction by cytochrome b5 (Yamazaki et al. 2001). Other studies found that cytochrome b5 was not involved in electron transfer to the cytochrome P450, but that it played an important role in the conformational effect (Loughran et al. 2001). The transfer of electrons by cytochrome b5 to cytochrome P450 was not a direct step, but involved apo-b5 in the presence of holo-b5 formation (Guryev et al. 2001). NADPH-cytochrome P450 reductase and cytochrome-b5 affect rate of metabolism and substrate specificity (Loughran et al. 2000). For example, cytochrome b5 is important for ω-hydroxylation of endogenous fatty acid, arachidonic acid, to its physiologically important product (20-HETE) by CYP4A4 and CYP4A7 (Okita and Okita 2001).

1.5 Mechanism of action

Based on a number of observations a cyclic reaction was proposed by Estabrook et al (1971) for cytochrome P450 catalysed oxygenation reactions. The reaction begins when the substrate binds to the active site of the cytochrome P450 to form a substrate ferric hem complex Fig 1.2. Two electrons are donated to cytochrome P450 by the reducing co-factor NADPH. NADPH is a two electron donor but cytochrome P450 can only accept one electron at a time. NADPH-cytochrome P450 reductase which possesses two flavins prosthetic groups, has the ability to accept two electrons from NADPH and simultaneously transfer one electron each to two different cytochrome P450 molecules. The ratio of NADPH-cytochrome

P450 reductase to cytochrome P450 molecules present in liver microsomes is about 20:1.

It is during the first electron donation that the iron atom present in the cytochrome P450 is reduced to the ferrous state (Fe^{+2}) and molecular oxygen binds to the cytochrome P450 complex. A second electron is then donated to the cytochrome P450 from NADPH-cytochrome P450 reductase. This is followed by electron rearrangement, insertion of an oxygen atom and product release with the generation of water or hydrogen peroxide (Guengerich et al. 1998) (Plant 2003)

The P450 absorption can be detected by measuring the difference in the spin state of heme iron (Josephy 1997). As a result of heme ligation, cytochrome P450 has two types of states known as low spin state and high spin state(Josephy 1997).

It is known that the heme group is a very strong chromophore and is affected by the nature of the heme ligand, the iron oxidation state and the presence of the protein substrate. A change from the low spin state to the high spin state leads to a blue shift of maximal absorption in the optical spectrum (Jefcoate 1978) (Ortiz de Montellano 1997). Based on the P450 spectrum differences, three types of P450 difference spectrum have been identified, Type-I, Type-2 and Type-3 (Lewis 2001). The Type-I difference spectrum has a maximum absorption at approximately 385-390 nm and a trough at approximately 420 nm (Josephy 1997). This difference spectrum represents the binding stage of a compound that displaces the distal ligand (H_2O) and causes a low to high spin shift (Josephy 1997). The Type-2 difference spectrum has a maximum absorption of approximately 425-435 nm and trough at 390-405 nm and represents the compound coordinating with the ferric heme.

The Type-III difference absorption is a reversal of Type-I (mirror image) with a maximum absorption at approximately 420 nm and a trough at 388-390 nm (Josephy 1997).

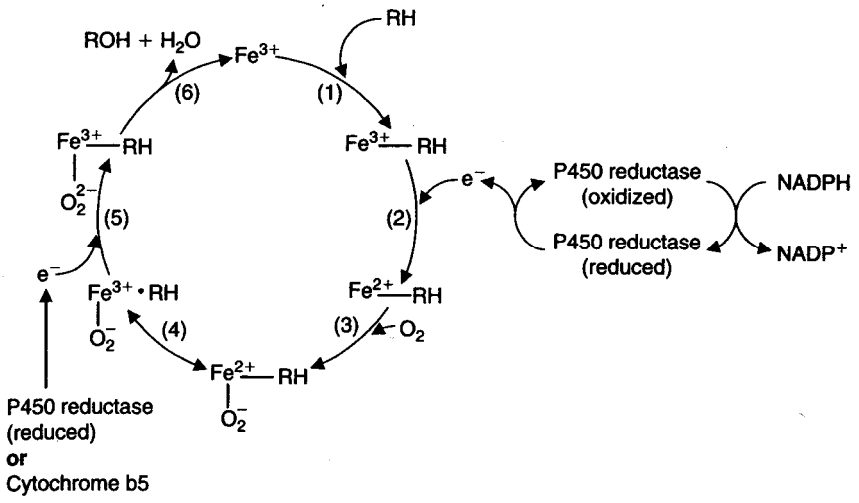


Figure. 1.2 Catalytic cycles for cytochrome P450 reactions (adapted from Plant N. 2003).

Six steps in cytochrome P450 catalytic reactions are shown by numbers. Fe^{3+}= oxidised cytochrome P450, Fe^{2+}= reduced cytochrome P450, b5= cytochrome b5, RH=substrate, ROH=product, and e^- = electron.

1.6 Classification of cytochrome P450 enzymes

Cytochrome P450 enzymes are classified according to their protein sequence similarity (Nebert and Nelson 1991) and divided into gene families and subfamilies.

265 families have now been identified, of which 18 families and 43 subfamilies are from mammals (http:// drnelson.utmem.edu/famcount.html.). Cytochrome P450 enzymes can be divided into three classes on the basis of the redox partner.

Class I P450s use a flavoprotein which contains FAD and iron-sulfur protein as electron transporters and this class is found in some bacteria and most mammalian mitochondrial P450s. Class II P450, need a flavoprotein which contains FAD and FMN as electron transporters and this class is found in microsomes. Class III P450s can gain electrons from the substrate without interacting with redox partners (self-contained system) and can be found in bacteria, mammals and plants (Ortiz de Montellano 1995) (De Mot and Parret 2002). Class IV P450 has recently been identified. In these, the substrate has a flavoprotein reductase domain, an iron-sulfur cluster and a cytochrome P450 enzyme (Roberts et al. 2002).

In the last decade CYP450 studies have been expanded rapidly and hence it was essential to classify the CYP450s to families and subfamilies

1.7 Cytochrome P450 4 family

CYP4 family is one of the oldest cytochrome P450 families and is thought to have evolved about 1.25 billion years ago (Simpson 1997) after the formation of the steroid biosynthetic genes (Simpson 1997) (Okita and Okita 2001). CYP4 proteins were highly conserved during evolution and have been isolated from both mammals and invertebrates (Nelson 1998). A highly conserved sequence of 13 residues at around position 315. (I helix) is a shared characteristic of this family (Bradfield et al. 1991).

The cytochrome P450 gene family was first identified after the sequencing and cloning of CYP 4A1 by Hardwick et al (1987) (Hardwick et al. 1987).

In humans, the CYP4 family consists of six sub-families (CYP4A, CYP4B, CYP4F, CYP4V, CYP4X and CYP4Z) (Demontellano et al. 1992) (http:// drnelson.utmem.edu/famcount.html.) (Capdevila et al. 2000) (Rieger et al. 2004).

This family is thought to be related to cholesterol metabolizing enzymes, which are involved in maintaining membrane integrity in early eukaryotes (Simpson 1997). In mammals, the CYP4 enzymes are thought to be involved in the metabolism of endogenous substances including arachidonic acid, prostaglandins (PGs), and leukotrienes (LTs) (Capdevila and Falck 2001), (Kikuta et al. 2002).

To date, the CYP4 family contains 18 subfamilies of which three were identified in mammals; CYP4A, CYP4B and CYP4F (Nelson et al. 1996) (Simpson 1997).

Most CYP4 genes are expressed in liver, kidney and lung and their expression is often known to be induced by peroxisome proliferators.

Members of the CYP4A family are responsible for the metabolism of fatty acids to ω and ω-1 hydroxylated forms, which is a preferable site for all CYP4 family (Roman 2002) (Needleman et al. 1986), and are involved in drug biotransformation like CYP2D6, 2C19 and 3A4 (van der Weide and Steijns 1999). CYP4 family is divided into subfamilies which of three subfamilies, CYP4A, CYP4B and CYP4F, are identified in mammalian species, Table 1.1. Furthermore, two uncharacterized new members of the CYP4 family have been identified in this laboratory and named CYP4X1 and CYP4Z1 (Savas et al. 2005). Two genes of CYP4Z1 sub-family were identified and isolated from human tissues (4Z1, 4Z2P), which one are a gene and the other pseudogene (Rieger et al. 2004). The position of these genes (CYP4Z1 and CYP4Z2P) in the chromosome were found to be localized on chromosome 1P33 in head to head orientation in a distance of 166 kb (Rieger et al. 2004). CYP4V subfamily has been identified by EST (expressed sequence tag) analysis as additional mammalian subfamily exists in the CYP4s. Recently, CYP4X1 has been identified in rats with a unique location in the brain and it is thought to play an important role in neurovascular function(Bylund et al. 2002).

CYP4 Family	Species Located
CYP4A	Human, Rabbit, Rat, Mouse, Guinea Pig
CYP4B	Human, Rabbit, Rat, Mouse
CYP4F	Human, Rat

Table 1.1 The mammalian CYP4 family. Members of the CYP4 family and the species as described by Lewis (2001).

1.8 The CYP4A subfamily

This subfamily is the well-studied among all of the CYP4 family subfamilies since it was identified by Hardwick et al 1987**,** due to its inducibility by many foreign chemicals (Omiecinski et al. 1999). The first CYP4 protein, CYP4A1 was isolated and characterised by Gibson et al in 1982 (Gibson et al. 1982) formerly designated as P452. In the liver it forms only 1-4% of the total P450 isozymes (Sharma et al. 1988).

Currently, 24 proteins of the CYP4A subfamily have been identified in mammalian species including human(Kawashima et al. 1992), rabbit (Johnson et al. 1990) (Matsubara et al. 1987), rat (Kimura et al. 1989) (Imaoka et al. 1990), mouse and guinea pig (Bell et al. 1993), Table 1.2.

The CYP4A subfamily are known to metabolize endogenous fatty acids, eicosanoids and prostaglandins (PGs) (Lewis and Lake 1999) (Capdevila et al. 2002), and also some exogenous fatty acids such as phthalate ester mono-(2-ethylhexyl)-phthalate (Lewis 2001). Moreover, the CYP4A subfamily is involved in the metabolism of arachidonic acid to the active metabolite, hydroxyeicosatetraenoic acid (HETEs), an ω-hydroxylated metabolite of arachidonic acid, and cis-epoxyeicosatrienoic acids (EETs) which have physiological importance in the blood flow of the kidneys, cornea and brain (Kroetz et al. 1997; Capdevila et al. 2000; Capdevila and Falck 2001; Okita and Okita 2001). Although endogenous fatty acids, such as lauric acid are the main substrates of CYP4A forms, it has been reported that some non-fatty acids are

metabolised by CYP4A forms, e.g. all trans-retinoic acids can be metabolised to 18-hydroxy retinoic acid by CYP4A11(Marill et al. 2000). It was found that CYP4A1 gene expression can be induced by peroxisome proliferators, e.g. clofibrate (2-(4-chlorophenoxy)-2-methylpropionate), and that the major expression was in the liver and the kidney.

Recently, a new member of CYP4A subfamily CYP4A21 was identified and isolated from pig liver and found to hydroxylate a steroid nucleus, particularly the alpha-hydroxylation of chenodoxycholic acid (Lundell et al. 2001).

Table 1.2: Members of the CYP4A subfamily identified in different species

	Species	GenEMBL	References
CYP4A1	Rat	M14972 M33937 and M57718	(Hardwick et al. 1987) (Kimura et al. 1989)
CYP4A2	Rat	M33938 and M57719	(Kimura et al. 1989)}
CYP4A3	Rat	M33936	(Kimura et al. 1989)
CYP4A4	Rabbit	J02818 L04758	(Palmer et al. 1993) (Matsubara et al. 1987)
CYP4A5	Rabbit	M28655 X57209	(Johnson et al. 1990) (Yokotani et al. 1991)
CYP4A6	Rabbit	M28656 L04755	(Johnson et al. 1990) (Muerhoff et al. 1992)
CYP4A7	Rabbit	M28657	(Johnson et al. 1990)
CYP4A8	Rat	M37828	(Stromstedt et al. 1990)
Cyp4a10	Mouse	X71478	(Bell et al. 1993)
CYP4A11	Human	S67580	(Imaoka et al. 1993)
Cyp4a12	Mouse	X71479	(Bell et al. 1993)
CYP4A13	Guinea Pig	X71481	(Bell et al. 1993)
Cyp4a14	Mouse	Y11639-Y11642	(Heng et al. 1997)
CYP4A15	Koala	AF252263	(Ngo et al. 2000)
CYP4A16	Cat	U91789	Gebremedhin, Lange *et al.*, unpublished
CYP4A17	Hamster	No accession number	Bell, unpublished
CYP4A18	Hamster	No accession number	Bell, unpublished
CYP4A19	Hamster	No accession number	Bell, unpublished
CYP4A20*	Human	AC026935 (replaced by AL450996, from genome)	Mclay, unpublished
CYP4A21	Pig	AJ278474	(Lundell et al. 2001)
CYP4A22	Human	AL390073 (from genome)	Mclay, unpublished
CYP4A23	Pig	AF384031	Van Es *et al.*, unpublished
CYP4A24	Pig	AJ318096	(Lundell 2002)
CYP4A25	Pig	AJ318097	(Lundell 2002)

*Now named 4Z1.

Twenty-four CYP4A members are summarised in the Table (1.2). The accession numbers of CYP4A members are also shown in the table. The accession numbers of CYP4A20 and CYP4A22 are from the human genomic sequence.

Typically, CYP4A is involved in the metabolism of endogenous fatty acids via ω-hydroxylation of long or medium fatty acids to short chain dicarboxylic acids which helps to prevent fatty acid accumulation in the liver (Okita and Okita 2001). The majority of this oxidation in the fatty acid was found to be in the omega (ω) site and to some extent in the ω-1 site.

The most important catalytic oxidation by the CYP4A subfamily is that involved in the oxidation of the long chain poly unsaturated endogenous fatty acid, arachidonic acid. Arachidonic acid can be metabolised by CYP4A1 to produce 20-HETE, a vasoconstrictor, which is thought to play an important role in physiological functions (Powell et al. 1998; Nguyen et al. 1999; Capdevila and Falck 2001; Okita and Okita 2001) and is also involved in the pathogenesis of hypertension (Roman et al. 2000). Inhibition of CYP4A expression (CYP4A1, CYP4A2 and CYP4A3) was found to reduce male rat hypertension which indicates a strong correlation between CYP4A expression and hypertension (Wang et al. 2001).

In vivo, the hydroxylation of medium and long chain saturated fatty acids represents an important secondary pathway for fatty acid metabolism in hepatocytes during times when there is an increase in lipolysis and fatty acid transport to the liver (Okita and Okita 2001).

In the kidney, the arachidonate CYP4A metabolites are hypothesised to be involved in the regulation of renal salt and water excretion (Lapuerta et al. 1988) (Schwartzman et al. 1990). This makes the CYP4A subfamily the most well studied of all the CYP4 subfamilies.

In an *E.coli* expression system, it was found that the CYP4A enzyme activity was very high on long chain fatty acids and prostaglandins (PGE1) with a low rate of lauric acid metabolism (Roman et al. 1993). Furthermore, studies in COS-1 cells revealed hydroxylation of PGE1 and PGA1 by CYP4A4 (Roman et al. 1993; Okita and Okita 2001). The rabbit CYP4A5 was found to metabolize lauric acid and palmitic acids (Hosny et al. 1999). CYP4A6 expressed in COS cells exhibited metabolism of lauric acid, but not prostaglandins (Roman et al. 1993). CYP4A7 was able to metabolise lauric and arachidonic acids with low activity to PGE1 and PGA1 (Okita and Okita 2001).

In humans, it is reported that CYP4A11 is the predominant lauric acid ω-hydroxylase in human liver, but the major ω-hydroxylation of arachidonic acid was catalysed by CYP4F2 and to a lesser extent by CYP4A11 in human kidney (Powell et al. 1998) (Lasker et al. 2000). CYP4A21 has been identified to catalyze the 6α-hydroxylation of chenodeoxycholic cid to 3α, 6α and 7α-trihydroxy-5β-cholanoic cid in pig liver (Lundell et al. 2001). Furthermore, CYP4A21 was not able to hydroxylase lauric acid (Lundell et al. 2001; Okita and Okita 2001).

Regulation of the CYP4A sub-family appears to be controlled by an orphan nuclear receptor, the peroxisome proliferators activated receptor (Llinas et al.) and most inducers of these enzymes are also involved in peroxisomal proliferation (Honkakoski and Negishi 2000) (Jeffery et al. 2004). The CYP4A sub-family has been identified to be induced by many compounds that have been known as PPs.

The PPs compounds include hypolipidemic agents (clofibrate and ciprofibrate), phthalate plasticizers (diethylhexylppthalate DEHP), solvents and lubricants

(perfluorooctanoic acid), non-steroidal anti-inflammatory drugs (aspirin and ibuprofen) and the steroid dehydroepiandesterone (DHEA)(Okita and Okita 2001). The induction of CYP4A sub-family is mediated by the activation of the PPARα, which heterodimerizes with RXR and binds to response elements in the target genes (Issemann et al. 1993) (Issemann et al. 1993).

So far, no induction of CYP4A in brain has been found with ligands for PPARα. (Although most of this ligand is acids, which don't cross the BBB)

1.9 CYP4B and CYP4F subfamilies

The CYP4B subfamily contains the least members (4B1) of all the CYP4s (Nelson et al. 1996). CYP4B1 has been expressed in human (Nhamburo et al. 1989), mouse (Imaoka et al. 1995), rats and rabbits (Gasser and Philpot 1989). In mouse, the cyp4b1 was cloned from kidney cDNA library (Imaoka et al. 1995). In rabbit, CYP4B found to be induced in the liver by phenobarbital (PB). However, no expression of CYP4B was found in rat liver (Gasser and Philpot 1989). CYP4B1 protein has been reported to hydroxylate short and medium chain length fatty acids (Fisher et al. 1998). In mouse, it is thought that cyp4b1 is responsible for carcinogenesis in renal microsomes of male mice (Imaoka et al. 1995).

Several forms of CYP4F subfamily have been identified in human and rat (Nelson et al. 1996), sea bass (Sabourault et al. 1999) (Bylund et al. 1999).

The typical function of CYP4F is hydroxylation of a wide variety of fatty acid substrates such as eicosanoids including leukotrienes (LTBs) and prostaglandins

(PGs), arachidonic acid and HETEs (Kikuta et al. 1999) (Bylund et al. 2000) (Hashizume et al. 2001) (Bylund et al. 2003).

1.10 CYP450 in the brain

Brain tissue contains a very high amount of fat which causes difficulties in detecting low amounts of protein. The brain can be exposed to neurotoxicant products such as xenobiotic metabolism from the liver and neurotransmitter system products (Lowndes et al. 1994).

CYP450s (CYP4A and CYP2C) were found in brain tissue at very low levels (Warner et al. 1991).

The content of CYP450 proteins in the brain is 0.5-2% of that in the liver (Hedlund et al. 2001) (Alexander et al. 1998). In most brain regions, the yield of P450 after extraction by hydrophobic chromatography is between 20 and 50 pmol/g and it is higher in cerebellum (300 pmol) (Warner et al. 1991).

Many cytochrome P450s are detected in the brain using *in situ hybridization,* immunohistochemical, catalytic activity and RT-PCR analysis with Southern blot quantitation have been the most used techniques (Table 1.3), which can show the tissue-specific brain P450 expression instead of general distribution (Strobel et al. 2001).

The low level of cytochrome P450 means that Western and immuno histochemical analysis have frequently given misleading results, because of the low signal, and confusion of noise with signal.

Family	CYP1		CYP2	CYP3	CYP4
Subfamily	1A1	1B1	2A 2B 2C 2D 2E 2F 2G 2J	3A	4A 4B 4F
Number of member	2	1	3 1	5	4 1 4
Northern blot detection				3A9	4F4 4F5 4F6
RT-PCR detection	1A1 1A2	2B1 2B2	2C6 2D1 2E1 2C7 2D4 2C11 2D5 2C12 2D18 2C13 2C23	3A1 3A2	4A2 4A3 4A8
Western blot and protein sequence detection			2D1 2E1 2D4		4A3 4A8

Table 1.3 CYPs of families 1-4 in Rat Brain (Adapted from Hedlund E, 2003).

Detection of CYP450 in the brain tissues was not possible in the past. However, it is now easily detected using several techniques such as RT-PCR (Warner et al. 1991), Northern blotting techniques, Immunohistochemistry, RNase protection assay and *In situ hybridization* techniques.

The presence of CYP450 at low levels in brain tissue does not mean that these enzymes are unimportant in the physiology or toxicology of the brain.

For example Aromatase, a cytochrome P450 enzyme which catalyses the conversion of androgens to estrogens in a specific brain area is present at a very low levels in the brain but play an important role in brain physiology (Sanghera et al. 1991) (Warner and Gustafsson 1994).

Cytochrome P450 plays an important role in biosynthesis and metabolism of neurosteroids, vasoactive eicosanoids, retinoids and cholesterol in the brain (Hedlund et al. 2001) (Lund et al. 1999) as well as metabolism of dopamine and anandamide, brain neurotransmitter (Bornheim et al. 1995; Hiroi et al. 1998).

A high amount of fatty acids are present in the brain, including polyunsaturated fatty acids docosahexaenoate and arachidonate, which are of physiological importance in the brain (Bylund et al. 2002).

Several CYP activities or mRNA have been reported in the nervous system, predominantly of types metabolising fatty acids and xenobiotics (sub-classes CYP2C,-2D, -2E and –4). CYP450s (CYP2C7, 2C11, 2E1, 4A3, and 4A8) can be induced by solvents such as ethanol (Warner and Gustafsson 1994).

A new CYP450 subfamily, CYP46, has also been identified in multiple subregions of mouse brain (Lund et al. 1999).

Different CYP450s isoform and amounts are expressed in different cell types in the brain (Farin and Omiecinski 1993; Lowndes et al. 1994). For example, CYP2E1 was found to be expressed in neurons but not in glia (Hansson et al. 1990) (Anandatheerthavarada et al. 1993).

In human, only two subfamilies of CYP4 have been identified in the brain, CYP4A and CYP4F (Bylund et al. 2002). CYP4A protein identification was carried out by Western blotting and it was found that CYP4A protein function could be detected using

CYP4A antiserum in brain and prostate (Stromstedt et al. 1994). It is also known that antisera can cross-react, whereas RT-PCR/ protections are much more specific.

CYP4A is expressed in the brain at low levels (Warner and Gustafsson 1994) and (Stromstedt et al. 1994). However, fatty acid hydroxylation (ω-side chain) occurs at high levels in the brain so another, as yet unidentified, P450 enzyme may be responsible for this.

The amount of the CYP4A content in the brain is 0.1% of that in the liver (Stromstedt et al. 1994).

The members of the CYP4 family present in brain include CYP4A1 (isolated from human libraries by PCR), and CYP4A3 (Yun et al. 1998). Both of these genes are expressed in brain tissues such as cerebral cortex, cerebellum and brain stem.

Finally, CYP4X1 expression has been identified in the brain stem, hippocampus, cortex, cerebellum and vascular endothelial cells of Sprague-Dawley rat (Bylund et al. 2002). In the same study, CYP4X1 shows 41-51% identity with that of CYP4A, CYP4B and CYP4F subfamily. CYP4X1 was found to be highly and specifically expressed in the rat brain tissues (Bylund et al. 2002). The role of CYP4X1 is not yet known, but it may play a role in neurovascular function.

1.11 General P450s expressed in brain tissue

Apart from the CYP4 family, several other P450s has been identified in brain. CYP2C and CYP2D are expressed in the brain and CYP2D6 is involved in the metabolism of neurotransmitters such as dopamine and anandamide (Hiroi et al. 1998) (Smith and Sachse 2001). One gene from the CYP7 family (CYP7B) has been found to be expressed in rat and mouse hippocampus (Stapleton et al. 1995) (Warner et al. 1989) (Akwa et al. 1993) (Rose et al. 1997). CYP7B has been shown to preferentially catalyse the hydroxylation of the neurosteroid DHEA, with a significant activity against pregnenolone. CYP7B1 is strongly expressed in rat and mouse hippocampus and is responsible for the metabolism of DHEA, pregnenolone and 25-hydroxycholesterol to 7α-hydroxy product. Another

CYP450 which is highly expressed in mouse brain is CYP2J9 (Qu et al. 2001) and the role of this P450 is as yet unknown.

A specific expression of CYP4X1 has been identified in neurons such as the brain stem, hippocampus, cortex, cerebellum and vascular endothelial cell (Bylund et al. 2002).

The function of CYP4X1 is not known yet, but it thought that this P450 might play un important role in neurovascular function (Bylund et al. 2002).

1.12 Expression of CYP4A genes

CYP4A1 is expressed in the liver and kidney of the rat and can be induced by peroxisome proliferators such as clofibrate [2-(4-chlorophenoxy)-2-methylpropionate]. The CYP4A subfamily genes are between 10-15 kb in size and transcribe to mRNAs of about 1.5 kb long. Expression of CYP4A may be tissue-specific and sex specific.

1.12.1 Expression of CYP4A genes in Rat

CYP4A was first identified and isolated in the rat (Tamburini et al. 1984). The CYP4A family has four members, CYP4A1, CYP4A2, CYP4A3 and CYP4A8.

Two of them have been identified from genomic clones (CYP4A1, CYP4A2) and the other two are cDNA (CYP4A3 and CYP4A8). The rat CYP4A1, CYP4A2 and CYP4A3 are found in the liver and kidney tissues and can be induced by exposure to peroxisome proliferators such as clofibrate. The high induction of this gene is found in the liver (Kimura et al. 1989). In the liver, CYP4A1 and CYP4A3 mRNA expression is found in the centrolobular region (Bell and Elcombe 1991), whereas in the kidney, expression is localised in the proximal convoluted tubule (Bell and Elcombe 1991). CYP4A1 is also expressed in extrahepatic tissues such as lung at very low levels (Stromstedt et al. 1994). However, this does not agree with work by Bell et al (Bell et al. 1992).

The CYP4A2 gene is expressed in a sex-specific manner in rats, with no expression in the female liver or kidney (Sundseth and Waxman 1992).

In males, CYP4A2 is expressed at low levels in the liver and induced strongly by clofibrate. In male kidney, CYP4A2 is highly expressed in untreated animals with low levels of induction by clofibrate(Kimura et al. 1989).

The sex specificity of CYP4A2 is regulated by the different growth hormone profiles between the sexes (Waxman et al. 1991). In the male liver, continuous exposure to growth hormone leads to inhibition of the CYP4A2 gene (Sundseth and Waxman 1992).

CYP4A3 is expressed in the lung, prostate, cerebellum, brain cortex, hypothalamus-preotic area and brainstem, but is not expressed in the seminal vesicle (Stromstedt et al. 1994). CYP4A3 is expressed in liver and kidney and is strongly induced by peroxisome proliferators (Kimura et al. 1989).

CYP4A8 is expressed in the prostate, kidney and retina (Stromstedt et al. 1994). Expression of CYP4A8 was found to be localised in the proximal tubules of the rat kidney using in situ hybridisation methods. This gene is not expressed in the liver (Stromstedt et al. 1994). Stromstedt et al, used castrated rats and androgen releasing implants to induce the CYP4A8 gene. In castrated rats, CYP4A8 expression in the prostate was completely abolished, however expression was restored by testosterone treatment.

1.12.2 Expression of CYP4A genes in Mouse

To date, three members of the cyp4a family (cyp4a10, cyp4a12, and cyp4a14) have been identified in the mouse (Henderson et al. 1994) (Bell et al. 1993) (Heng et al. 1997). Cyp4a10 was identified by Bell et al (1993) using PCR to clone a partial murine cyp4a10 cDNA fragment. Specific primers were used to generate PCR product from a highly conserved sequence (exon 8 and 12) homologue to the CYP4A1 gene. A full-length rat CYP4A1 cDNA probe was used to isolate the full-length murine cyp4a10 cDNA from mouse liver and it was found to be identical to the partial PCR fragment cloned by Bell et al, 1993). It was found that cyp4a10 was highly induced using methylclofenapate (Sanghera et al.) in mouse liver and kidney (Bell et al. 1993). The deduced cDNA of cyp4a10 encodes 509 amino acids and the full-length cDNA was approximately 1.6 kb.

Sequence alignment showed that mouse cyp4a10 and cyp4a14 were similar to rat CYP4A8 and CYP4A2 respectively.

The other cyp4a genes expressed in the mouse liver are cyp4a12 and cyp4a14.

The mouse cyp4a12 gene is highly expressed in the male liver and kidney and is not induced by treatment with MCP. In female liver and kidney, the cyp4a12 is expressed at low levels, but it is highly induced following treatment with MCP (Bell et al. 1993). This means a cyp4a12 shows a sex specific expression pattern. Cyp4a12 is found on chromosome 4 in a region synteric with human chromosome 1.

1.12.3 Expression of CYP4A genes in Rabbit

Four rabbit CYP4A genes (CYP4A4, CYP4A5, CYP4A6 and CYP4A7) have been identified (Nelson et al. 1996). CYP4A1 is expressed in the aorta of rabbits and is not inducible by treatment with ciprofibrate (Irizar and Ioannides 1995). CYP4A4 is expressed in liver, lung, small intestine and uterus of pregnant rabbits (Palmer et al; 1993a) and is induced by treatment with progesterone (Matsubara et al. 1987).

CYP4A5 is expressed in liver and kidney and is induced at very low levels in the liver following treatment with clofibrate (Roman et al. 1993).

Induction of CYP4A5 in the liver by clofibrate was found to be slightly induced, but no induction of this gene was found in the kidney (Roman et al. 1993).

CYP4A6 was found to be highly induced by treatment with clofibrate (Yokotani et al. 1989) (Roman et al. 1993).

CYP4A7 is expressed in the liver, kidney and small intestine, whereas induction of this gene by clofibrate leads to expression of this gene in the liver but not kidney (Yokotani et al. 1989) (Roman et al. 1993).

1.12.4 Expression of CYP4A genes in Human

A single gene in the human CYP4A family (CYP4A11) has been identified so far. Human CYP4A11 is located on chromosome 1 (Bell et al. 1993) and the cDNA was isolated from a kidney cDNA library (Imaoka et al. 1993) (Palmer et al. 1993). Human CYP4A11 is highly expressed in the kidney and also found in liver and lung (Palmer et al. 1993) (Imaoka et al. 1993).

1.12.5 Expression of CYP4A genes in Guinea Pig

Only a partial cDNA clone of CYP4A13 has been cloned from guinea pig liver (Bell et al. 1993). The sequence of the cloned guinea pig CYP4A13 is similar to rat and mouse genes, 77% and 82% respectively. CYP4A13 was found to be non-inducible in both male and female guinea pig treated with peroxisome proliferators (Bell et al. 1993).

1.13 The novel P450s 4Z1and 4X1

Our laboratory has cloned two novel human cytochrome P450, known as CYP4X1 and CYP4Z1. However, nothing is known of the function of these two P450s; the substrate specificity of these P450s is likely to be of interest.

1.14 Black Widow Spider Venom (BWSV)

Over the last decade the venom of black widow spider has been intensively studied. This is because the black widow spider (BWS) venom has a component named α-latrotoxin, which has been shown to be extremely useful for studying synaptic transmission. Clinically, black widow spider bites are not a significant health problem for humans because they rarely cause serious disease. In severe cases, the patient has a generalized muscle pain, abdominal cramps, blood pressure, and tachycardia (Muller 1993). The active components of the venom of black widow spider are high molecular weight proteins compared with other spider venoms (Frontali et al. 1976). Seven separate venom components were isolated by further ion exchange and hydrophobic fractionations(Krasnoperov et al. 1997). These components are named α-latrocrustatoxin (α-LCT), α-latroinsectotoxin, β-latroinsectotoxin (β-LIT), γ-latroinsectotoxin (γ-LIT), δ-latroinsectotoxin (δ-LIT), ε-latroinsectotoxin (ε-LIT) and α-latrotoxin (α-LTX). Only α-LTX is a vertebrate toxin (Knipper et al. 1986). α-latrotoxin (α-LTX) is a high molecular weight protein (120 kDa) on SDS-PAGE (Sudhof 2001) (Frontali et al. 1976).

α-latrotoxin stimulates exocytosis only after binding to specific neuronal receptors (Tzeng and Siekevitz 1979). Two classes of receptors for α-latrotoxin have been identified in brain, neurexin (Ushkaryov et al. 1992) (Ushkaryov et al. 2004) and latrophilin (CIRL)(Davletov et al. 1996) (Krasnoperov et al. 1996) (Tobaben et al. 2002). Neurexin binds α-LTX in the presence of Ca^{2+} , whereas latrophilin or CIRL (Calcium Independent Receptor for Latrotoxin) binds α-LTX without the

presence of Ca^{2+} [calcium independent] (Sugita et al. 1998) (Ichtchenko et al. 1999) (Petrenko et al. 1999).

Recently, a third α-LTX receptor, protein-tyrosine phosphatase-σ, has been identified that binds toxin in a Ca^{2+} -independent manner (Krasnoperov et al. 2002).

The mechanism of the interaction between the latrotoxin and these receptors (latrophilin and neurexin) has still not been fully understood. It was proposed that these receptors serve as targeting sites to bring the toxin to the necessary location on the cell surface. The nematode *C.elegans* is used as a model system to examine the relationship between the BWSV and the latrophilin receptors.

1.14.1 Latrophilin/CIRL (LPH)

α latrophilin is a neurotoxin from black widow spider venom, which causes massive exocytosis of synaptic vesicles (Lelianova et al. 1997). The Latrophilin receptor was isolated from rat (Bittner 2000) and bovine brain membranes by affinity chromatography (Davletov et al. 1996) (Krasnoperov et al. 1996). Three closely related forms of latrophilin are expressed in vertebrates (Sugita et al. 1998). These forms of latrophilin are latrophilin-1, latrophilin-2, and latrophilin-3. When these forms are expressed in various tissues, only latrophilin-1 and latrophilin-2 bind to α-LTX at nanomolar concentrations, whereas the latrophilin-3 protein cannot bind the toxin (Ichtchenko et al. 1998). LpH 1 and LpH 3 are highly expressed in mammalian brain, wherease LpH 2 is expressed ubiquitously (Sugita et al. 1998) (Ichtchenko et al. 1999) (Matsushita et al. 1999). All of the latrophilins are G-protein coupled receptors with large intra and extracellular

domains and all have the same domain structure (Lelianova et al. 1997) (Henkel and Sankaranarayanan 1999). However, the *C.elegans* latrophilin is uncharacterised. Fig 1.3, shows the structural domains of latrophilin (Sugita et al. 1998) (Modified from Sugita et al, 1998). Latrophilin consists of four domains: lectin-like domain, olfactomedin-like domain, the domain which shows homology with G-protein coupled receptor BAI1-3 a brain specific angiogenesis inhibitor and cysteine rich domain (Sugita et al. 1998) (Danilevich et al. 1999).

The cysteine rich sequence is thought to be responsible for a proteolytic cleavage signal during the maturation of G-protein-linked receptor (Krasnoperov et al. 1999) (Ichtchenko et al. 1999). All of these domains are located in the extracellular plasma membrane and contains ~ 500 amino acids. The intracellular part has seven transmembrane regions of latrophilin, similar to the calcitonin/secretin family of G-protein coupled receptors. It also contains a long intracellular C-terminal tail, which is approximately 500 amino acids in length.

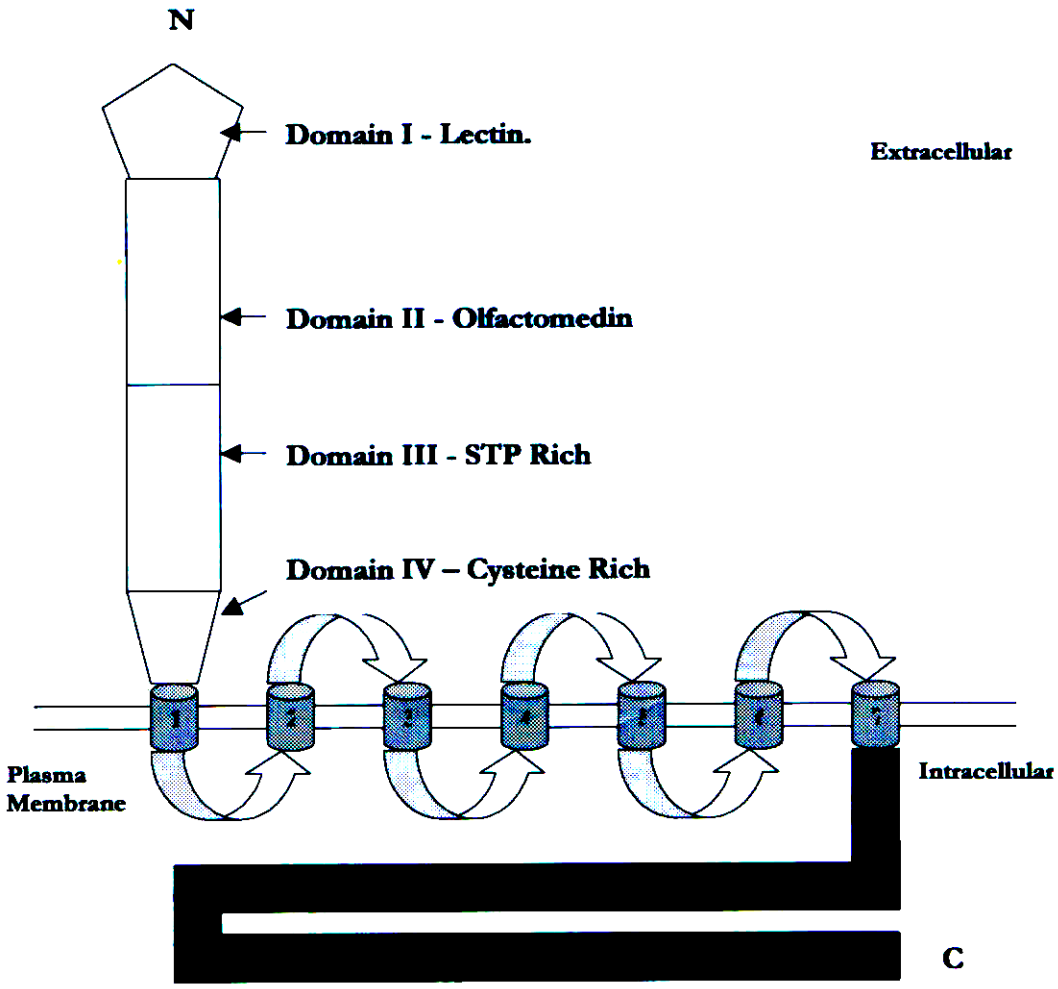


Figure 1.3 Domain structures of latrophilin/CIRL
Four domains are present in the extracellular region, while no domains in the intracellular region. The seven transmembrane regions are similar to those in the calcitonin/secretin receptor family. [Modified from Sugita et al, 1998].

Latrophilin and neurexin Iα are known to bind the mammalian-specific α-LTX. Therefore, homologues of these receptors in *C.elegans* may be responsible for the latrotoxin effects in *C.elegans.*

The *C.elegans* latrophilin homologue was compared with the rat latrophilin homologue and showed several conserved domains. Figure 1.4, shows alignment of the *C.elegans* latrophilin domains and rat latrophilin domains. These domains

are the galactose-binding lectin at residues (51-133), a hormone receptor motif at residues (181-240), a GPS (G-protein-coupled receptor protease cleavage site) motif at residues (493-541), a seven-transmembrane domain of the secretin family at residues (548-799), and a sequence of four conserved cysteine residues at (815-870).

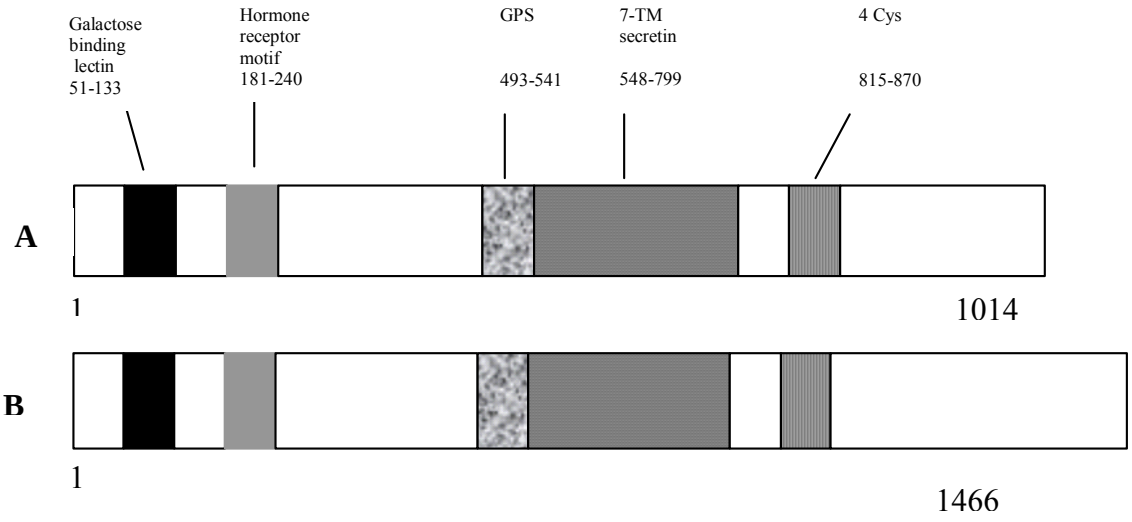


Figure 1.4 Domain structure of the latrophilin.
(A) shows the *C.elegans* homologue. A galactose-binding lectin domain (51-133 is shown by a black box, hormone receptor motif (181-240) is shown by solid grey box. The G-protein-coupled receptor proteolytic site domain (493-541) is shown by black colour on a grey background. The seven transmembrane domain (7-TM secretin) (548-799) is shown by black dots on a white background, that similar to those of the calcitonin/secretin receptor family. A domain of four conserved cysteine residues (815-870) is shown by black lines on a white background. **(B)** shows the rat latrophilin homologue. Protein conserved region is shown by alignment of image **(A)** and **(B).** Labelling numbers (1-1014) and (1-1466) show total amino acid size of *C.elegans* and rat latrophilin respectively.

In order to understanding the function of α-latrotoxin, LpH 1 and neurexin 1 knock-out mice has been produced and demonstrated that both LpH 1 and neurexin were a major latrotoxin receptors in brain (Tobaben et al. 2002).

The role of latrophilin in vivo and the natural ligand of the receptors are unknown. Recent work in this laboratory has shown that it mediates the action of black widow spider venom, and so it is important to find out if latrophilin has a similar mechanism of action in *C.elegans*. The development of antibodies against this protein is an essential first step for subsequent functional analysis.

1.15 Aryl hydrocarbon receptor (AhR)

Aromatic hydrocarbons can be classified into two types based on their chemical structure. Type-I are halogenated aromatic hydrocarbons (HAHs) and Type-II are non-halogenated polycyclic aromatic hydrocarbons (PAHs).

The AhR gene is present in all vertebrates and even in *Caenorhabditis elegans* (Hahn 2002) (Nebert et al. 2004).

The Aryl hydrocarbon receptor is a soluble protein present in most organs and cells that shows high affinity specific binding to certain planar aromatic compounds, such as polycyclic aromatic compounds (*e.g.*, 3-methylcholanthrene) and halogenated aromatic compounds (*e.g.*, TCDD [2,3,7,8 tetrachlorodibenzo-p-dioxin]) and initiates the coordinated expression of human CYP1A1, CYP1A2 and CYP1B1 (Hedlund et al. 2001) (Bell and Poland 2000) (Poland et al. 1991) (Poland and Glover 1990) (Poland and Glover 1987).

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor belonging to the basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) family of transcription factors.

This bHLH/PAS family plays an important role in the regulation of circadian rhythms, activation of xenobiotic responses and cell fate determination. The PAS represents the first letters of the three members of the family PER (product of Drosophila Period gene), ARNT (AH receptor nuclear translocator) and SIM (product of Drosophila Single-minded protein) (Crews 1998) (Korkalainen et al. 2004).

The most potent inducing agent for AhR is the environmental pollutant and carcinogen TCDD, which is a very strong CYP4A1 inducer. It has also been known some endogenous ligand such as flavonoids in the dietary (Ashida 2000), indols and tryptophan

derivatives and benzoflavones (Denison and Nagy 2003). Furthermore, some urine products such as indirubin and indigo were found to be very potent inducers of AhR than TCDD in a yeast assay system (Adachi et al. 2001).

AhR can be induced by either environmental pollutant such as TCDD (Huff et al. 1994) or diet (Denison and Nagy 2003) (Denison et al. 2002).

Typically, the mechanism of AhR action starts when the ligand binding to AhR receptor induces nuclear translocation (Gasiewicz and Bauman 1987) and then makes a complex with bHLH/PAS protein, ARNT (heterodimerization) (Reyes et al. 1992). This heterodimerization recognizes and binds specific DNA sequence, dioxin responsive elements (DREs), within gene promoter regions and modulates subsequent gene transcription (Fujisawasehara et al. 1987).

Recently, a number of endogenous AhR ligands have been reported to have a high affinity toward AhR. Most of these chemicals are tryptophan derivatives (Fujii-Kuriyama and Mimura 2003).

AhR ligands such as TCDD are involved in the induction of some CYP450s such as CYP1A1 and CYP1B1 in most body tissues, but in brain it was found to be at very low levels (Hedlund et al. 2001).

It was found that both AhR and ARNT are expressed in the brain (Dolwick et al. 1993) (Li et al. 1994) (Kainu et al. 1995).

TCDD can penetrate poorly into rat brain and the concentrations found are far lower than in the liver or adipose tissue (Pohjanvirta et al. 1990). However, after repeated exposure TCDD induces more significant oxidative damage in brain than in liver (Hassoun et al. 2000).

TCDD may also penetrate into the brain, where a high region concentration was in the hypothalamus (Pohjanvirta et al. 1990).

The molecular size of AhR shows a variation between species. It was 95 kDa in mouse, 101 kDa in chicken, 103 kDa in guinea pig, 104 kDa in rabbit, 106 kDa in rat, 106 kDa in human, 113 kDa in monkey and 124 kDa in hamster (Poland and Glover 1987).

It has also been reported that variation was found within rat strains where 106 kDa of inbred strain and 101 kDa and/or 106 kDa allelic form of outbred Long-Evans rats (Poland and Glover 1987).

In liver cytosol, two AhR molecular weight, 95 kDa and 70 kDa, were produced indicating the 70 kDa was due to proteolytic product of the bigger peptide (90 kDa) (Poland and Glover 1987).

It has also been reported that mouse variation of an AhR molecular size found within allele forms. The Ah^{b1} allele has 95000 kDa, Ahb^{2} has 104 kDa and Ahb^{3} has 105 kDa (Poland and Glover 1990).

In the brain, it was found that TCDD neurotoxicity was mediated by the AhR in the mouse cortical endothelial cells and astrocytes (main cellular components of the BBB) (Filbrandt et al. 2004).

1.6 Aims of the thesis

Our laboratory has cloned two novel human cytochrome P450, known as CYP4X1 and CYP4Z1. However, nothing is known of the function of these two P450s. In order to address this problem, this project will aim to delineate the site of expression of these proteins. As a first step to addressing this, specific antisera against these cytochrome P450s will be prepared, prior to western and immunohistochemical approaches.

Specific objectives include:

1) Characterising the antisera for sensitivity and selectivity of CYP4X1 and CYP4Z1.
2) Characterising the recombinant antigen of CYP4X1
3) Expression and Tissue-specific localization of CYP4X1

Whilst characterising the antisera against CYP4Z1, high sensitivity antisera against recombinant antigens of AhR and *C. elegans* latrophilin were obtained, and subsequently used to characterise the full-length, native proteins.

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Chapter 2 Materials and Methods

2.1 Materials

2.1.1 General Materials

All general chemicals used for experiments were obtained at the highest grade. The radioactivity [α-32P]-CTP (800 Ci/mmol) was obtained from ICN Flow Company. DNase-free RNase 1U/µl was obtained from Promega. Oligonucleotides were synthesised by J.Keyte of the Polymer Synthesis and Analysis Unit, Department of Biochemistry, University of Nottingham. UHP grade water >13MΩ/cm³ was produced by using Purite Select Bio system. ProSTAR UltraHF RT-PCR for RT-PCR Kit of 1st strand cDNA synthesis was purchased from Stratagene. Plasmid mini-prep kit, maxi-prep kit and gel extraction kit were obtained from QIAGEN Ltd. X-ray film was manufactured by Fuji and obtained from Amersham International plc. Developer and Fixer were obtained from Ilford. DEPC was obtained from Sigma. DEPC-treated water was prepared by adding 1 ml of Diethyl Pyrocarbonate (dissolved in 9 ml absolute ethanol) to 1 litre of UHP water and the mixture has incubated in a fume hood for 1 hour and then autoclaved for 20 minutes at 120ºC. Sequa gel stock acrylamide solution (19:1 acrylamide: bisacrylamide) was obtained from National Diagnostics. Triazol reagent was obtained from (Invitrogen), RNA quantitation reagent and kit were obtained from Molecular Probes, Restriction enzymes were obtained from Biolab, pGEMT-easy vector was obtained from Promega, pRSET-a his-tag expression vectors was obtained from Invetrogen, yeast tRNA was obtained from Sigma. SP6 polymerase (20,000 U/ml) was obtained from Biolab, T7 polymerase (50,000 U/ml) was obtained from Biolab,

RNase A (80 ng/µl) + T1 (250 U/µl) Mix was from PharMingen , DNase I was obtained from Sigma, DNA was obtained from Sigma, IPTG was obtained from BRL, Acrylamide stock solution (30% acrylamide w/v, 37.5 : 1 bis-acrylamide) was obtained from Severn Biotech LTD. Transcription Buffer was obtained from Boehringer Mannheim, 1 kb marker DNA ladder was obtained from Gibco-BRL, Bacterial strains BL21 (DE3) and BL21 (DE3) plysS were obtained from Novagen, RNA guard RNase inhibitor was obtained from Amersham Pharmacia Biotech Inc, Mouse genomic DNA was obtained as a gift from Dr. David Bell, Nottingham university, Goat anti-rabbit IgG conjugated secondary antibody was obtained from BIO-RAD, Bacterial strain JM109 was obtained from Promega, His.Bind resin was obtained from Novagen, T4 ligase and buffer were obtained from Biolab, *pfu* Turbo DNA polymerase (2.5 U/µl) was obtained from Stratagene,1.1 x Reddy Mix PCR Master Mix (1.5 mM MgCl2) was obtained from ABgene, Ribonuclease Protection Assay (RPA) Kit was obtained from PharMingen.

2.1.2 pRSET-a-mcyp-4x1 plasmids

A genomic fragment of mouse *CYP*4X1-exon 12 was cloned into pGEMT-easy (Promega) in our laboratory to generate the plasmid pGEMT-m-*CYP*4X1-ex12 (section 2.2.3.12). This plasmid contains coding and non-coding region of amplified PCR fragments of exon12 from the genomic mouse *CYP*4X1. The PCR generates a product of 329 b.p; 191 b.p are derived from exon 12 and 138 b.p are intron as shown in Figure 2.1. A search of the mouse genome using the BLAST algorithm (28/07/05) with this sequence detected no other sequences with significant sequence identity to this region of the Cyp4x1 clone.

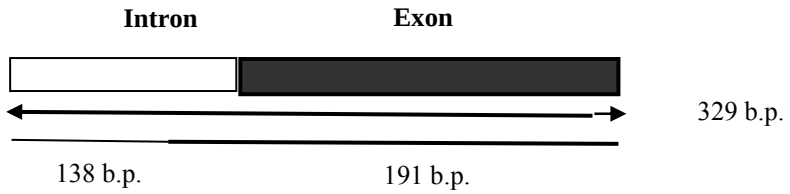

Figure 2.1. PCR of partial sequence of a mouse CYP4X1-exon 12 gene.
The full length of this sequence is 329 b.p including both exon and intron. The PCR was predicted to generate a 329 b.p. fragment on genomic mouse DNA.

A PCR fragment of full-length m-*CYP*4X1 cDNA (section 2.2.4.1) was cloned into pRSET-a for expression to give the plasmid pRSET-a-m-*CYP*-4X1. The plasmid pRSET-a-m*CYP*-4X1 contains the T7 promoter, with a six-histidine tag at the N-terminus of the fusion protein. The sequence of the protein shows that there are 49 amino acids from the pRSET-a vector, while the inserted DNA was 497 amino acids as shown in the Figure 2.2.

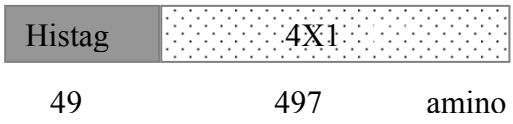

Figure 2.2 Diagram of protein produced from pRSET-a-mcyp-4x1.
The shaded region represents the six-histidine tag fusion and the black dots on a white background show the full-length of mcyp-4x1 protein region cloned into expression vector (pRSET-a).

2.1.3 pRSET-b-hCYP-4z1 plasmids

A fragment of the human CYP4Z1 (residues 177-493) was inserted as a XHOI-HindIII fragment into pRSET-b vector (pRSET-b-hCYP-4z1) for expression in *E.coli.* The oligonucleotides 5'AACAAATGGGAGGAACACATTGCCCAAAACTCGAGTCTGGAGCTC TTTCAACATGTCTCC3' and 3'GTGAGTTCCGGAGGGGTCGGACAAGCAGTTCAACAGGAGTTCACT TCCGAACCTTAGGTA 5' were used to PCR the sequence encoding amino acids (177-493) of the human 4Z1 cDNA. The amino acid sequence shows 318 amino acids of 4Z1 protein and 36 amino acids from the vector as shown in the Figure 2.3. The calculated molecular weight is 39,700 Da.

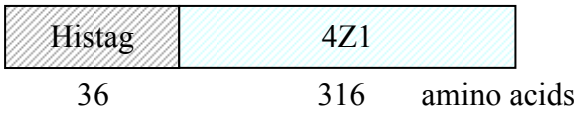

Figure 2.3. Diagram of protein produced from pRSET-b-hCYP-4z1.
The shaded region represents the six-histidine tag fusion and the plain region represents the partial cDNA of human CYP-4z1 protein cloned into expression vector (pRSET-b).

2.1.4 pRSET-AhR285-416 plasmids

A fragment of the mouse AhR b-1 allele (residues 285-416) clone '5g´ (J, Tao. Thesis, 2004) was inserted by BamHI digestion into the pRSET-c vector. The cloned DNA construct pRSETc-AhR285-418 contains 168 amino acids of AhR and the calculated molecular weight is 19,267 Da. The plasmid pRSET-AhR285-418 contains the T7 promoter, with a six-histidine tag at the N-terminus of the fusion protein.

The sequence of the protein shows that there are 35 amino acids from the pRSET-c vector, while the inserted DNA was 133 amino acids. Figure 2.4, shows a diagram of the mouse AhR domain structure.

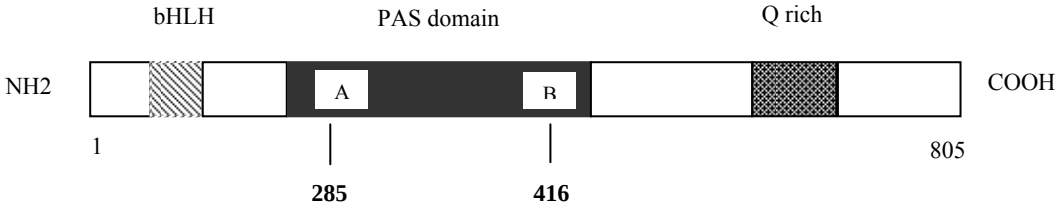

Figure 2.4. Domain structure of mouse AhR.
(b) represents the basic region, HLH represents the helix-loop-helix. PAS represents the first letters of the finding PAS domain membrane in *Drosophila* **P**eriod clock protein, the vertebrate **A**ryl hydrocarbon receptor nuclear translocator and *Drosophila* **S**ingle-minded protein. The highlighted numbers (285 and 416) represent the region of the cloned mouse AhR b-1 allele fragments inserted in the expression vector (pRSET-c).

2.1.5 pRSET-c-LpH plasmids

Latrophilin (LpH) is the receptor for toxic proteins produced by Black Widow spider. A partial *C.elegans* LpH cDNA was cloned into pRSET-c for expression as a histidine-tagged fusion containing 373 amino acids of LpH, from 168 to the end of amino acid number 542. This partial clone of LpH protein contains the hormone receptor motif (181-240) and the G-protein-coupled receptor protease cleavage site (493-541) of the extracellular domain as shown in Figure 2.5.

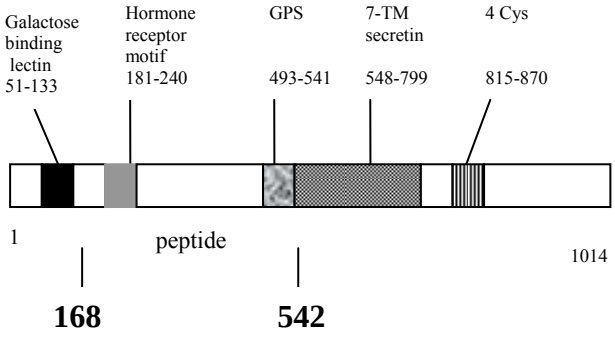

Figure 2.5. Domain structure of the latrophilin.
GPS represents the G-protein-coupled receptor proteolytic site domain. 7-TM secretin represents the seven transmembrane regions that similar to those of the calcitonin/secretin receptor family. The highlighted numbers (168-542) represent the site of the partial sequence of LpH protein that amplified to generate the cloned LpH cDNA inserted into pRSET-c expression vector.

2.2 Methods

2.2.1 Animals

Adults (male) C57 BL/6N and 129S4/SvJae strains mice were obtained from Queen Medical Centre (QMC). Rabbits (Female New Zealand White rabbits ~ 4 Kg) were obtained from Queens Medical Centre (QMC) of Nottingham University. The animals were allowed to eat ad libitum and had access to drinking water at all times.

2.2.1.1 Dosing of 129S4/SvJae strain mice

The treated control group were given a single dose by gavage of vehicle control, 10ml/kg of corn oil containing 2.5% (v/v) p-dioxane. The TCDD group was given a single dose by gavage of 10ml/kg of corn oil containing 2.5% (v/v) of p-dioxane and 50μg/kg TCDD. Dosing for the treated control and the TCDD mice group were performed once on day 1 and then animals were sacrificed in the morning of day 3. The Phenobarbital group was dosed daily by intraperitoneal injection with 80mg/kg PB in saline (0.75ml) for three days and sacrificed on the following day.

The Ciprofibrate group were dosed daily by gavage with 50mg/kg in corn oil (0.75ml) for three days and sacrificed on the following day. The Dexamethasone group were dosed daily by gavage with 50mg/kg in corn oil as a suspension for three days and sacrificed on the following day. 129S4/SvJae wild type (+/+) mice and knock-out (-/-) mice for PPARα were used as untreated control group (Akiyama T. E, et al; 2001).

The untreated control group were allowed to eat ad libitum and had access to drinking water for three days and then sacrificed on the following day. Animal work was done by Declan Brady. The animals were sacrificed by cervical dislocation and liver, kidney, brain; heart, spleen, lung and aorta were weighed and harvested quickly and then flash frozen in liquid nitrogen. The frozen tissues were then stored at -80°C until use.

2.2.2 Antiserum Production

The production of the antibodies was carried out by injection of female New Zealand White rabbits (~ 4 Kg) subcutaneously with purified recombinant proteins. An initial test bleed (5 ml) was taken as a preimmune bleed sample prior to starting the immunization course. The test bleed was taken from the peripheral ear vein of each rabbit. In order to produce polyclonal antibodies, rabbits were injected with approximately 100µg of recombinant protein as a 50% emulsion in Freunds incomplete adjuvant. One injection of the antigen was done every month for each rabbit. The antisera were titred after each immunization to check the sensitivity of the antisera, and immunization was repeated periodically until a good signal was obtained. A terminal bleed was taken after obtaining a high potency antiserum.

The terminal bleed was then incubated in a fridge at 4ºC for overnight. The blood was then centrifuged at 3000 rpm for 15 minute. The plasma was taken, aliquoted in eppendorf tubes and then stored at –20°C.

2.2.3 General Molecular Biology Techniques

2.2.3.1 Bacterial growth media

E.coli JM 109 strain was used in all experiments and was obtained from Stratagene.

The following components of the bacterial growth media were used:

Luria-Bertania (LB) per litre:

10 gm Tryptone

5 gm Yeast extract

10 gm NaCl

All the materials above were made up to 1 litre with UHP water and autoclaved for 20 minutes at 120°C (15lb/in²). LB-agar media was made by adding 15gm of Bactoagar to 1 litre LB-Broth and autoclaved for 20 minutes at 120°C (15lb/in²). For preparing LB agar plates, the media was allowed to cool and then an appropriate antibiotic, e.g. Ampicillin to a final concentration of 50 µg/ml was added and the media was poured into 10 cm Petri dishes.

2.2.3.2 Transformation

2.2.3.2.1 Preparation of electro competent cells

A single colony from freshly streaked JM109 cells was grown in 5 ml of LB broth overnight on the incubator shaker (220 rpm) at 37°C. 50 ml of LB broth was added to the overnight culture of cells and placed on the shaker for 2-3 hours or until $O.D_{600}$ of 0.5-0.8 was reached. The cells were then cooled on ice and then centrifuged in a JA20 rotor at 23700xg for 10 minutes at 4°C. The supernatant was removed and the pellets were then washed 4 times with 20 ml of ice-cold UHP water and were then centrifuged at 23700xg for 10 minutes at 4°C. The washed water was decanted and the pellets were resuspended in1 ml of ice-cold water. The wash water was again decanted and the pellets were weighed and resuspended with two volume of ice-cold water. The cells were then placed on ice until use for electrotransformation.

2.2.3.2.2 Plasmid transformation into electro competent cells

Electroporation cuvettes (0.2 cm Bio-Rad genepulser cuvette) were placed on ice. Aliquots of 40ul competent cells were placed on ice and 1µl of plasmid DNA was added and mixed thoroughly. The mixture of the plasmid DNA and cells was then transferred to the prepared cuvette and electroporated at 1.8 kv. The cells were then immediately resuspended in 1ml of LB broth and then transferred to a new eppendorf tube. The cells were then incubated in a water bath at 37°C for 30 minute. Selection of the transformed cells was obtained by

growing the transformed cells on 1% LB glucose agar plate containing Ampicillin (50 μg/ml) antibiotic.

2.2.3.3. Phenol preparation

500 gm of crystalline phenol was melted at 69°C and then 0.1 % (w/v) hydroxyquinoline was added. The melted phenol was then mixed with an equal volume of 500 mM Tris-HCl, pH 8.0 and stirred for 15 minutes at room temperature. The mixture was then allowed to separate into two phases. The aqueous phase was removed and the organic phase was then equilibrated with an equal volume of 0.1 M Tris-HCl (pH 8.0) with continuous stirring for 15 minutes. The mixture was then allowed to settle into two phases. Again the aqueous phase was removed and the organic phase was treated several times with 0.1 M Tris-HCl until the phenol reached a pH of 8.0. The equilibrated phenol under the 0.1 M Tris-HCl pH 8 was then stored in the dark at 4°C.

2.2.3.4 Phenol/Chloroform extraction

In order to remove any protein contaminants, phenol/chloroform extraction method was used. One volume of phenol/chlorophorm mixture was added to the nucleic acids. The mixture was then vortexed thoroughly into an emulsion for 1 minute and then centrifuged at 20817xg for 5 minutes in a bench top centrifuge at room temperature. Two separated layers (phenolic and aqueous) and an interface were obtained. The aqueous layer contains the nucleic acids whereas the interface layer contains the contaminant protein. The aqueous layer was taken carefully into a fresh eppendorf tube.

2.2.3.5 Ethanol precipitation of DNA

Nucleic acids were precipitated by adding two volumes of ice-cold absolute ethanol (100%) and 0.25 volume of 4M ammonium acetate. The solution was mixed vigorously by hand for 15 second and then placed at –20°C for 30 minutes. The tube was then centrifuged at 23700xg in a microfuge for 15 minute at room temperature. The supernatant was decanted and the pellets were then washed with 90% ice-cold ethanol (2 volumes) and centrifuged at 23700xg for 5 minutes. The supernatant was decanted and the pellets were then air-dried for 5 minutes to remove any remaining ethanol. The pellets were resuspended in DEPC-treated water.

2.2.3.6 Isolation of plasmid DNA (small-scale, crude preparation)

Alkaline lysis is a method for isolating plasmid DNA. This method is based on Sambrook. et al (1995). Bacterial colonies were inoculated in 15 ml LB broth containing an appropriate antibiotic and incubated with shaking overnight at 37ºC. One ml of each bacterial culture was centrifuged at 20817xg for 1 minute in bench top centrifuge. The supernatant was decanted and the cell pellets were resuspended in 100µl of solution I [50 mM glucose, 25 mM Tris-HCl pH 8, 10 mM EDTA and RNase A (100µg/ml)]. To this, 200ul of solution II [200 mM NaOH, 1% SDS], freshly prepared, was added to each eppendorf tube and the solution were mixed by inversion. The samples were then allowed to stand for 5 minutes at room temperature. 150 µl of ice-cold solution II [5 M potassium acetate, 1.5% glacial acetic acid] was then added to each eppendorf and mixed by inversion and then placed on ice for 10 minutes to allow the

bacterial lysate to precipitate. The samples were then centrifuged 20817xg for 10 minutes. The supernatant was taken to a new eppendorf tube and extracted with one volume of phenol/chloroform and then centrifuged at 20817xg for 5 minutes. The aqueous layer was taken to a new eppendorf and the DNA was precipitated by addition of ¼ volume of 4M ammonium acetate and 2 volume of 100% ethanol (section 2.2.3.5). The sample was then mixed gently and then incubated at -80ºC for 30 minute. The tube was then centrifuged in a microcentrifuge at 20817xg for 15 minute at 4ºC. The supernatant was decanted and the pellets were then washed with 75% ethanol and centrifuged at 20817xg in a microcentrifuge for 5 minute at 4ºC. The supernatant was removed and the pellets were then allowed to air-dry for 5 minutes. The plasmid DNA pellets were then resuspended in 50µl of DEPC water and stored at -20 ºC.

2.2.3.7 Isolation of plasmid DNA Qiagen miniprep method

This method is based on QIAGEN Handbook. 1.5 ml of an overnight culture of bacteria was centrifuged at 20817xg for 1 minute at 4ºC in a minicentrifuge.

The supernatant was decanted and the pellets were resuspended in 250µl of buffer P1 (resuspension buffer). To this 250µl of buffer P2 (lysis buffer containing NaOH) was added and the tubes mixed gently by inversion for 4-6 times and then 350µl of neutralization buffer N3 (guanidine HCL, acetic acid) was added. The tubes were then mixed gently by inversion again for 4-6 times and then centrifuged at 20817xg for 10 minute in a bench top centrifuge. The supernatant was transferred immediately onto the Qiagen column and then centrifuged at 20817xg for 1 minute.

The flow-through was decanted and the column was washed with 0.5 ml of PB (phosphate buffer) and then centrifuged at 20817xg for 1 minute. The column was then washed with 0.75 ml of elution buffer PE and centrifuged at 20817xg for 1 minute. To elute all the PE buffer, the column was centrifuged again at 20817xg for another 1 minute. The plasmid DNA was eluted from the column using 50µl of UHP distilled water and incubated at room temperature for 1 minute prior centrifuged at 20817xg for 1 minute and eluted in a clean eppendorf tube. The plasmid DNA was then stored at -20ºC until use.

2.2.3.8 Isolation of plasmid DNA Qiagen maxi prep method

This method is based on QIAGEN Handbook 10 ml of the overnight cell culture was prepared as mentioned in section (2.2.3.7). The overnight culture of bacteria was diluted with 100 ml LB broth containing antibiotic and then grown overnight at 37ºC on the shaker (220 rpm). The culture of cells was then centrifuged at 6000xg on a JA10 rotor for 20 minutes at 4ºC. The supernatant was decanted and the pellets were resuspended in 10 ml of buffer P1 (50mM Tris-HCl, pH 8). To this, 10ml of lysis buffer P2 (200 mM NaOH, 1% SDS w/v) were added and mixed thoroughly 6 times by inversion and then incubated at room temperature for 5 minutes. After incubation, 10 ml of neutralisation buffer P3 (3 M potassium acetate, pH 5.5) was added and mixed by inversion 6 times and then incubated on ice for 20 minute. The cells were then centrifuged at 20000xg on a JA20 rotary for 30 minute at 4ºC.

The supernatant was taken to a clean tube and placed on ice. A Qiagen-tip 500 was equilibrated with 10 ml of buffer QBT (750 mM NaCl, 50 mM MOPS, pH 7.0, 15% isopropanol v/v, 0.15% Triton® X-100 v/v). The supernatant was transferred to the Qiagen-tip column and allowed to pass through using the

gravity force to pull the supernatant through. The column was then washed twice with 30 ml of wash buffer QC (1.0 M NaCl, 50 mM MOPS, pH 7.0, 15% isopropanol v/v). To elute the DNA, 15 ml of elution buffer QF (1.25 M NaCl, 50 mM Tris-HCl, pH 8.5, 15% isopropanol v/v) was added to the column and the DNA was collected in a clean tube. The DNA was then precipitated by adding 10.5 ml of isopropanol and then mixed and centrifuged at 15000xg in a JA20 rotor at 4ºC for 30 minute. The supernatant was decanted and the pellets were washed with 5 ml of 70% ethanol and then centrifuged at 15000xg in a JA20 rotor at 4ºC for 10 minutes. The ethanol was decanted and the pellets were resuspended in 1 ml of DEPC water. The DNA sample was then stored at -20ºC until use. The DNA concentration was determined as mentioned in section (2.2.3.16).

2.2.3.9 RNA extraction from mouse tissues

Total RNA was extracted with TRIAZOL reagent kit (Invitrogen). TRIAZOL commercial reagent kit contains a solution of phenol and guanidine isothiocyanate.

The extraction was carried out as described in the manufacturer's instructions and all the glassware and the tubes were cleaned and autoclaved. Typically, approximately 0.1 gm of mouse tissue was homogenised in a Potter-Elvehjem glass homogenizer with a Teflon pestle with 1 ml TRIAZOL reagent in a glass tube. The mixture was centrifuged at 12000xg for 10 minutes at 4ºC in a microfuge. This is an optional centrifugation step to remove cell debris. The cleared homogenate was then transferred to a clean eppendorf and incubated for 5 minutes at room temperature to allow complete dissociation of nucleoprotein complex. 0.2 ml of chloroform was added to the solution and

shaken vigorously by hand for 15 second. The solution was then incubated at room temperature for 3 minutes. The solution was then centrifuged at 12000xg for 15 minutes at 4ºC. Three layers were obtained and only the aqueous layer was taken. This aqueous layer was taken into a fresh eppendorf and 0.5 ml of isopropanol / 1 ml Triazol reagent was added to precipitate the RNA. The samples were then incubated at 4ºC for 10 minutes. The samples were then centrifuged at 12000xg for 10 minutes at 4ºC. The supernatant was decanted and the samples were then washed by 1 ml ethanol. The samples were mixed and centrifuged at 7500xg for 5 minutes at 4ºC. The samples were then dried for 5 minutes and then resuspended with 100 µl DEPC-treated water. The samples were stored at –80ºC until use.

2.2.3.10 Purification of DNA from an agarose gel

Amplified PCR products were purified by using the QIAquick gel extraction kit (QIAGEN), as described in the manufacturers instructions. The PCR fragments were visualised under visible Light using a Dark Reader (Clare Chemical Research) and the PCR fragment was then excised with a sharp scalpel. The gel slice was then weighed and transferred to a clean eppendorf tube. Three volumes of solubilization buffer QG (guanidine thiocyanate) was added to the gel slice and then incubated at 50ºC for 10 minutes with inverting every 2-3 minutes. The melted gel slice was then applied into the QIAquick column and centrifuged at 20817xg for 1 minute at 4ºC.

The flow-through was decanted and the column placed to the same collection tube. The column was then washed with 0.75 ml of buffer PE and centrifuged at 20817xg for 1 minute at 4ºC. The flow-through was discarded and the column was then centrifuged again at 20817xg for another 1 minute at 4ºC.

For eluting the DNA, the column was placed on a clean 1.5 eppendorf tube and then 50 µl of distilled water were added to the centre of the QIAquick membrane. The column was then incubated at room temperature for 1 minute. The column was then centrifuged at 20817xg for 1 minute at 4ºC. The eluted DNA was then stored at – 20ºC until use.

2.2.3.11 Restriction digestion of DNA

Restriction digestion was carried out according to the standard protocol of Sambrook et al, 1989, but using digest buffers provided by the manufacturer. The final volume of the reaction takes place in 100µl in an eppendorf tube (1.5 ml) at 37ºC for 2 hours. The digested reaction contains of the plasmid DNA mixed with 4µl of a restriction enzyme and an appropriate 10X restriction enzyme buffer to a final volume of 100µl distilled water. The mixture was then mixed by vortex and then quick centrifuged for 10 second on the bench top at room temperature. The reaction was then incubated at 37ºC for 2 hrs. Ethanol precipitation was carried out to stop the reaction as described in section 2.2.3.6.

2.2.3.12 Ligation of PCR products pGEMT-easy vector.

Ligation is the process of the formation of phosphodiester bonds in the presence of ATP between double-strand DNAs with 3-hydroxyl and 5-phosphate termini. The insert DNA was gel purified as described in the section (2.2.3.10). The ligation reaction was carried out for both the experimental and the control samples by adding the following components:

Component	Experimental	Control
2X Rapid ligation reaction	5µl	5µl
pGEMT-easy vector (50ng/µl)	1µl	1µl
PCR product	2ul	----
T4 DNA ligase (3 U/µl)	1µl	1µl
Distilled water	1µl	3µl

Rapid ligation buffer components:

60 mM Tris-HCl, pH 7.8

20 mM MgCl2

20 mM DTT

2 mM ATP 10% PEG

The Ligation reaction was placed in a clean 1.5ml eppendorf tube and vortexed gently prior to brief centrifugation for a few seconds. The ligation reaction was then incubated at room temperature for 2 hours.

2.2.3.13 Agarose gel electrophoresis of DNA (non-denaturing)

The buffers were used in agarose gel as follow:

1% agarose gel components:

0.5 g agarose
50 ml of 1xTBE
1 µl of ethidium bromide (10 mg/ml)

10xloading buffer: [0.25% Bromophenol, 30%glycerol, 0.25% Xylene Cyanol FF]

10xTBE buffer in 1 litre
10 gm Tris-HCl
55 gm Boric acid
40 ml of 0.5M EDTA

DNA agarose gel electrophoresis was carried out using a horizontal Pharmacia GNA-100 gel electrophoresis kit. Prior to preparing the agarose gel, the apparatus was washed with 30% hydrogen peroxide to remove any DNA, dirt and grease contaminants. A 1% agarose gel was prepared by adding 0.5 gm agarose to 50 ml of 1xTBE buffer. The mixture was then incubated in a microwave for several minutes until all the agarose crystal are dissolved in the solution. The solution was then allowed to cool gradually at room temperature. Once the agarose solution had cooled to 55ºC, 1 µl of ethidium bromide (10 mg/ml) was added. The mixture was then poured into the gel tray containing the well-forming comb and allowed to gel at room temperature. The gel was then transferred and placed on the horizontal Pharmacia GNA-100 gel electrophoresis kit. The gel was completely covered with 1xTBE buffer. Prior to loading the samples, wells were flushed with 1XTBE buffer. The samples were then prepared in 1x loading buffer and run at 90 v/cm for 45 minute. The

gels were then illuminated with UV light and fluorescence photographed using a Gel-Doc (BIO RAD).

2.2.3.14 denaturing agarose gel electrophoresis of RNA

RNA denaturing agarose electrophoresis was performed on a Pharmacia GNA-100 electrophoresis apparatus. RNA can form complex secondary structure; hence it needs to use a denaturing condition such as 0.1% SDS to ensure that its separation was not due to secondary structure. The denaturing agarose gel is the same agarose gel preparation with addition of SDS to 0.1% (w/v) into the gel preparation prior to pouring it to the gel tray. The gel was then analysed as mentioned in section (2.2.3.13).

2.2.3.15 RNA quantification

The concentration of the isolated total RNA was determined by the Fluorescence spectrophotometer (Varian). The special reagents were the Ribogreen RNA Quantitation reagent Kit (Molecular probes). The components of this assay are as follows:

Ribogreen RNA quantitation reagent (component A)
1 ml solution in DMSO (Dimethylsulfoxide)
20xTE (component B)
25 ml of 200 mM Tris-HCl
20 mM EDTA, pH 7.5 (20xTE) in DEPC-treated water

Ribosomal RNA standard (16S and 23S rRNA from *E.coli* (component C)
Five vials, each containing 200 µl of a 100 µg/ml solution in TE

The concentration of the RNA samples was analysed as described in the manufacturing protocol. Typically, high range assay was chosen and the standard curve was carried out and prepared by diluting the concentrated buffer (component B) 20-fold with DEPC-treated water. RiboGreen reagent was prepared by diluting an aliquot of the concentrated DMSO stock solution into 1xTE. To minimize the photobleaching effects, all the samples of the standard curve and sample analysis were diluted in 1 ml of the RiboGreen reagent at the same time. The spectrophotometer was zeroed using DEPC-treated water as a blank. The RNA standard curve was then carried out by preparing 2µg/ml solution of RNA in 1xTE and 1 ml of the RiboGreen solution was added to each cuvette. The fluorescence samples of the standard curve were then measured at excitation ~ 480 nm and emission ~ 520 nm.
The concentration of the RNA was determined on the basis of absorbance at 260 nm (A260) in a cuvette with a 1 cm path length; an A260 of 0.05 corresponds to 2 µg/ml RNA.

Table 2.1. The protocol for preparing high-range standard curve.

Volume (µl) of TE	Volume (µl) of 2 µg/ml RNA stock	Volume (µl) of 200-fold diluted RiboGreen reagent	Final RNA concentration in RiboGreen assay
0	1000	1000	1 µg/ml
500	500	1000	500 ng/ml
900	100	1000	100 ng/ml
980	20	1000	20 ng/ml
1000	0	1000	blank

2.2.3.16 DNA quantification

DNA quantification was carried out by the Fluorescence spectrophotometer.

In determining DNA concentration, a dye called Hoechst 33258 was used.

The solutions for this assay were first prepared as follow:

- 1 ml of Hoechst 33258 dye (10 mg/ml) was diluted with 9 ml of distilled water to a final concentration 1mg/ml. The dilution was then filtered with 0.45 um filter paper and stored at 4ºC in a protected tube until use.
- 10x TNE buffer stock solution were prepared by dissolving the following ingredients in 800 ml of distilled water:

12.11 gm Tris base [Tris (hydroxymethyl) aminomethane]
116.89 gm Sodium chloride
3.72 gm EDTA

The buffer was then adjusted pH to 7.4 with concentrated HCl and completed with distilled water up to 1000 ml. The buffer was then filtered with a 0.45 µm filter and stored at 4ºC until use.

- DNA stock solution (100µg/ml)

DNA stock solution was prepared by diluting 10 µl of stock DNA preparation
(10 mg/ml) with 990 µl of 1xTNE solution to a final concentration 100 µg/ml.

After preparation of all the solutions above, low range assay (5-500 ng final DNA concentration) was chosen for the standard curve at excitation 350 nm and emission 450 nm. For running the standard curve, Quartz cuvette was used and the spectrophotometer was zeroed at these conditions using distilled water as a blank.

Briefly, 1 ml of the Hoechst reagent (1 mg/ml) was added to each standard curve samples containing DNA stock solution (10 mg/ml) and the unknown sample concentrate. The samples were protected from the light in a dark place for 5 minutes and the reading was then taken.

2.2.3.17 1st strand cDNA preparation from the total RNA

Total RNA of mouse brain was used as a template. The ProSTAR UltraHF RT-PCR Kit was used to generate the first strand cDNA and the reaction was carried out as the manufacturer's instructions. All the components were mixed in a 0.5 ml tube and quickly centrifuged before using. The control and the experimental reactions were carried out as follow:

Control Reaction

33.5µl of RNase-free water (Not DEPC-treated water)
5ul of 10x StrataScript RT buffer
3µl of oligo (dT) primer (100 ng/µl)
5µl of dNTP mix (40mM)
1µl of control mRNA

Experimental reaction

33.5µl of RNase-free water (Not DEPC-treated water)
33.5µl of RNase-free water (Not DEPC-treated water)
3µl of oligo (dT) primer (100 ng/µl)
5µl of dNTP mix (40mM)
1µl of (1:10) mouse brain RNA

The samples were incubated at 65ºC for 5 minutes and then allowed to cool at room temperature to allow the primers to anneal to the RNA. In order to prevent heat inactivation, 2.5ul of StrataScript RT (20 U/µl) was added to each reaction. The samples were then incubated at 42ºC for 90 minutes. The

samples were then placed on ice until using for PCR amplification. The cDNA now synthesised and ready for PCR amplification.

2.2.3.18 Polymerase chain reaction (PCR)

The polymerase chain reaction is a molecular biological method widely used for amplifying DNA. This procedure provides a very large amount of the DNA from a small amount of molecules. Two oligonucleotides are used as primers in the presence of a DNA polymerase for amplifying a known sequence in the target DNA region. PCR products were amplified by using 1.1 ReddyMix PCR Master Mix (1.5 mM $MgCL_2$) solutions. The composition of this solution was:

125 units of Taq DNA polymerase
75 mM Tris-HCL (PH 8.8 at 25 ºC)
20 mM $(NH4)_2SO_4$
1.5 mM $MgCL_2$
0.01% (v/v) Tween 20
0.2 mM each of dATP,dCTP,dGTP and dTTP

The PCR reaction was prepared for both negative and positive control to a final PCR reaction volume of 50ul as follow:

Component	Positive control	Negative control
5'-Primer 1 (~ 70ng)	1µl	1µl
3'-Primer 2 (~ 70ng)	1µl	1µl
DNA template	1µl	-
Reddy Mix	47µl	47µl
UHP water	-	1µl

The components were mixed thoroughly and quick centrifuged to collect all the mixture in the 0.5 ml tube and then one drop of mineral oil was added to prevent evaporation of the components. The PCR reaction was then carried out on a Perkin Elmer DNA Thermal Cycler 480 as follow:

94ºC for 2 minutes
60ºC for 1 minute
72ºC for 45 second x 35 cycles
94ºC for 0.45 second
60ºC for 10 minutes
4ºC end

PCR reaction was then analyzed on1% agarose gel electrophoresis and stored at -20ºC.

2.2.3.19 Primers for PCR amplification

Primers were used in all experiments as shown in the Figure 2.6. The primers 4x1-12-p*F* and 4x1-12-p*R* were annealed into pGEMT-easy to produce 329 b.p., whereas the primers 4x1-f-p*F* and 4x1-f-pR were annealed into pRSET-a to produce 507 amino acid of mouse cyp4X1.

Primer Name	Sequence (5'-3')
4x1-12-p*F*	CATGGACATAAGTCCTTTTCCCTTCCTCCT
4x1-12-p*R*	AAACATAAATTTCGCCATTTCTCCTAGTAT
4x1-f-p*F*	ATGGAGGCCTCCTGGCTGGAGACTCGTTGG
4x1-f-p*R*	AAACATAAATTTCGCCATTTCTCCTAGTAT

Figure 2.6. **List of primers for generation mcyp-4x1 PCR**.
All the primers were synthesized by J. Keyete, Dept. of Biochemistry, University of Nottingham. 4x1-12-p*F* represents forward primer and 4x1-12-p*R* represents reverse primer were used for PCR amplification of genomic mouse cyp-4x1 exon 12. 4x1-f-p*F represents* forward primer and 4x1-f-p*R* represents reverse primer were used for PCR amplification of cDNA mouse cyp-4x1.

2.2.3.20 Microsome preparations

Composition of reagents:

Buffer A (20% glycerol (w/v), 0.1 M KH2PO4, pH 7.4)

Buffer B (0.15 M NaCl, 10 mM KH2PO4, pH 7.4)

Mice Sv/129 strain were sacrificed by cervical dislocation. The liver was perfused with ice-cold buffer B and the inflated gall bladder then was removed. The liver was then washed and minced by 10 ml of ice-cold buffer B and transferred to a universal tube (30 ml) containing 20 ml of ice-cold buffer B. After rinsing the liver, the solution was decanted into a glass homogenisation tube and homogenised with 3 ml of buffer B by a motor–driven loose-fitting pestle on ice. The homogenised liver was divided into four eppendorf tubes so that each one contained 1 ml. The eppendorf tubes were then centrifuged at 43,300 x g for 10 minutes at 4°C. The supernatant was removed and centrifuged at 444,000 x g in MLA80 rotor for 30 minutes at 4°C.

Finally, the pellet was resuspended in 1 ml of buffer A, and the microsomes stored at –80°C.

2.2.3.21 Mouse cytosol preparation for AhR assays

This method was carried out as described in Molecular Pharmacology protocol, (34: 229-237) and the composition of reagents as follow:

(MEN)

25 mM MOPS

0.02% Sodium Azide

1 mM EDTA, pH 7.5

(MDENG)

1 mM Dithiothreitol

10% (w/v) glycerol

One mouse was killed by cervical dislocation. 5 ml of MDENG was injected into the portal vein of the liver. The liver was excised and placed in a tube containing MDENG, and then cut into small pieces. The MDENG solution was then decanted. The liver pieces were transferred into a glass homogeniser with 9 ml of MDENG solution and were homogenised. The solution was mixed and transferred into a two test tubes in equal amount. The solution was centrifuged in an MLA-80 rotor for 20 minutes at 444,000 x g at 4ºC. The supernatant was transferred and divided into two test tubes and centrifuged again at 444,000 x g for 40 minutes. The supernatant was then aliquoted and stored at –80°C until use.

2.2.4 Protein methodologies

2.2.4.1 Bradford assay

The protein concentration was carried out as described by Bradford (1967) assay method (Bradford, 1976). Typically, 5x Bradford dye concentrates were prepared by dissolving100 mg of coomassie brilliant blue G-250 in 50 ml 95% ethanol, and 100 ml of concentrated phosphoric acid. The solution was completed by addition of 200 ml of deionised water. The protein standards were from 0 to 40 µg of bovine serum albumin (BSA) freshly prepared from a 1µg/µl stock solution of BSA. For each sample duplicate aliquots were diluted to a volume 50 µl. 1x Bradford dye reagent was prepared by diluting 11 ml of 5x Bradford stock dye with 44 ml distilled water. The solution was then filtered through 3MM paper (Whatman). One ml of 1x Bradford reagent was added to each sample, and the samples were then mixed and incubated at room temperature for five minutes. The absorbance of the samples was measured at 595 nm. A standard curve of bovine serum albumin (BSA) was used to determine the protein concentration, which covers the range of 0-40 µg protein. Non-linear regression analysis in Prism v 3.0 was used to calculate the unknown sample concentrations.

2.2.4.2 Coomassie staining of SDS-PAGE gels

Coomassie blue R 250 was used to stain the gels after electrophoresis to visualise the resolved protein bands. Before use, it is filtered through Whatman 3MM paper to remove any undissolved particles. The gel was immersed in coomassie blue staining solution (0.25 g coomassie R 250, 90 ml methanol: water (1:1 v/v), 10 ml glacial acetic acid) for 1 hr with agitation.

The staining solution was decanted and can be reused again. It can be used several times without any change in the quality of staining and can be stored at room temperature. The gel was washed with destain solution, which consists of 30% methanol and 10% glacial acetic acid. The destain solution was changed until bands visualise clearly. After the gel has been completely destained it is placed onto Whatman 3MM paper and dried under vacuum at 80ºC for 2 hrs using a Bio-Rad 583 gel dryer.

2.2.4.3 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

This technique was described by Laemmli (1970), which depends on the separation of protein according to their molecular weight under denaturing conditions. A negative charge produced by binding of sodium dodecyl sulphate (SDS) with amino acid chain of the protein and consequently, the proteins are separated according to their length
(i.e. molecular weight).

2.2.4.4 Gel preparation

Protein gel was prepared and run by using a Bio-Rad Mini-Protein II electrophoresis cell (Bio-Rad). Table 2.2, shows different gel concentration for separation of different molecular size proteins. All the glass plates were washed with ethanol and distilled water to remove any contaminant prior to fitting on the apparatus. The resolving gel (5 ml) was mixed gently with 50 µl of fresh 10% ammonium persulphate (APS) to initiate polymerisation, Table 2.3. The solution was then poured immediately between the glass plates.

To complete the polymerisation of the gel, the solution was covered with 100 µl of 0.1% SDS solution for 30 minutes at room temperature until the polymerisation occurred.

Table 2.2. Acrylamide Gel Percentages for Resolving of Proteins

% Acrylamide	Protein size /Range
8%	40-200 kDa
10%	21-100 kDa
12%	10-40 kDa

Table 2.3. Resolving Gel for Tris-glycine SDS-Polyacrylamide Gel Electrophoresis (For preparing 100 ml).

Component	**Resolving gel**			**Stacking gel**
	8%	10%	12%	5%
	(ml)	(ml)	(ml)	(ml)
Water	46.4	39.6	33	68
30% Acrylamide mix	26.6	33.4	40	17
1,5 M Tris (pH 8.8)	25	25	25	12.5
10% SDS	1	1	1	1
10% APS	50 µl	50 µl	50 µl	40µl
TEMED	0.06	0.04	0.04	0.10

Once resolving gel had completely polymerised, 2 ml of a stacking gel (5% stacking gel) were prepared and mixed with 20 µl of APS and poured on the top of the resolving gel (Table 2.3). The comb was then inserted into the stacking gel and left > 1 hour at room temperature. The gel can be used immediately or covered by saran wrap and stored at 4ºC until use next day. The tank was filled to the top of the gel with electrophoresis buffer, which consists of (25 mM Tris, 250 mM glycine).

The protein samples were prepared by mixing with 5x loading buffer and water to a total volume of 15 µl. The samples were then denatured in water bath at 90ºC for 10 minutes. The samples were quickly centrifuged for 5 seconds and then loaded and run at 110 v for 90 minutes.

2.2.4.5 Loading buffer preparation

The component of the loading buffer as follow:

5x Load dye (5x loading buffer):
6 mls of 0.5 M Tris pH 6.8
2 mls glycerol
2 mls 10% SDS
5 mg Bromophenol blue

This buffer can be stored at room temperature. Before use, 77 mg DTT per ml was added to an aliquot of loading buffer and can be stored at –20ºC.

2.2.4.6 Western blotting

Western blotting is a method, which transfers the proteins from the gel to the membrane by using an electric field to bind the protein onto a membrane, and then antibodies can detect the immobilised proteins. It is used to detect specifically trace proteins on an immobilizing membrane and therefore enables further characterization.

2.2.4.6.1 Electrophoretic transfer of proteins onto PVDF membrane

Composition of reagents:

5XTris-glycine electrophoresis buffer: 15 gm Tris, 94 gm Glycine, 5 gm SDS per 1 litre (Running buffer)
1XTris-glycine electrophoresis buffer: 200 ml of 5XTris-glycine electrophoresis buffer + 800 ml distilled water
Blocking solution: 10% Marvel dried milk powder made with 1xTBS solution.
5x TBS: 12.1 gm Tris-HCl (pH 7.6), 40 gm NaCl per litre.
1xTBS: 200 ml of 5xTBS + 800 ml distilled water
1x TTBS: 1XTBS containing 0.5 ml Tween20 (v/v) in 1 litter.
Primary antibody: Rabbits 91, 92, A, B, C, D, E, F, 189 and 190
Secondary antibody: Goat anti-rabbit IgG (H+L) conjugated to horseradish peroxidase (Bio-Rad).
ECL Western blotting detection kit (Amersham Life Science)
1x developer solution: (Ilford) 1:9
1 fixing solution (Ilford) 1:4 and leave it 2 minutes before use
Methanol (Fisher Scientific corp.)
PVDF membrane (Millipore Immobilon-P, 0.45 μM pore size)
Whatman paper 3MM
UHP water

Protein samples (20 μg) were resolved by SDS-PAGE on a 12% gel and then transferred onto the Polyvinylidene Difluoride (PVDF) membrane for

immunoblotting. Whatman paper (3MM) and 7x10 cm Polyvinylidene Difluoride (PVDF) membranes were prepared and immersed in 100% methanol for 5 minutes. The methanol was decanted and the membrane was wetted with transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% methanol (v/v), 0.1% SDS w/v) for 10 minutes. The gel was then placed carefully on the Whatman paper. The PVDF membrane was placed on top of the gel. Any air bubbles were removed between the PVDF membranes and the gel. Another two Whatman papers were placed on the membrane to sandwich the gel and the membrane. The gel sandwich was placed in a blotting cassette where the protein transfers to the PVDF membrane in transferring buffer (25 mM Tris-HCl, 192 mM glycine, 20% methanol v/v, 0,1% SDS w/v) for 1 hr at 90 v.

2.2.4.6.2 Incubation of transferred protein with antibody

After transfer of protein, the membrane was placed in blocking solution (10% marvel solution (w/v) in 1 x TBS) and incubated at room temperature overnight. The blot was then incubated with 40 ml of 1x TTBS containing the selected primary antibody at a dilution 1:500 for one hour with agitation. The blot was washed 5 times for 5 minutes with 1x TTBS solutions. The blot was then incubated in 50 ml of 1x TTBS solutions containing 5 µl of secondary anti-body 1:10000 (GAR Ig G-HRP) for one hour with agitation. The blot was then washed 5 times for 5 minutes with 1x TTBS solutions and developed with ECL kit as mentioned in the manufacturer's instructions (Amersham Biosciences). The membrane was then exposed to ECL film (Amersham Life Science) for different intervals of time and then each film developed by 1x developer solution (Ilford) until bands appear. The film was washed in water

for 2-3 minutes and fixed in 1X fixing solution (Ilford 1:4) until the film becomes clear. The film was then dried at room temperature.

2.2.4.7 Expression of proteins in BL21 (DE3)

A fresh colony was picked from a plate of BL21 (DE3) bacteria and inoculated in 10 ml of 1% glucose LB broth containing Ampicillin (100µg/ml). The culture was incubated at 37ºC on the shaker (220 rpm) overnight. 200 ml of 1% glucose LB broth containing Ampicillin were inoculated with the overnight culture of bacteria and incubated on the shaker (220 rpm) for 3 hrs at 37ºC or until an OD_{600} of 0.4-0.8 was reached. 5 ml of this culture were taken onto a clean universal tube as un-induced bacteria. 200µl of 1M IPTG was added. The cultured cells were then incubated at 37°C on the shaker (220 rpm) for 3 hrs. After induction, 5 ml of the induced cells were taken onto a clean universal tube as induced cells. The remainder of the cells were then centrifuged at 4360xg for 20 minutes at 4°C. The cells were then resuspended in 1/10 of the original volume of 50 mM sodium phosphate pH8. The suspension was then sonicated for five bursts of 30 seconds and placed immediately on ice for 30 second. The sonicated pellets were then centrifuged at 4360xg for 20 minutes at 4°C on a JA10 rotor. The supernatant was decanted into a fresh tube and stored at -20°C as induced total cell supernatant. The pellets were resuspended in 6 M urea, 100 mM Tris-HCl (pH 8). The suspension was incubated for 10 minutes at room temperature, and then centrifuged at 4360xg for 20 minutes at 4°C. The supernatant was decanted into a fresh tube and labelled as supernatant of soluble pellets. This supernatant, which contains the soluble protein, was centrifuged twice at 4360xg for 20 minutes on a JA2-21 rotor to

remove any particles material. The supernatant was then poured into a fresh tube and stored at -20°C until use. The protein concentration was then determined as described in section (2.2.4.1). Proteins were then analysed on denaturing Polyacrylamide gel electrophoresis.

2.2.4.8 Protein diafiltration

Protein samples from 2.2.4.7 were diafiltered prior to affinity chromatography. An aliquot of protein samples (200µl) were stored as a control sample before dilution and dialysis. One ml of LpH protein was diluted into 10 mls of 0.5 M NaH2PO4, 0.1% SDS solution. For mouse cyp4x1 protein, 90 ml of mcyp4x1 protein were diluted into 200 ml of 6 M urea, 0.1% SDS solution. The diluted solution was then dialysed in a stirred ultra filtration cells from Millipore company (model 8200) at 5 LB/in² using nitrogen gas using YM10 exclusion membrane. If the solution during dialysis fell below half the original dilution solution, the diafiltration chamber was then filled again with a dilution solution. The dialysis was continued until the solution becomes clear. The dialyzed solution was stored at 4ºC until use.

Bradford assay was used to determine the protein concentration before and after dialysis.

2.2.4.9 Nickel column affinity purification

The purification of the m-4x1 protein was carried out on the nickel affinity chromatography column according to the manufacturer's instructions (Novagen). All the following buffers were used and diluted to 1x prior to use in the affinity column:

Component	Binding buffer [8x]	Elute buffer [4x]	Wash buffer [8x]	Strip buffer [4x]	Charge buffer [8x]
Imidazole	40 mm	4 M	480 mM	-	-
NaCl	4 M	2 M	4 M	2 M	-
Tris-HCl (pH 7.9)	160 mM	80 mM	160 mM	80 mM	-
EDTA	-	-	-	400 mM	-
NiSO4	-	-	-	-	400 mM

The column was prepared by insertion of a glass wool and His.Bind chelating resin (Novagen). 2.5 ml of His**.**Bind resin (Novagen) was loaded into 20 ml syringe tube. 7.5 ml of distilled water was used to wash the column to remove the ethanol in the resin. The resin was then charged with 12.5 ml of 1x charge buffer (50 mM NiSO4). The column was then equilibrated in 7.5 ml of 1x binding buffer containing 8 M urea. The sample was loaded in the column and poured gently into the column. To remove unbound protein, 25 ml of 1x binding buffer containing 8 M urea were loaded and followed by 15 ml of a mixture solution of 11 ml of 1x binding buffer (8 M urea) and 4 ml of

1x washing buffer (8 M urea). The 2-3 ml of un-bound protein were collected in a fresh tube and stored at -20ºC. The bound protein was eluted from the column with 15 ml of 1x elution buffer (8 M urea). The eluted protein was collected in clean eppendorf tubes at each 1 ml elution. All un-bound and eluant fractions were analysed by SDS-PAGE. After all the eluant fractions had been collected, the column was washed with 15 ml of 1x strip buffer followed by 10 ml of deionised water.

Finally, the column was then washed with 10 ml of 20% ethanol and then stored at 4ºC.

2.2.4.10 Manipulation of Latrophilin protein

2.2.4.10.1 *C.elegans* preparations

This method is based on Ian A. Hope (1995). All the preparations solution were made up to 1 litre of UHP water and sterilized by autoclaving.

Component of the reagents:

S basal

5.85 g NaCl

1 g K2HPO4

6 g KH2PO4

1 ml Cholesterol (5mg/ml in ethanol)

1M potassium citrate (pH6):

20 gm citric acid monohydrate

293.5 gm tri-potassium citrate monohydrate

Trace metals solution:

1.86 g Disodium EDTA (5mM)

0.69 g $FeSO_4.7H_2O$ (2.5mM)

0.2 g $MnCl_2.4H_2O$ (1mM)

0.29 g $ZnSO_4.7H_2O$ (1mM)

0.025 g $CuSO_4.5H_2O$ (0.1mM)

1M $CaCl_2$:

55.5 g $CaCl_2$

1M $MgSO_4$:

120.4 g $MgSO_4$ (anhydrous)

S medium:

1 litre S basal

10 ml 1M potassium citrate, pH 6

10 ml trace metals solution

3 ml 1M $CaCl_2$

3 ml $MgSO_4$

The S medium components were not autoclaved.

E.coli cells (OP50) was grown in 1 litre LB broth media at 37 ºC overnight. The bacteria cells were centrifuged at 2830xg for 15 minute. The supernatant was decanted and the pellets were resuspended in S-basal medium. *C.elegans* worms were grown at 25ºC overnight. 4 ml of the M9 buffer were used to transfer the growing worms to the S-basal medium containing *E.coli* cells. The mixture was incubated at 25ºC on the shaker (3000 rpm) for 2 days. 2 mls of the worms culture was taken and viewed under the microscope. The worm culture was centrifuged at 145xg for 2 minutes and then resuspended in 4 ml of

M9 buffer. The worm culture was centrifuged again at 145xg for 2 minutes. The supernatant was decanted and the pellets were then transferred to a clean measuring cylinder. 25 ml of ice-cold sucrose (60% w/v sucrose) were added and mixed immediately. The worms were then centrifuged at 484xg for 5 minutes. The worms which floated on the top of the solution were then pipetted and transferred to a new tube, mixed with M9 solution and centrifuged at 145xg for 5 minutes. The supernatant was decanted and the pellets were transferred to a clean eppendorf tube. The pellets were then briefly centrifuged and the supernatant was removed. The pellets were then resuspended in 2 volumes of M9 buffer and stored at –80ºC until use.

2.2.4.10.2 Preparation of *C.elegans* fractions

Frozen *C.elegans* worm pellets were thawed on ice for a few minutes. Protease inhibitor (50µl) was added to the pellets, along with 20 µl of 0.1 M phenyl methyl sulfonyl fluoride (PMSF) per ml worms. The samples were aliquoted and frozen at - 80°C until thoroughly frozen. Samples were then thawed rapidly on ice and sonicated for 6 bursts of 30 seconds, with one minute periods on ice in between each sonicated burst. Total homogenate sample (T) was taken after sonication and the samples were then centrifuged at 5000xg for 5 minutes at 4°C. The supernatant was transferred to a clean eppendorf tube (5K super) and the pellets (5K pellet) were resuspended in 0.5 ml of the buffer (20mM Tris-HCl pH 8, 10% glycerol, 150mM NaCl) and stored at -80°C. The supernatant (5K super) was then centrifuged at 15000xg for 30 minute at 4°C. The supernatant was taken to a clean eppendorf tube (15K super) and the

pellets were then resuspended in 0.5 ml of the buffer (20mM Tris-HCl pH 8, 10% glycerol, 150mM NaCl) and stored at -80°C.

2.2.4.10.3 Dilution of *C-elegans* LpH protein in 10% emulgen 913 (0.6%, 0.8%, 1%, 2%)

Composition of reagents:

Buffer solution (20 mM Tris pH8, 10% (w/v) glycerol, 150 mM NaCl)

10% emulgen 913

2% emulgen 913

C.elegans membrane protein (15 k pellets) (14mg/ml) were prepared and stored at -80ºC. The samples were thawed on ice and one ml was stored at -80ºC as a control sample. 1ml was diluted into 7 ml of buffer (20 mM Tris pH8, 10% (w/v) glycerol, 150 mM NaCl) to get a final concentration of 2 mg/ml. Several percentages of 10% emulgen detergent (0.6% (w/v). 0.8% (w/v), 1% (w/v)) were made to dissolve the LpH protein in the solution. Each solution was stirred on ice for one hour. The samples were centrifuged at 20,817 x g for 20 minutes at 4ºC. The supernatant of the samples was taken and stored at -80ºC. The pellets were resuspended in the same amount and the concentration of the detergent solution (0.6% (w/v), 0.8% (w/v), and 1% (w/v)). The pellet suspension of each sample was then stored at - 80ºC until use. Two percent emulgen was prepared and the pellet samples were stirred with 2% emulgen solution for 1 hr at 4ºC. The solution was centrifuged at 793,000 x g for 20 min. The supernatant was then taken and stored at 4ºC. The pellets were resuspended in 1 ml of the buffer (20 mM Tris pH8, 10% (w/v) glycerol, 150 mM NaCl) and stored at 4ºC.

2.2.4.10.4 Dilution of *C-elegans* LpH protein in dodecylmaltoside detergent (0.8%, 1%, 1.2%)

The dodecylmaltoside detergent was added to the membrane pellets to a final concentration of 0.8% (w/v), 1% (w/v) and 1.2% (w/v) respectively. The samples were then mixed gently at 4ºC for 1 hour. The samples were then centrifuged at 20,817 x g for 10 minutes at 4ºC. The supernatant was taken to a clean tube and stored at 4ºC. The pellets were then resuspended in an equal amount of original buffer (20 mM Tris pH8, 10% (w/v) glycerol, 150 mM NaCL2).

2.2.4.10.5 Ammonium sulphate precipitation

In order to extract and prepare LpH antisera for purification on an affinity column, ammonium persulphate was used for precipitation.

An aliquot (300 µl) of LpH antisera was taken as a control sample and stored at 4 ºC. Six ml of LpH antisera were centrifuged at 20,817 x g at 4ºC for 5 min. The supernatant was taken and placed in a universal tube and stirred gently on ice. Saturated ammonium sulphate solution (2.85 ml) was pipetted into the antibody solution dropwise. The antibody solutions were incubated at 4ºC for 6 hr. The antibody solutions were then centrifuged at 20,817 x g for 5 min to give a 25% ammonium sulphate pellet, and supernatant. The supernatant of the antibody solution was transferred into a clean universal tube and stirred with a magnetic stirrer at room temperature. Ammonium sulphate (2.85 ml) was poured slowly into the antibody solution. The solution was incubated at 4ºC for at least 6 hr or overnight. The antibody solution was centrifuged at 10,621 x g for 10 min. The supernatant was decanted in a fresh tube (50% NH_4SO_4 supernatant). The pellets were then resuspended by 1 ml of PBS and shaken gently to avoid any air bubble (50% NH_4SO_4 pellets). The 50% NH_4SO_4 pellet was dialysed in stirred ultra filtration cell from Millipore Company at 5 LB /in². The 50% NH4SO4 pellets was then stored at 4°C until use. The 50% NH_4SO_4 supernatant was dialysed in stirred ultra filtration cell from Millipore Company at 5 LB /in² and then stored at 4°C until use.

2.2.4.10.6 Dot blot assay

Dot blot assay is a clear guide to show how much antibody purification was achieved and how much is in each fraction.

Procedure:

A sheet of a nitrocellulose (Hybond ECL) was prepared by drawing a circle for each sample site on the membrane. The samples were diluted in the phosphate buffer saline (PBS) for each sample 10^{1}, 10^{2}, 10^{3}, 10^{4}, 10^{5}, 10^{6}, 10^{7}, 10^{8} respectively. One µl of each sample was spotted onto the membrane and left to dry. The membrane was immersed in a blocking solution 10% (w/v) Marvel dried milk for 2 hours at room temperature. The blocking solution was decanted and the membrane then incubated in 50 ml of 1x TTBS solutions containing 5 µl of secondary anti-body 1:10000 (GAR Ig G-HRP) for one hour with agitation. The blot was then washed 5 times for 5 minutes with 1x TTBS solutions and developed with ECL kit as mentioned in the manufactures instructions. The membrane was then exposed to ECL film (Amersham Life Science) for different intervals of time and then each film developed by 1x developer solution until bands appear. The film was washed in water for 2-3 minutes and fixed in 1X fixing solution (Ilford 1:4) until the film becomes clear. The film was then dried.

2.2.4.10.7 Activated bead preparation

0.5 ml of N-Hydroxyl succinimide activated agarose (beads) was prepared in a fresh eppendorf tube. The beads were shaken gently and briefly centrifuged for a short time. The alcohol supernatant was decanted and the residue was placed on ice. The beads were washed twice with 1 ml of pure ice-cold water and centrifuged at 20,817x g at room temperature. The water supernatant was removed and the residue was placed on ice.solutions and materials:

2.2.4.10.8 Affinity purification with LPH protein

This method was carried out as described in The Antibodies, a laboratory manual (Harlow, E.D; Lane, D. 1988)

Composition of reagents:

0.5 M Sodium phosphate pH (7.5)
1M Sodium chloride, 0.05 M Sodium phosphate pH (7.5)
4 ml of LpH protein
50 ml of 100 mM Glycine, pH 2.5
50 ml of 10 mM of NaH_2PO_4, pH 6.8
50 ml of 1 M NaH_2PO_4, pH 8

Procedure:

4 ml of LpH protein (0.6mg/ml) in (20 mM Tris pH8, 10% (w/v) glycerol, 150 mM NaCl) were centrifuged at 20,817 x g for 2 min at room temperature. The supernatant was decanted in fresh universal tubes. 1ml of LpH protein (0.14mg/ml) in (20 mM Tris pH8, 10% (w/v) glycerol, 150 mM NaCl) was pipetted onto the beads. The protein-bead solutions was shaken gently and left on the shaker overnight.

The protein-bead solution was then left on the bench for 10 min until all the beads settled. The supernatant was decanted and the beads were transferred into a fresh eppendorf. The beads were washed twice with 1 ml of 0.5 M NaH_2PO_4 (pH 7.5) and once with 1 M NaCl, 0.05 M NaH_2PO_4 (pH 7.5). The beads were then washed with 10 volumes of 100 mM ethanolamine (pH 7.5) and incubated at room temperature for 4 hr with gentle mixing. The beads were centrifuged and washed twice with 0.5 M phosphate buffer saline (PBS), 2 ml of 100 mM glycine, 1 M NaH_2PO_4 (pH 8) and 1 ml x 4 of PBS. This process yields beads containing covalently bound LPH protein.

0.6 ml of the 50% NH_4SO_4 pellets was mixed with protein-beads solution and left overnight on the rocker at 4°C. The Ab-beads solution were loaded into the column and washed with 20 ml of PBS. The beads were then washed with 10 ml of 10 mM NaH_2PO_4. Before the protein elution, eppendorfs were prepared which each one contains 20 µl of 1M NaH_2PO_4 (pH 8) and then eluted by 10 x 200 µl of 100 mM glycine (pH 2.5). The column was then washed with 20 ml of PBS and then 2 ml of 0.1 % sodium azide.

2.2. 5 Gene expression analysis

2.2.5.1 Denaturing Poly acrylamide gel electrophoresis (6%)

Composition of reagents

Sequa gel concentrates:

237.5g/litre acrylamide

12.5g/litre methylene bis-acrylamide

500g/litre 8.3M urea

Sequa gel diluents:

500g/litre 8.3M urea

10xTBE buffer

0.89M Tris base

0.89M boric acid pH 8.0

20mM EDTA

RNA can form secondary structure and consequently fails to show linear separation with increasing mass. To address this, denaturing conditions such as urea was used in RNA gel electrophoresis using BioRad protein II vertical electrophoresis apparatus.

The glass plates and the spacer (0.4mm) were firstly washed with absolute ethanol and then cleaned with water to remove any dirt or acrylamide contaminants.

The poly acrylamide gel preparation was prepared based on the following formula:

1/10 X total gel volume = volume of 10XTBE

% acrylamide gel required X volume of gel / 25 = volume of Sequa gel concentrate

volume of gel – (1/10th volume of gel + volume of concentrate) = volume of diluents

Based on the formula above, to prepare 6% poly acrylamide gel of 10ml total volume gel 2.4ml of Sequa gel concentrate, 1ml of 10xTBE and 6.6ml of Sequa gel diluents were mixed in a universal tube. To this mixture, 6ul of TEMED and 100ul of APS were added and mixed gently by hand. The mixture was then pipetted immediately between the glass plates until the glass plates fully filling by the mixture. The comb was then inserted in between the glass plates and the gel allowed polymerising for 1 hour. The polymerised gel was placed in the gel tank and 1xTBE was used to fully cover the anode and cathode compartment. The wells were then flushing by 1xTBE running buffer to remove any precipitant of unpolymerised acrylamide and Urea crystallization form. The samples were loaded and the gel was run at 120 v/cm for 90 minute. The gel was fixed in 10% methanol and 1% acetic acid and placed on the shaker for 1 hour. The solution was decanted and the gel then washed by water and placed on the shaker for 1 hour. The washed water was decanted and the gel again washed by water for 15 minute on the shaker. The gel was then removed and placed on 2x3MM paper and then dried under the vacuum. The dried gel was transferred to an intensifying apparatus and exposed to Kodak BioMax MS film for overnight at – 80ºC. The intensifying apparatus was removed from the – 80ºC and allowed to warm in a dark room at room temperature. The film was then treated by the fixer and the developer solutions as described in section (2.2.9.2).

2.2.5.2 RNase protection assay

Ribonuclease protection assay (RPA) is a high sensitive and specific method to detect and quantitative RNase. This method is based on a method described by Melton et al (1984). The RNase protection assay used was obtained from PharMingen Ltd, commercially available as Riboquant®. The assay was performed as manufacturer's instructions.

2.2.5.3 Generation of the DNA template

Before starting the RNase protection assay, the plasmid DNA (pGEMT-easy) containing the inserted mouse 4X1 cDNA was used as a template for the RNase protection assay. The plasmid DNA was linearised by a restriction digest (NcoI) and analysed on 1% agarose gel. The DNA was then precipitated by two volume of absolute ice-cold ethanol and 0.25 volume of 7MM ammonium acetate for 30 minute at – 20ºC. The sample was centrifuged at 20817xg for 15 minute at 4ºC. The supernatant was decanted and the precipitant pellets were then washed with 70% ice-cold ethanol. The sample was centrifuged again at 20817xg for 5 minutes at 4ºC. The pellets were resuspended in 30ul of DEPC-treated water.

2.2.5.4 Synthesis of 170 b.p labelled [P32] Marker

The marker was generated from a synthesized plasmid DNA (pGEMT-easy) containing a known sequence of an inserted PCR fragments. The inserted fragments were 124 b.p. The marker was synthesised as described in section 2.2.13.2.4.1.

2.2.5.5 Incorporation test

This is a method for estimating the amount of radioactivity that incorporates into the probe. Whatman DE81 filter paper was used for this test. The DE81has positive charge which strongly bind to nucleic acids. In brief two DE81 (Whatman) filter paper were labelled unwashed and washed, with a pencil. One microlitre of probe was pipetted onto each paper and allowed to dry. One DE81 filter paper was then washed five times for five minutes with 10 ml of 0.5M $NaHPO_4$, five times for five minutes with 10 ml of distilled water and five times for five minutes with 10 ml ethanol. The filter was then allowed to air-dry at room temperature. Unincorporated nucleotides are easily removed by the washing process with 10 ml of 0.5M $NaHPO_4$.

The measuring of the radioactivity for both filter papers (the washed and unwashed filters) was carried out by a Geiger counter. The incorporated radioactivity nucleotides rate into the probe was then calculated by the difference between the two readings using the formula:

$$\frac{\text{CPS of washed filter}}{\text{CPS of unwashed filter}} = \text{Incorporation rate (\%)}$$

2.2.5.6 RNA probe synthesis

The RNase protection assay was carried out using Riboquant probe kit from PharMingen Ltd. . The Riboquant probe kit contains the following reagents:

1xHybridization buffer

1xRNase buffer

RNase A+T1 (80ng/ml, 250U/µl)

1xProteinase K buffer

Proteinase K (10mg/ml)

Yeast tRNA (2mg/ml)

4M ammonium acetate

1xLoading buffer

In order to prepare the RNA probe for the RNase protection assay, the transcription of this probe was performed by adding 1ul of RNA guard, 4µl of 5xTCB, 2µl of DTT, 2µl of A,G,UTP (3 mM), 10µl of [α-^{32}P] CTP (specific activity 800Ci/mmol), 1µl of distilled water, 1µl of linearised DNA, and 1µl of Sp6 or T7 polymerase enzyme.

The reaction above was added in order and mixed by gentle pipetting or flicking and then quick centrifuged on the bench top centrifuge. The reaction was then incubated at 42ºC for Sp6 or 37ºC for T7 polymerase for 1 hour. The reaction sample was tested to DE81 assay to measure the incorporated labelled nucleotide into the synthesised RNA as described in section (2.2.13.2.3). The incorporation test to be accepted it should be at least 10% other wise rejected. The assay was then terminated by adding 3ul of DNase and then mixed gently prior to quick centrifuged into a bench top centrifuge. The assay sample was

then incubated at 37ºC for 30 minute and then 26µl of EDTA (20mM), 25µl of Tris-saturated phenol, 25µl of Chloroform:Isoamyl alcohol (50:1), and 2µl of yeast tRNA (2µg/µl) were added in order. The reaction was mixed by vortex into an emulsion and then centrifuged for 5 minutes at room temperature on bench top centrifuge. The upper aqueous layer was taken to a new eppendorf tube and 50µl of Chloroform/Isoamyl alcohol (50:1) were added and mixed by vortex and then centrifuged for 2 minute at room temperature on a bench top centrifuge. The aqueous layer was then transferred to anew eppendorf tube and 50µl of 4M ammonium acetate and 250µl of ice-cold 100% ethanol were added mixed by inversion and then incubated for 30 minute at -80ºC to precipitate the RNA probe. The reaction was centrifuged at 20817xg for 15 minute at 4ºC. The supernatant was decanted and the pellets were washed with 100µl of 90% of ice-cold ethanol and then centrifuged for at 20817xg for 5 minutes at 4ºC. The supernatant was removed and the pellets were air-dried for 5-10 minutes. The pellets were resuspended in 50µl of hybridisation buffer by gentle vortex for 20 second and then quick centrifuged to collect the components in the tube. The probe was then ready to use or stored at - 20ºC until use within 2 days after preparing.

2.2.5.7 RNA samples preparation and hybridisation

30µg of each RNA sample were precipitated as described in section (2.2.3.5) and then resuspended with 8µl of hybridisation buffer. The samples were solubilized by gentle vortex and then briefly centrifuged in a bench top centrifuge. To this, 1ul of the synthesised probe (0.3µl probe) was hybridised with 30µg of each RNA sample and then mixed by pipetting. One drop of oil was added to each sample to prevent component evaporation. The samples

were placed on a water bath at 90ºC for 3 minutes and then immediately the temperature was turned down to 56ºC and incubated overnight. The temperature was then turned down to 37ºC for 15 minute prior to the RNase treatments. The samples were treated with 100ul of RNase cocktail pipetted under the oil layer except the positive control (-ve tRNA) where treated with 100ul of RNase buffer. The samples were centrifuged and incubated for 45 minute at 42ºC. Meanwhile this incubation, 18µl of Proteinase K cocktail were added to each a new eppendorf tube. The RNase digestion was taken and transferred to the new eppendorf tubes containing the Proteinase k cocktail. The samples were mixed by vortex and then incubated for 15 minute at 42ºC. A 65µl of Tris-saturated phenol and 65µl of Chloroform: Isoamyl alcohol (50:1) were added and mixed by vortex to each sample and then centrifuged at room temperature for 15 minute.

The aqueous layer was transferred to a new eppendorf tube and then 120µl of 4M ammonium acetate and 650µl of 100% of ice-cold ethanol were added and mixed by inversion 6-12 times. The samples were then incubated at -80ºC for 30 minute and then centrifuged at 20817xg for 15 minute at 4ºC. The supernatant was removed and the pellets were resuspended in 100µl of 90% ice-cold ethanol. The supernatant was decanted and the pellets were air-dried for 5 minutes. The pellets were then resuspended in 5µl of 1x loading buffer and then brief centrifuged on a bench top centrifuge. The samples were heated at 95ºC for 3 minutes on a water bath and then placed immediately on ice. The samples were loaded on 6% Polyacrylamide gel and electrophoreted at 120 v/cm until the loading buffer dye reach the bottom of the gel.

The gel was then treated with destaining solution (10% of glacial acetic acid and 10% of methanol) for 1 hour. The gel was dried under the vacuum drier for 2 hours and then exposed to Kodak BioMax MS film at -80ºC for overnight. The film was then treated by the fixer and the developer solutions as described in section (2.2.4.6.2).

2.2.6 Immunohistochemistry

Solutions:

0.2M phosphate buffer (buffer A and buffer B)

Buffer A (0.2M KH_2PO_4)

13.61 g of potassium dihydrogen orthophosphate

Buffer B (0.2M $Na_2HPO_4.12H_2O$)

35.81 g of disodium hydrogen orthophosphate

Buffer A and B were each made up to 500 ml with UHP water and then mixed together and the mixture was adjusted to pH 7.2 at 20ºC.

0.01M sodium citrate buffer (pH 6.0):

2.9 g sodium citrate

3.4 ml HCl

The buffer was made up to 500 ml with UHP water and adjusted to pH 6.0 at 20ºC.

Tris-HCl, pH 7.6 (TBS):

8.1 g sodium citrate

0.6 g Tris-HCl hydroxymethylamine

3.8 ml HCl

The buffer was made up to 500 ml with UHP water and adjusted to pH 7.6 at 20ºC.

2.2.6.1 Immunohistochemistry fixative

Immunohistochemistry fixative solution was made by mixing one part of 8% paraformaldehyde with one part 0.2 M phosphate buffer.

2.2.6.2 Mouse perfusion

Adult male S129 mice were anesthetized using halothane and then perfused using perfusion solution (50:50 paraformaldehyde and 0.2M phosphate buffer) for 20 minutes. The brain tissue was kept in fixative solution overnight at 4ºC.

2.2.6.3 Paraffin-embedded sections

Brain tissue was embedded in paraffin because it provides excellent morphological detail and resolution. Paraffin is a mixture of paraffin wax and resin. Paraffin-embedded sections was prepared by Leica TP1020 automatic tissue processor as follow:

70% alcohol for 90 minute
80% alcohol for 90 minute
96% alcohol for 90 minute
100% alcohol for 60 minute
100% alcohol for 60 minute
Xyleene for 90 minute
Xyleene for 90 minute
Paraffin for 2 hours
Paraffin for 2 hours

Brain tissue was then blocked (10x10x3 mm) in paraffin and placed on a cool plate prior to store at 4ºC until use. The block was cut with a cryotome (Leica CM1900) at 10 µm thickness. The paraffin sections were then placed on poly-L-lysine coated slides and stored at 4ºC.

2.2.6.4 Immunohistochemistry for NFT and GFAP on paraffin sections

Brain paraffin slides were placed in a black plastic slide rack and deparaffinzed, and then hydrated as follows:

Xylene	2x5 minutes
100% Ethanol	2x5 minutes
90% Ethanol	2x5 minutes
70% Ethanol	2x5 minutes
50% Ethanol	2x5 minutes
Running tap water	5 minutes

The slides were transferred to a plastic container containing approximately 300 ml of fresh 0.01M citrate buffer (pH 6.0) and heated by microwave for 10 minutes. The slides were allowed to cool for 20 minutes and then immediately placed in cold running water. The sections were wiped with tissues and outlines of them were drawn using a hydrophobic pen. The slides were placed in hydrogen peroxide solution (59 ml hydrogen peroxide and 1 ml of ethanol) for 10 minutes to quench, endogenous peroxidases. The slides were washed in TBS and placed into a hydrophobic chamber prior to adding protein blocker. The slides were incubated with protein blocker for 30 minutes at room temperature. The slides were then drained and incubated with diluted primary antibodies (1:1000) for two hours at 4ºC. The slides were washed using TBS

and incubated with link No.3 from the LSAB2 kit for 20 minutes at room temperature. The slides were washed again and incubated with link No.4 from the LSAB2 kit for 20 minutes at room temperature. The slides were washed in TBS and then 300 µl of DAB solution (one ml of peroxidase substrate solution and one drop of chromogen) were added to the sections for approximately 10 minutes at room temperature. To stop the DAB reaction, the slides were washed using water. The slides were dried around the sections, mounted in aqueous mounting media and then sealed around the edges with nail varnish.

Chapter 3 Results

3.1 Sensitivity of antisera against CYP4Z1

Native human CYP4Z1 in tissue samples cannot be specifically detected and, hence characterisation relies on Western blot analysis. Therefore, it was necessary to develop a sensitive human CYP4Z1-specific antibody.

A partial cDNA of human CYP4Z1 was cloned into pRSET-b for expression as a histidine-tagged fusion protein, containing 319 amino acids (residues 177-493) of 4Z1. The purified protein was approximately 39 700 Da. The integrity of the protein was confirmed by SDS-PAGE (not shown), and the protein concentration was 0.3 mg/ml as determined by Bradford assay (not shown).

Polyclonal antibodies were produced by injection of female New Zealand White rabbits (~ 4 kg) with purified CYP4Z1 recombinant protein. Rabbits were initially injected with the CYP4Z1 antigen (100 μg) as a 50% emulsion in Freunds incomplete adjuvant and bleeds were taken approximately two weeks later (all animal work done by D. Brady). Subsequent immunisations were at approximately monthly intervals. Antisera were obtained from four rabbits; 91, 92, C, D. Antisera were titred for reactivity against 4Z1 by western blotting using various amount of protein (e.g. 10, 3, 1, 0.3 ng). The protein was blotted and detected with primary antiserum at a dilution of 1:500, Figure 3.1. The antigen CYP4Z1 is easily visualised in the left hand panel of Figure 3.1, in lanes 10, 3, 1 and 0.3 ng. Three bands were detected by the antisera from each rabbit. The size of the CYP4Z1 antigen was determined by Prism v 3.0 program and it was 39 700 Da, and this is shown by an arrow; higher molecular

weight bands were visible in a coomassie-stained gel of this antigen; these may be artefacts, possibly arising from protein aggregation.

The terminal antiserum from rabbit D detected 0.3 ng of antigen, whereas the terminal antiserum from rabbit C detected 1 ng of antigen. Therefore, the antiserum from rabbit D was more sensitive than that from rabbit C.

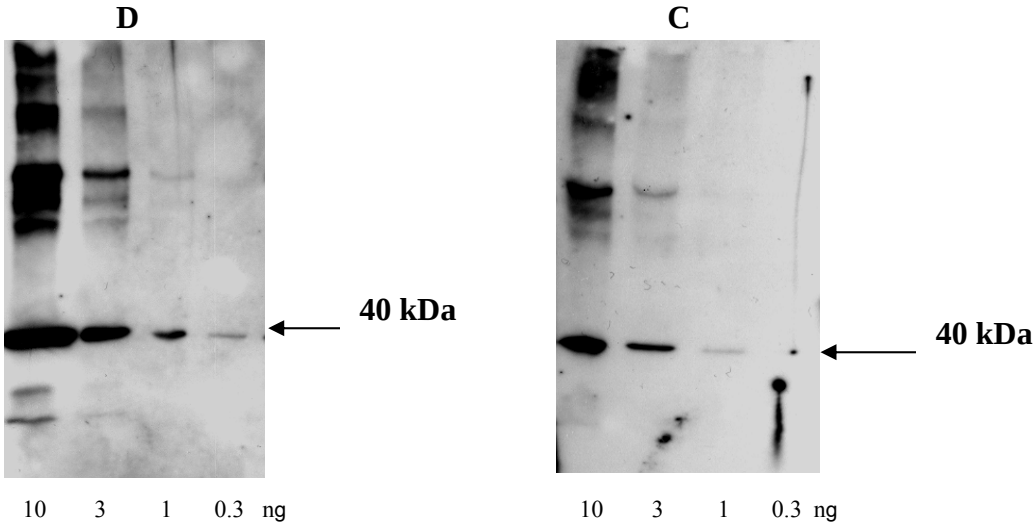

Figure 3.1. Sensitivity of antisera against CYP4Z1.
The indicated amount (0.3, 1, 3 or 10 ng) of CYP4Z1 protein was run on 12% SDS-PAGE then blotted to PVDF membrane as described in the methods. The membranes were incubated with antiserum (from rabbit C or D) at a concentration of 1:500, and the blots were developed with ECL as described in the methods. Film was exposed for thirty minutes. The arrow shows the protein at ~ 40 kDa.

Thus the western blotting technique provides a characterisation of the sensitivity of the antiserum for antigen. The terminal antisera from rabbits 91 and 92 were less sensitive, and detected 10 ng of antigen (data not shown).

The development of antisera over time was followed by western blotting of sequential bleeds. Time course injections were given at four week intervals and once a sufficient antibody titre was obtained a terminal bleed sample were

collected. Figure 3.2, shows the sensitivity of antiserum for antigen over the course of several immunizations.

The sensitivity of antiserum increased with each immunisation of the rabbit, achieving sensitivity at the 0.3 ng level after 6 boosters, at which point the terminal bleed was taken.

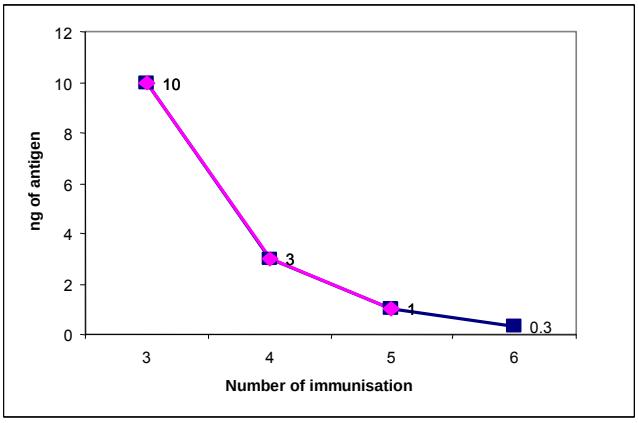

Figure 3.2. **Development of antisera against CYP4Z1.**
Rabbits were immunised six times for western blotting at monthly intervals. Both antisera detected 1 ng of antigen was diluted 1:500. The diamond represent the antisera of rabbit C and the square represent the antisera of rabbit D, which had been collected at the indicated date, characterised as shown in Figure 3.1. The data points represent the minimum amount of antigen detected.

The sensitivity of the antisera against hCYP4Z1 was shown to be sufficient for subsequent characterization of CYP4Z1. The detection limit was 0.3 ng but a lengthy course of immunisations was required to achieve this level of sensitivity.

3.2 Characterisation of antisera against the Aryl hydrocarbon-Receptor (AhR)

Native AhR in tissue samples and after recombinant expression was too low to detect by coomassie blue staining on SDS-PAGE and hence characterisation relies on Western blot analysis or functional analysis (ligand-binding assay). Therefore, it was necessary to produce antisera specific for the mouse AhR.

A fragment of the mouse AhR b-1 allele (residues 285-416) (J.Fenlon, M. Q. Fan, and DR. Bell) was inserted by BamHI digestion into the pRSET-c vector (clone '5g´).

The cloned DNA construct pRSETc-AhR285-416 encodes 131 amino acids of AhR and the calculated molecular weight of the protein is 19,267 Da. Recombinant protein was provided by John Fenlon. The protein was shown to be intact by SDS-PAGE and the concentration was determined to be 0.76 mg/ml by Bradford assay (not shown).

Two rabbits were injected with the recombinant AhR fragment to generate antisera against the AhR recombinant protein. Time course injections were given at four week intervals and once sufficient antibody titres were obtained, terminal bleed samples were collected.

Bleeds from rabbits A and B were collected after each immunisation, and gave strong and clear bands against the antigen (AhR), Figure 3.3. The minimum amount of antigen detected was 0.3 ng at a primary antibody dilution of 1:500; this gave a strong band in both rabbit bleeds. Figure 3.4, shows a graph of the sensitivity of antisera over time. In the initial titration, the sensitivity of the antisera was 3 ng, whereas the sensitivity of the terminal bleeds for both rabbits

was less than 1 ng. This indicates an improvement in the sensitivity of the antisera from these rabbits against the antigen over the course of several immunizations and shows that the antisera can be used to sensitively detect the AhR LBD protein of mouse.

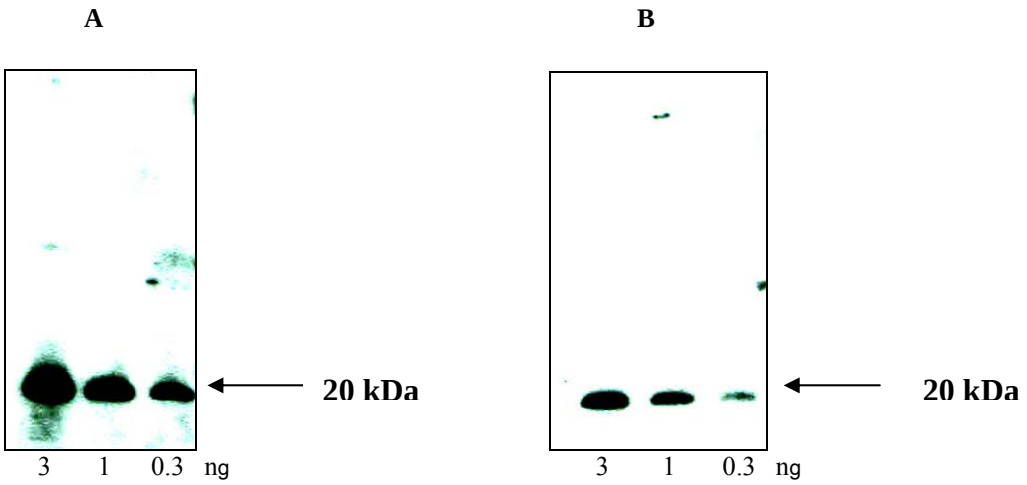

Figure 3.3. **Sensitivity of antisera against AhR**.
The indicated amount (3, 1, 0.3 ng) of AhR protein was run on an 18% SDS-PAGE gel, then blotted to PVDF membrane as described in the methods. The membranes were incubated with the indicated antiserum (A or B) at a concentration of 1:500, and the blot developed with ECL as described in methods. Films were exposed for thirty minutes.

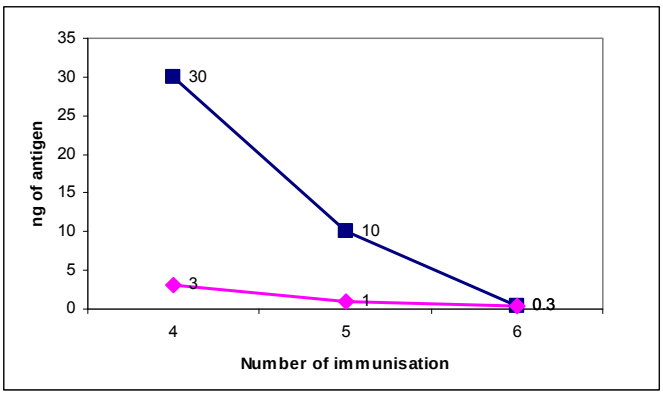

Figure 3.4. Development of antisera against AhR protein.
Rabbits were immunized at monthly intervals for six months. Antisera from rabbits A and B were collected at the indicated time, diluted 1:500, and incubated with antigen for 1 hr. Diamond points represent rabbit B and the square points represent rabbit A, these show the minimum amount of AhR antigen detected at the indicated time.

The detection limit of the antisera for AhR LBD was 0.3 ng. This was sufficient for further characterization of the AhR protein in mouse and rat cytosol.

The antisera were tested for cross-reactivity and specificity against native liver proteins. The AhR is known to be expressed in rodent liver, and so cytosol was prepared from the livers of C57BL/6 mice (male) and Wistar rats (male) as described in the methods. Cytosolic proteins from the livers of mice and rats were western blotted and incubated with antiserum, Figure 3.5. Several minor bands of reactivity were seen in both mouse and rat cytosol possibly due to proteolysis because it is also known that the AhR is sensitive to proteolytic degradation (Poland et al, 1990). However, there was a strong band of AhR protein at a size of ~111kDa in mouse cytosol and ~110 kDa in the rat cytosol. This corresponds approximately to the expected migration of the AhR protein on SDS-PAGE, 104 kDa in C57BL/6 mice and 106 kDa in rats (Poland et al, 1987, 1990). The lower molecular weight bands were found to be strong in the mouse cytosol, whereas these bands in the rat cytosol were faint. Furthermore, the intensity of the higher molecular weight bands was greater in the rat cytosol than in the mouse.

The antisera against mouse LBD detected the rat AhR due to high sequence identity.

The percentage amino acid identity of mouse AhR and rat AhR LBD is 93% and only five residues are different.

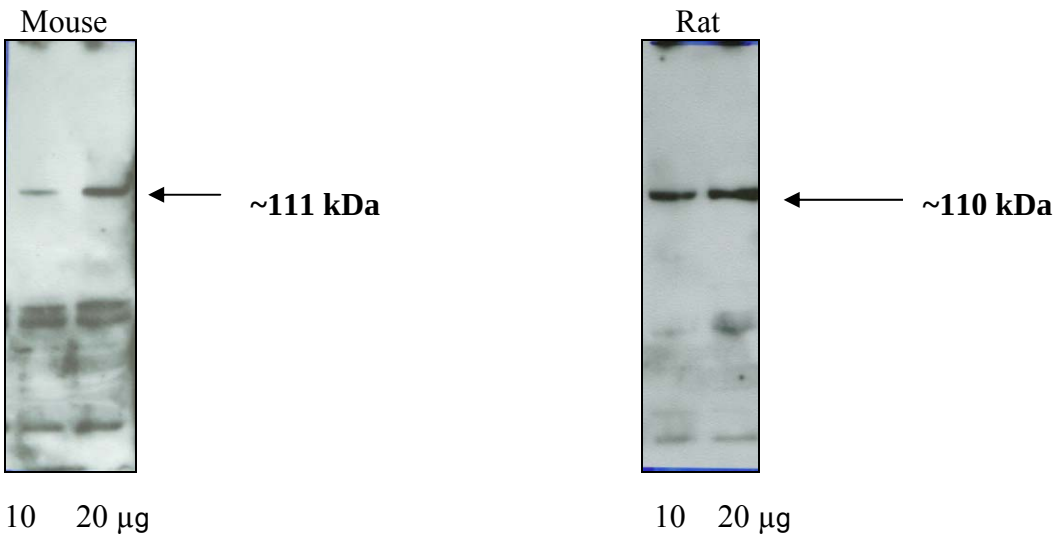

Figure 3.5 Western blotting of mouse and rat cytosol.
The indicated amount (20, 10 µg) of C57BL/6 and wistar cytosol protein was run on 8% SDS-PAGE gel, then blotted to PVDF membrane as described in the methods. The membranes were incubated with antiserum A at a concentration of 1:500, and the blot developed with ECL as described in methods. Films were exposed for 30 minutes. The arrows show the AhR protein at a size of ~ 111 kDa in mouse cytosol and ~110 kDa in rat cytosol.

Characterisation of the antisera against native liver proteins of mouse and rat showed that it was sufficient to detect the AhR in 10 µg of cytosol protein. The signals produced in rat cytosol were stronger than in mouse cytosol.

3.3 Characterisation of antisera against Latrophilin (LpH)

Latrophilin (LpH) is the receptor for toxic proteins produced by Black Widow spiders. A partial *C.elegans* LpH cDNA was cloned into pRSET for expression as a histidine-tagged fusion protein containing 374 amino acids of LpH (residues 168 -542)**,** with a predicted mass of 46 447 Da. The integrity of the protein was confirmed by SDS-PAGE (not shown), and its concentration was determined to be 3.3 mg/ml by Bradford assay (not shown).

In order to characterise the purified and native LpH protein, antibody specific to LpH was required. Two New Zealand white female rabbits (E and F) (~ 4 kg) were injected with ~ 100 µg of LpH antigen. Booster injections were given at four week intervals and once sufficient antibody titre was obtained, terminal bleed samples were collected.

An example of a Western blot to detect LpH protein is shown in Figure 3.6.

This Figure shows a good titre with antiserum, which was collected from rabbits E and F. Different amounts of protein were blotted and visualised using antiserum E and F to determine the sensitivity of the antiserum. Both antisera show a high sensitivity to the LpH (0.3 ng) at a dilution of 1:500. There are no differences in the intensity of protein bands detected using antisera from rabbit E or rabbit F.

The rabbits were injected with the antigen once a month and bleed samples were tested for antibody titre, shown in Figure 3.7. The minimum amount after the first injection of the antigen was 3 ng and the sensitivity of antisera increased to detect 0.3 ng after six months.

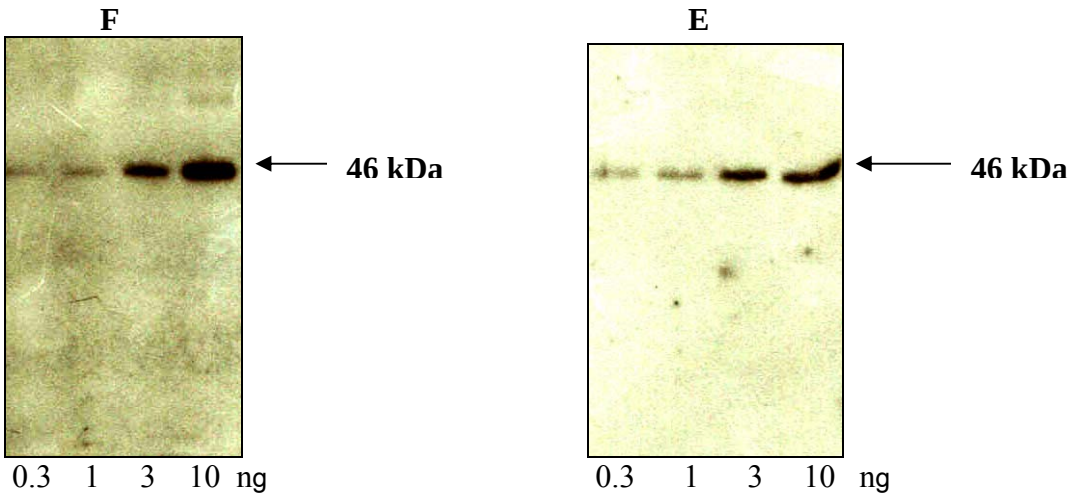

Figure 3.6. **Sensitivity of antisera against LpH**.
The indicated amount (10, 3, 1, 0.3 ng) of LpH protein was run on a 12% SDS-PAGE gel, and then blotted to PVDF membrane as described in the methods. The membranes were incubated with the indicated antiserum (E or F) at a concentration of 1:500, and the blot developed with ECL as described in methods. Films were exposed for thirty-minutes.

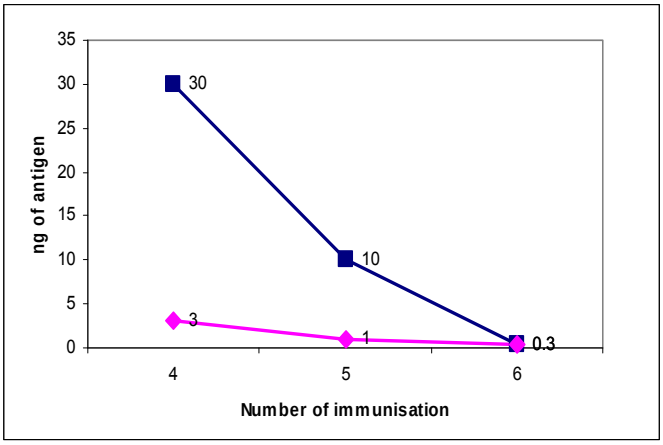

Figure 3.7. **Development of antisera against LpH.**
Rabbits were immunized at monthly intervals for six months. Antisera of rabbits E and F were collected at the indicated times diluted 1:500, and incubated with antigen for 1 hr. Diamond points represent the amount of antisera rabbit F was detected at the indicated time. The square points represent the amount of antisera rabbit E was detected at the indicated time.

Antiserum against latrophilin was sensitive enough to detect 0.3 ng of antigen, sufficient for use in further studies.

3.3.1 Characterisation of latrophilin (LpH) protein

The LpH protein is 1014 amino acids in length and is predicted to be a G-protein coupled receptor (Poland et al; 1991), with seven membrane-spanning domains, on the basis of its primary structure. This protein would therefore be predicted to be localised in a membrane compartment. In order to test this hypothesis, different sub-cellular fractions of *C.elegans* were isolated. *C.elegans* were purified by sucrose density gradient purification to remove contaminating bacterial debris, which otherwise gave immunoreactive bands (data not shown). The *C.elegans* was lysed by extensive sonication, and initial separation was a 5,000rpm (3,020 x g) spin for five minutes to remove worm carcasses and nuclei (the 5k pellet). The 5,000rpm (3,020 x g) supernatant (5k super) was fractionated by centrifugation at 15,000rpm (27,200 x g) for thirty minutes to yield a 15k pellet and 15k supernatant (15k super).

The15k pellet and supernatant were blotted and probed with antiserum F. Figure 3.8, shows that several bands were found in these fractions. The 15k pellet fraction has a band at approximately ~ 113 kDa which represents the expected size of the LpH protein, whereas no band of this size was found in the 15k supernatant. Therefore, LpH protein is found in the cell membrane compartment. Furthermore, several bands of reactivity were seen in both fractions (15k pellet and 15k supernatant), these are possibly due to protein cleavage at the GPS site, but it is also possible that they are non-specific cross-reactivity.

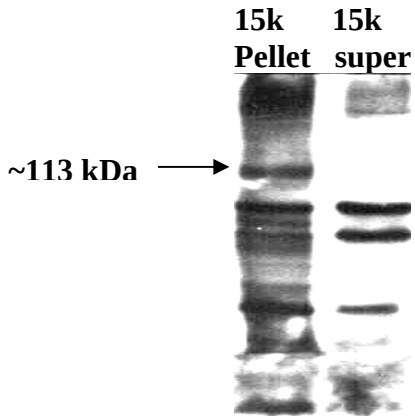

Figure 3.8. **Western blotting of sub-cellular fractions of *C.elegans* (15k pellet and supernatant).**
20 µg of each fraction was run on a 12% SDS-PAGE gel, and then blotted to PVDF membrane as described in the methods. The membrane was incubated with 1:500 antiserums F for 1 hr, and the blot was then developed with ECL as described in the methods. A film was exposed for thirty minutes. The arrow shows a protein at ~113 kDa.

3.3.2 Wild type and latrophilin knockout *C.elegans*

As a further control for the specificity of the LpH antiserum, we used the antiserum to probe protein extracts from wild-type and LpH null worms. The LpH null worms are derived from strain VC965, which are heterozygous for the ok1465 allele (http://aceserver.biotech.ubc.ca/cgi-bin/generic/allele?class=Allele;name=ok1465), and were used to generate homozygous ok1465 null worms (DRB, unpublished data).

The ok1465 allele has an extensive deletion of the LpH gene, including the entirety of the region covered by the LpH antigen (Figure 3.9). These worms should therefore be a negative control for the LpH antiserum.

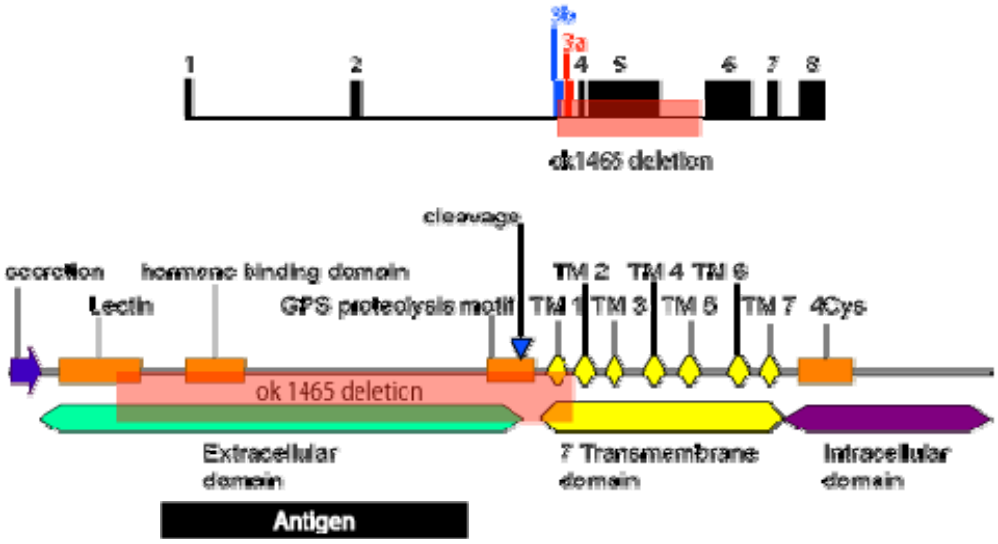

Figure 3.9. Cartoon of the ok1465 deletion. The top figure shows the exons (numbered) of the LpH gene, and the alternatively spliced exons 3a and 3b are shown in red and blue, respectively. The region of the deletion is marked by a red box. The bottom figure shows the domain organisation of the *C. elegans* LpH protein. The secretion signal (secretion), Lectin binding domain (lectin), Hormone binding domain, GPS proteolysis motif and cleavage site, transmembrane regions 1-7 (TM1-7), and the 4 cystein motif (4Cys), are indicated, together with the putative extracellular, transmembrane and intracellular domains. The deletion is marked as a red box, and this position of the antigen used for immunisation by a black box (Antigen).

The two strains of *C.elegans* were sonicated and pelleted as described in section 2.2.4.10.2, the purity of the culture was checked by PCR analysis on the worms to demonstrate the presence and absence of the respective alleles (DRB, unpublished). Figure 3.10 shows that several low molecular weight bands are present in both worm samples, and these are therefore non-specific binding of the antibody. However, the latrophilin knockout *C.elegans* did not contain a specific band of ~113 kDa in the 12k pellet, whereas the wild type did contain this band, which is the expected size of the LpH protein. The band at 113kDa is smeared, and covers a size range. This is typical for the mammalian LpH proteins, where the smearing is a result of glycosylation of

the protein. This shows that the LpH antiserum was able to specifically detect native LpH protein, and suggests that the *C. elegans* LpH is glycosylated.

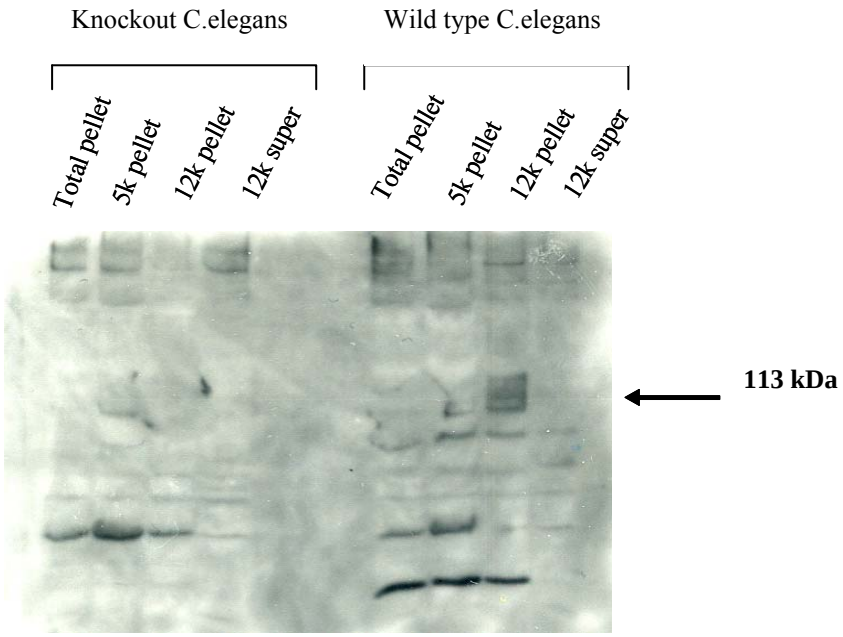

Figure 3.10. Western blotting of wild type and LpH knockout C.elegans.
20 µg of each LpH knockout fraction and (12,20, 20,6.5 µg) of wild type C.elegans fractions(total pellet, 5k pellet, 12k pellet, and 12K supernatant respectively) were run on a 8% SDS-PAGE gel, and then blotted to PVDF membrane as described in the methods. The membrane was incubated with 1:500 antiserums F for 1 hr, and the blot was then developed with ECL as described in the methods. A film was exposed for twenty minutes. The arrow shows a protein at ~113 kDa.

LpH was found in the cell membrane compartment. In order to liberate this protein from the cell membrane into solution, solubilization by detergents was performed using Emulgen 913 and 2% Dodecylmaltoside.

3.3.3 Solubilisation of latrophilin (LpH)

LpH protein was solubilized from the 15k pellet fraction with Emulgen 913 detergent at 0.6%, 0.8%, or 1% concentrations. A Western blot of the resulting fractions shows that little protein was detected in the resulting solutions.

However, an intense immunoreactive band was detected in the pooled, detergent-insoluble fractions of the 15k pellet, Figure 3.11. A strong band of 113 kDa was seen in the lane containing the pooled detergent insoluble protein, Figure 3.11. Therefore, LpH protein was not solubilized in 0.6%, 0.8% or 1% Emulgen 913.

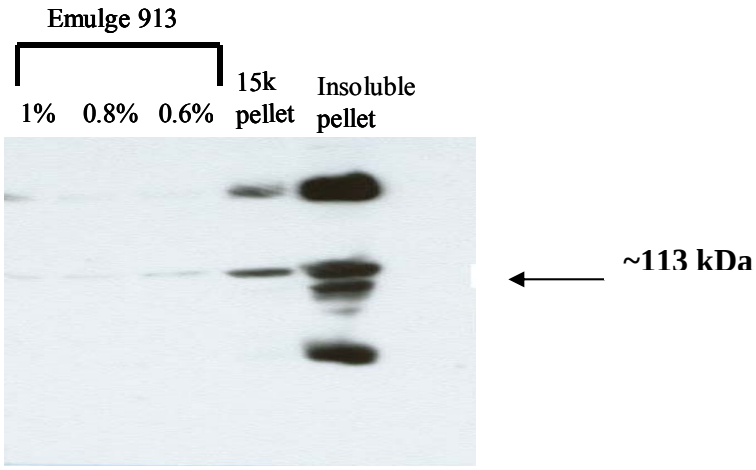

Figure 3.11. Solubilization of LpH.
The 15k pellet (15k) was treated with 0.6, 0.8, or 1% Emulgen 913 to yield soluble fractions, and a pooled pellet (pellet); fractions were run on a 10% SDS-PAGE gel, then western blotted with antiserum F as described in the methods. Film was exposed for thirty minutes. The arrow shows a protein at ~113 kDa.

The 15k pellet was also treated with 2% dodecylmaltoside. In order to optimise protein solubility, the sub-cellular fractions were incubated in 2% dodecylmaltoside for varying lengths of time as shown in Figures 3.12 and 3.13.

A fresh 15k supernatant, treated with 2% dodecylmaltoside detergent, showed a strong immunoreactive band at ~113 kDa, Figure 3.12. However, after storage of these samples at – 80ºC, an intense band at 66 kDa was detected in addition to faint lower molecular bands, Figure 3.13.

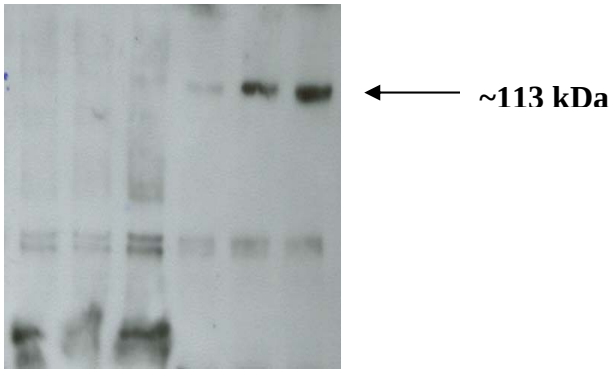

Figure 3.12. Solubilization of a fresh sub-cellular fraction of C.*elegans* (15K pellet and supernatant) in 2% dodecylmaltoside detergent.
The fractions were incubated in 2% dodecylmaltoside for different length of time (10, 20, 60 min). 20 µg of each fraction was run on a 10% SDS-PAGE gel, and blotted to PVDF membrane as described in the methods. The membrane was incubated with 1:500 antiserums F for 1 hr, and the blot was then developed with ECL as described in the methods. (P) represents the pellet fraction and (S) represents the supernatant fraction. Film was exposed for thirty minutes. The arrow shows the protein at ~ 113kDa.

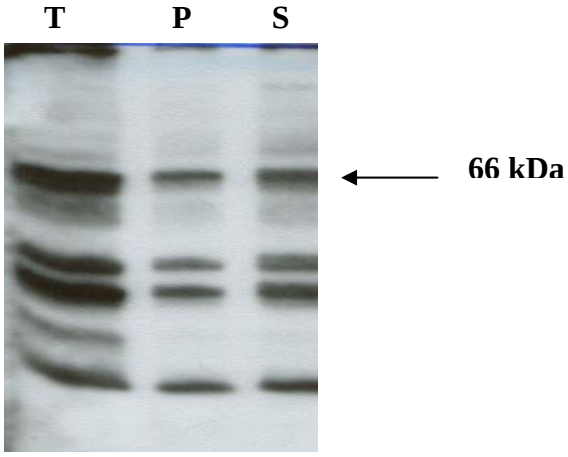

Figure 3.13. **Solubilization of sub-cellular fractions of C.*elegans* (pellet and supernatant) in 2% dodecylmaltoside detergent.**
The fractions were incubated in 2% dodecylmaltoside and left on a shaker at 4ºC overnight. 20 µg of each fraction was run on a 10% SDS-PAGE gel, and blotted to PVDF membrane as described in the methods. The membrane was incubated with 1:500 antiserum F for 1 hr, and the blot was then developed with ECL as described in the methods. (T) represents total homogenised LpH protein. (P) represents the pellet fraction and (S) represents the supernatant fraction. Film was exposed for thirty minutes. The arrow shows a part of this protein at 66 kDa.

The intense band at ~ 66 kDa may be due to the presence of a G protein coupled receptor proteolysis site (GPS) between positions C497 and M536 of LpH, which is a characteristic feature in the LpH protein family as shown in Figure 3.14. After cleavage at the GPS site between L529 and T 530, the predicted N-terminal fragment would be a 529 amino acid protein of 58 513 Da.

Figure 3.14. The amino acid sequence of the G protein coupled receptor site of LpH.
All amino acids under line are conserved and the cleavage site is between L529 and T530 as indicated by the arrow.

C.elegans Latrophilin GPS sequence was compared with the rat latrophilin GPS (accession No. 2239297) to determine if there was sequence identity. This comparison showed an amino acid sequence (Figure 3.14) that is conserved (41%) between rat and *C.elegans* Latrophilin GPS sequence. The conserved residues are also known to be conserved in other GPS proteins (Krasnoperov et al, 1997). The cleavage site of LpH between L529 and T 530 is present in both rat and *C.elegans* Latrophilin GPS sequence. Therefore, the GPS domain represents important key for proteolytic process and functionally significant of GPS superfamily.

Fractionation of antiserum was performed to improve the specificity of antiserum for LpH. Ammonium sulphate precipitation of the antiserum was performed using 25% or 50% saturated solutions of ammonium sulphate. The resulting samples were analysed by SDS-PAGE and dot blotting to determine how much of the antibody was present in each fraction. Figure 3.15, compares serial dilutions of the serum and fractions from the ammonium sulphate steps and shows that most of the antibodies were successfully precipitated in the 25% and 50% pellet fractions. Furthermore, no antibodies were seen in the 50% supernatant (fraction), Figure 3.15, track 4, and the amount of precipitated antibodies in both pellet fractions (25% and 50%) was equal.

Antiserum treated with 50% saturated ammonium sulphate contained low amounts of other proteins and hence was selected for sensitivity and selectivity testing.

The coomassie stained gel in Figure 3.16, shows that several bands were present in the 25% and 50% pellets. A strong band of ~ 66 kDa, which is probably albumin, was mostly removed from the 25% and 50% pellet fractions. Therefore, the data show that albumin concentration was reduced in the fraction containing the antibodies.

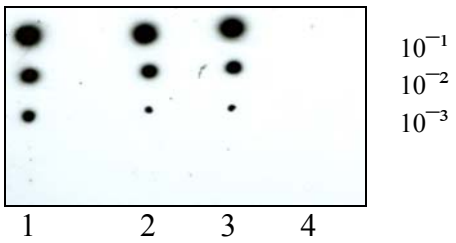

Figure 3.15. **Ammonium sulphate precipitation of antibodies**.
Antiserum was treated with several dilutions of saturated ammonium sulphate, yielding 25% and 50% pellets, and the 50% soluble fraction. Fractions were analysed by dot blotting onto a nitrocellulose membrane, the membrane was incubated with antirabbit antibody and developed with ECL as described in methods. Track (1) represents untreated of the total antiserum of rabbit F. Track (2) represents the pellet of the treated antiserum of rabbit F with 25% saturated ammonium sulphate. Track (3) represents the pellet of the treated antiserum of rabbit F with 50% saturated ammonium sulphate. Track (4) represents the supernatant of the treated antiserum of rabbit F with 50% saturated ammonium sulphate. Dilutions are indicated the right of the Figure.

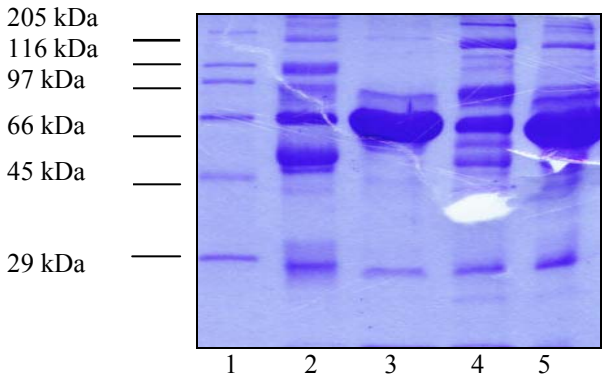

Figure 3.16. **A coomassie stained gel of ammonium sulphate precipitation of antibodies**.
The fractions of solubilized antibodies were loaded and run on a 10% SDS-PAGE gel. Track (1) represents the protein marker (sigma SDS 6H). Track (2) represents the pellet of the treated antiserum of rabbit F with 25% saturated ammonium sulphate. Track (3) represents the supernatant of the treated antiserum of rabbit F with 50% saturated ammonium sulphate. Track (4) represents the pellet of the treated antiserum of rabbit F with 50% saturated ammonium sulphate. Track (5) represents untreated of total antiserum of rabbit F.

The antibodies were further purified using an affinity column, to which the LpH protein was attached. LpH protein was coupled to agarose (beads), and then antibodies binding LpH were purified on the beads. In order to confirm that the prepared column was working properly, LpH protein concentration was determined by Bradford assay before and after binding of the antigen to the LpH protein. The concentration of LpH protein before binding was 0.6 mg/ml, whereas the concentration of the protein after binding was 0.01 mg/ml. Therefore, most of the LpH protein binds to the column. Figure 3.17, compares serial dilutions of the purified antibodies from the affinity column. The purified antibodies from the column represented > 50% of the original antibodies in the antiserum. The purified antibodies had a lesser sensitivity for LpH protein (10 ng), Figure 3.18.

When the protein extracts from Figure 3.8 were probed with antiserum F, several bands appeared. However, the purified antiserum gave selective principal bands at 113 kDa and 66 kDa, Figure 3.19.

Clarification of antiserum was carried out by 25% and 50% saturated ammonium sulphate precipitation. Pellet (50%) fraction was further purified using an affinity column and the antiserum selectivity was improved.

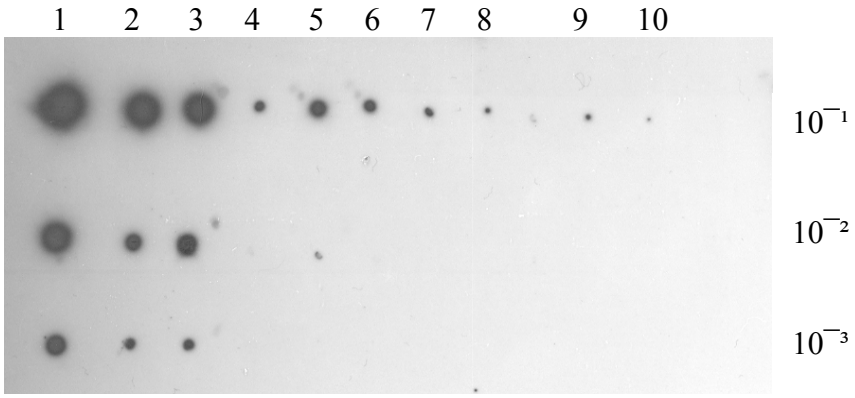

Figure 3.17. **Antibodies purified by affinity column**.
Antibodies were purified from antiserum using affinity column containing immobilized LpH binding protein. Fractions were analysed by dot blotting onto a nitrocellulose membrane. The membrane was incubated with antirabbit antibody and developed with ECL as described in Methods**.** Track (1) represents the 50**%** saturated ammonium sulphate antiserum pellet. of rabbit F. Tracks (2-10) represent the eluted fractions of purified antibodies. Dilutions are indicated to the right of the figure.

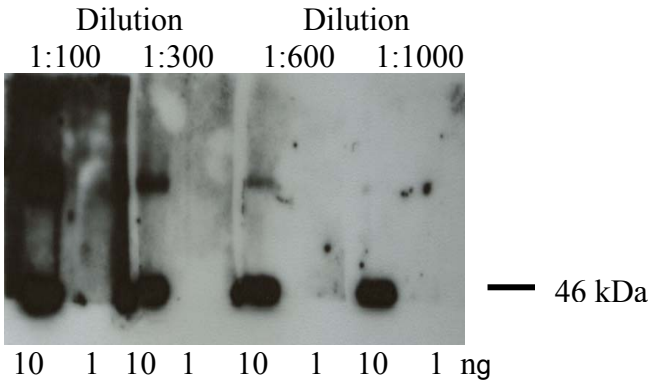

Figure 3.18. Sensitivity of purified antisera against LpH.
1 ng or 10 ng of LpH protein was run as indicated amount on a 12% SDS-PAGE gel, then blotted to PVDF membrane as described in the methods. The membrane was incubated with purified antiserum F diluted as shown, and the blot was developed with ECL as described in methods. Film was exposed for thirty minutes.

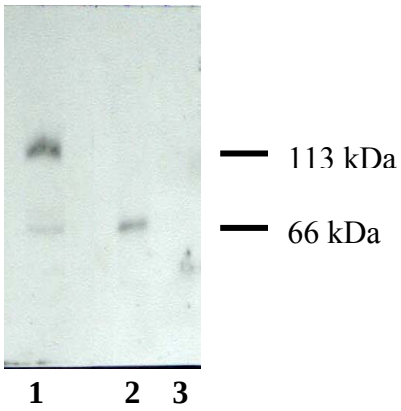

Figure 3.19. Western blot showing sub-cellular fractions of *C.elegans,* probed with purified antiserum F.
The fractions of *C.elegans* were run on a 12% SDS-PAGE gel, and then blotted to PVDF membrane as described in the methods. The membrane was incubated with purified antiserum F for 1 hr, and the blot was developed with ECL as described in the methods. Film was exposed for thirty minutes. Track (1) represents 15K of the total homogenate *C.elegans* lysate. Track (2) represents 15k pellet of the sub-cellular fraction of 15k *C.elegans* lysate. Track (3) represents 15k supernatant of the sub-cellular fraction of 15k *C.elegans* lysate.

3.4 Gene expression studies

3.4.1 Extraction of total mouse RNA

Total RNA was isolated from mouse brain and liver using the Triazol Method (Invitrogen) as described in the Methods, section 2.2.3.9. The yield of total RNA from mouse brain and liver tissues were 1.8 mg/g and 4.3 mg/g respectively. RNA concentration was determined using the RiboGreen quantitation kit as described in the Methods, section 2.2.3.15 and the integrity of the RNA samples was analysed by agarose gel electrophoresis. Figure 3.20, shows that RNA integrity was maintained as shown by intact 18S and 28S ribosomal RNA bands.

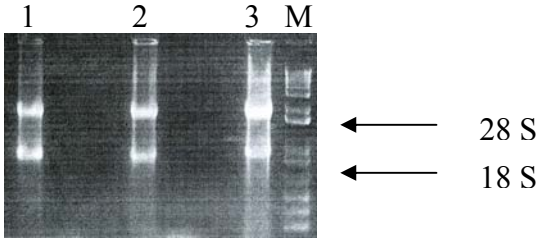

Figure 3.20. Agarose gel electrophoresis of mouse brain RNA. RNA was extracted using TRIAZOL reagent.
Brain samples were run on a 1% agarose gel (containing 0.2% SDS and 0.2 µg/ml ethidium bromide) at 125 v for 1 hr with 1XTBE. Lanes (1-3) represent 5 µg; 10 µg and 15 µg of RNA were loaded respectively. M represents marker 1kb DNA. The gel was photographed under UV transillumination. The arrows show the position of intact 28S and 18S rRNA bands.

3.4.2 Specificity of m4x1 probes

Determination of the probe specificity is an essential step prior to using the RNase protection assay. The specificity of the probe was determined by the inclusion of two control yeast tRNA samples, positive and negative controls, which were hybridised to the probe. The positive control was carried out by hybridisation of the probe with yeast tRNA in the presence of RNase A/T1 (+ve control). The negative control was carried out by hybridisation of the probe with yeast tRNA and no RNase was added (-ve control). Figure 3.21, shows the specificity of the assay. It demonstrates that the probe in the negative control was full-length and intact. In the positive control that included RNase A/T1, the probe was degraded and no bands can be seen.

Therefore, the probe specificity on the control samples confirms that signals that were obtained from mouse RNA samples were due to specific protection of the probe.

For optimisation of the riboprobe, several probes were prepared and diluted 4-fold and 10-fold as shown in Figure 3.21. Figure 3.21, shows that the intensity of these probes decreased according to the dilution range as expected. 10-fold dilution probe was subsequently prepared for all samples because intensity was sufficient at this dilution and low level of background noise.

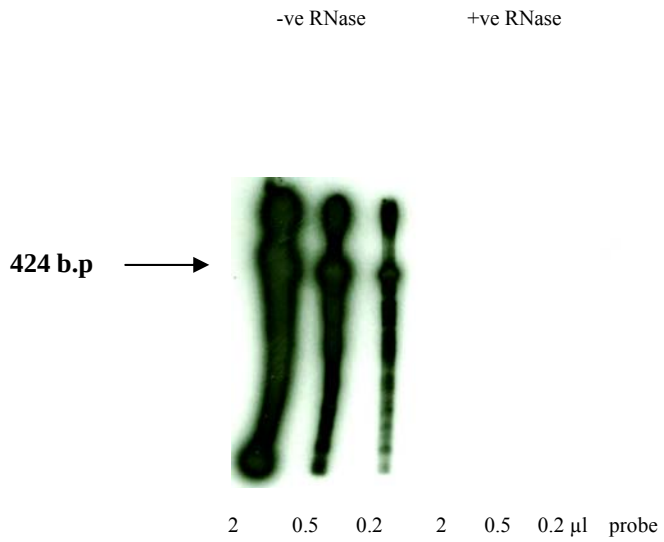

Figure 3.21. RNase protection assay specificity and optimisation.
The specificity of the assay was shown by hybridisation of the riboprobe to yeast tRNA with and without RNase A/T1 treatment. To demonstrate probe integrity, control yeast tRNA (20 µg) samples were hybridised to the probe, with or without RNase A/T1 (+ve or -ve). No detectable signal of the riboprobe (+ve control) was observed after yeast tRNA hybridisation with the probe and RNase treatment indicating that the radioactivity signal obtained in test samples was due to specific hybridisation of probe to RNA. The full-length of the riboprobe is 424 b.p. Numbers (2, 0.5 and 0.2) represent the volume of probe used.

3.4.3 PCR amplification of m-4x1 (exon 12)

The mouse *CYP*4X1 gene has 12 exons and putatively encodes a protein. In order to prepare a probe for use in RNase protection assay for cyp4X1, PCR amplification of m-cyp4x1 (exon 12) fragments was performed. PCR of genomic DNA (C57 Bl/6) generated a 330 b.p. product which contained 177 b.p. of exon and 153 b.p. of intron. Amplified PCR fragments were then subcloned into pGEMT-easy as described in section 2.2.3.12. Genomic DNA from adult (male) C57 Bl/6 mice was obtained as a gift from Dr. D.Bell (Biology School, Nottingham University). Thirty mer primers were designed to amplify a region of the m-cyp4X1 gene within the sequence in accession number AL731651 of XX-PAC129 on chromosome 4.

A region (121000-121330 b.p) of exon 12 of the mouse cyp4X1 gene was amplified from the genomic DNA. A PCR fragment (330 b.p) was generated from genomic DNA. Figure 3.22, shows the position of the primers designed to amplify mcy4X1-12 genomic DNA.

The PCR was tested with annealing temperature of 55ºC, 60ºC, 63ºC and also with different concentrations of magnesium (1.5mM Mg^{2+}, 2.5mM $Mg^{2+}$and 3.5mMg^{2+}). The negative control of the PCR sample was also carried out at the same conditions.

CATGGACATAAGTCCTTTTCCCTTCCTCCTGAAAAGAATGTTTCAATCA
GAGGAAACTGCTAAGCAGCTGTGGACTCCAGACTTATTCTAGAAACTTAC
CCCTCTCTCTCCCTTGGTGTCTACAGGAACTGCATCGGGCAGCAGTTTGCC
ATGCTGGAGCTAAAGGTGGCCATTGCCTTGATTCTGCTCCACTTTCAAGTA
GCTCCAGACCTCACCAGGCCTCCTGCCTTCTCCAGCCACACTGTCCTCAGA
CCCAAGCATGGAATCTATTTGCACCTGAAGAAACTCCTTGAGTGTTAG**ATA**
CTAGGAGAAATGGCGAAATTTATGTTT

Figure 3.22. Mouse cyp-4x1 (exon 12) clone for RNase protection assay.
PCR of genomic DNA (C57BL/6) generated a 330 b.p. fragment. a genomic. Amplified PCR fragments were then sub-cloned into pGEMT-easy vector. Bold nucleotides represent the forward primer and the reverse primer. The shaded region represents exon fragments (177 b.p.), whereas the unshaded region represents intron fragments (153 b.p.).

The PCR amplified a 330 b.p. fragment. The amplified PCR fragments were analyzed on 1% agarose gel electrophoresis as shown in Figure 3.23 and were successful at all annealing temperatures and magnesium concentrations, track 2-5, whereas no band was observed in the negative control, track 1. The optimum amounts of PCR product were obtained at a temperature of 60ºC with 2.5mM magnesium. Gel electrophoresis showed that the primers amplified a 330 b.p product, which is the size expected from the sequence. The fragment was then excised from the gel, purified using the QIAquick gel extraction kit

(QIAGEN). The amplified PCR fragment of cyp4X1 (exon 12) was then sub-cloned into pGEMT-easy and the gel shows that the plasmid DNA was inserted correctly as shown in Figure 3.24.

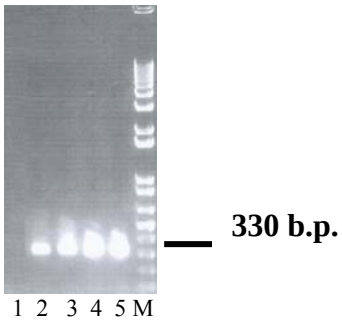

Figure 3.23. PCR of cyp4x1 (exon 12) genomic DNA.
The DNA was amplified using cyp4x1 primers of exon 12 for 35 cycles. The samples (10 µl) were run on 1% agarose gel at 125 V for 1 hr. Lane 1 shows the –ve control. Lanes (2-4) show the PCR products obtained in 2.5mM MgCl and a different annealing temperature (55ºC, 65ºC and 60ºC respectively). Lane 5 shows the PCR product obtained in 1.5mM MgCl at 60ºC annealing temperature. M shows 1 Kb DNA ladder. The gel was photographed under U.V. transillumination.

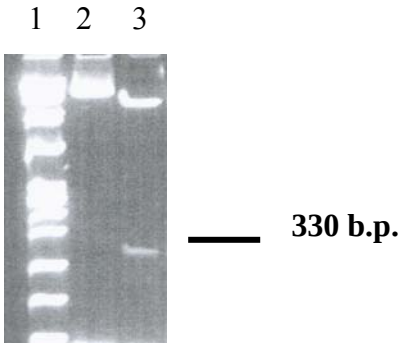

Figure 3.24. Restriction digest of cyp4x1 (exon 12) inserted into pGEM-T easy.
DNA was restriction digested with EcoRI and run on 1% agarose gel at 120 V for 1 hr. The DNA was visualized by Ethidium bromide staining. Lane 1 shows 1 Kb ladder. Lane 2 shows uncut plasmid DNA and lane 3 shows digested pGEM-T easy containing inserted 4x1 genes (exon 12).

Six clones of the transformed pGEM-T easy containing the inserted DNA were obtained and analyzed on agarose gel electrophoresis. One sample was purified using Qiagen mini prep column and sent for sequencing. Figure 3.22, shows the sequence result of the PCR m-cyp4x1 fragments. The sequencing PCR fragments data shows complete identity when comparing with the sequence of exon 12 of mouse genomic DNA (accession number AL731651). Therefore, the sequencing confirmed that the plasmid DNA of the mcyp-4x1 (exon 12) was cloned successfully from the genomic mouse DNA and was in the correct antisense orientation necessary for the generation of a riboprobe. The exon 12 clone was then used for RNase protection assay.

3.4.4 PCR amplification of the full-length mouse 1st strand cDNA

Although the genomic sequence of cyp-4X1 is known (AJ297131), the cDNA sequence is not known and there are possible ambiguities as to where the intron/exon junctions are. Therefore, cyp4X1 was cloned from mouse brain as a full-length open reading frame from the PCR product. While the native sequence has putative 5' and 3' untranslated sequences, these were not examined in this study. In order to synthesise the full-length mcyp4X1, 1st strand cDNA was prepared using ProSTAR UltraHF RT-PCR kit (Stratagene) from mouse (S129) brain RNA. Primers were designed from the beginning and end of the known human CYP4X1 cDNA sequence, and from the sequence of mouse genomic DNA. Figure 3.26, shows the amino acid alignment of the regions of the mcyp-4X1 used to design the PCR primers. The sense primer (4x1-f-p*F*) was 5'-ATGGAGGCCTCCTGGCTGGAGACTCGTTGG-3' and the antisense primer

(4x1-f-p*R*) was 5'-AAACATAAATTTCGCCATTTCTCCTAGTAT-3'**.** PCR was carried out as described in section 2.2.3.18. Figure 3.25, shows a cartoon of the strategy used to produce the amplified 1.6 kb mcyp-4X1 cDNA.

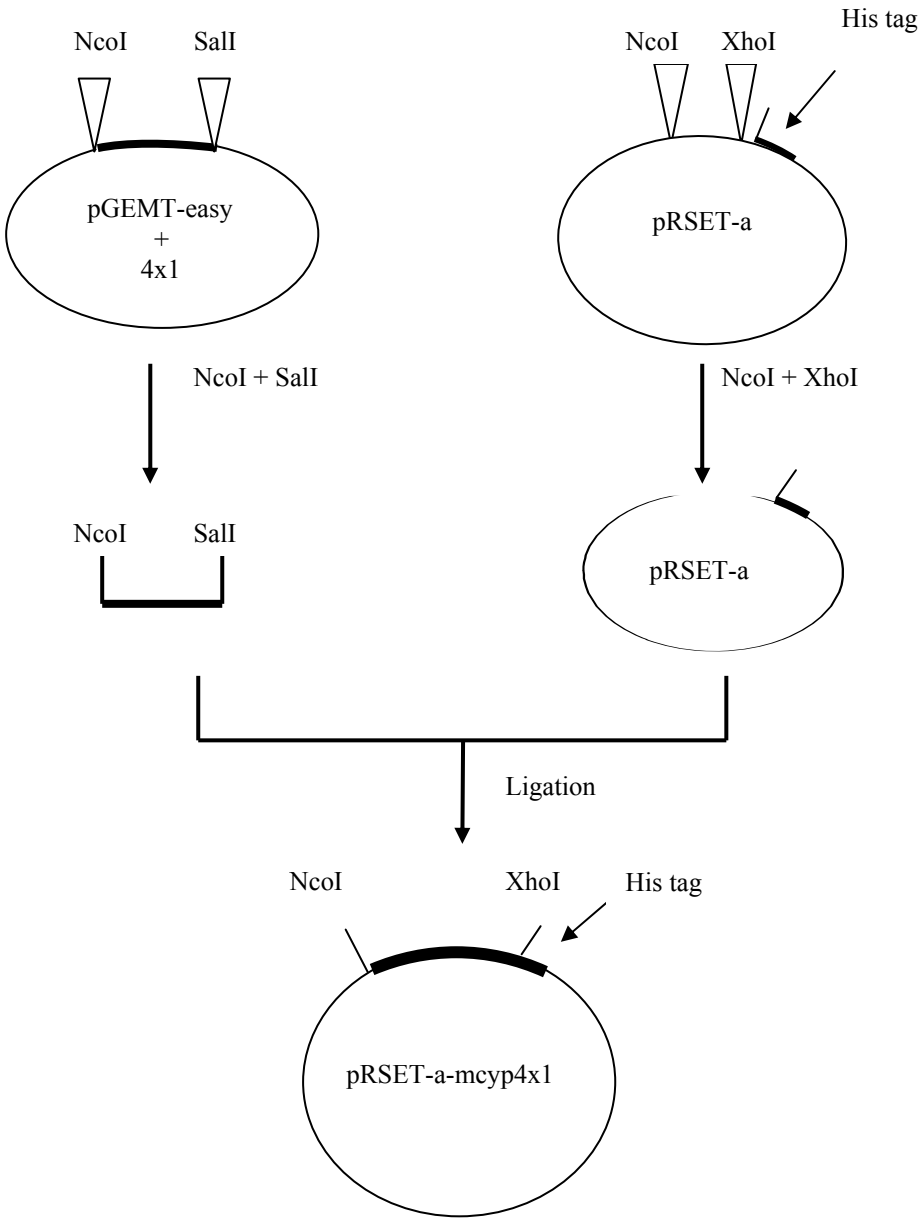

Figure 3.25. **Cartoon of the full-length mouse cyp4x1 cDNA**.
The mouse cyp4x1 fragment (The solid black line) was obtained by NcoI and SalI and then subcloned into plasmid pRSET-a.

The resultant PCR fragments were analyzed on a 1% agarose gel as shown in lane (S) in Figure 3.27. The Figure shows that small amounts of a 1620 b.p. fragment of the full-length mouse 4x1 were produced and it was of the predicted molecular size. This means that the cDNA was generated successfully from the total mouse brain RNA. Because the cDNA was faint, the PCR product of lane (S) was reamplified using the same primers and a Reddy Mix kit as shown in lanes (1-3) of Figure 3.28.

The reamplification of the PCR resulted in an increase in the PCR fragments intensity when analyzed on 1% agarose gel. The reamplified PCR product was excised and purified as described on section 2.2.3.10. The excised and purified cDNA was then sub-cloned into pGEMT-easy cloning vector as described in section 2.2.3.12 and designated pGEMT-f-mcyp-4x1.

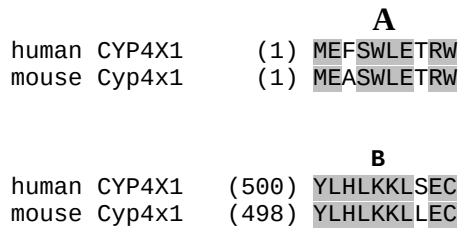

Figure 3.26. Amino acid alignment for PCR primer design.
Primers were designed from the beginning of exon 1 and the ending of exon 12 of human CYP4x1 sequence. **(A)** shows amino acids alignment for region of forward primer. **(B)** shows amino acids alignment for region of reverse primer.

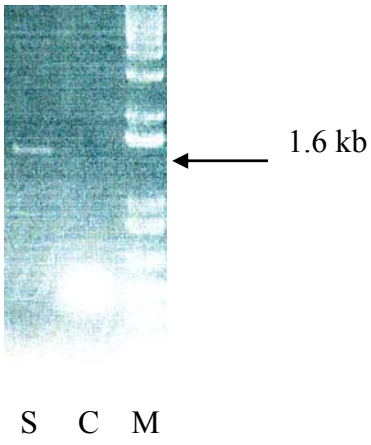

Figure 3.27. **PCR amplified fragments of mcyp4x1 cDNA**.
The mcyp4x1 cDNA was generated by reverse transcription of the total RNA brain sample using UltraHF RT-PCR kit. The sample was then run on 1% agarose gel at 120v for 1 hour. The amplified fragments were visualised under dark reader U.V light. M shows marker (1 kb ladder). C shows control sample and S shows mcyp4x1 cDNA amplified sample.

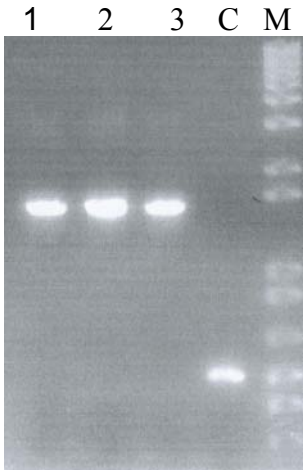

Figure 3.28. **Agarose gel showing reamplified PCR of cDNA**.
The mcyp4x1 cDNA fragments were reamplified using full-length mcyp4x1 PCR primers for 35 cycles with Reddy Mix kit. The mcyp4x1 cDNA was amplified using different concentrations of MgCl and run on 1% agarose gel electrophoresis at 90v for 1 hour. M shows the marker (1kb ladder). (C) Shows the control sample of amplifying cDNA kit reaction. Lanes (1-3) show amplified mcyp4x1 cDNA at several MgCl concentrations (1.5mM, 2.5mM, 3.5mM MgCl respectively).

To confirm the presence of the insert cDNA in the vector, several colonies were obtained and double restriction digests were carried out with NcoI and SalI and then analyzed on a 1% agarose gel. The cloned cDNA was then purified by Qiagen mini prep and two samples were sent for double stranded sequencing and both clones had an identical sequence as shown in Figure 3.29. The deduced mouse cyp4x1 cDNA was submitted to Genbank under accession number AJ786769. The DNA sequence and deduced amino acid sequence of the cDNA are shown in Figure 3.29 and Figure 3.30 respectively. Translation of the full-length mouse cyp4x1 1524 b.p. gives an open reading frame of 507 amino acids.

```
1       ATGGAGGCCT CCTGGCTGGA GACTCGTTGG GCGCGGCCCT TGCACCTGGC
        TTTGGTGTTC TGCCTGGCGC TGGTGCTGAT GCAGGCCATG  AAGCTCTACC

101     TGCGGAGACA GCGACTGCTG CGCGACCTGA GCCCCTTCCC GGGGCCTCCC
        GCCCACTGGC TCCTGGGACA CCAGAAGTTT CTTCAGGAGG  ATAATATGGA

201     GACGCTTGAT GAGATTGTCA AAAAGCACCC TTGTGCCTTC CCCTGCTGGG
        TAGGGCCCTT CCAGGCATTT TTCTACATCT ATGACCCAGA  CTATGCGAAG

301     ATATTTCTGA GCAGAACAGA CCCAAAGATG CAGTATCTGC ACCAGCTCCT
        GACTCCATGC ATTGGACGAG GACTCCTGAA TTTAGATGGG  CCCAGGTGGT

401     TCCAACACCG CTGCCTCCTA ACTCCTGCAT TCCATCAGGA CATCCTAAAA
        CCATGTGTGG ACACGATGGC CCACTCTGTG AAAGTGATGC  TGGATAAATG

501     GGAGAAGATG TGGACCACTC AGGAAACAAC CATCGAGGTT TTTGAACACA
        TCAACTTGAT GACCCTGGAC ATAATAATGA AATGTGCTTT  TGGCCAGGAG

601     ACCAACTGCC AGATAAACGG CACCTATGAG TCTTACGTGA AGGCAACATT
        TGAACTTGGT GAAATCATAT CTTCTCGCTT GTACAATTTC  TGGCATCATC

701     ATGACATAAT TTTCAAACTC AGTCCTAAGG GCCACTGCTT CCAGGAGTTA
        GGCAAAGTGA TACATCAATA CACAGAAAAG ATAATCCAGG  ACAGAAAGAA

801     AATCCTCAAG AATCAAGTAA AGCAGGATGA CACTCAGACG TCTCAAATTT
        TTCTGGATAT TGTTCTTTCT GCACAGGCTG AAGATGAACG  AGCCTTCTCA

901     GATGCTGACC TTCGGGCTGA GGTGAACACC TTCATGTGGG CAGGACATGA
        TGCCTCTGCA GCTAGCATCT CCTGGCTCCT TTACTGCCTG  GCTCTAAATC

1001    CCGAGCATCA GGACAGATGC CGGACAGAGA TCAGGAGCAT CCTGGGAGAT
        GGGTCTTCCA TCACCTGGGA ACAGCTGGAT GAGATGTCAT  ACACCACAAT

1101    GTGCATCAAG GAGACACTCC GCTTGATTCC TCCTGTCCCA TCCATCTCCA
        GGGAGCTCAG CAAACCCCTT ACCCTCCCAG ATGGACACTC  ACTGCCTGCA

1201    GGTATGACTG TGGTTCTTAG TATTTGGGGT CTCCACCACA ACCCTGCTGT
        CTGGAACGAC CCAAAGGTCT TTGACCCCTT GAGATTCACT  AAGGAGAATT

1301    CTGATCAGAG ACACCCCTGT GCCTTCCTAC CATTCTCCAG TGGTCCAAGG
        AACTGCATCG GGCAGCAGTT TGCCATGCTG GAGCTAAAGG  TGGCCATTGC

1401    CTTGATTCTG CTCCACTTTC AAGTAGCTCC AGACCTCACC AGGCCTCCTG
        CCTTCTCCAG CCACACTGTC CTCAGACCCA AGCATGGAAT  CTATTTGCAC

1501   CTGAAGAAAC TCCTTGAGTG TTAG
```

Figure 3.29. **Nucleotide sequence of the mcyp-4x1 cDNA**.
The deduced mcyp-4x1 cDNA shows that the full-length of the cyp4X1 open reading frame (1524 b.p. long). Sequence analysis was carried out by J. Keyte, Biochemistry Dept., Nottingham University (Acc. No. AJ786769).

```
  1   MEASWLETRW ARPLHLALVF CLALVLMQAM KLYLRRQRLL RDLSPFPGPP
 51   AHWLLGHQKF LQEDNMETLD EIVKKHPCAF PCWVGPFQAF FYIYDPDYAK
101   IFLSRTDPKM QYLHQLLTPC IGRGLLNLDG PRWFQHRCLL TPAFHQDILK
151   PCVDTMAHSV KVMLDKWEKM WTTQETTIEV FEHINLMTLD IIMKCAFGQE
201   TNCQINGTYE SYVKATFELG EIISSRLYNF WHHHDIIFKL SPKGHCFQEL
251   GKVIHQYTEK IIQDRKKILK NQVKQDDTQT SQIFLDIVLS AQAEDERAFS
301   DADLRAEVNT FMWAGHDASA ASISWLLYCL ALNPEHQDRC RTEIRSILGD
351   GSSITWEQLD EMSYTTMCIK ETLRLIPPVP SISRELSKPL TLPDGHSLPA
401   GMTVVLSIWG LHHNPAVWND PKVFDPLRFT KENSDQRHPC AFLPFSSGPR
451   NCIGQQFAML ELKVAIALIL LHFQVAPDLT RPPAFSSHTV LRPKHGIYLH
501   LKKLLEC
```

Figure 3.30. The amino acid sequence of mcyp4x1 cDNA.
The deduced amino acid sequence shows that the cDNA encodes a protein of 507 amino acids.

After this sequence was deposited in Genbank, two other mouse sequences of 4x1 were found. NM_001003947 is identical, and is a cDNA prediction based on genomic sequence, and my sequence, AJ786769. It was also found that this sequence has high identity (94%) with the published rat CYP4x1 (AF439343) cDNA sequence.

The deduced mcyp4x1 protein sequence shows a high level of similarity but not complete identity when compared with rat and human sequences as shown in Figure 3.31. This Figure shows that identity is 94 % and 79.2 % between the full-length mouse 4x1 sequence and rat (AF439343), and human (Q8N118) respectively. Therefore, the alignments of the mouse CYP4X1 amino acid sequence with the rat and human CYP4X1, showed a high level of homology between them.

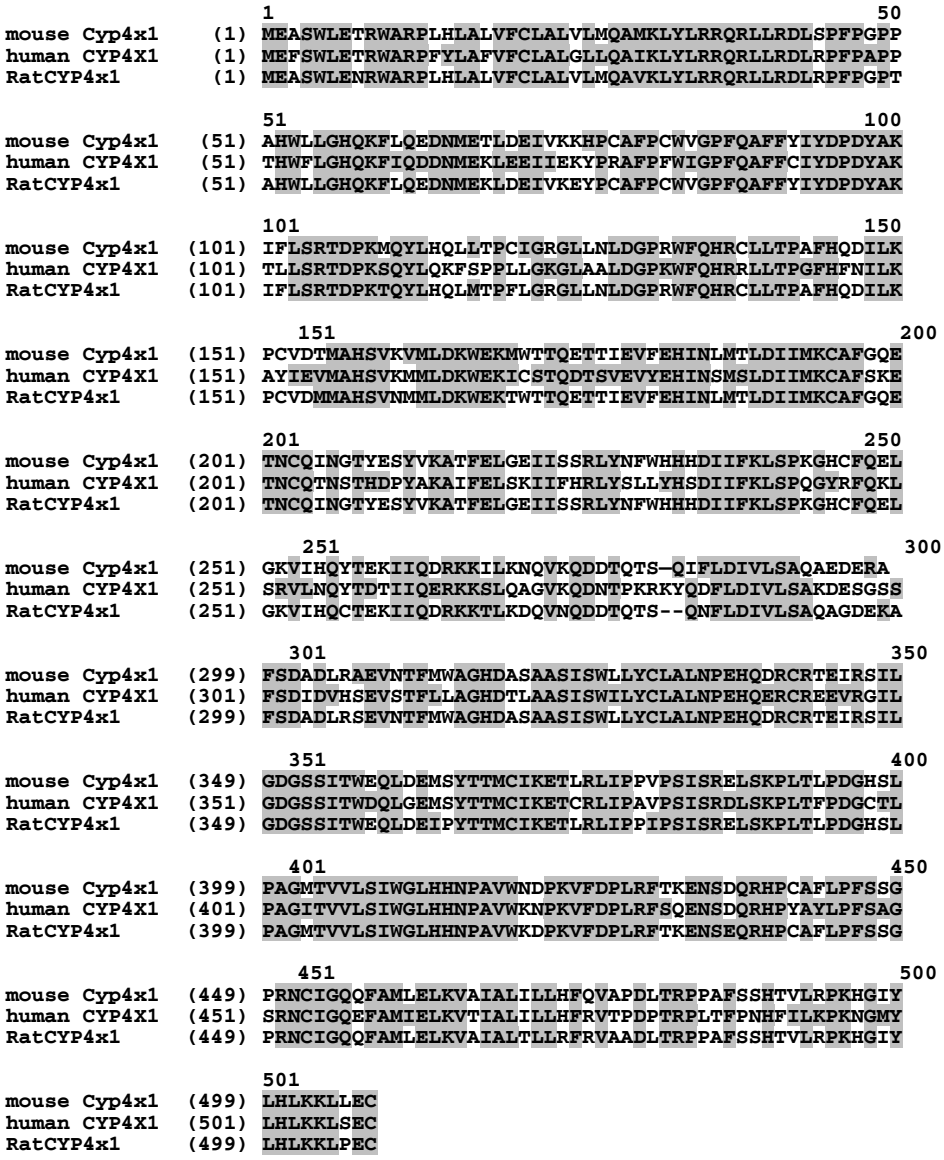

Figure 3.31. **Alignment of the deduced amino acid sequence of mcyp-4x1 cDNA with that of rat and human 4x1 sequences**.
The alignment shows high similarity of 94% and 79.2% between mouse cyp-4x1 (AJ786769) and rat (AF439343), human 4x1 (Q8N118) respectively.

Alignment of the deduced amino acid sequence of the mouse cyp4x1 cDNA with that of rat (AF439343) and human (Q8N118) CYP4x1, show high similarity of 94% and 79% respectively. The length of the primary sequence of mouse cyp4x1 cDNA was equal to that of rat and human CYP4x1 respectively. Furthermore, Exon 11 and exon 12 were found to code a highly conserved amino acid, PFSGG(A/S)RNCIG, which comprises the haem-binding domain which is essential for all cytochrome P450 proteins (Gonzalez, 1989). Therefore, comparison of the deduced amino acid sequences of the cloned mouse cDNA with known 4x1(rat and human) demonstrate that the cDNA encodes a 4x1 protein.

3.4.5 Comparison of the mouse cDNA with genomic DNA and intron/exon boundaries

The intron and exon junction site was determined by direct comparison of the 4X1 cDNA against the genomic sequence of XX-Pac129 on chromosome 4 (see Figure 3.32). The intron and exon junction of exon 1 to 12 of the genomic mouse cyp4x1 cDNA are summarised in Table 4.

Exon-Intron No.	Intron	Exon	Exon	Intron
1			……AAG	GTA
2	TAG	TTT……	……CAG	GTA
3	CAG	ACC……	……TTG	GTA
4	AAG	GAC……	……CTG	GTA
5	CAG	GAT……	……CGG	GTC
6	CAG	CAC……	……CAG	GTA
7	CAG	AAA……	……CAG	GTA
8	TAG	GCT……	……CTG	GTA
9	CAG	GGA……	……CAG	GTC
10	CAG	GTA……	……AAG	GTA
11	CAG	GTC……	……AAG	GTG
12	CAG	GAA……		

Table 3.1. The intron-exon junction's boundaries of exon 1 to exon 12 of mouse cyp4x1.

Determination of the intron/exon junctions is listed in Table 5, a comparison of the consensus sequence of intron/ exon junctions in mouse cyp4x1 gene with the published intron/exon consensus (Introduction to Genetic Analysis, Ed. Griffiths et al). The data in Table 5, demonstrate that the intron/exon junction is CAG/GNN, whereas in the published is CAG/GN and the exon/intron junction is CAG/GTA, whereas in the published is (A/C)AG/GT(A/G).

Nucleotide	Intron %	Exon %	Exon %	Intron %
A	9 100 0	18 45 36	27 64 0	0 0 73
C	73 0 0	9 18 36	64 0 0	0 0 18
G	0 0 100	64 9 0	0 9 100	100 0 9
T	18 0 0	9 27 27	9 27 0	0 100 0
	C A G	G N N	C A G	G T A

Table 3.2. Consensus sequence of intron/ exon junctions in mouse cyp4x1 gene.

96129
GCTCAGAGTG**ATGGAGGCCTCCTGGCTGGAGACTCGTTGGGCGCGGCCCTTGCACCTGGCTTT**
GGTGTTCTGCCTGGCGCTGGTGCTGATGCAGGCCATGAAGCTCTACCTGCGGAGACAGCGACT
GCTGCGCGACCTGAGCCCCTTCCCGGGGCCTCCCGCCCACTGGCTCCTGGGACACCAGAAGGT
AGATGGAG 96305

102524
TTTACTATAG**TTTCTTCAGGAGGATAATATGGAGACGCTTGATGAGATTGTCAAAAAGCACCC**
TTGTGCCTTCCCCTGCTGGGTAGGGCCCTTCCAGGCATTTTTCTACATCTATGACCCAGACTA
TGCGAAGATATTTCTGAGCAGAACAGGTAAGGAATG 105764

103501
GGTTCTGCAG**ACCCAAAGATGCAGTATCTGCACCAGCTCCTGACTCCATGCATTGG**TATGTAT
GTG 103546

105637
TTCCTCAAGG**ACGAGGACTCCTGAATTTAGATGGGCCCAGGTGGTTCCAACACCGCTGCCTCC**
TAACTCCTGCATTCCATCAGGACATCCTAAAACCATGTGTGGACACGATGGCCCACTCTGTGA
AAGTGATGCTGGTAAGTGAAAC 105764

108127
CTCTGGCAGG**ATAAATGGGAGAAGATGTGGACCACTCAGGAAACAACCATCGAGGTTTTTGAA**
CACATCAACTTGATGACCCTGGACATAATAATGAAATGTGCTTTTGGCCAGGAGACCAACTGC
CAGATAAACGGGTCAGTGATG 108253

108331
CTTCTTGCAG**CACCTATGAGTCTTACGTGAAGGCAACATTTGAACTTGGTGAAATCATATCTT**
CTCGCTTGTACAATTTCTGGCATCATCATGACATAATTTTCAAACTCAGTCCTAAGGGCCACT
GCTTCCAGGAGTTAGGCAAAGTGATACATCAATACACAGGTAAAAACAC 108485

109854
TTCTGTGCAG**AAAAGATAATCCAGGACAGAAAGAAAATCCTCAAGAATCAAGTAAAGCAGGAT**
GACACTCAGACGTCTCAAATTTTTCTGGATATTGTTCTTTCTGCACAGGTAAGTGCACC
109955

110485
GTGTTGTAGG**CTGAAGATGAACGAGCCTTCTCAGATGCTGACCTTCGGGCTGAGGTGAACACC**
TTCATGTGGGCAGGACATGATGCCTCTGCAGCTAGCATCTCCTGGCTCCTTTACTGCCTGGCT
CTAAATCCCGAGCATCAGGACAGATGCCGGACAGAGATCAGGAGCATCCTGGGAGATGGGTCT
TCCATCACCTGGTAAGATCCAC 110675

117040
TCCTTACAGG**GAACAGCTGGATGAGATGTCATACACCACAATGTGCATCAAGGAGACACTCCG**
CTTGATTCCTCCTGTCCCATCCATCTCCAGGGAGCTCAGCAAACCCCTTACCCTCCCAGATGG
ACACTCACTGCCTGCAGGTCTTCACATTC 117174

118298
TGTCTCAGGT**ATGACTGTGGTTCTTAGTATTTGGGGTCTCCACCACAACCCTGCTGTCTGGAA**
CGACCCAAAGGTAAGATTGTCT 118362

119869
CGTTTCAGGT**CTTTGACCCCTTGAGATTCACTAAGGAGAATTCTGATCAGAGACACCCCTGTG**
CCTTCCTACCATTCTCCAGTGGTCCAAGGTGAGATGAAC 119950

121127
TGTCTACAGG**AACTGCATCGGGCAGCAGTTTGCCATGCTGGAGCTAAAGGTGGCCATTGCCTT**
GATTCTGCTCCACTTTCAAGTAGCTCCAGACCTCACCAGGCCTCCTGCCTTCTCCAGCCACAC
TGTCCTCAGACCCAAGCATGGAATCTATTTGCACCTGAAGAAACTCCTTGAGTGTTAGATACT
AGGAG 121300

Figure 3.32. Intron/exon junctions of the mouse cyp4x1 gene.
Bold nucleotides represent exons, whereas faint nucleotides represent introns.

3.4.6 Analysis of amino acid sequence of mouse cyp4X1

The sequence of exon 1 contains an ATG codon which is the translation start codon. The translation stop codon is at the end of exon 12. The presence of the stop codon at the end of exon 12 suggests that exon 12 is the final exon of the mcyp4X1. The open reading frame of mcyp4X1 codes a protein of 507 amino acids.

The deduced amino acids sequences encoded by each exon were compared with the corresponding published amino acid sequences of the rat (AF439343) and human (Q8N118) as shown in table 6. By comparing amino acid sequence of mouse cyp4x1 with other rat and human CYP4X1 genes, it showed that mouse cyp4X1 is highly similar to rat (94%) and human (71%) CYP4X1. From the comparison, it was found that amino acid sequences corresponding to exon 2, 5, 8 and 11 are highly similar between mouse cyp4X1 and rat and human CYP4x1 genes among other exons. Furthermore, exon 1 and exon 3 are highly similar between mouse cyp4X1 and human CYP4X1, whereas exons 3, 6 and 7 of the human amino acid sequences are very dissimilar.

Exon No.	Rat (% identity)	Human (% identity)
1 (1-58)	96.4	84.2
2 (59-106)	97.9	73.4
3 (107-121)	80	46.6
4 (122-164)	93	67.4
5 (165-207)	97.6	72
6 (208-258)	98	56.8
7 (259-292)	91.6	58.3
8 (293-356)	96.8	70.3
9 (357-401)	93.3	82.2
10 (402-423)	95.4	90.9
11 (424-449)	96.1	76.9
12 (450-507)	91.3	72.4

Table 3.3. Percent identity of amino acid sequences by exons between mouse cyp4x1 and that of rat and human CYP4x1.
Exons were deduced from mouse cyp4x1 cDNA amino acid sequences and compared with the corresponding published amino acid sequences of the rat and human CYP4x1.

Alignment of intron/exon junction sites between mouse cyp4x1 cDNA (Acc.No AJ786769) and published mouse cyp4b1 (Acc.No. BC008996) and rat CYP4a1 (Acc. No. M57718M33937) was determined (see Figure 3.33). The comparison show that very close intron/exon junction site between mouse cyp4X1 and mouse cyp4b1, whereas, more diversion between mouse cyp4X1 and rat CYP4a1.

```
Mouse cyp4x1   MEASWLET-----RWARPLHLALVFCLALVLMQAMKLYLRRQRLLRDLSPFPGPPAHWLL 55
Mouse cyp4b1   MALSFLSP-----SLSRLGLWASVVILMVTVLKLLSLLFRRQKLARALDSFPGPPKHWLF 55
Rat   CYP4a1   MSVSALSSTRFTGSISGFLQVASVLGLLLLLVKAVQFYLQRQWLLKAFQQFPSPPFHWFF 60

Mouse cyp4x1   GHQKFLQEDN-METLDEIVKKHPCAFPCWVGPFQAFFYIYDPDYAKIFLSRTDPKMQYLH 114
Mouse cyp4b1   GHALEIQKTGGLDKVVTWTEQFPYAHPLWLGQFIVFLNIYEPDYAKAVYSRGDPKAAYVY 115
Rat   CYP4a1   GHK-QFQGDKELQQIMTCVENFPSAFPRWFWGSKAYLIVYDPDYMKVILGRSDPKANGVY 119

Mouse cyp4x1   QLLTPCIGRGLLNLDGPRWFQHRCLLTPAFHQDILKPCVDTMAHSVKVMLDKWEKMWTTQ 174
Mouse cyp4b1   DFFLQWIGKGLLVLEGPKWFQHRKLLTPGFHYDVLKPYVAIFAESTRVMLDKWEKKAS-E 174
Rat   CYP4a1   RLLAPWIGYGLLLLNGQPWFQHRRMLTPAFHYDILKPYVKNMADSIRLMLDKWEQLAG-Q 178

Mouse cyp4x1   ETTIEVFEHINLMTLDIIMKCAFGQETNCQINGTYESYVKATFELGEIISSRLYNFWHHH 234
Mouse cyp4b1   NKSFDIFCDVGHMALDTLMKCTFGK-GDSGLSHSDNSYYLAVSDLTLLMQQRIDSFQYHN 233
Rat   CYP4a1   DSSIEIFQHISLMTLDTVMKCAFSHNGSVQVDGNYKSYIQAIGNLNDLFHSRVRNIFHQN 238

Mouse cyp4x1   DIIFKLSPKGHCFQELGKVIHQYTEKIIQDRKKILKNQVKQDDTQTS--QIFLDIVLSAQ 292
Mouse cyp4b1   DFIYWLTPHGRRFLRACQIAHDHTDHVIRQRKAALQDEKEQKKLQERRHLDFLDILLGAR 293
Rat   CYP4a1   DTIYNFSSNGHLFNRACQLAHDHTDGVIKLRKDQLQNAGELEKVKKKRRLDFLDILLLAR 298

Mouse cyp4x1   AEDERAFSDADLRAEVNTFMWAGHDASAASISWLLYCLALNPEHQDRCRTEIRSILGDGS 352
Mouse cyp4b1   DESGIKLSDADLRAEVDTFMFEGHDTTTSGISWFLYCMALYPMHQQRCREEVREILGDRD 353
Rat   CYP4a1   MENGDSLSDKDLRAEVDTFMFEGHDTTASGVSWIFYALATHPEHQQRCREEVQSVLGDGS 358

Mouse cyp4x1   SITWEQLDEMSYTTMCIKETLRLIPPVPSISRELSKPLTLPDGHSLPAGMTVVLSIWGLH 412
Mouse cyp4b1   SFQWDDLAQMTYLTMCMKECFRLYPPVPQVYRQLSKPVTFVDGRSLPAGSLISLHIYALH 413
Rat   CYP4a1   SITWDHLDQIPYTTMCIKEALRLYPPVPGIVRELSTSVTFPDGRSLPKGIQVTLSIYGLH 418

Mouse cyp4x1   HNPAVWNDPKVFDPLRFTKENSDQRHPCAFLPFSSGPRNCIGQQFAMLELKVAIALILLH 472
Mouse cyp4b1   RNSAVWPDPEVFDPLRFSPENMTGRHPFAFMPFSAGPRNCIGQQFAMNEMKVVTALCLLR 473
Rat CYP4a1     HNPKVWPNPEVFDPSRFAPD--SPRHSHSFLPFSGGARNCIGKQFAMSEMKVIVALTLLR 476

Mouse cyp4x1   FQVAPDLTRPPAFSSHTVLRPKHGIYLHLKKLLEC--- 507
Mouse cyp4b1   FEFSPDPSKIPIKVPQLILRSKNGIHLYLKPLGPGSGK 511
Rat   CYP4a1   FELLPDPTKVPIPLPRLVLKSKNGIYLYLKKLH----- 509
```

Figure 3.33. Alignment of mouse cyp4X1 protein with mouse cyp4b1 and rat CYP4a1. Colors represent each exon in legend.

In order to study protein expression, a sub-cloning strategy for putting the mcyp-4X1cDNA into an expression vector was formulated as shown in Figure 3.25. The first step was excision of the inserted mcyp-4X1cDNA with NcoI and SalI restriction enzymes (1620.bp.) and then sub-cloned into pRSET-a cut by NcoI and XhoI restriction enzymes, an expression vector for bacterial expression, as described in section 2.2.3.11.

Several clones were isolated and the positive clones were identified by double digestion with NcoI and NdeI (Data not shown). The insertion of mcyp4X1cDNA into pRSET-a was confirmed by DNA sequencing using forward primer (4X1-f-p*F)* 5`ATGGAGGCCTCCTGGCTGGAGACTCGTTGG 3` and reverse primer (4X1-f-p*R)* 3` TATGATCCTCTTTACCGCTTTAAATACAAA 5 `as shown in Figure 3.29. The sequencing data confirmed that the full-length m4X1 cDNA had been produced successfully and cloned correctly.

3.4.7 Expression and localisation of mcyp-4X1 mRNA

It has been shown in this lab that human 4x1 is expressed in aorta (Bell D.R, et al 2005), and that the cyp4X1 gene is expressed in rat brain (Bylund et al, 2001), but nothing is known about its expression in mouse. Hence, mouse tissues were investigated.

In order to analyse the expression of this gene, RNase protection assays were performed on total RNA (30µg) from mouse brain using a riboprobe for mcyp4X1, Figure 3.34, and total RNA from liver was used as a control. The probe was synthesised as described in section section 3.5.3. The probe was generated from the Sp6 promoter of Nco1 digested template and a full-length mcyp4X-12 probe of 424 b.p was produced.

The integrity of the isolated RNA was analysed by 1% agarose gel electrophoresis. The protected RNA fragments were run on 6% poly

acrylamide gels and then film exposed overnight. The principle of this experiment is that the hybridisation of riboprobe to the m4X1 mRNA forms a double-stranded RNA structure, which is resistant to degradation by RNase. In Figure 3.34, the specificity of the probe was determined by the inclusion of two control yeast tRNA samples, positive and negative control, hybridised to the probe (see lane 1 and 5 of Figure 3.34). This is an essential step to confirm that the probe is selective and works properly. The *CYP*4X1 (exon 12) probe was shown the full-length (RNase –ve track) and gave 424 b.p. as shown in lane 5 of Figure 3.34. The RNase positive track shows an absence of signal when hybridised to yeast tRNA, which shows that, in the absence of complementary RNA, the probe RNA is fully degraded. Therefore, the protected fragments in the samples must be due to protection of the probe by complimentary RNA sequences.

In this study expression of cyp4X1 was investigated in the brain and liver of mice. RNase protection assay was carried out on total RNA using a riboprobe for cyp4X1 (Figure 3.34). The riboprobe generated is 330 b.p. long as described in section 3.5.3. The probe will give a 177 b.p. fragment for RNA, but a much longer fragment (327 b.p.) for genomic DNA.

The protection assay in Figure 3.34, lane 3, shows that a strong signal was produced at 177 b.p. Furthermore, two high molecular weight bands were also produced. However, since these are the same length as the full length probe, they do not correspond to the fragment size of protected RNA, and were not considered to reflect specific protection of the probe. The size of the protected fragment in mouse brain sample was shorter than the full-length probe, which shows that RNase treatment has removed 247 b.p. of unprotected probe.

Figure 3.34, shows that the brain samples have a specific protected fragment of 177 b.p, which corresponds to the expected size of the cyp4X1-12 RNA, whereas no signal was produced in the liver RNA sampleas shown in Figure 3.34, lane 4. Therefore, the murine CYP4X1 is not expressed in control liver, but is expressed in the brain (Figure 3.34) and the signal was protected due to hybridization of the mcyp4X1 probe to endogenous 4X1 transcripts in brain.

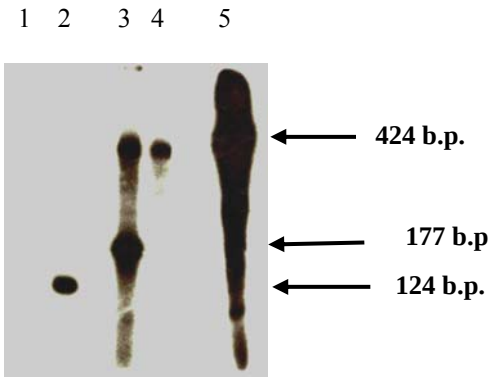

Figure 3.34. RNase protection assay of CYP4X1.
The protection assay was performed against 30 µg of total RNA extracted from mouse brain and liver tissues. The full-length probe (b.p.) was generated by in vitro transcription of NcoI cut mcyp4x1-12 plasmid with Sp6 RNA polymerase and then hybridised to the samples. Lane 1 shows a +ve control, lane 2 shows a marker probe (124 b.p). Lane 3 and 4 show a protection assay on brain and liver RNA samples respectively. The arrows indicate a protected fragment (177 b.p.) of CYP4X1 (exon 12) and the marker size (124 b.p.).

3.4.8 mcyp4X1 RNA expression in wild type (+/+), knock-out (-/-) and control

It is known that cyp4a genes are regulated by PPARα (Bell et al, 1993) and the availability of PPARα knock-out mice which do not contain a functional PPARα gene offers an ideal system to study any role of PPARα in the expression of mcyp4X1. Hence, the expression of cyp4X1 expression in wild type and knock-out mice was investigated. Figure 3.35, shows expression of mcyp4X1 in total brain RNA extracted from wild type and knock-out mice.

The wild type mice and the knock-out mice all showed strong mcyp4X1 gene signals as shown in Figure 3.35. The signals produced by wild type and knock-out mice show no intensity differences between them as shown in Figure 3.35. Therefore, brain cyp4X1 expression was similar in both wild type and knock-out and PPARα has no effect on expression of this gene in brain.

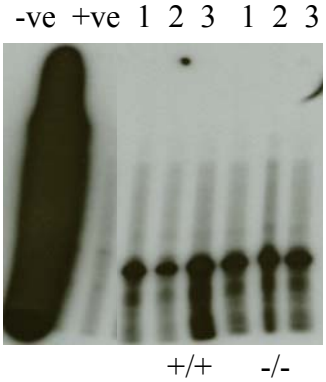

Figure 3.35. Expression of cyp4x1 in wild-type and knock-out mice brain tissues.
RNase protection assays were performed on mouse total RNA samples using [α-^{32}P] labelled antisense riboprobe to mcyp4x1. The control mice were dosed using corn oil (vehicle) as described in section 2.2.1.1. The wild type (+/+) and knock-out (-/-) mice were given a normal diet (untreated mice). Antisense riboprobe was generated by transcribing NcoI cut mcyp4x1-12 plasmid with Sp6 RNA polymerase. Full-length probe was 424 b.p and the protected fragment of the samples was 177 b.p long. Total RNA was isolated as described in section 2.2.3.9 and 30 µg of each RNA sample was hybridized with [α-^{32}P] CTP labelled probe. Two yeast tRNA control samples were hybridized to the probe, one sample treated with the RNase A/T1 (+ve control) and the other sample without RNase A/T1 (-ve control) (10 µg/ml). The numbers (1, 2, and 3) indicate the number of each mouse. M shows the marker (124 b.p). RNase protection assay was run on 6% denaturing poly acrylamide gel in 1xTBE at 120v for 1hour. Gels were then fixed, dried and exposed to Hyperfilm at -80ºC overnight.

RNA expression in other mice tissues (lung, heart, and kidney) was also investigated as shown in Figure 3.36. Figure 3.36, shows that protected bands was produced after probe hybridisation for these tissues. The protected signals were detected only after prolonged exposure of autoradiography (four days). Therefore, cyp-4X1 gene is expressed in these tissues but at very low levels.

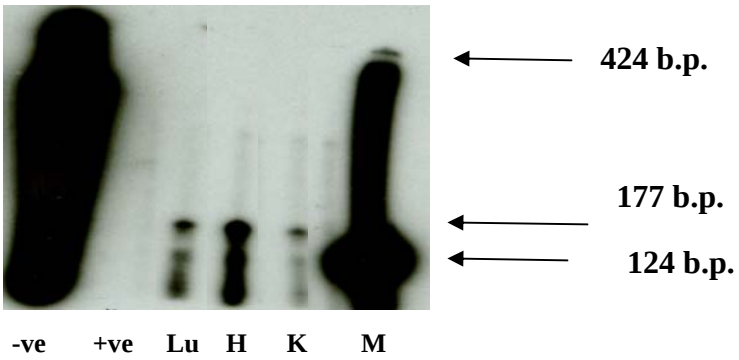

Figure 3.36. **.Expression and induction of cyp4x1 in mouse lung, heart, and kidney tissues**. RNase protection assays were performed on mouse total RNA samples using [α-^{32}P] labelled antisense riboprobe to mcyp4x1. Antisense riboprobe was generated by transcribing NcoI cut mcyp4x1-12 plasmid with Sp6 RNA polymerase. Full-length probe generated was 424 b.p long. Total RNA was isolated as described in section 2.2.3.9 and 30 µg of each RNA sample was hybridized with [α-^{32}P] CTP labelled probe. Two yeast tRNA control samples were hybridized to the probe, one sample treated with the RNase A/T1 (+ve control) (10 µg/ml) and the other sample without RNase A/T1 (-ve control). Lu shows lung, H shows heart, K shows kidney. M shows the marker (124 b.p). RNase protection assay was run on 6% denaturing poly acrylamide gel in 1xTBE at 120v for 1 hour. Gels were then fixed, dried and exposed to Hyperfilm at -80ºC for four days.

3.4.9 Expression of mcyp4X1 in Aorta tissues

It is known that CYP4X1 is predominantly expressed in human aorta and induced by the PPARα agonist Wy14643 in HepG2 cells (Savas U et al, 2005). However, nothing is known in mouse aorta. In order to address this issue, aorta tissues were pooled from 13 mice and aorta RNA was extracted as described in section 2.2.3.9. RNase protection assay was performed as described in section 2.2.5.2.

Ciprofibrate was used to induce cyp4X1 gene. Figure 3.37 shows expression and induction of this gene in both treated and untreated mice. The RNase protection assay shows that a specific protected band was produced in the total aorta tissue and no protected band was present in the positive control. Two protected bands were observed, of which the lower protected band was possibly due to probe degradation. The protected bands produced in both treated and untreated mice were at a similar level. Therefore, cyp4X1 gene is expressed in Aorta tissue, but at low level. Furthermore, there was zero or little induction of cyp4X1 produced in aorta in response to dosing with the inducing agent (ciprofibrate).

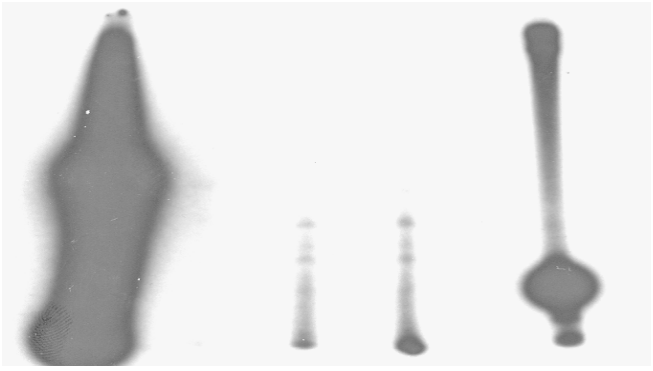

Figure 3.37. **Expression and induction of cyp4x1 in Aorta tissues**.
The protection assay was performed against 30 µg of total RNA extracted from mouse aorta tissues. The mice were dosed with ciprofibrate as described in the methods. The full-length probe (424 b.p.) was generated by in vitro transcription of NcoI cut mcyp4x1-12 plasmid with Sp6 RNA polymerase and then hybridised to the samples. Two yeast tRNA control samples were hybridized to the probe, one sample treated with the RNase A/T1 (+ve control) and the other sample without RNase A/T1 (-ve control) (10 µg/ml). Track (AU) represents a protection assay on untreated mice (control). Track (AT) represents a protection assay on treated mice. The arrows indicate a protected fragment (b.p.) of cyp4x1 and the marker (M) size (124 b.p.). RNase protection assay was run on 6% denaturing poly acrylamide gel in 1xTBE at 120v for 1 hour. Gels were then fixed, dried and exposed to Hyperfilm at -80ºC overnight**.**

3.4.10 Induction of mcyp4X1

A study of tissue-specific expression of mcyp4X1 RNA was performed by treating the mice with inducing agents such as peroxisome proliferators (PPs). Peroxisome proliferators are known to induce the CYP450s enzymes. The induction was carried out by dosing 129S4/SvJae mice as described in section 2.2.1.1.

Each group consisted of three mice and the samples were carried out on 30µg for each sample. These were seven groups of mice: the control group, TCDD group, Dexamethasone group, Phenobarbital group, Ciprofibrate group. All total RNA brain samples from these groups were isolated as described in section 2.2.3.9 and probed with m-cyp4X1-12 probe. The integrity of the total

RNA was analysed by 1% agarose gel electrophoresis. The tissues studied were brain, liver, heart, kidney, aorta, lung and spleen.

3.4.11 Induction of mcyp-4X1 RNA in brain tissues

RNase protection assays were performed on 30 μg of total RNA using a riboprobe for mcyp4x1, Figure 3.38. Samples from all mice in each group were hybridized using the same riboprobe at the same time. Figures 3.38, show that the brain samples had a specific protected fragment of 177 b.p. which corresponded to the expected size of the cyp4X1-12 RNA. Induction of mcyp4X1 RNA in the brain samples was assessed roughly by comparing the signal intensity of the induced samples with that produced from the control samples. In Figure 3.38, the mcyp4X1-12 probe was almost shown to be in full-length (RNase –ve tract). The RNase positive lane demonstrated an absence of signal when hybridised to yeast tRNA, which indicates the specificity of the signal in the samples. A protected single band was produced in all protection reactions. Figure 3.38, shows the protected bands were produced after probe hybridisation for these tissues.

The size of the protected brain fragment in mouse samples was shorter than the full-length probe, which indicates the RNase removal of 247 b.p. of unprotected probe.

Therefore, the murine cyp4X1 is strongly expressed in all brain samples protected by the probe as shown in Figure 3.38. However, there was no evidence of any significant differences in the level of m4X1 observed in either control or mice treated with TCDD, Dexamethasone, Phenobarbital and

Ciprofibrate. Therefore, there was no induction of mcyp4X1 RNA in any of treated mice in response to dosing with inducing agents.

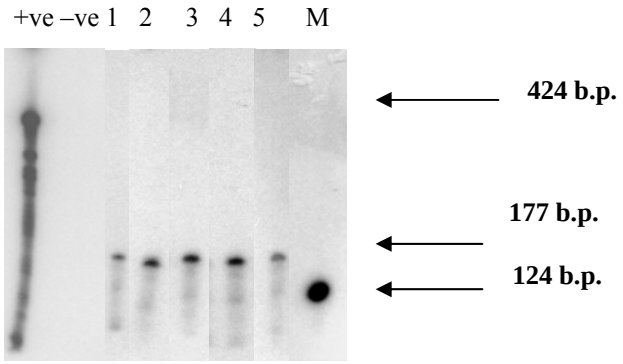

Figure 3.38. **Expression and induction of cyp4x1 in brain tissues**.
The protection assay was performed against 30 µg of total RNA extracted from mouse brain tissues. Animals were treated with TCDD, PB, Dex and Cipro as described in the methods. The full-length probe (424 b.p.) was generated by in vitro transcription of NcoI cut mcyp4x1-12 plasmid with Sp6 RNA polymerase and then hybridised to the samples. Two yeast tRNA control samples were hybridized to the probe, one sample treated with the RNase A/T1 (+ve control) and the other sample without RNase A/T1 (-ve control) (10 µg/ml). Track (1) represents a control mouse. Track (2) represents induced mice by TCDD. Track (3) represents induced mice by Dexamethasone. Track (4) represents induced mice by PB. Track (5) represents induced mice by ciprofibrate. M shows the marker (124 b.p). RNase protection assay was run on 6% denaturing poly acrylamide gel in 1xTBE at 120v for 1 hour. Gels were then fixed, dried and exposed to Hyperfilm at -80ºC overnight**.**

3.4.12 Induction of mcyp4X1 in liver tissues

The effect of treatment of the mice with inducing agents was also investigated using liver samples. It is known that cyp4a genes are regulated by PPARα in liver (Bell et al, 1993). The mice were treated as described in the methods. Liver RNA was extracted and RNase protection assays were carried out as described in section 2.2.5.6. Figure 3.39, shows that only faint expression bands were produced from these tissues.

All the induced samples when compared with the control mice show the same level of protected fragment. This means that no induction of mcyp4X1 RNA was produced in liver in response to dosing with the inducing agents. The autoradiography film was exposed for five days, so there are many non-specific signals as the 4X1 signal in liver is very low.

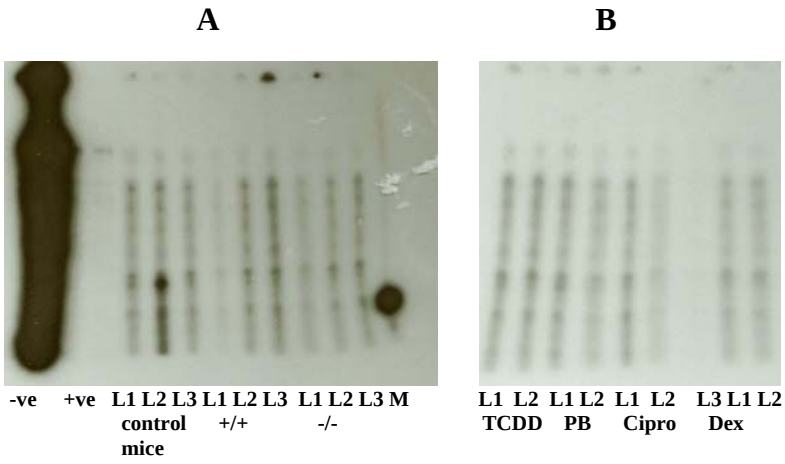

Figure 3.39. **Expression and induction of cyp4x1 in mouse liver tissues**.
RNase protection assays were performed on mouse total RNA samples using [α-^{32}P] labelled antisense riboprobe to mcyp4x1. The control mice were dosed using corn oil (vehicle) as described in section 2.2.1.1. The wild type (+/+) and knock-out (-/-) mice were given a normal diet (untreated mice). Antisense riboprobe was generated by transcribing NcoI cut mcyp4x1-12 plasmid with Sp6 RNA polymerase. Full-length probe was 424 b.p. and protected the fragment of the samples were 177 b.p. long. Total RNA was isolated as described in section 2.2.3.9 and 30 µg of each RNA sample were hybridized with [α-^{32}P] CTP labelled probe. Two yeast tRNA control samples were hybridized to the probe, one sample treated with the RNase A/T1 (+ve control) and the other sample without RNase A/T1 (-ve control) (10 µg/ml). **A** shows expression of cyp4x1 in a control, wild type (+/+) and knock-out mice liver samples. **B** shows induction of cyp4x1 in liver mice samples by ciprofibrate. L shows liver while the numbers show the mice number in the treated group. M shows the marker (124 b.p). RNase protection assay was run on 6% denaturing poly acrylamide gel in 1xTBE at 120v for 1 hour. Gels were then fixed, dried and exposed to Hyperfilm at -80ºC for 5 days.

Ciprofibrate is a known inducing agent in mice liver. Livers were weighed and extracted from control mice and ciprofibrate treated mice. The effect of ciprofibrate treatment on the liver weight was determined by liver: body weight ratio as shown in Figure 3.40. It demonstrated that there was a significant difference ($p<0.05$) between the ciprofibrate induced mice liver and the control. A statistically significant liver weight change was produced after ciprofibrate administration to mice, which therefore demonstrates that ciprofibrate was having a pharmalogical effect at this dose level.

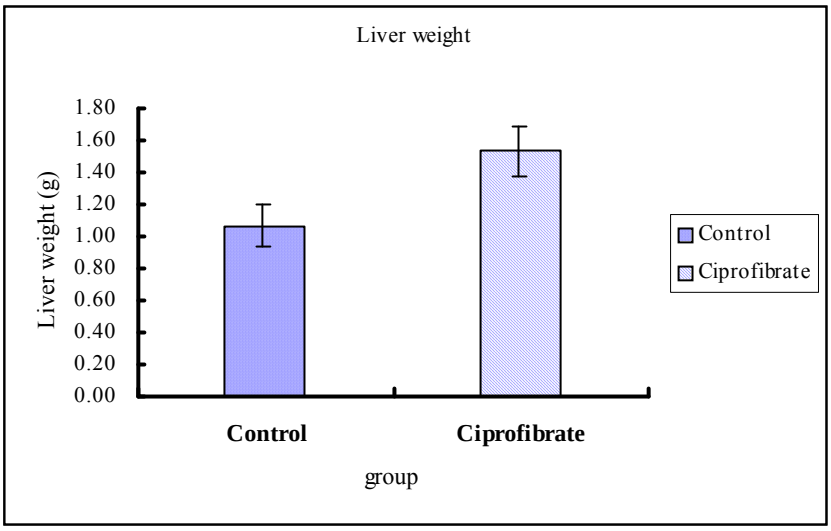

Figure 3.40. **Induction of ciprofibrate in total mice liver**.
The control mice and the ciprofibrate treated mice were dosed as described in the methods. Three mice were used per group. All values are given as number of liver weight. Each bar shows the STD of the three samples in that group. Error bars shows the standard error of the samples in each group. Data shows a significant liver weight increase was observed in ciprofibrate treated mice, whereas no liver weight increase in that of control mice ($p<0.05$).

3.4.13 RNase protection assay of Ciprofibrate induction

Several tissues such as heart, kidney, lung and spleen were isolated and the total RNA was extracted as described in section 2.2.3.9. The effect of ciprofibrate treatment on the level of cyp4X1 was determined by RNase protection assay of total homogenate of heart, kidney, lung and spleen samples as shown in Figure 3.41. RNA extracts from untreated heart tissue were used as a control, tract H (1-3) of Figure 3.41.

The tissues, which were induced with ciprofibrate, show no evidence of any signal induction. Therefore, mcyp4X1 is not induced in these tissues following treatment with ciprofibrate. The control samples from untreated heart tissue also show that no bands were produced. The autoradiography films were exposed for five days, so there are many non-specific signals

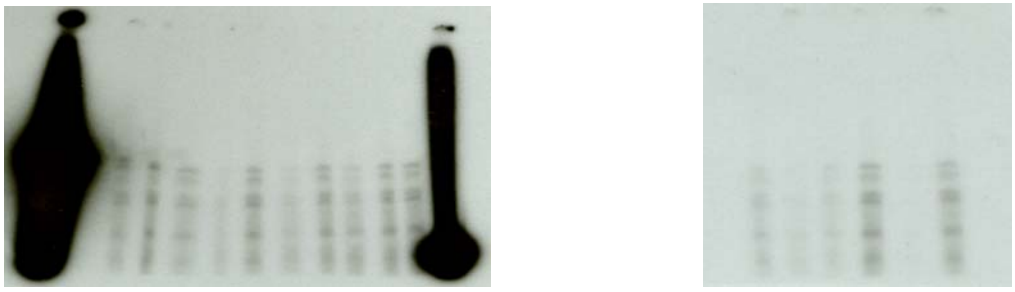

Figure 3.41. **Expression and induction of cyp4x1 in several mouse tissues**.

RNase protection assays were performed against 30 µg of total RNA extracted from mouse heart, kidney, lung and spleen tissues. Total RNA was isolated as described in section 2.2.3.9. The treated mice were dosed with ciprofibrate as described in section 2.2.1.1. Antisense riboprobe was generated by transcribing NcoI cut mcyp4x1-12 plasmid with Sp6 RNA polymerase and labeled with [α-^{32}P] CTP. Full-length probe generated was 424 b.p. long. Two yeast tRNA control samples were hybridized to the probe, one sample treated with the RNase A/T1 (+ve control) and the other sample without RNase A/T1 (-ve control) (10µg/ml). RNA heart samples (H) were used as a control (untreated mice). H shows heart (control), h shows heart (treated mice), K shows kidney, Lu shows lung and S shows spleen, while the numbers show the mice number in the group. M shows the marker (124 b.p). RNase protection assay was run on 6% denaturing poly acrylamide gel in 1xTBE at 120v for 1 hour. Gels were then fixed, dried and exposed to Hyperfilm at -80ºC for 5 days.

3.4.14 Expression of m-4X1 protein

pRSET-a-m4X1 plasmid DNA was transformed into BL21 (DE3) and BL21(DE3)pLysS *E.coli* strain. Small scale expression was used to optimise protein samples for SDS-PAGE analysis. Both types of *E.coli* transformed cells were induced with a final concentration of 1mM IPTG, as described in section 2.2.3.2.1. The induced and uninduced cells were then sonicated and centrifuged as described in section 2.2.4.7. The proteins from the induced and un-induced cells were analysed by SDS-PAGE as shown in Figure 3.42. Figure 3.42, shows that a specific band of ~ 62 kDa was expressed in both cells, BL21 (DE3) and BL21 (DE3) pLysS, after IPTG addition, whereas no protein expression was seen in the uninduced cells, or cells with pRSET-a vector, (BL21) DE3 and BL21 (DE3) pLysS. Therefore the induction of protein production was successful. Furthermore, the induced protein was produced at a higher level in BL21 (DE3) cells than that in the pLysS cells. Therefore, BL21 (DE3) cells were used in future experiments.

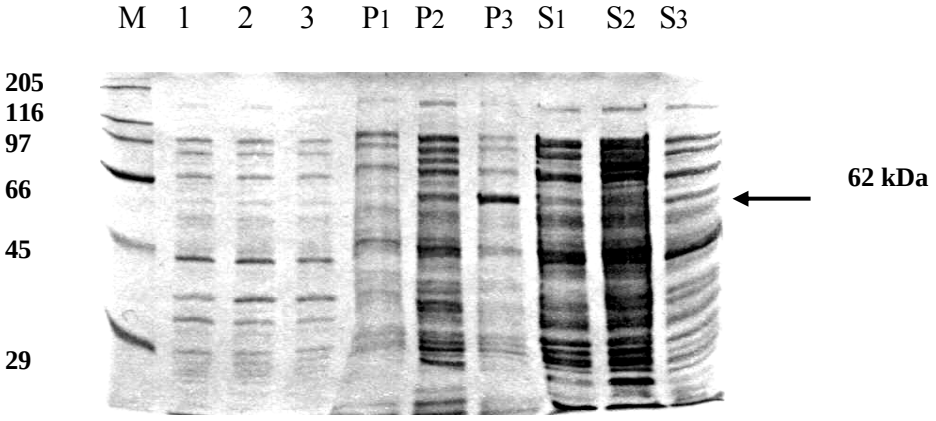

Figure 3.42. **The expression and induction of m-cyp4x1 recombinant protein in BL21 (DE3) pLysS and BL21 (DE3).** The cells were induced using a final concentration of 1mM IPTG for three hours. The pellets (P) were lysed by sonication and fractionated by centrifugation (S) as described in the methods. The supernatant (S) and the pellets were analysed on 10% SDS-PAGE and then visualized by coomassie blue staining. Tracks labelled **1** have pRSET-a, **2** are pRSET-4x1 in BL21 (DE3) pLysS cells and **3** are pRSET-4x1 in BL21 (DE3) cells. Track P1 shows pellet of uninduced pRSET-a. Track P2 shows pellet of induced BL21 (DE3) pLySs cells. Track P3 shows pellet of induced BL21 (DE3) cells. Track S1 shows supernatant of induced pRSET-a. Track S2 shows supernatant of induced BL21 (DE3) pLysS cells. Track S3 shows supernatant of induced BL21 (DE3) cells. M shows protein marker. The size of the molecular weight markers are shown in kDa, and position of the induced band is shown by the arrow.

In Figure 3.42, the majority of induced protein was found in the pellet fraction (insoluble), whereas a very low amount, if any, was found in the supernatant fraction (soluble). Therefore, further sonication and solubilization of pellets with 8M urea, 0.1% SDS was required to solubilise the protein. The solubilisation of the pellet with 8M urea, 0.1% SDS is illustrated in Figure 3.43. Figure 3.43, shows that the recombinant protein existed in both pellet and supernatant of the solubilized DL21 (DE3) pellet with 8M urea, 0.1% SDS. Some recombinant protein in the pellet was found in the supernatant fraction, whereas the majority of the recombinant protein was in the pellet. Therefore, approximately half of the recombinant protein in the pellet was solubilized in the solution and a strong band of molecular weight ~ 62 kDa can be seen as shown in Figure 3.43, whereas no bands were observed in BL21 (DE3) control cells. The cyp4X1 cDNA contains 507 amino acids. The N-terminal fusion peptide from pRSET-a contains 49 amino acids including a six-histidine tag. Therefore, the whole cyp4X1 fusion protein contains 556 amino acids with a predicted molecular mass of 62, 808 Da. The size of the detected band at the position of ~ 62 kDa on SDS-PAGE was consistent with the predicted value. The size of the protein produced was slightly lower than the predicted molecular size of the m-4X1 protein. The protein was therefore successfully expressed in the cells and it was ready for purification using an affinity column. In order to purify large amounts of expressed protein, 1 litre of BL21 DE3-full-length-m4X1 was prepared under the same conditions as described in section 2.2.4.7.

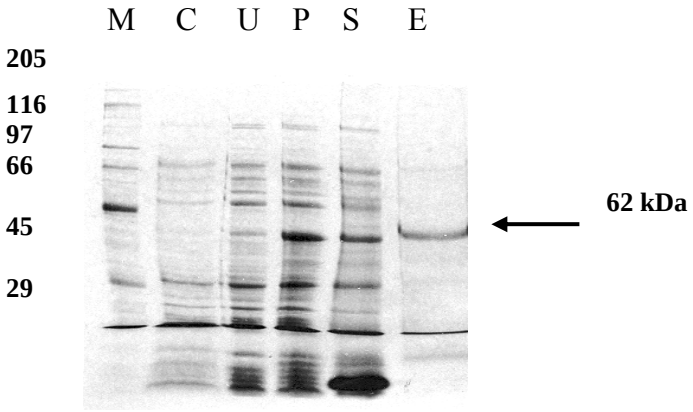

Figure 3.43. The expression and induction of m-cyp4x1 recombinant protein in BL21 (DE3). The cells were induced using a final concentration of 1mM IPTG for three hours. The pellet was prepared from induced BL21 (DE3) and treated with 8M urea, 0.1% SDS (PT) and fractionated by centrifugation (ST) as described in the methods. Track C shows the control of untransformed pRSET-a. Track U shows uninduced BL21 (DE3) expression vector. Tracks P and S show pellet and supernatant of solubilized BL21 (DE3) pellet with 8M urea, 0.1% SDS. The induced and uninduced BL21 (DE3) were analysed on 10% SDS-PAGE and then visualized by coomassie blue staining. M shows protein marker of sigma SDS 6H. Track E shows purified cyp4x1 protein after His.Bind Nickel affinity column.

3.4.15 Affinity purification and diafilter of mcyp-4X1 protein

The induced protein was solubilized in 8M urea, 0.1%SDS. In order to purify this protein, the His.Bind Nickel affinity column was used. Purification of the expressed protein was carried out on the affinity column as described in section 2.2.4.9 after diafiltration on YM10 exclusion membrane as described in section 2.2.4.8.

The solubilized protein fraction was passed through the His.Bind affinity resin. The unbound eluted protein and bound protein fractions were collected sequentially. Figure 3.44, shows SDS-PAGE analysis of 1ml eluted fractions several protein bands can be seen in the eluted fractions. Figure 3.44, demonstrates that the majority of the solubilized protein passed through the

nickel column, whereas a few bands of bound protein were obtained in the eluted fractions (2-5).

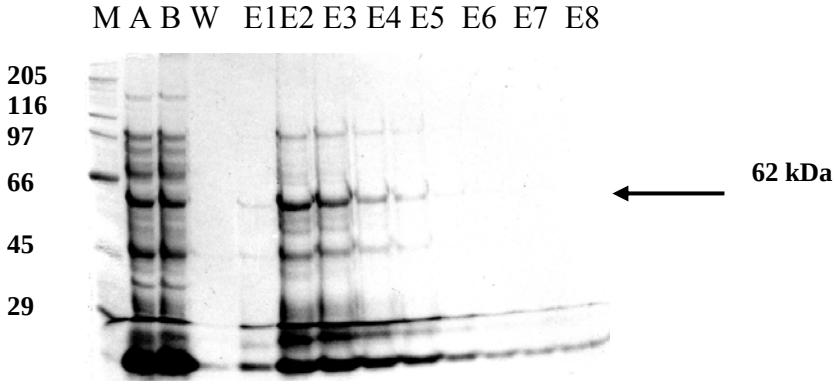

Figure 3.44. **Affinity purification of mcyp-4x1 recombinant protein in Nickel affinity column**.
The soluble protein (S) was diafiltered on stirred ultrafiltration cells as described in section 2.2.4.8. The purified recombinant mcyp-4x1 protein was analysed on 10% SDS-PAGE and then visualized by coomassie staining blue. M shows protein marker. (**A)** shows soluble protein before Nickel affinity purification. (**B)** shows protein flow through after passing through the column. **(W)** shows wash buffer. **E** (1-8) shows the elution protein fractions respectively.

In the eluted protein fractions, there are more than three bands in each fraction. The majority of eluted protein was a recombinant mcyp-4X1 and it was in the expected molecular size of m-4X1 (62 kDa), whereas the faint band at ~ 110 kDa is possibly due to protein aggregation and the smaller protein fragments may be due to proteolysis. These bands were obtained from the early eluted fractions. However, no bands were observed in the late eluted fractions (fraction 6-8). This means that most of the protein was eluted in three mls as shown in Figure 3.44, (E2-E4), and the concentration of the first eluted fraction was 1.4 mg/ml. Therefore the purification of the protein was successful and the bands were very clear.

3.5 Characterisation of antisera against mouse cyp4X1

Native mouse cyp4X1 in tissue samples cannot be specifically detected and, hence characterisation relies on Western blot analysis. The antibody against human CYP4X1 did not cross react with mouse cyp4x1 protein (see Figure 3.45). Therefore, it was necessary to develop a sensitive mouse cyp4x1-specific antibody.

Antisera against human CYP4X1 were described earlier and were used to probe the mouse cyp4X1 recombinant protein, Figure 3.45. Antisera were titred for reactivity against mouse cyp4X1 by Western blotting using various amounts of protein (100, 30, and 10 ng). The protein was blotted and detected with primary antiserum at a dilution of 1:500. Mouse cyp4X1 antigen was detected by the human cyp4X1 antiserum at a sensitivity of 100 ng, Figure 3.45. A lower molecular weight band was also detected using human antisera, but this is non-specific antibody binding.

Therefore, the mouse cyp4X1 antigen has very low sensitivity or no cross-reactivity with the human CYP4X1 antiserum.

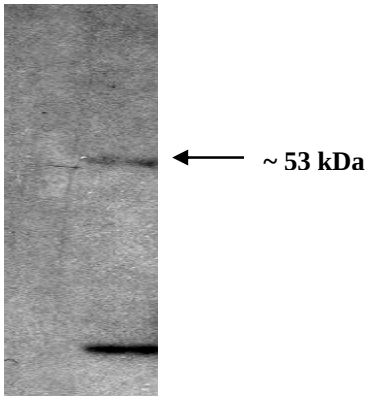

Figure 3.45. Sensitivity of human CYP4x1antisera against purified recombinant cyp4x1 protein.
The indicated amounts (100 and 30 ng**)** of mouse cyp4x1 protein were run on 10% SDS-PAGE then blotted to PVDF membrane as described in the methods. The membrane was incubated with humanCYP4x1 antiserum at a concentration of 1:500, and the blot was developed with ECL as described in the methods. Film was exposed for 2 hours. The arrow shows the protein at ~ 53 kDa.

A full length cDNA encoding mouse cyp4X1 was cloned into pRSET-a for expression as a histidine-tagged fusion protein, containing 507 amino acids of cyp4X1.

The deduced sequence of the fusion protein shows that there are 49 amino acids from the pRSET-a vector, while the inserted DNA was 497 amino acids. pRSET-a-m4X1 plasmid DNA was transformed into BL21 (DE3) *E.coli* strain and induced to a final concentration of 1mM IPTG (section 2.2.4.7). The induced cells were then sonicated and centrifuged as described in section 2.2.4.7. A 62 kDa protein was observed when the cells were exposed to 1 mM IPTG. The protein was solubilized in 8M urea, 0.1 SDS and then purified by affinity chromatography on a nickel column.

The protein contains 546 amino acids with a predicted molecular mass of 62,808 Da and the purified protein was approximately 62 kDa on SDS-PAGE.

Therefore, the detected protein band at the position 62 kDa was consistent with the predicted value, and identified as the recombinant mouse cyp4X1. The purified protein concentration was 0.38 mg/ml as judged by Bradford assay (not shown).

Two New Zealand white female rabbits (189 and 190) (~ 4 kg) were injected with ~ 100µg of mcyp4X1 antigen. Booster injections were given at four week intervals and once sufficient antibody titre was obtained, terminal bleed samples were collected.

Bleeds from rabbits 189 and 190 were collected after each immunisation and titred for reactivity against mouse cyp4X1 by Western blotting using various amount of protein (e.g. 10, 3, 1 and 0.3 ng) as shown in Figure 3.46.

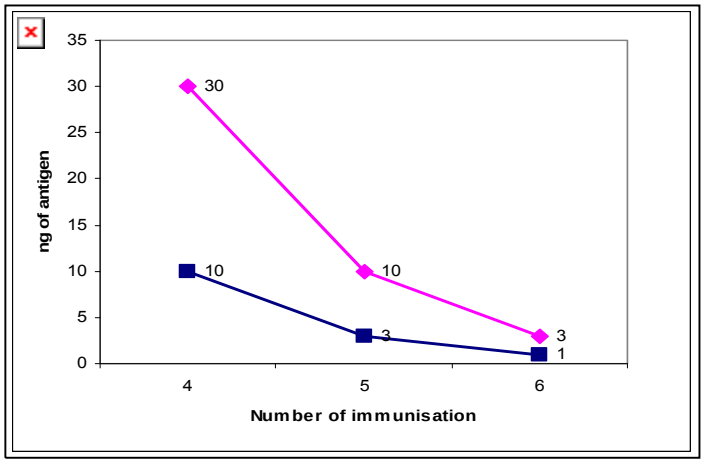

Figure 3.46. Development of antisera against mouse cyp4x1protein.
Rabbits were immunized at monthly intervals for six months. Antisera from rabbits 190 and 180 were collected at the indicated time, diluted 1:500, and incubated with antigen for 1 hr. Diamond points represent rabbit 190 and the square points represent rabbit 180, these show the minimum amount of cyp4x1 antigen detected at the indicated time. The data shows an improvement in the sensitivity of the antisera from these rabbits against the antigen over the course of several immunizations

The antigen cyp4X1 is clearly visible in Figure 3.47 (A), in lanes 1, 2 and 3.

Only one band at 53 kDa in three tracks was detected by the antisera from rabbit 190. The antisera from rabbit 189 detected only one clear band at 53 kDa in three tracks and the others were less clear as shown in Figure 3.47 (B). The streaky bands produced in Figure 3.47 (B) show a clear band at 10ng, but indistinct bands at 3, 1, and 0.3 ng. Although titration of the antiserum from rabbit 189 was repeated three times, the streaky banding was consistently reproduced.

The antiserum from rabbit 190 detected 1 ng of antigen at a primary antibody dilution of 1:500, whereas the antiserum from rabbit 189 detected 3 ng of antigen, but not 1 ng.

Therefore, the antiserum from rabbit 190 was more sensitive than that from rabbit 189 and the antisera can be used to sensitively detect the mouse cyp4X1 protein. Therefore, the antiserum from rabbit 190 was used for further characterization of native cyp4X1.

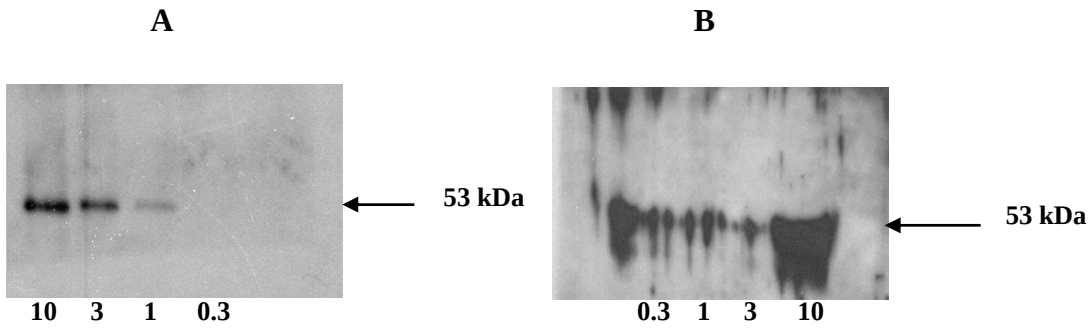

Figure 3.47. Sensitivity of antisera against mouse cyp4x1.
The indicated amount (0.3, 1, 3 or 10 ng) of mcyp4x1 protein was run on 12% SDS-PAGE then blotted to PVDF membrane as described in the methods. The membranes were incubated with antiserum (from rabbit 189 (B) or 190 (A)) at a concentration of 1:500, and the blots were developed with ECL as described in the methods. Film was exposed for 2 hours. The arrow shows the protein at ~ 53 kDa.

3.5.1 Specificity of antisera against the native mouse cyp4X1 protein

The mouse cyp4X1 RNA was found to be expressed in brain, but not in liver, and hence specificity tests of antisera against the native cyp4X1 protein relies on these tissues.

In order to test the specificity of the antiserum to mcyp4x1 protein in tissue extracts, immune and pre-immune sera were tested against microsomal and cytosolic extracts from adult male 129S4/SvJae mouse brain and liver on Western blots. Cyp4X1 antigen was used as a positive control on the blots. Figure 3.48, track 1 and 2, shows that a strong band at 53 KDa was detected in mouse brain microsomes and cytosol using the cyp4X1 antisera, whereas in liver this band was not detected.

Minor cross-reactivity bands were detected in brain and liver. However, the protein bands detected in brain fractions were not observed after probing with preimmune serum. Furthermore, several bands were detected in liver microsomes and cytosol after probing with preimmune serum and were the same bands that were detected by the immune serum in liver. These confirm that the antisera specifically detected cyp4X1 but not other proteins in mouse brain microsomes and cytosol.

The size of the protein detected (~ 53 kDa) by mouse cyp4X1 antibody in brain microsomes and cytosol was slightly lower than that of the purified recombinant protein, consistent with the 49 amino acid N-terminal fusion. Therefore, the protein detected in brain microsomes and cytosol samples has the expected size for the mcyp4X1 protein.

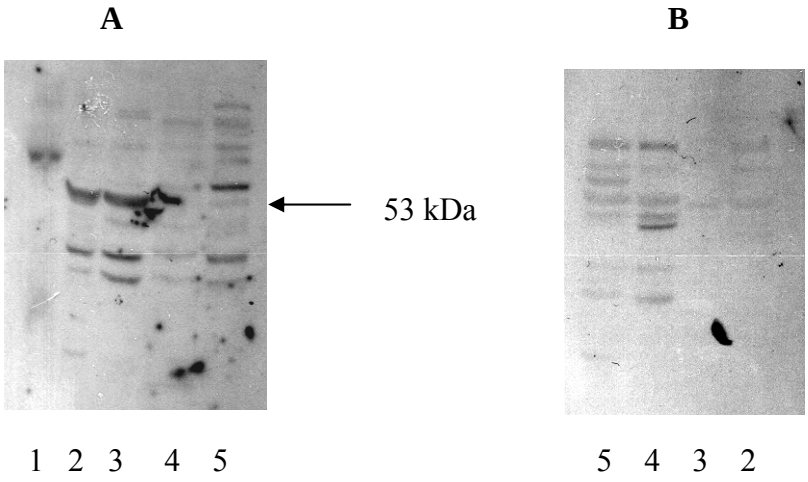

Figure 3.48. **Western blots showing brain and liver protein extracts probed with antiserum from rabbit 190**.
20 µg mouse brain and liver microsomes and cytosol and 10 ng purified recombinant mcyp4x1 protein were run on 12% SDS-PAGE gel and then blotted to PVDF membrane as described in the methods. The membranes were incubated with antiserum from rabbit 190 (A) or pre-immune serum (B) at a concentration of 1:1000, and the blot was developed with ECL as described in the methods. Films were exposed for five seconds. Track (1) in A represents antigen protein (+ve control). For both Figures, track (2) represents brain microsomes, track (3) represents brain cytosol, track (4) represents liver microsomes and track (5) represents liver cytosol. The arrows show the cyp4x1 protein at a size of ~ 53 kDa in mouse brain microsomes and cytosol.

Unexpectedly, Figure 3.49 showed that 4X1 was present in both microsomes and cytosol. To confirm that mouse cyp4X1 was located in brain cytosol, microsomes and cytosol were extracted from three adult female mice (S129) and analyzed on SDS-PAGE. The microsome preparations and cytosol were centrifuged twice at 328000 xg for thirty minutes at 4°C. Liver microsomes and cytosol were used as a control. Figure 3.49, shows that cyp4X1 (~ 53 kDa) was detected in all mice, but not in the control liver fractions. After the first centrifugation of the microsomes, strong bands and lower molecular weight bands were observed in all mice. However, these bands were less strong after

double centrifugation, with no lower molecular weight bands. Furthermore, the microsome preparations and cytosol were also centrifuged once at 568000 xg for one hour at 4°C (Figure 3.50).

Although cyp4X1 bands were also detected in all cytosol samples after double centrifugation, these are probably due to artifacts.

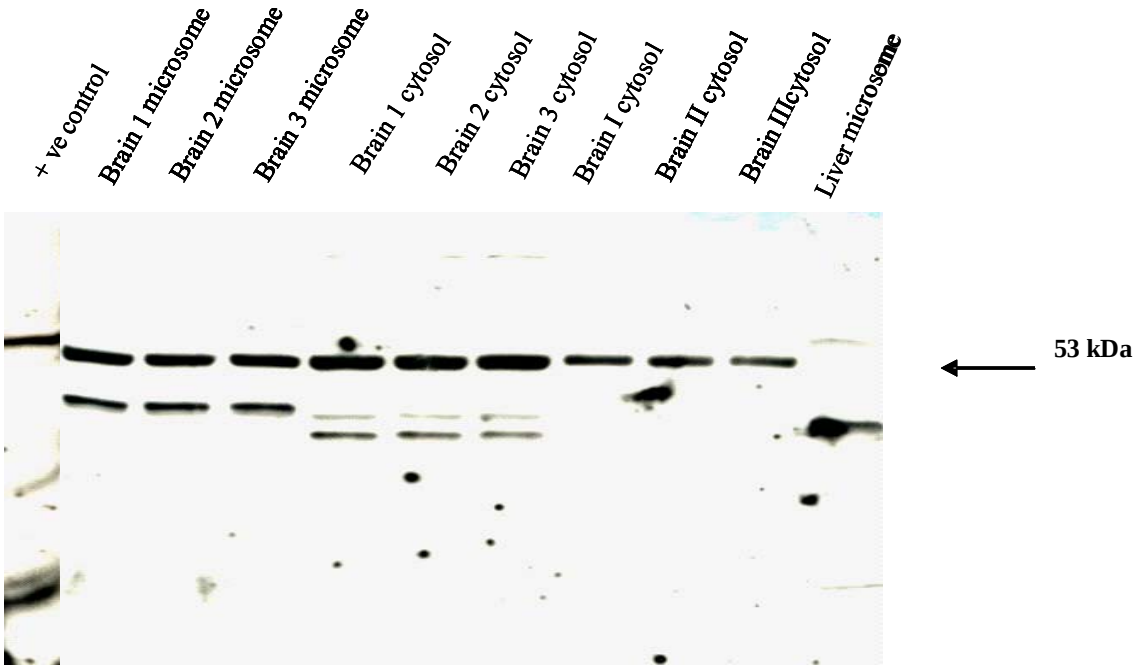

Figure 3.49. **Western blots showing double centrifugation of mice brain microsomes and cytosol probed with antiserum 190**.
The brain microsomes and cytosol were double centrifuged at 328000 xg for thirty minutes as described in the methods. 10 µg mouse brain and liver microsomes and 10 ng purified recombinant mcyp4x1 protein were run on 12% SDS-PAGE gel and then blotted to PVDF membrane as described in the methods. The membrane was incubated with antiserum (rabbit 190) at a concentration of 1:1000, and the blot was developed with ECL as described in the methods. Film was exposed for 2 minutes. +ve control represents antigen. Numbers (1-3) refer to mice 1-3. Roman Figures (I, II and III) represent cytosol double centrifugation.

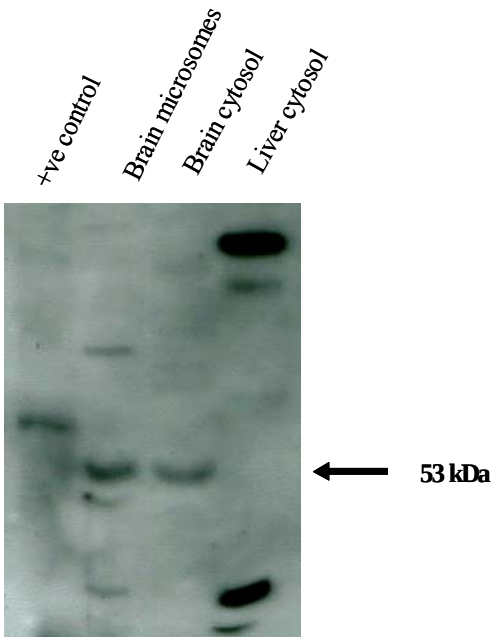

Figure 3.50. **Western blots showing centrifugation of mouse brain microsomes and cytosol probed with antiserum 190**.
The brain microsomes and cytosol were centrifuged at 568000 xg for one hour as described in the methods. 20 µg mouse brain and liver microsomes and 30 ng purified recombinant mcyp4x1 protein were run on 12% SDS-PAGE gel and then blotted to PVDF membrane as described in the methods. The membrane was incubated with antiserum (rabbit 190) at a concentration of 1:1000, and the blot was developed with ECL as described in the methods. Film was exposed for 20 minutes. +ve control represents antigen. Numbers (1-3) refer to mice 1-3. Roman Figures (I, II and III) represent cytosol double centrifugation.

As a further test of specificity, cyp4X1 antiserum was also blocked by adding a large excess of purified recombinant cyp4X1 protein and tested against the purified protein.

In order to confirm that the signals produced in Figure 3.48 were due to the specificity of the antiserum to cyp4X1, normal cyp4X1 antiserum and antiserum pre-incubated with purified cyp4X1 protein were tested against brain and liver microsomes.

Figure 3.51, shows that specific bands were detected in both the positive control, cyp4X1 antigen, and in brain microsomes using the antisera.

However, these protein bands were not observed in the samples after probing with blocked immune serum, confirming that the antisera specifically detected cyp4X1. Liver microsomes were also probed using immune serum, Figure 3.51 (A), and preincubated serum, Figure 3.51 (B). Two bands were detected in liver microsomes after probing with cyp4X1 antisera, whereas the same bands are detected after probing with preincubated serum. This indicates that the cyp4X1 antiserum is able to differentiate between cyp4X1 and other proteins.

Therefore, the specificity of the antisera for cyp4X1 was confirmed.

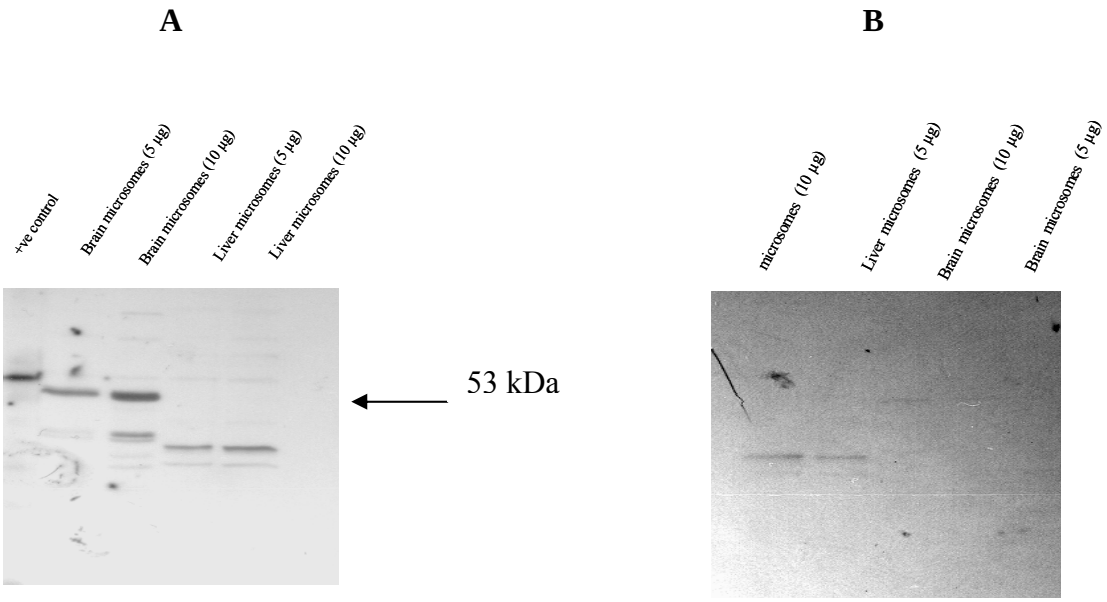

Figure 3.51. **Selectivity of the mouse cyp4x1 antiserum**.
Microsomal proteins from mouse brain, liver (5 µg and 10 µg of protein respectively) and 30 ng purified recombinant mcyp4x1 protein were resolved on 12% SDS-PAGE gels and then blotted to PVDF membranes as described in the methods. Pre-incubated antiserum was prepared by incubation of the cyp4x1 antisera with purified cyp4x1 protein for 1 hour at room temperature. The membranes were incubated with normal antiserum 190 (A) or pre-incubated antiserum 190 (B) at a concentration of 1:1000, and the blot was developed with ECL as described in the methods. Films were exposed for one minute. The arrows show the cyp4x1 protein at a size of ~ 53 kDa in mouse brain microsomes.

3.5.2 Quantitation of microsomal mouse cyp4X1

Quantitation of microsomal mouse cyp4X1 was carried out by Western blotting. Figure 3.52, shows that the signal intensity of microsomal cyp4X1 in the brain microsomal sample (1µg) was approximately equal to the signal intensity from 10 ng of 4X1 antigen. The total amount of cyp4X1 in brain microsomes was calculated to be 0.2 n mol per mg of microsomal protein.

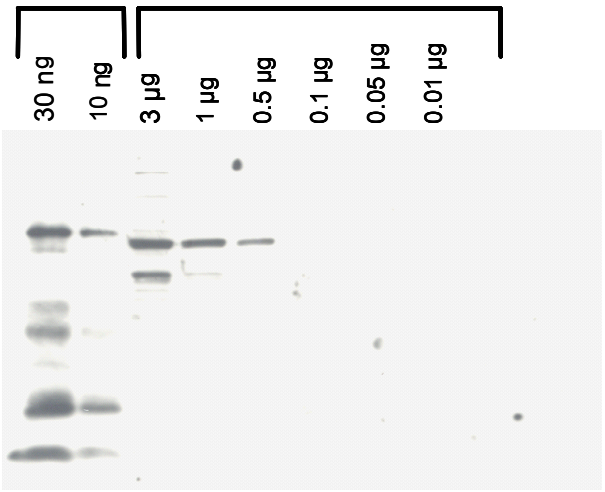

Figure 3.52. **Mouse cyp4x1 quantitation**.
Several brain microsomal dilutions (3, 1, 0.5, 0.1, 0.05, and 0.01 µg) and purified cyp4x1 (30 and 10 ng) were subjected to Western blotting on 12% SDS-PAGE gel and then blotted to PVDF membrane as described in the methods. The membrane was incubated with antiserum (rabbit 190) at a concentration of 1:1000, and the blot was developed with ECL as described in the methods. Film was exposed for 10 minutes.

3.5.3 Cyp4X1 protein localization

In order to determine the cyp4X1 protein localization in mouse brain tissues, immunohistochemistry was carried out using female mouse brain (129S4/SvJae) sections (Figure 3.53). The negative control of brain tissue stained in the absence of primary antiserum (Rabbit 190) (Panel A) showed that no cells were immuno-stained, which indicates that the stained cells observed in the test samples were due to the presence of cyp4X1 protein. Figure 3.53, panel B shows that the cyp4X1 protein was localized in the cytoplasm of cells of the cerebellum. These appear to be neurons based on their size and shape. Definitive neuronal localization as opposed to glial staining must await double labelling experiments using GFAP and NF antibodies to identify the cell type. Blood vessels were stained showing that the vascular endothelial cells also contained cyp4X1 protein. Interestingly not all neurons of the cerebellum were stained; as the Purkinje cells identified by their large size and layered distribution did not contain the P450 protein (Panel D).

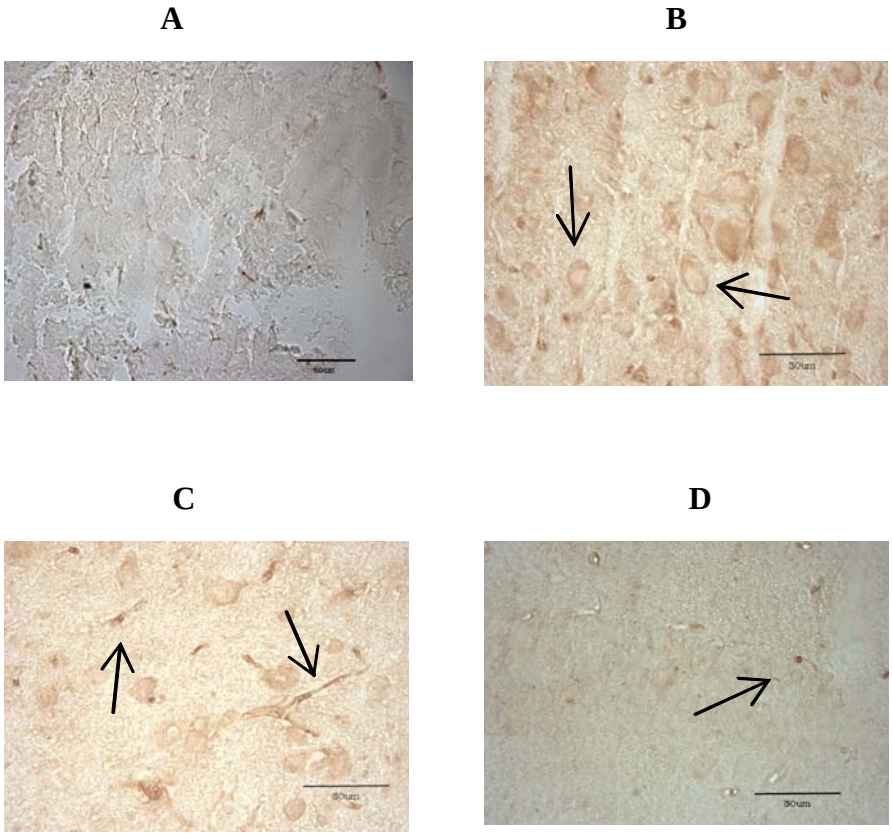

Figure 3.53. Immunohistochemistry of mouse brain tissue
Brain tissue was perfused and fixed as described in the methods. The sections were incubated with primary antiserum (from rabbit 190) at a concentration of 1:1000, and stained with DAB solution as described in the methods. (**A**) shows unstained brain tissue without primary antiserum (scale bar 50 µm). (**B**) shows stained neuron cells (scale bar 30 µm). (**C**) shows stained blood vessels (scale bar 30 µm). (**D**) Purkinje cells were not stained by the antibody to cyp4x1 (scale bar 30 µm). Arrows indicate the cells in each cerebellum section.

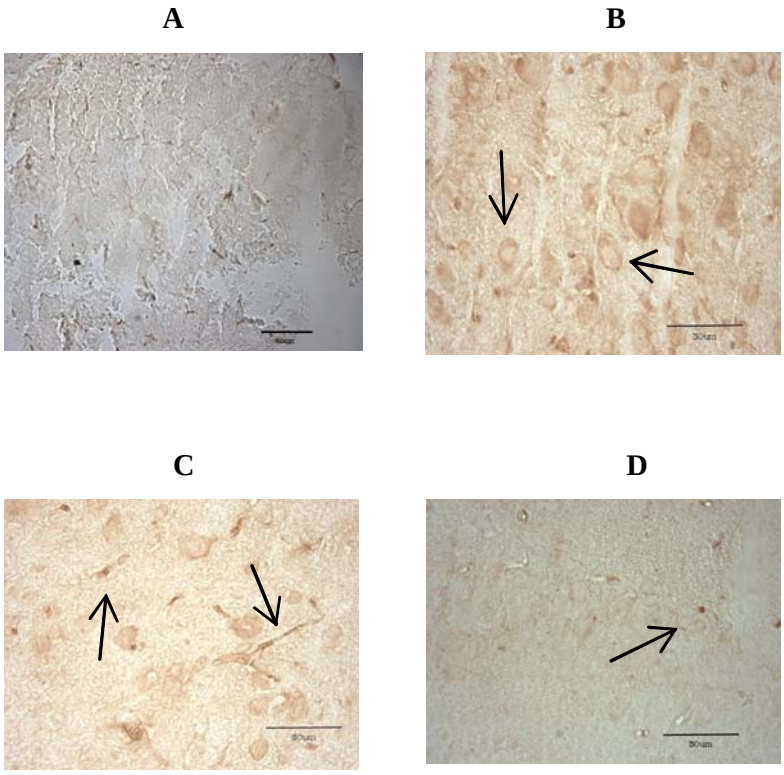

Figure 3.53. Immunohistochemistry of mouse brain tissue
Brain tissue was perfused and fixed as described in the methods. The sections were incubated with primary antiserum (from rabbit 190) at a concentration of 1:1000, and stained with DAB solution as described in the methods. (**A**) shows unstained brain tissue without primary antiserum (scale bar 50 µm). (**B**) shows stained neuron cells (scale bar 30 µm). (**C**) shows stained blood vessels (scale bar 30 µm). (**D**) Purkinje cells were not stained by the antibody to cyp4x1 (scale bar 30 µm). Arrows indicate the cells in each cerebellum section.

Chapter 4 Discussion

One of the aims of this present study was to generate antisera against, and subsequently characterize, human CYP4Z1, mouse AhR LBD and *C. elegans* latrophilin. Antisera were successfully produced, as discussed below.

4.1 Characterisation of CYP4Z1

Antisera against human CYP4Z1 were produced by immunising two rabbits (denoted "C" and "D"), both of which responded. The sensitivity of antisera improved with increased numbers of immunizations, and the minimum detection limit obtained was 1 ng.

The antisera detected the protein antigen at 40 kDa on SDS-PAGE, which is the predicted molecular size of the CYP4Z1 antigen. CYP4Z1 antiserum has not been used to detect native protein in human tissues yet. However, it has recently been shown that CYP4Z1 RNA is expressed in human tissues (Rieger 2004), and that the antiserum detects the full-length recombinant protein expressed in cells. In the present study, it was not possible to obtain human tissue; thus the CYP4Z1 antiserum was not tested against native human protein. The characterization of CYP4Z1 antisera confirms that the antisera are sensitive and are ready to use in detection of the native human CYP4Z1 protein. The characterisation of CYP4Z1 protein expression and immunohistochemical localisation would be a substantial advance in understanding the biology of the CYP4Z1 protein in human.

4.2 Characterisation of AhR LBD

The aim of this experiment is to produce antibodies against the LBD domain of the mouse AhR^{b-1} protein and then to test the specificity and selectivity of these antibodies. An antibody specific for the AhR LBD is an important reagent for studying the recombinant expression of the mouse AhR^{b-1} AhR LBD (T. Jiang, PhD thesis, 2004).

The antisera produced against the AhR LBD were more sensitive than those against CYP4Z1, detecting as little as 0.3 ng of antigen. The antisera could specifically detect native AhR in 10 μg of liver cytosol from both mouse and rat. The AhR is reportedly found at ~100 fmol/mg of cytosolic protein (Poland et al, 1987, 1990), or ~10ng of AhR/ mg cytosolic protein, equivalent to 100pg/ 10 μg of cytosol, confirming that the antisera are highly sensitive. Thus, these results confirm that the antisera are highly sensitive.

The specificity of these antisera was for AhR tested by immunodetection of AhR in rodent liver cytosol preparations. Clear bands were detected in hepatic cytosol from mouse and rat at 110 kDa and 111 kDa respectively, when preparation were probed with the antisera. Previous workers have determined the size of the AhR protein to be 104 kDa (C57B1/6 mouse) and 106 kDa (rat) (Poland et al, 1987, 1990). This confirms that the antisera detect native AhR at the predicted molecular weight. It was not surprising that antisera against mouse AhR LBD can detect the rat AhR protein, given the high sequence identity (5 mismatches in 192 amino acids) in AhR LBD between mouse and rat. Indeed, this antisera detects recombinant human AhR (Ming Qi Fan, unpublished data).

It has been reported that the variation in AhR molecular weight between mouse and rat is due to genetic polymorphism (Poland et al., 1990). In the current study, a slight variation in the apparent molecular weight of AhR between mouse and rat cytosol was also seen and this is likely to be for the same reason. Poland et al (1990) also reported that the cytosolic AhR can undergo proteolytic degradation, so the detection of low molecular weight bands in mouse liver cytosol may be due to the presence of AhR fragments following proteolysis. The data presented here confirm that the antisera produced against AhR are both sensitive and specific in mouse and rat.

4.3 Characterisation of LpH

Our laboratory has used *C. elegans* as a model system for studying the effects of BWSV. The *C. elegans* latrophilin homologue, B0457.1 (LpH), mediates the toxic effects of black widow spider venom, since *C.elegans* subject to an RNAi knock-out of latrophilin was resistant to the toxic effect of BWSV (Mee et al., 2004). For studying the native LpH protein in *C.elegans*, characterisation relies on Western blotting. Therefore it was necessary to develop a sensitive latrophilin-specific antibody. The antisera raised against latrophilin improved in sensitivity over time, detecting as little 0.3 ng of the 46 kDa LpH antigen. However, several bands were detected in sub-cellular fractions of *C.elegans*.

C. elegans were homogenised, then fractionated into a 5k pellet (nuclei and carcasses), a 15 k pellet (cell membranes and mitochondria) and 15k supernatant (endoplasmic reticulum and cytosol). Western blots of the 15k pellets were probed with both immune and pre-immune sera. When immune

sera were used to probe 15k pellets of C.elegans, a clear band of reactivity with the antisera was detected at a size of ~113 kDa, which was absent from other fractions. Several other bands were also detected in the 15k pellet fractions. However, none of the bands detected by the immune serum appeared after probing with preimmune serum, which argues that all of the bands detected by the immune serum are specific. However, the antigen was purified from *E. coli*, and was contaminated with small amounts of *E. coli* protein; given that *C elegans* eats *E. coli*, this does not exclude the possibility that some of the proteins detected by the immune serum may be unrelated to latrophilin.

The detected band at ~ 113 kDa is likely to be LpH, because the primary sequence of the LpH cDNA predicts a protein of 113 kDa. The sub-cellular localisation of this protein in the membrane fraction, together with its apparent molecular mass being consistent with the predicted molecular mass of LpH, suggest that this protein is the *C. elegans* LpH.

Previous studies have reported that the mammalian latrophilin has a molecular weight of 116 kDa (Lilianova et al., 1997), or 120 kDa (Krasnoperov et al., 1996) (Daveltov et al., 1996) (Itchenenko et al., 1999). It has also been reported to have two subunits (120 kDa and 80 kDa) (Krasnoperov, V., et al 2002). In the same study above, the 120 kDa band represents the intracellular latrophilin fragment, whereas the 80 kDa protein was due the glycosylated extracellular domain of the LpH protein.

Several lower molecular weight bands were also detected in the membrane and cytosolic fractions (15k pellet and supernatant) and these bands were possibly due to protein cleavage at the GPS site or non-specific cross-reactivity.

To test these possibilities, the antibodies were further purified using an affinity column and subsequently showed improved selectivity for the LpH protein. However, there was a loss of sensitivity: purified antibody detected 10 ng of the antigen, whereas the antisera before purification could detect 0.3 ng. The band detected using purified antibody was very strong at an antibody dilution of 1:1000, which indicates that a large quantity of antibody was purified.

The purified antibody, consistent with the antisera, detected a strong band at 113 kDa and a faint lower molecular weight band in total *C.elegans* lysate, whereas a single band was detected at ~ 66 kDa in the 15 k sub-cellular pellet. The lower molecular weight bands (~ 66 kDa) may be due to proteolysis.

The majority of the latrophilin protein was found at ~ 113 kDa in the 15k pellet (membrane proteins) with antisera and hence to solubilise the protein, emulgen 913 and 2% dodecylmaltoside were used. In these detergents, LpH was more soluble in 2% dodecylmaltoside than in 2% emulgen 913.

A fresh 15k supernatant treated with 2% dodecylmaltoside showed a strong band of LpH protein at ~113 kDa. However, this 113kDa band was absent following overnight storage of samples at – 80ºC. Figure 3.13 shows that an intense band at ~66 kDa was detected in these samples following storage, which must therefore be due to degradation of the ~113 kDa LpH protein.

Improved methods for sample preparation and storage need to be developed for further studies, given the apparent instability of LpH.

It is known that mammalian latrophilin is a member of the secretin family of G protein-coupled receptors with two fragments, one of which has a molecular size of 116 kDa (Lelianova et al., 1997). The full-length latrophilin protein is subject to proteolytic degradation and cleavage occurs between residues

Leu (837) and Thr (838) in the extracellular region of mammalian latrophilin close to the first transmembrane segment (Krasnoperov et al., 2002). In the same study, multiple homologous sequences to this cleavage site have been demonstrated in other G protein coupled receptors.

In the data presented here, several faint lower molecular weight bands were detected as shown in Figure 3.12 and a strong band was seen at 66 kDa as shown in Figure 3.13.

The presence of these lower molecular weight bands may be attributed to the presence of a GPS (G protein-coupled receptor proteolysis site) between positions C497 and M536 of LpH. This GPS is a characteristic feature of the LpH protein family.

Recently, a functional role for the GPS domain in receptor cleavage has been proven in CIRL 1 (latrophilin 1), where mutations in the GPS domain render the molecule resistant to intracellular cleavage (Krasnoperov et al., 2002).

After cleavage at the GPS site between L529 and T530, the predicted N-terminal fragment would be a 529 amino acid protein of 58 513 Da. This fragment would be detected by the antibody, which was raised against a recombinant expressed fragment containing amino acids 169-536 of the *C. elegans* LpH protein. The presence of LpH and LpH fragments in the 15k pellet indicates that this protein is localized in the membrane compartment.

The sub-cellular localisation of this protein in the membrane fraction, together with its apparent molecular mass being consistent with the predicted molecular mass of LpH, strongly suggest that the detected protein is indeed *C. elegans* LpH. Furthermore, the presence of a protein at 113 kDa with a proteolytic fragment at 66 kDa is in agreement with published studies on latrophilin.

In summary, antisera were produced against latrophilin and these detected several protein bands in *C.elegans.* Purified antibody still detected more than one band in the membrane compartment of *C.elegans* sub-cellular fractions, but the molecular weights of the detected proteins were consistent with GPS cleavage of LpH.

4.4 Sequence analysis of the cloned mouse cDNA

Human CYP4X1 and CYP4Z1 have been cloned (Savas et al., 2005; Rieger et al., 2004). However, we do not know where these CYP450s are expressed in human and it is difficult to obtain human tissue, so further investigation of these proteins in human is difficult. Therefore, I sought to establish a mouse model to study the expression of these CYP450s. However, nothing is known of mouse cyp4X1; the expression and function of this P450 is of interest. Hence, the next aim of the study was to generate mouse cyp4X1 probes. Mouse cyp4X1 was therefore cloned and characterized as described below.

For the cloning of mouse cyp4x1, two primers, 4x1-f-p*F* and 4x1-f-p*R*, were designed from the aligned protein sequences from human CYP4X1 (Figure3.26). These primers were designed from the beginning and end of the known human CYP4X1 cDNA sequence, and from the sequence of mouse genomic DNA (Fig 3.28).

The primers were used to amplify a cDNA fragment of ~1600 bp, the predicted size of the murine cyp4x1 cDNA. The PCR product is shown in Figure 3.28.

The cloned cDNA was purified, cloned and multiple independent clones sequenced to confirm its identity (Fig 3.29). The sequence of the cDNA was 1524 b.p., with an open reading frame of 507 amino acids (Fig 3.30).

In the nucleotide sequence of the mcyp4X1 cDNA, the forward primer (4x1-f-p*F*) and the reverse primer (4x1-f-p*R*) have been reproduced, which indicates that cDNA amplification and cloning was successful.

The predicted protein from the cDNA sequence was 507 amino acids, starting with a methionine translation start site (Figure3.30). It has been reported that mouse cyp4b1 and rat CYP4a1 each have 12 exons (Acc BC008996, Acc M57718M33937). The presence of the stop codon at the end of exon 12 indicates that this is also the final exon for the mouse cyp4x1. This result is similar to the exon number of other CYP4 family genes.

The deduced mcyp4x1 protein sequence shows a high level of similarity but not complete identity when compared with rat and human sequences as shown in Figure 3.31. This Figure shows that identity is 94 % and 79.2 % between the full-length mouse 4X1 sequence and rat (AF439343), human (Q8N118) respectively. The alingment shows that mouse cyp4x1 is more similar to rat CYP4X1 protein than to the human CYP4X1, and the length of the three proteins is identical. This high sequence identity with the rat defines the mouse as a new member of the CYP4X cytochrome P450 subfamily. Moreover, the genomic sequence suggests that there is only one mouse cyp4X gene, and hence this is the mouse cyp4X1 gene.

Table 6 shows that amino acid sequences corresponding to exon 2, 5, 8 and 11 show higher similarity between mouse cyp4X1 and rat and human CYP4X1 genes than the other exons. This higher conservation of these exons suggests that they might encode functionally important regions of the protein.

Exon 4 encodes for a highly conserved motif GRGLLNLDGPRWFQHRCILLTPAFHQDILKPCVDTMAHSVKVML, located between residue sites 121 and 165 in the cyp4X1 (Figure 3.33).

When using nucleotide sequence of this motif against blast search, it found that only the CYP4 subfamily was matched against this motif. This indicates that this motif is conserved in all the CYP4 family and subsequently it suggests that it might play an important role in the enzyme activity of this CYP4 family.

Exon 8 putatively encodes LRAEVNTFMWAGHDAS, a 16 amino acid motif. This motif contains a stretch of 13 amino acids and has been proposed to be a signature for the CYP4 family due to its high conservation, even in cockroach (Blaberus discoidalis) CYP4C1 (Bradfield et al., 1991).

Furthermore, Exon 11 and exon 12 were found to encode a highly conserved amino acid motif, PFSGG (A/S) RNCIG, which contains the haem-binding cysteine which is essential for all cytochrome P450 proteins (Gonzalez, 1989).

In conclusion, the mouse Cyp4X1 protein contains several motifs, which are know to be highly conserved within the CYP4 family, and required for function. It is therefore likely that the mouse Cyp4X1 encodes a functional cytochrome P450. Therefore, comparison of the deduced amino acid sequences of the cloned mouse cDNA with known 4X1 (rat and human) demonstrate that the cDNA encodes a 4X1 protein.

The enzyme activity of cyp4X1 has not been characterized, but it is likely that it metabolizes fatty acids by ω and ω-1 oxidation due to its high similarity to mouse cyp4b1 and rat CYP4A1 proteins of known enzyme activity (Okita and Okita., 2001).

4.5 Analysis of intron/exon junction boundaries in mouse cyp4X1

The intron and exon junction sites (1-12) were determined by direct comparison of the 4X1 cDNA against the genomic sequence of XX-Pac129 on chromosome 4 (see Figure 3.32). To determine the intron/exon junction, there are several rules, and these are described at http://www.cc.ndsu.nodak.edu/instruct/mcclean/plsc731/transcript/transcript4.htm. The junctions of the mouse cyp4X1 gene are in agreement with the published rules for >90% of this data.

Mouse cyp4a genes have been localized to chromosome 4 (Bell et al., 1993., Henderson et al., 1994), adjacent to the cyp4b subfamily (Heng et al., 1997). Moreover, all three mouse cyp4a genes are shown to be physically linked and are phylogenetically related to the Cyp4b subfamily (Heng et al., 1997). The present study amplified mouse cyp4x1 cDNA based on the genomic sequence of XX-Pac129 on chromosome 4 (see Figure 3.32). Hence, the cyp4X1 cDNA was produced from chromosome 4 and this is consistent with the locations of other CYP4 family members.

Alignment of intron/exon junction sites between mouse cyp4X1 cDNA and published mouse cyp4b1 (Acc.No. BC008996) and rat CYP4a1 (Acc. No. M57718M33937) was carried out (see Figure 3.33). The sequence of exon 1 contains a methionine at the start in all alignments using the published CYP4 family members discussed above.

The alignment showed that intron/exon junction sites were in very similar positions between mouse cyp4X1 and mouse cyp4b1, whereas, these was greater divergence between mouse cyp4X1 and rat CYP4A1 (Figure 3.33).

However, the protein sequences of cyp4a10 and cyp4b1 have similar % identity to cyp4x1, at 49 and 47% respectively. Cyp4b1 is the most distant member of the cyp4 cluster from cyp4X1, suggesting a greater evolutionary distance from cyp4X1 (Figure 3.54). In summary, neither the position of the intron/exon junctions, the % identity, nor the organization of the genes immediately provide obvious clues as to the evolution of the mouse cyp4 family.

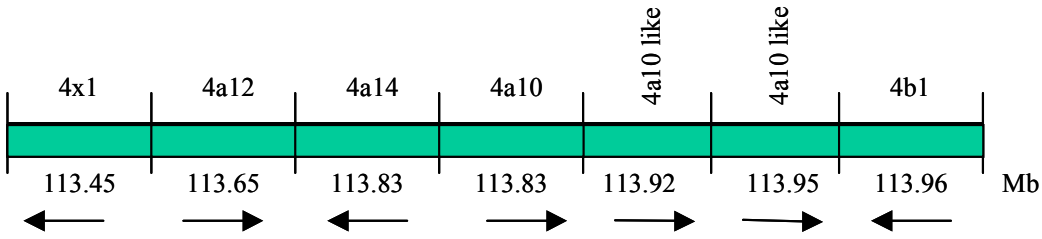

Figure 3.54. Cartoon of cyp4 genes on chromosome 4, showing orientation of gene (arrow), and the position of the gene in Megabases (Mb).

Figure 3.54 demonstrates that all cyp4a genes are closely linked to cyp4X1, head to head on chromosome 4, whereas the cyp4b1 gene is located far away from cyp4X1, but is closely linked to cyp4a pseudogene.

4.7 Expression and purification of mouse cyp4X1 in BL21 (DE3) *E.coli* cells

A full length cDNA encoding mouse cyp4X1 was cloned into pRSET-a for expression as a histidine-tagged fusion protein, containing 507 amino acids of cyp4X1.

pRSET-a-m4X1 plasmid DNA was transformed into BL21 (DE3) and plysS *E.coli* strains and induced using a final concentration of 1mM IPTG (section 2.2.4.7).

Figure 3.42 demonstrated that a protein 62 kDa was expressed in cultures treated with IPTG. Cultures grown without the addition of IPTG did not express any protein. This protein is dependent upon IPTG induction, and is the predicted molecular mass for the histidine-tagged Cyp4x1 fusion protein; it can therefore be concluded that this band is the cyp4X1. The induced protein was produced at a higher level in BL21 (DE3) cells without the plysS plasmid. Cultures of BL21 (DE3) cells treated with IPTG produced a strong band of induced protein (62 kDa), whereas the BL21 (DE3) plysS cells produced a fainter induced protein (62 kDa) band.

Figure 3.42 demonstrates that the majority of induced protein was found in the pellet fraction (insoluble protein) and hence the insoluble protein was pelleted and resuspended in 8M urea, 0.1% SDS. The cyp4X1 protein was soluble in the urea/SDS solution, but still had several faint bands of contamination after centrifugation. The solution was diafiltered on YM10 exclusion membranes prior to purification using His.Bind Nickel affinity column (Figure 3.44).

In the eluted fractions, the majority of the protein was recombinant mcyp-4X1 at 62 kDa, in addition to several minor lower molecular weight bands. The faint band at ~ 110 kDa was possibly due to protein aggregation, whereas the lower molecular weight bands were possibly due to proteolysis. The results presented here show that the protein was highly purified and resolved at the same molecular weight before and after purification.

4.8 Expression of cyp4X1 in aorta tissue

It has been reported (Savas et al., 2005) that human CYPX1 RNA is expressed in aorta tissue and these authors suggested it might contribute to the oxidation of arachidonic acid. Furthermore, Irizar and Ioannides (1995) demonstrated that the expression and inducibility of P450 protein in rabbit aorta by using polyclonal antibodies to CYP4A1. This study shows that the rabbit aorta expresses a CYP4A immunoreactive protein; noticeably, it has a different mobility to CYP4A1. It is tempting to speculate that this antiserum shows cross-reactivity with the CYP4X1 protein. Hence it may be that the CYP4X proteins play an important role in regulation of cardiovascular blood pressure.

In order to study the expression of cyp4X1 in aorta tissue, RNA was extracted as described in section 2.2.3.9. An RNase protection assay was performed and ciprofibrate was used to determine if the cyp4X1 gene was inducible by treatment of mice with peroxisome proliferators. Figure 3.37 shows expression and induction of this gene in both treated and untreated mice. The RNase protection assay showed that a specific protected band was produced in total aorta tissue and no protected band was present in the negative control. It is known that CYP4X1 is expressed in the aorta in humans and induced by the PPARα agonist Wy14643 in HepG2 cells (Savas et al, 2005). However, nothing is known about regulation of cyp4X1 expression in mouse aorta.

In the present study protected bands were observed in both ciprofibrate treated and untreated mice, at a similar level. The aortic cyp4X1 protein was not inducible by peroxisome proliferators in this study. This data shows for the first time that expression of cyp4X1 has been demonstrated in the aorta in

mice. Furthermore, the experiment demonstrated no induction effect of ciprofibrate in treated mice in aorta.

The data presented here are otherwise consistent with previous reports and support the hypothesis that CYP4X1 might contribute to the oxidation of arachidonic acid in mice.

4.9 Expression and induction of cyp4X1 in brain tissue in mice

Cytochrome P450s have been shown to be involved in the biosynthesis and metabolism of neurosteroids, vasoactive eicosanoids, retinoid, cholesterol and in the metabolism of neurotransmitters (Hedlund et al., 2001; Compagnone et al., 2000; Whl et al., 1993; Roman et al., 2002; White et al., 2000; Lund et al., 1999; Hiroi et al., 1998; Bronheim et al., 1995). A recent study has demonstrated that CYP4x1 is expressed in rat brain (Bylund J. et al., 2002). However, nothing is currently known about its expression in the brains of mice. In order to study cyp4X1 gene expression in mouse brain tissues, samples were analysed using a sensitive RNase protection assay.

Mice were also treated with ciprofibrate to study the induction effect of this agent. Expression of the cyp4X1 gene was first identified in the brain samples prior to studying the induction effect (Figure 3.34). The probe length in the experiment was 424 b.p. and the marker was 124 b.p. The data in Figure 3.34 showed that a specific protected band at 177 b.p was seen in the brain sample, whereas no band was seen in the control sample (Liver). Two high molecular weight bands were also produced: since these were the same length as the full length probe, they did not correspond to the fragment size of protected RNA, and were not considered to reflect specific protection of the probe.

The size of the protected fragment in the mouse brain sample was shorter than the full-length probe, which showed that RNase treatment removed 247 b.p. of unprotected probe. This indicated that cyp4X1 was expressed in brain tissue, but not in liver. The detection of cyp4X1 expression in brain tissue is noteworthy, because of the low content of total P450 in brain. Furthermore, most of the P450 isoform detected in the brain are also expressed at high levels in extracranial tissues (Bylund et al., 2002; Hedlund et al., 2001), but mouse cyp4x1 is not found in the liver. It had been reported previously that CYP4X1 had a unique localization to the rat brain, whereas the data presented in section 3.3.9 demonstrated that mouse cyp4X1 was also expressed in the aorta. The physiological functions of the 4X1 genes are not yet known. However, the data presented here together with previous studies suggest that the 4x1 gene may be physiologically important enzyme in neuron function.

It is known that cyp4a genes are regulated by PPARα (Bell et al, 1993) and the availability of PPARα knock-out mice that do not contain a functional PPARα gene offers an ideal system to study any role of PPARα in the expression of mcyp4X1. Hence, expression of cyp4x1 expression in wild type and knock-out mice was also investigated. Figure 3.35 shows expression of mcyp4X1 in total brain RNA extracted from wild type and knock-out mice.

This study has found that both wild type and knock-out mice express cyp4X1 at a similar level. This was surprising, because expression of the cyp4a subfamily is regulated by PPARα (Bell et al., 1993), and suggests that the cyp4X1 gene is regulated not by PPARα, but by alternative mechanisms.

The induction effects of peroxisome proliferators on the cyp4X1 gene were also investigated. It has been reported that ciprofibrate induces mitochondrial

3-hydroxy-3-methylglutaryl-CoA synthase (mHS) in the brain, and that it must therefore be bioavailable in the brain (Cullingford et al., 2002). Figure 3.38 shows the induction effect of these agents on the expression of cyp4X1 in mouse brain. None of the peroxisome proliferators tested caused significant induction of cyp4X1. This indicates that cyp4X1 gene is not induced by ciprofibrate and this consistent with the fact that ciprofibrate induced only the CYP4A subfamily.

In summary, the mouse cyp4x1 gene is expressed in the brain, but not in the liver. However, mouse cyp4X1 expression is not uniquely localized in the brain, but is also found in the aorta. There was no difference in cyp4X1 expression levels between wild type and PPARα knockout mice, and cyp4X1 expression was not induced by peroxisome proliferators.

4.10 Tissues distribution of cyp4X1 expression and induction

Several mouse tissues were isolated and RNA was extracted in order to study the tissue distribution of cyp4X1 gene expression. Figure 3.36 shows the evidence for cyp4X1 expression in mouse lung, heart, and kidney tissues.

Protected bands were produced after probe hybridisation in these tissues.

The protected signals were detected only after prolonged exposure of autoradiography (four days). Therefore, the cyp4X1 gene may be expressed in these tissues but at very low levels.

Because the protected fragment was detected at very low levels and only after prolonged exposure times, it cannot be said for sure that cyp4x1 is expressed in these tissues. Cyp4X1 is definitely expressed in brain and aorta but further

study of the other tissues is necessary to obtain convincing proof of more widespread cyp4X1 expression.

4.11 Sensitivity of mouse cyp4X1 antisera

The next aim of the study was to produce antisera against mouse cyp4X1 and characterise this antisera using recombinant and native mouse cyp4X1 proteins. Antisera were successfully produced, as discussed below.

Antiserum against human CYP4X1 (A gift from Dr. Bell) was initially tested against recombinant mouse cyp4X1protein, and the minimum detection limit obtained was 100 ng (Figure 3.45). The antisera against human cyp4X1 therefore had low and insufficient sensitivity against the mouse recombinant protein. There is only 79.2% identity between the mouse and human cyp4X1proteins, which explains the low level of cross-reactivity between them. For studies of mouse cyp4X1, a mouse-specific antiserum was therefore required.

Antisera against mouse cyp4X1 were produced using two rabbits (denoted "189" and "190"). Sensitivity against the antigen was improved with increasing numbers of immunizations in rabbit 190, and the minimum detection limit obtained was 1 ng, whereas repeated immunization in rabbit 189 failed to improve its sensitivity beyond 3 ng.

When antisera against mouse cyp4X1 were blotted against recombinant antigen, a clear band at 53 kDa was detected in each track as expected (Figure 3.47 A). The primary sequence of the cyp4X1 cDNA predicts a protein of 62,808 Da, which is slightly higher than the observed molecular size of the recombinant mouse cyp4X1 (53 kDa). This is consistent with the literature,

since most P450s run at 52-55 kDa on SDS-PAGE gels yet have a predicted molecular mass of ~60 kDa. The antisera from rabbit 190 consistently detected a single band at 53 kDa, as expected. However, the antisera from rabbit 189 detected a single clear band at 53 kDa in only one of the samples tested (Figure 3.47 B). When 10 ng of antigen was probed, a single band was detected, as expected, but lower quantities of antigen (3, 1, and 0.3 ng) consistently produced streaky, indistinct bands. The reasons for this are not known.

4.12 Selectivity of mouse cyp4X1 antisera

Once the sensitivity of the antisera had been determined, the selectivity was tested. This testing was carried out using brain and liver tissues from mice, since cyp4X1 RNA was known to be expressed in brain but not liver, as discussed above.

The specificity of the antisera for mouse cyp4X1 was tested by immunodetection using microsomal and cytosolic preparations from mouse brain and liver (Figure 3.48 A).

Western blots of microsomal and cytosolic extracts were probed with both immune and pre-immune sera. When immune sera were used to probe mouse brain microsomes and cytosol, a strong band was detected at 53 kDa, but this band was not detected in liver preparations. Several other bands were also detected in both brain and liver fractions. However, only the 53 kDa band detected in brain extracts failed to appear after probing with preimmune serum. This indicated that the immune serum was able to discriminate between cyp4X1 and other proteins. Pre-immune sera did not detect cyp4X1 at 53 kDa so this demonstrates that the 53 kDa band is specific. The size of the protein

detected (~ 53 kDa) by mouse cyp4X1 antisera in brain microsomes and cytosol was slightly lower than that of the purified recombinant protein, consistent with the presence of a 49 amino acid N-terminal fusion on the recombinant protein.

The data presented here in the current study show that highly sensitive and selective antibodies were present in the mouse cyp4X1 antisera. However, in addition to detecting the 53 kDa cyp4X1 band, antisera detected several minor bands. These were also detected by pre-immune sera and were therefore due to non-specific interactions. In order to prove that the minor contaminating bands were due to non-specific antibodies present in the antisera, antisera were blocked by incubation with an excess of purified recombinant cyp4X1 protein.

Normal and blocked cyp4X1 antisera were used to probe brain microsomes. Only normal antisera detected the 53 kDa cyp4X1 band, but both antisera detected the minor bands, indicating that these minor bands were due to the presence of non-cyp4X1 specific antibodies in the antisera.

The detection of cyp4X1 in both brain microsomes and cytosol was surprising, because CYP450 is usually found in the microsomes, but not in the cytosol.

To check this finding, double centrifugation was carried out at 111000xg for thirty minutes and one centrifugation at 111000xg for one hour to obtain pure cytosol. Although the cyp4X1 protein was still present following double centrifugation for thirty minutes and one centrifugation for one hour, the band was less intense and no lower molecular weight bands were present (Figure 3.49 and 3.50). This result suggests that the detection of cyp4X1 in the cytosol may be an artefact caused by homogenisation or failure to completely remove

microsomes during centrifugation. Further investigation is therefore required to reliably determine the cellular localization of cyp4x1 in the mouse brain.

The total content of P450 in rat brain tissue is approximately 100 pmols/mg microsomal protein ((Bhagwat,et al 1995). In this study, the calculated amount of cyp4X1 protein in brain was ~200 pmol/mg, which demonstrates that the cyp4X1 content in brain is potentially a major component of the total brain cytochrome P450 (Figure 3.52). It is important to note that all of the immunoreactive protein is not necessarily present as functional holoenzyme. However, the very high levels of cyp4X1 present strongly suggest an important role of this P450 in brain function.

4.13 Cyp4X1 protein localization

It has been reported that CYP4X1 has a unique localization specific in the rat brain (Bylund et al., 2002). In the same study it was suggested that CYP4X1 was mainly expressed in neurons. In the present study it was found that the cyp4X1 protein is localized in the cytoplasm of cerebellar cells in mouse brain tissue (Figure 3.53). A negative control in the absence of primary antiserum showed no immunostaining, demonstrating that positive staining, in the test samples was due to the presence of cyp4X1 protein. Furthermore, the fact that Purkinje cells did not stain for cyp4X1 demonstrated that there was specific staining by the antisera in cerebellar cells.

In Figure 3.53 (panel B), it was not absolutely confirmed that the stained cells were neuron cells, but based on their size and shape this was a reasonable conclusion. To confirm that the stained cells were neurons and not glial cells,

double labelling experiments using GFAP and NF antibodies would be required.

Blood vessels were also stained indicating that vascular endothelial cells also contained cyp4X1 protein. This result was surprising because it was the first time that cyp4X1 was seen in the blood vessels, but it supports, the hypothesis that this P450 may play an important role in neurovascular function.

The data presented here show that the cyp4X1 protein is expressed in brain tissue, and characterised the localisation of this protein to specific cell types. Given the high-level expression of this P450, it is of great interest to determine the function of this P450.

4.13 Future work

The present study has characterized anti-human CYP4Z1 antisera and showed that they are capable of detecting <1ng of recombinant antigen. However, the antibody against CYP4Z1 has not been tested against human tissue, due to a shortage of human tissue (e.g. aorta, heart). It is of interest to raise human CYP4Z1 antibody against human tissue in order to test antibody specificity and selectivity against a native human CYP4Z1.

To date, nothing is known of the function and expression of this P450. It is of interest to investigate the site expression and function of human CYP4Z1. The present study showed that mouse cyp4X1 is expressed in brain tissue, but not other tissues (e.g liver). However, nothing is known the function of this P450.

For studying of 4X1 functions, its endogenous substrate needs to be determined. Furthermore, it has been reported that this P450 may have an

important role in regulation of the blood pressure. Hence, it is of interest to investigate the enzyme activity of cyp4X1.

The present study showed the expression of mouse cyp4X1 in mouse tissues, but not in human tissues. It is of interest to investigate the expression of the 4X1 gene in human tissues.

The present study showed that mouse cyp4x1 is expressed in brain tissue, but nothing is known about mouse cyp4x1 localization. It is of interest to investigate the localization of 4X1gene in mouse brain tissues.

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