The role of PPARα in

Cytochrome P450 gene expression and DNA synthesis

By

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A thesis submitted in partial fulfilment of

the requirements for the degree of

Doctor of Philosophy

University of Nottingham

October 2001

Abstract

Cytochrome family P450 4A encode a major group of enzymes which are involved in the mechanism of peroxisome proliferation in rodents. Induction of CYP4A expression by peroxisome proliferators is due to transcriptional activation, mediated via the peroxisome proliferator activated receptor alpha. Cyp4a enzymes catalyse the ω-hydroxylation of fatty acids and eicosanoids, and it has been suggested they thereby play a pivotal role in blood pressure regulation. Murine *Cyp4a10*, *Cyp4a14* and *Cyp4a12* genes have been reported by Bell *et al* (1993) and Heng, *et al* (1997). There is contradictory evidence in the literature concerning the expression of lauric acid hydroxylase (LAH) and Cyp4a-related protein in mouse liver and kidney. We have previously shown that *Cyp4a12* is expressed at high level in a male-specific fashion in liver and kidney of mouse (Bell *et al* 1993). However, various workers have reported the presence, or absence, of Cyp4a proteins, or LAH, in male mouse liver. Work by Hiratsuka *et al* (1996) demonstrate that ddY mice show a male specific expression of LAH and Cyp4a-related protein in the liver, while other strains Balb/c and C57BL/6 exhibit no sex difference in neither enzyme or protein. In the kidney, ddY, Balb/c and C57BL/6 all show sexual dimorphism in the expression of both LAH and Cyp4a related protein. The aim of this project was to characterise the expression of mouse *Cyp4a12* and resolve the conflicting results in the literature.

Findings demonstrate that there is a male specific expression of *Cyp4a12* in male liver and kidney. The data differs from Hiratsuka *et al* in that their

findings present no sex difference in Balb/c and C57BL/6 expression of Cyp4a proteins. Hiratsuka *et al* also determined that in the kidney of ddY, Balb/c and C57BL/6 there is no sex difference in expression of neither enzyme or protein. Data here agrees with these findings and suggests that the sexual dimorphism exhibited by the LAH and Cyp4a related protein in the kidney is due to constitutive expression of *Cyp4a12*. Thus it appears that there is still a discrepancy between the *Cyp4a12* hepatic expression pattern presented here and that of the enzyme and protein determined by Hiratsuka *et al*. An explanation may infer that the LAH and Cyp4a-related protein measured by Hiratsuka *et al* is not only *Cyp4a12* but also another member of the Cyp4a family. Further work is required to establish what Cyp4a is expressed predominately in the female. Work by Henderson *et al* (1994) supports the case that this Cyp4a candidate may be *Cyp4a10*. Continuing studies will clarify the expression pattern of the Cyp4a and also investigate the mechanism of regulation of the Cyp4a family genes and the expression of male specific genes.

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Acknowledgements

In the 3 years I spent in Nottingham I was lucky enough to work with people who are now very good friends. In alphabetical order (and the order in which they can handle their drink), Chris, Neill, and Sunny, have made the process of doing a Phd a little more bearable. Thanks for your support over the past few years and I am glad we escaped staying at her majesty's pleasure!

I would like also like to thank, Munim, Declan, Mark, Rebecca, Simon, Yee, Dianne, Fatima, Brian, Neil, Nick and Kate all who provided me with advice, technical help, a roof over my head, the opportunity to show the world my great football talent and the excuse to go to the pub once or twice.

I would especially like to thank Dr. Tim Gray for the technical, and financial support help he and his colleagues at Sanofi-Synthelabo have provided. I am also indebted to my colleagues at the Food Standards Agency. Cate and Tom have shown a lot of patience with me by allowing me to write up on the job.

Dr. David Bell has as a supervisor, shown immeasurable patience, and excellent support throughout my PhD. Thanks for all the hard work you put in!

Julia has a pillar of strength over the duration, and I also like to thank her for her perseverance and support.

Finally this thesis is dedicated to my Mum, Dad and Sister who have loved and made me what I am today!

Chapter 1 Introduction

Section 1.1 Peroxisome proliferation

Section 1.1.1 Peroxisomes and their function

Peroxisomes are spherical organelles, which are single membrane bound and present in almost all eukaryotic cells (Baudhuin *et al* 1965, De Duve and Baudhuin 1966). Amongst the enzymes they contain are those that use molecular oxygen to remove hydrogen atoms from specific organic substrates to produce hydrogen peroxide. Peroxisomes are approximately 0.5μm in diameter and contain high concentrations of catalase and urate oxidase (Wilcke *et al* 1995). In the liver and kidney, peroxisomes are found in high numbers with 600 individual organelles present in one cell and representing 2% cell volume (Masters and Crane 1995).

Mammalian peroxisomes contain more than 50 proteins associated with lipid metabolism (Mannerts and van Veldhoven, 1993), and are essential elements of the glucose fatty acid cycle, which metabolises carbohydrate and fatty acids to generate ATP (Reddy and Hasimoto 2001). During periods of starvation, energy is generated from the metabolism of carbohydrates to free fatty acids and their ketone derivatives (Salway *et al* 1998). The essential role of peroxisomes in this process is emphasized when their function is impaired.

Zellwegers syndrome is an inheritable genetic disorder that results in a deficiency of functional peroxisomes. This disorder impairs β-oxidation of fatty acids (due to the lack of peroxisomal â -oxidation enzymes) leading to accumulation of long chain fatty acids in tissues and blood of patients. (Schutgens *et al* 1986; Moser 1987; Lazarow and Moser 1989). Another disorder which results in long chain fatty acid accumulation is X-linked adrenoleukpdystrophy which effects the peroxisomal enzyme very-long-chain acyl-CoA (VLCFA-CoA) synthase (Wanders *et al* 1988; Lazo *et al* 1988).

Section 1.1.2. β-oxidation of fatty acids in peroxisomes

β-oxidation is the predominant mechanism of fatty acid oxidation. This may occur either in the peroxisomes of mitochondria however the enzymes involved are different (table 1.1).

Table 1.1: Enzymes in the peroxisomal fatty acid β-oxidation system

Very-long-chain acyl-CoA synthetase	Branched-chain acyl-CoA oxidase
Long chain acyl-CoA synthetase	L-Bifunctional protein
Carnitine octaoyltransferase	D-Bifunctional protein
Carnitine acetyltransferase	3-Ketoacyl-CoA reductase
Straight-chain acyl-CoA oxidase	Sterol carrier protein 2/3-ketoacyl-CoA thiolase
2,4-dienoyl-CoA reductase	
△3, 2-Enoyl-CoA isomerase	
△3,5,2,4-Dienoyl-CoA isomerase	

Peroxisomes work in concert with mitochondria but contain enzymes that perform additional reactions such as very-long chain acyl CoA synthetase responsible for the metabolism of VLCFA's ($>C_{20}$). Peroxisomal β-oxidation

also metabolises long chain dicarboxylic acids, eicosanoids, and bile acid precursors. Two pathways are thought to exist for peroxisomal β-oxidation (figure 1.1). Oxidation of straight-chain fatty acids is catalysed by the enzymes AOX, L-PBE and thiolase. 2-methyl branched fatty acids and the bile acid intermediates, di- and trihydroxycoprostanoic acids, occurs via a second pathway (figure 1.1).

Fatty acids in the liver are predominantly non-esterified fatty acids transported by serum albumin and triglycerides carried by chylomicrons. Triglyerides are hydrolysed to glycerol and fatty acids by lipoprotein lipase (LPL). Before entering the peroxisomes fatty acids are activated to acyl-CoA thioesters by removal of two carbon atoms. These are converted to acetyl CoA, which is returned to the cytosol for recycling. The flux of fatty acids into mitochondria is regulated by a carnitine-dependent transport system of which carinitine octanoyltransferase is a critical component. Acyl-CoA derivatives of fatty acids are carried to the peroxisomes where they are subjected to oxidation by acyl-CoA oxidases. Acyl-CoA oxidases contain FAD as a prosthetic group that use molecular O_2 to oxidize fatty acids, yielding hydrogen peroxide as a byproduct (Van den Bosch *et al*., 1992; Schultz 1991)

Bifunctional enzyme catalyses the hydration and dehydrogenation in the subsequent steps of β-oxidation, although it may also possess 3,2-enoyl- CoA isomerase, 2-enoyl-CoA hydratase and 3-h ydroxyacyl-CoA dehydro-genase activities (Palosaari and Hiltunen, 1990). Peroxisomosal â -oxidation is completed by the thiolytic cleavage of oxo-acyl-CoA (3-ketoacyl-CoA) by CoA-

SH catalysed by 3-keoa-cyl-CoA thiolase (Salway 1987, figure 1.1 shows an example of peroxisomal â -oxidation of a fatty acids)

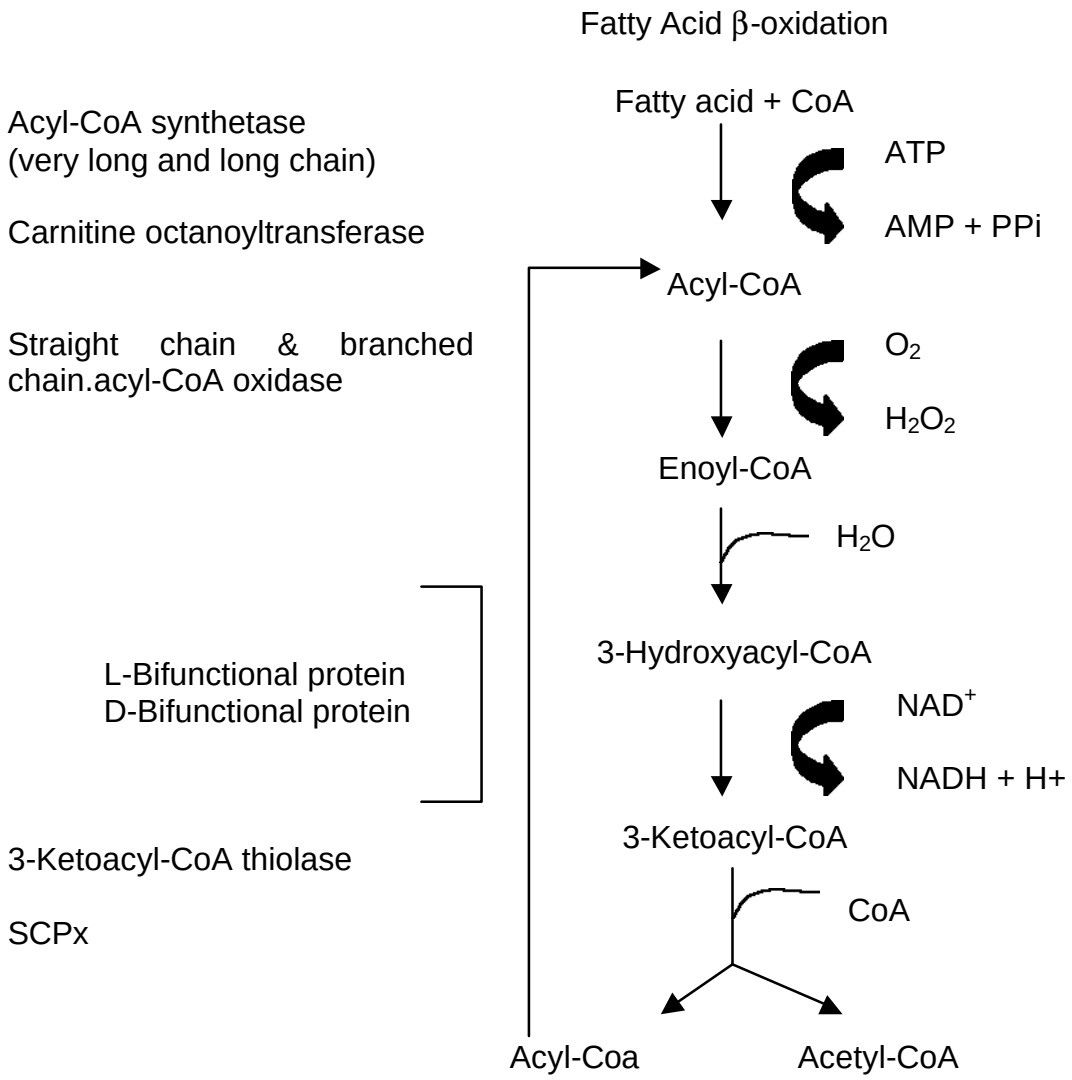


Figure 1.1. Enzymology of peroxisomal β-oxidation systems in humans: Inducible straight chain and the non-inducible branched chain fatty acid β-oxidation. SCPx- sterol carrier protein x., Modified by Reddy and Hashimoto (2001)

Section 1.1.2 Peroxisome Proliferators

Peroxisome proliferators are a class of structurally diverse chemicals (figure 1.2) that cause a dramatic increase in the size and number of peroxisomes in

susceptible species (Reddy *et al*., 1980; Lock *et al*., 1989). In addition these compounds cause a range of biochemical and morphological changes. These include the induction of both peroxisomal and microsomal fatty acid-oxidising enzyme activities (Lake 1995).

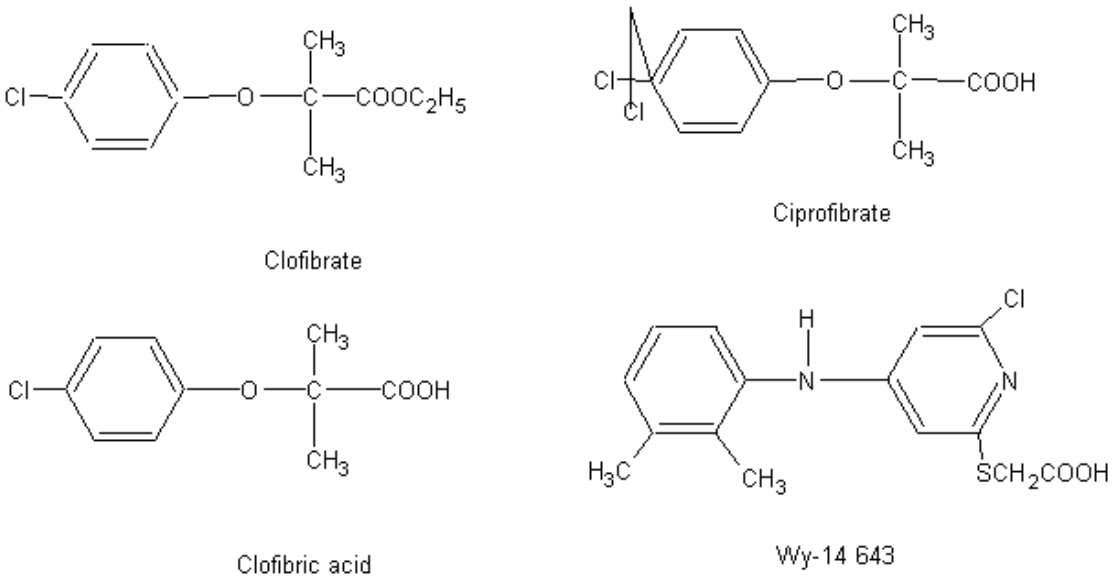


Figure 1.2. Chemical structures of some peroxisome proliferators.

Although their structures are diverse, many contain a functional group that is either carboxylic or acidic. For example clofibrate is metabolised to clofibric acid. The acidic group of the peroxisome proliferators may be an integral part of the parent structure or it may be a metabolite. However phase II metabolism such as glucoronidation is not thought to generate peroxisome proliferators in rodents (Lake and Lewis 1993).

Figure 1.2 shows the structure of some known peroxisome proliferators. Peroxisome proliferators form a group of chemicals that have a wide range of uses, including hypolipidemic agents, herbicides, and plasticizers. (Moody *et*

al 1991, Bentely *et al* 1993). Peroxisome proliferation may also occur without exposure to these chemicals. Conditions including starvation, high-fat diets, cold temperature, and diabetes mellitus also result in peroxisome proliferation. Historically this phenomenon was first identified in rodents, and it is this species that peroxisome proliferation is best described (Chu *et al* 1996, Lock *et al* 1989, Reddy *et al* 1983).

Section 1.1.3 Acute effects of peroxisome proliferators

Exposure to PPs result in a set of responses in the liver, kidney and heart (Reddy and Lalwani et 1983). These include increased liver weight, due to hepatocyte hypertrophy and hyperplasia, and transcriptional increases of enzymes involved in the metabolism of fatty acids (Reddy and Lalwani et 1983). The increase in liver weight is rapid with administration of Wy14, 643, a potent PP, with organ weight reaching a maximum by day 14. (Marsman *et al* 1988). Hepatocyte hypertrophy is a result of an increase in the size of individual cells due to a proliferation in sub-cellular organelles such as smooth endoplasmic reticulum, golgi bodies, mitochondria and peroxisomes (Reddy *et al*., 1980; Lock *et al*., 1989). Hepatomegaly continues for the duration of PP exposure. Cessation of treatment results in the liver reducing to its original size (Cohen and Crasso 1981). Treatment with PPs increases peroxisome number and size to up to 25% of the cytoplasmic volume (Moody and Reddy 1976, Nemali *et al* 1989). Despite an increase in the number of hepatocyte peroxisomes, the organelles may lack cores containing urate oxidase (Stott 1988).

Section 1.1.4 Peroxisome proliferation - Biochemical characteristics

The major biochemical alterations consist of the induction of both peroxisomal and microsomal fatty acid oxidizing enzyme activities. (Hawkins *et al* 1987; Auwerex *et al* 1996; Lazarow and De Duve 1976, Lazarow, 1977; Norseth and Thomassen, 1983; Veerkamp and Van Moerker, 1986). Enzyme activity of peroxisomal β-oxidation maybe determined by measuring overall activity or by assaying the rate-limiting enzyme of the cycle i.e. acyl-CoA oxidase. Peroxisomal enzyme activities are differentially induced as enzymes such as acyl-CoA oxidase activity may increase 30-fold, while catalase increases by only 3-fold. (Reedy and Lalwani, 1983; Lock *et al*., 1989). Peroxisome proliferation also includes an increase in smooth endoplasmic reticulum and associated levels of microsomal lauric acid ù -hydroxylase activity resulting from high level of induction of cytochrome P-4504A family (Kimura *et al*., 1989, Bell and Elcombe, 1991a). Rodents administered methylclofenapate exhibit CYP4A1 induction (Bell *et al* 1991). Induction of CYP4A1 followed by peroxisomal acyl-CoA oxidase suggests that peroxisomal and microsomal genes are regulated in a coordinated manner and are frequently used as a marker of peroxisome proliferation. Table 1.2 shows a list of some of the enzymes induced by PPs.

Enzyme	Cellular Localization	Reference
Cytochrome P450 4A1	Smooth ER	Gibson *et al*., 1982
Carnitine Palmitoyl-CoA transferase	Total cellular	Sakuma *et al*., 1992
Acyl-CoA oxidase	Peroxisome	Osumi *et al*., 1987
Bifunctional enzyme	Peroxisome	Reddy *et al*., 1986
Thiolase	Peroxisome	Baumgart *et al*., 1990
Catalase	Peroxisome	Pacot *et al*., 1993
Urate oxidase	Peroxisome	Nemali *et al* 1988
HMG-CoA Synthase	Mitochondrial	Rodriguez *et al*., 1994
Malic enzyme	Cytosolic	Sakuma *et al*., 1992
Fatty acid binding protein	Cytosolic	Kaikaus *et al*., 1993
Epoxide hydrolase	Cytosolic	Schladt *et al*., 1987

Table 1.2 Enzymes induced by peroxisome proliferators.

Section 1.1.5 Hyperplastic response to peroxisome proliferators

Peroxisome proliferators induced hepatomegaly is a result of an increase in size and number of parenchymal cells. PPs mitogenic properties are demonstrated by the induction of DNA synthesis (Styles *et al*, 1988; Roberts *et al*., 1995; Marsman *et al*., 1988). DNA synthesis as measured by bromodeoxyuridine (BrdU) incorporation shows that relatively high doses of MCP (25mg/kg bw i.p.) result in maximal induction 20-30 hours after administration (Styles *et al* 1988). Induction of unscheduled DNA synthesis is limited to the periportal region of the liver acini (Roberts *et al* 1995, Barrass *et al* 1993). DNA synthesis by PPs occurs in the binucleated hepatocyte population which exhibit amitotic cytokinesis to form 4N cells following their stimulation to S-phase, since the proportion of 2x2N cells was reduced while the fraction of 4N cells was increased by MCP treatment (Styles *et al* 1988).

The molecular mechanisms responsible for PP-induced liver growth are not fully understood. Several workers have reported that the proto-oncogenes c-

fos, c-myc and c-jun are not induced after PP dosing. (Coni *et al* 1993 and Hasmall *et al* 1997) suggesting a different mechanism to that involved in compensatory hyperplasia induced by partial hepatectomy or CCl_4 (Goldsworthy *et al* 1994; Coni *et al*., 1993; Hasmall *et al*., 1997).

Section 1.1.6 Chronic exposure to peroxisome proliferators

Sustained exposure to synthetic peroxisome proliferators and the chronic induction of peroxisome proliferation in livers leads to the development of hepatocellular carcinoma in rats and mice (Gonzalez *et al* 1998, Rao and Reddy 1996, Reddy *et al* 1980). The carcinogenic potency of the PP MCP and Wy-14,643 is greater than that of DEHP, as studies have shown that while the former result in a 100% incidence of tumours in rat liver after 12 months, the latter requires higher doses with longer periods of exposure (>2 years. Reedy *et al*., 1982; Rao *et al*., 1984; Cattley *et al*., 1987; Kluwe *et al*., 1982).

Section 1.1.7 Peroxisome proliferators are non-genotoxic carcinogens

Conventional tests for genotoxicity of PPs have failed to detect any direct interaction or damage to DNA. PPs ability to induce hepatocarcinogenesis in rodents is not accompanied by a positive result in the Ames Salmonella mutagenesis assay (Warren *et al*., 1980; Reedy and Lalwani *et al*., 1983; Bentley *et al*., 1987). Furthermore study of the genotoxic properties of PPs have been examined using ^{32}P-post labelling assays for DNA adduct formation (Gupta *et al*., 1985), unscheduled DNA synthesis (Cattley *et al*.,

1986), chromosomal aberrations (Nilsson *et al*., 1991) and DNA repair assay (Butterworth *et al*., 1989). All these *in vitro* and *in vivo* assays for genotoxicity indicate that PPs are not direct mutagens and do not directly damage DNA (Ashby *et al* 1994). PPs have been termed non-genotoxic carcingens to reflect these results and it is unclear how chronic exposure to PPs result in hepatocarcinogenesis. Several hypotheses have been proposed but as yet none have been substantiated.

Section 1.1.8 Models of PP-induced hepatocarcinogenesis

Proposed mechanisms for the initiation of peroxisome proliferation in rodents include the role of the PPARα receptor, and the oxidative stress hypothesis. The identification and role of the PPARα receptor is discussed later (pg 30). The oxidative stress hypothesis proposes that peroxisome proliferation is an adaptive response to the alteration of lipid metabolism and involves the induction of CYP4A enzymes. Peroxisome proliferators have been shown to inhibit fatty acid oxidation (Lock *et al* 1989), form coenzyme A esters (Bentley *et al* 1993), increase levels of fatty acids (Lake and Lewis 1993), and fatty acids from cytosolic fatty-acid binding protein (Brandes *et al* 1990). Due to the differential induction of peroxisomal enzymes involved in the peroxisomal β-oxidation of fatty acids (e.g. acyl-CoA oxidase) by PPs the generation of H_2O_2 byproduct is not balanced by its degradation (Rao and Reddy, 1987 and 1991; Reedy and Rao, 1989, Lake 1993). Excess H_2O_2 may diffuse out of the peroxisome in the cell and form reactive oxygen species that react with DNA to form adducts.

H_2O_2 is normally degraded in the peroxisome by the enzyme catalase. However PPs induce catalase activity by only 2-3-fold above normal levels and under conditions of excessive H_2O_2 production is unable to degrade the reactive byproduct (Nemali *et al*., 1988, Reedy and Rao, 1989). Reactive oxygen species (ROS) generated from H_2O_2 are also cleared by other enzymes whose expression is reduced by PPs. This includes, superoxide dismuatse (Ciriolo *et al* 1982, Elliot and Elcombe 1987); glutathione-S-transferase (Foliot *et al*, 1986; Lake *et al*, 1989; Furukawa *et al*., 1985; Tamura *et al*., 1990b) and glutathione peroxidase (Tamura *et al*, 1990a and 1990b, Furukawa *et al*, 1985).

Weak evidence exists for the hypothesis that PPs increase H_2O_2 concentrations in the cell and lead to DNA adduct formation resulting in mutagenesis. A number of researchers have measured H_2O_2 and acyl-CoA oxidase concentrations under conditions of substrates saturation, and found that although the activity of the latter is high, there is a relatively small increase in intracellular H_2O_2. (Lake 1993, Foerster *et al*., 1981; Handler *et al*., 1988). Formation of DNA adducts after exposure to PPs is also very low. The adduct 8-hydro-2-deoxyguanosine (8-OHdG) was found at levels only 0.5-2.5-fold greater than controls (Kasai *et al*, 1989; Takagi *et al*, 1990 and 1991, Huang *et al*, 1994, Catley and Glover, 1993). In addition studies that use whole liver homogenates contain nuclear and mitochondrial DNA that is 16-fold more susceptible to oxidative damage, this is due to the extensive oxygen metabolism in these organelles (Richter *et al*., 1988). In whole liver homogenate treated with Wy-14,643 , 8-OhdG levels were 2-fold greater than

controls, but this increase included a 3-fold increase in mitochondrial levels. (Sausen *et al* 1995). Isolated hepatic nuclei also showed no increase in 8-OHdG after PP administration (Cattley and Glover 1993). Studies that have examined other measures of DNA damage have also showed that PPs do not signicantly increase the incidence of modified DNA bases or breaks (Elliott and Elcombe 1987, Tamara *et al*, 1991). This body of evidence does not support the mechanism of oxidative stress and DNA damage for PP-induced hepatocarcinogenesis

Section 1.1.9 Peroxisome proliferators and tumourgenesis

Perturbation in normal hepatocyte growth has also been suggested as a mechanism of PP-induced tumour growth in rodents. Hyperplasia as a result of amitotic cytokinesis by PPs maybe acute, chronic or result in preneoplastic lesions (Butterworth *et al* 1991). Increases in cell proliferation at all three stages could increase the frequency of spontaneous mutations and result in clonal expansion. Upon PP administration, rodent liver weights increase during a 2-7 day period as a result of a burst of hepatocyte replication that this localized in the periportal region (James and Roberts, 1996, Prince *et al*, 1992, Melchiorri *et al*, 1993, Ohmura *et al*, 1996, Styles *et al*, 1988, Lake *et al*, 1993; Lalwani *et al*, 1997, Eldridge *et al*, 1990; Styles *et al*, 1988). During PP-induced hepatomegaly there is suppression of apoptosis (Marsman *et al* 1988), which is restored after cessation of PP exposure (Roberts 1996). During PP exposure in rodents in concert with the proliferative response by hepatocytes (i.e. cell proliferation) it has also been shown to suppress

apoptosis in hepatocytes. PPs have been shown to affect the apoptotic process of the liver that may play an important role in the promotion of PP-induced liver carcinogenesis. PPs were found to suppress both spontaneous and TGFβ induced apoptosis in rat hepatocytes (Bayly *et al*., 1993; Roberts *et al*., 1995; James and Roberts, 1996) and in the FAO hepatoma cell line (Bayly *et al*., 1993). It is thought that PPs induce liver cancer by inducing cell proliferation and at the same time suppressing apoptosis. Thus, DNA damaged cells that are normally removed by apoptosis are allowed to survive, and upon PP-induced mitogenic stimulation they may proliferate (clonal expansion) and accumulate further genetic changes that may ultimately lead to liver cancer (Cattley *et al* 1994).

Chronic exposure to PPs also results in cell proliferation but the sustained exposure leading to tumour growth is dependent on PP potency, route of administration and the dose (Marsman *et al*, 1988; Eacho *et al*, 1991, Price *et al* 1992). For example high doses of MCP, nafenopin and Wy-14,643 do induce cell replication while lower doses fail to stimulate DNA synthesis (Price *et al*., 1992; Lake *et al*., 1993). Thus while for potent PPs the magnitude of the sustained increase in cell proliferation result in tumours, this is not the case when the level of exposure (time course) and PP potency is low (Eacho *et al* 1991, Barrass *et al* 1993, Tanaka *et al* 1992).

In models that use preneoplastic foci initiated by genotoxic carcinogens exposure to PPs result in the selective growth of hepatocytes (Cattley and Popp, 1989, Ward *et al*, 1983, 84 and 86; Glaurent and Clarke, 1989). In mice

pretreated with N-nitrosodiethylamine (DEN), DEHP was found to promote initiated focal hepatocellular proliferative lesions (Ward *et al* 1986), When DEN and clofibrate were co-administered to rats there was a significant increase in the number of hepatic tumours formed compared to rats administered DEN alone (Mochizuki *et al* 1983). Age is another determinant of PP-induced tumour progression in rodents. Older animals have a higher incidence of pre-neoplastic lesions and spontaneously initiated cells, which are thought to accumulate over a lifetime. PPs, nafenopin and Wy-14,643 both produce more liver adenomas and carcinomas in older rats than younger rats (Cattley *et al* 1991; Kraupp-Grasl *et al* 1991).

Section 1.2 Cytochrome P450 monooxygenase system

Cytochrome P450 were first identified by Klingenberg (1958) and Garfinkel (1958) as a pigment whose maximum absorption wavelength after binding carbon monoxide was 450nm. To date, 481 P450 genes have been reported. These genes have been described in 85 eukaryote and 20 prokaryote species. Of 74 gene families so far described, 14 families comprise 26 mammalian subfamilies. Each subfamily usually represents a cluster of tightly linked genes widely scattered throughout the genome (http://drnelson.utmem.edu/CytochromeP450.html).

The role of this pigment was later characterized as the terminal component of the microsomal mixed function monooxygenase system of which the other key enzymatic component is NADPH-cytochrome P450-oxidoreductase (Werck-

Reichhart & Feyereisen 2001). P450's metabolise a wide range of chemicals including endogenous substances such as vitamins, steroids, fatty acids and prostaglandins as well as xenobiotics. (Mansuy 1998) For example, P450s can convert fatty acids and to their ω and/or ω-1 hydroxylated form (by the CYP4 family, Gibson *et al* 1982 Bains *et al* 1985), or be involved in drug metabolism (by the CYP2D subfamily) (Distlerath *et al* 1985).

Cytochromes P450 are also found in other organelles such as the inner membrane of mitochondria where they are involved in steroid biosynthetic reactions and are mainly found in steroidogenic organs such as the adrenal glands. Cytochrome P450's catalyse phase I oxygenation reactions in detoxification, a prerequisite for further phase II reactions involving glucoronidation and conjugation with glutathione, sulphate or glycine.

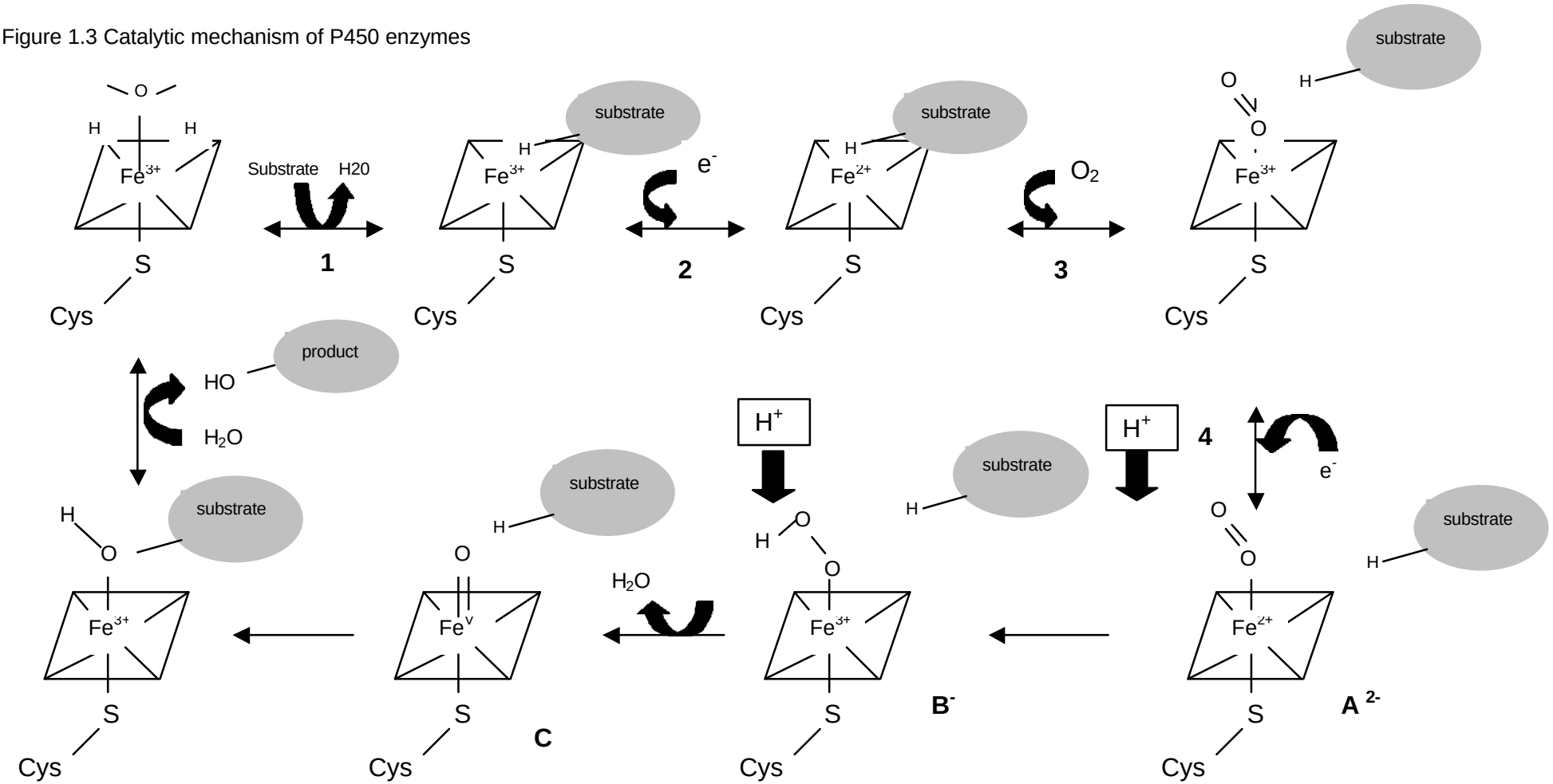
P450-mediated metabolism not only catalyzes regiospecific and stereospecific oxidation of hydrocarbons but other reactions such as oxidative and reductive dehalogenation, N-hydroxylation and N-oxidation, oxidative deamination, S-, N- and O- dealkylation and aliphatic and aromatic hydroxylation are possible depending on the substrate. The most intensely researched of P450's reactions is it's mono-oxygenase activity The active center for catalysis is the iron –protoporphyrin IX (haem) with the thiolate of the conserved cysteine residue as fifth ligand. In the resting state P450's are ferric. The catalytic cycle of the cytochrome P450 is shown in Figure 1.3.

Four steps are involved in the catalytic sequence. Substrate binding occurs first, which results in a change in the absorbance, redox potential and spin state of the haem group. Step 2 involves reduction of the iron atom to the ferrous state by the gain of an electron from NADPH via the reductase. The P450-substrate complex then binds molecular oxygen (step 3) whereby the superoxide is reduced by another electron, again from NADPH reductase. Complex rearrangement of the complex with the insertion of oxygen results in the final product, including either water or hydrogen peroxide.

Substrate specificity and the type of reaction catalysed are governed by the conserved regions of the cytochrome P450 protein and are not well understood.

Figure 1.3: Catalytic mechanism of P450 enzymes. Monooxygenase P450s catalyse the insertion of one of the atoms of molecular oxygen into a substrate, the second atom of oxygen being reduced to water. A major reaction catalysed by the P450s is hydroxylation (O insertion) involving the reactive and electrophilic iron-oxo intermediate (species [C], bottom row). The electrophilic hydroperoxo form of the enzyme (species, $[B^{-}]$) also catalyses OH^{+} insertion. Nucleophilic attack can be catalysed by species $[A]^{2}$ and [B] ; reduction, isomerisation or dehydration are catalysed by the oxygen-free forms of the enzyme. Copied from Werck-Reichhart and Feyereisen (2001)

Figure 1.3 Catalytic mechanism of P450 enzymes



Section 1.2.1 P450 Nomenclature

The P450 super-family are subdivided and classified by Nelson and coworkers (1996) who have created a nomenclature system that is constantly reviewed and updated, on the basis of amino acid identity, phylogenetic criteria and gene organization.

Originally discovered in rat liver microsomes (Klingenberg 1958) P450 genes exist in plants, animals and bacteria and yeast. P450 families are formed from proteins with 40% amino acid sequence identity. Subfamilies contain proteins that share 55% identity homology and P450 clans are formed from P450 genes that have diverged from a single common ancestor (Nelson *et al* 1996). Table 1.3 lists the current number of genes found in a number of organisms.

Organism	Number of P450 genes
Escherichia coli	0
Saccharomyces cerevisiae	3
Mycobacterium tuberculosis	20
Caenorhabditis elegans	80
Drosophilia melanogaster	83
Arabidopsis thaliana	286
Human	55 (25 pseudogenes)

Table 1.3: Approximate number of P450 genes found in organisms. The number of genes reflect the process of gene duplication, amplifications, conversions, genome duplications, gene loss and lateral transfers. E.coli lacks any P450 genes, probably due to it's origin in a oxygen free environment.

P450s maybe divided into four classes based on the source of their reducing electrons. Class 1 proteins require FAD-reductase and an iron sulfur redoxin,

class 2 require FAD/FMN-containing P450 reductase, class 3 proteins require no electron donor, and class 4 proteins receiver electrons from NADPH. Class 2 enzymes are the most common eukaryote P450 enzymes. P450 genes are classified first by the prefix CYP ('*Cyp*' for the mouse and *Drosophil*a) describing it as a cytochrome P450, an Arabic number assigning a P450 family, a letter for the subfamily and an Arabic numeral representing the individual gene.

Of the class 2 enzymes, the primary hepatic drug-metabolising enzymes currently consist of 4 gene families designated 1-4. Mouse *Cyp4a12* refers to the cytochrome P450 gene number 12 of sub-family 4A. Genes of other species are scribed using italics and uppercased names e.g. CYP4A2 (in the rat). cDNA, mRNA and protein for all species are represented by Non-italicized and all capital letters. Although P450 gene family are classified based on 40% or more amino acid identity, exceptions include: the CYP2D subfamily which includes distant members of the CYP2 family, CYP4C1 (in the cockroach) is 32-36% identical to mammalian CYP4A and CYP4B enzymes (Bradfield *et al* 1991), but shares a 13-amino acid peptide exclusive to CYP4As (Bradfield *et al* 1991) and CYP11A1 and CYP11B1 (mitochondrial genes) which share 34-39% identity to the family sequences.

CYP families in the human, rat, and mouse may also be split into two groups: those that show no variation in family or sub-family size (CYP1, CYP5 and higher) and those that exhibit variation (CYP2, 3 and 4). Most drug metabolising

enzymes are variable in size but those that are not are involved in the biosynthesis and metabolism of various endogenous compounds (Nelson 1999).

Section 1.2.2 Cytochrome P450 4A subfamily

The CYP4 family is related to the cholesterol metabolizing enzymes that are thought to have evolved about 1.25 billion years ago (Nerbert *et al* 1987). The Cyp4 gene family was first identified, after the sequence, and cloning of CYP4A1 by Hardwick *et al* (1987). CYP4A1 was found to be expressed in the liver and kidney of the rat and its expression was inducible by the PP, clofibrate [2-(4-chlorophenoxy)-2-methylpropionate]. The subfamily CYP4A encodes several cytochrome P450 enzymes that are capable of hydroxylating the terminal ω- and ω-1 carbon of saturated and unsaturated fatty acids and prostaglandins (Sharma *et al* 1989, Matsubara *et al* 1987). There are currently 26 genes in the CYP4A subfamily (table 1.4) although the current sequencing of the human genome is likely to identify several more CYP4 genes (Simpson *et al* 1997)

The CYP4A subfamily genes are between 10-15kb in size and transcribe to mRNAs of approximately 1.5kb long. CYP4A proteins are comprised of approximately 509 amino acids. Expression of CYP4A varies between tissues and maybe subject to sex or tissue specific patterns.

Table 1.4 CYP4A Subfamily

*: http://drnelson.utmem.edu/CytochromeP450.html

P450	Species	Tissue	References
4A1	Rat	Liver, kidney ileum	Kimura *et al* (1989). Hardwick *et al* (1987)
4A1	Rabbit	Aorta	Irizar & Ioannides (1995)
4A2	Rat	Liver Kidney	Kimura *et al* (1989).
4A3	Rat	Liver Kidney	Kimura *et al* (1989).
4A4	Rabbit	Lung, Kidney, Uterus, Placenta	Palmer *et al* (1993) Yoshimura *et al* (1990) Matsubara *et al* (1987)
4A5	Rabbit	Kidney, Liver, Small intestine	Johnson *et al* (1990) Yokotani *et al* (1991)
4A6	Rabbit	Liver, Kidney	Muerhoff *et al* (1992) Kikuta *et al* (1990) Yokotani *et al* (1991) Johnson *et al* (1990)
4A7	Rabbit	Kidney. Small intestine Liver	Yoshimura *et al* (1990 Acc. No. AB017785) Yokotani *et al* (1989) Johnson *et al* (1990)
4A8	Rat	Prostate, Kidney, Retina	Imaoka *et al* (1990) Stromstedt *et al* (1990)
4A9	Human	Liver	Kawashima *et al* 1994
4a10	Mouse	Liver Kidney	Henderson *et al* (1994) Bell *et al* (1993) Heng *et al* (1997) Yasumura *et al* (1998)
4A11	Human	Liver Kidney	Palmer *et al* (1993) Bell *et al* (1993) Imaoka *et al* (1993) Kawashima *et al* (1994) Kikuta *et al* (1994)
4a12	Mouse	Liver Kidney	Bell *et al* (1993) Heng *et al* (1997) Capdevila J. (1996) Abstract only
4A13	Guinea Pig	Liver	Bell *et al* (1993)
4a14	Mouse	Liver, Kidney	Heng *et al* (1997) Capdevila J. (1996)
4A15	Koala	Liver	Ngo *et al* (2000)
4A16	Cat	Brain	Gebremedhin *et al* (1997)*
4A17	Hamster	Liver	Bell *
4A18	Hamster	Liver	Bell*
4a19	Hamster	Liver	Bell*
4A20	Human		Gottgens B 2000*
4A21	Pig	Liver	Lundell *et al* (2001)*
4A22	Human		Kawashima *et al* (2000)*
4A?	Human		Grafham *et al* (2000)
4A23	Pig	Renal	Van *et al* (2001)*
4A24	Pig	??	Lundell (2001)*
4A25	Pig	??	Lundell (2001)*

Section 1.2.3 Expression of Rat CYP4A genes

CYP4A1 and 4A2 have been identified from genomic clones, while 4A3 and 4A8 are cDNA clones. The CYP4A1 mRNA exhibits constitutive and inducible expression in the adult rat liver and kidney (Hardwick *et al* 1987, Kimura *et al* 1989). CYP4A1 and 4A3 mRNA expression is induced by clofibrate, which like the constitutive expression is found in the centrilobular region (Kimura *et al* 1989, Bell *et al* 1991). However in the kidney the mRNAs of CYP4A1 and CYP4A3 are present at low levels, which are induced to liver levels after PP administration. In rat kidney CYP4A1 expression is localized in the proximal convoluted tubule (Bell *et al* 1991). Rat kidney CYP4A2 exhibits limited clofibrate inducibility (Kimura *et al* 1989). In addition to renal expression CYP4A2 has also been detected in male seminal vesicles, prostate, brain cerebellum, cortex and stem (Stromsteidt *et al* (1994). CYP4A2 is also expressed in a sex-specific manner with CYP4A2 mRNA not expressed in the liver or kidneys of female rats (Sundseth and Waxman 1992). Hepatic CYP4A2 expression is suppressed by exposure to continuously high levels of growth hormone, which is present in female animals. In the kidney CYP4A2 expression is also male-specific but appears to be regulated by tesotosterone (Sunseth and Waxman 1992).

Of the remaining rat CYP4A genes, 4A3 has been found in extraheptic tissues excluding the seminal vesicle (unlike 4A2), and 4A8 has been detected in the prostate, kidney and retina (Stromstedt *et al* 1994 and 1990).

Section 1.2.4 Expression of Mouse Cyp4a genes

Henderson *et al* (1990) determined that mouse hepatic liver microsomes exhibit ω-hydroxylase activity in both control and MCP treated animals. Subsequent immunoblotting using a CYP4A1 antibody, detected an immunoreactive protein. The mouse homologue of CYP4A1, *Cyp4a10* was identified by Bell *et al* (1993) using PCR to generate a cDNA clone. A full-length cDNA was determined later by Henderson *et al* (1994) using a full-length rat CYP4A1 cDNA probe to screen a mouse liver cDNA library. Methylclofenapate (MCP), a PP, was found to induce *Cyp4a10* mRNA in mouse liver and kidney (Bell *et al* 1993). *Cyp4a12* and *Cyp4a14* were identified in mouse liver and found to be homologues of rat CYP4A8 and 4A2 respectively. *Cyp4a14* is highly inducible by PPs in both male and female mouse liver (Heng *et al* 1997).

CYP4A4, 4A5, 4A6 and 4A7 have been isolated in the rabbit and genomic sequences have been determined for CYP4A4 and 4A6. Rabbit 4A6, liver and kidney expression is low while CYP4A7 exhibits high constitutive expression in the liver, kidney and small intestine (Yokotani *et al* 1989; Roman *et al* 1993). Expression is induced by PPs in the liver and kidney for CYP4A6, CYP4A5 and liver for CYP4A7. In the guinea pig a partial cDNA clone was identified by Bell *et al* (1993), this was found to be non-inducible by MCP. Human CYP4A11 is expressed in the liver, kidney and lung (Palmer *et al* 1993, Imaoka *et al* (1993).

The mouse *Cyp4a12* gene is expressed at high levels in the male liver and kidney and was not induced by treatment with MCP. However, there was a low level of expression of *Cyp4a12* mRNA in the female liver and kidney, which was greatly induced by treatment with MCP (Bell *et al*, 1993).

There is conflicting evidence about the sex-specific expression of the Cyp4a protein and LAH activity (table 1.4)

Cyp4a isoform/protein/ LAH activity	Mouse strain	Constitutive expression pattern	Author
Cyp4a12 RNA	CFLP	Male-specific	Bell *et al* (1993)
Cyp4a10 RNA	CFLP	Low level in both sexes	Bell *et al* (1993)
Cyp4a protein	C57BL/6	Male specific	Henderson *et al* (1994)
Cyp4a protein	ddY	Male specific	Hiratsuka *et al* (1996)
Cyp4a protein	C57BL/6 & Balb/c	Found in both sexes	Hiratsuka *et al* (1996)
LAH activity	ddY	Male specific	Hiratsuka *et al* (1996)
LAH activity	C57BL/6 & Balb/c	Found in both sexes	Hiratsuka *et al* (1996
Cyp4a14 RNA	NMC	Low level in both sexes	Heng *et al* (1997)

Table 1.4. Mouse liver Cyp4a isoform, protein or LAH activity. A review of the literature indicates that Cyp4a RNA or protein expression is sex-dependent in a number of studies. However studies that determine Cyp4a protein levels use a non-specific polyclonal antibody to the rat CYP4A1 (Henderson *et al* 1994, Hiratsuka *et al* (1996). Conflicting evidence is derived from those studies that use a ribobrobe specific to a Cyp4a RNA isoform (Bell *et al* 1993 and Heng *et al* 1997).

In ddY mice the hepatic level of Cyp4a related protein and LAH activity was higher in male mice than in female mice. No sex difference was observed in expression of Cyp4a related protein and LAH activity in mouse strains Balb/c and C57BL/6 (Hiratsuka *et al* 1996a). Renal Cyp4a related protein and LAH activity is greater in males than female mice although strain differences were not seen in the animals examined (ddY, Balb/c, C57BL/6, C3H/HeN and C3H/HeJ (Hiratsuka *et al* 1996b and Henderson *et al* 1990). In addition Henderson & Wolf (1991) also

described a female specific Cyp4a related protein detected in little testicular feminized mice (Tfm) when administered pulsatile growth hormone. Tfm animals possess a defunct androgen receptor, producing phenotypically female, yet genotypically males. Therefore not only it is unclear which protein is expressed but is it is also unclear how these genes are regulated.

Male specific expression of hepatic Cyp4a related protein in ddy mice, absence of sex-specific expression in other strains, sex specific renal expression of Cyp4a protein without strain differences and the detection of a female specific Cyp4a protein in a transgenic animals suggest that the Cyp4a gene family is regulated in a sexually differentiated manner. It has been shown previously by RNase protection assay that *Cyp4a12* is expressed at high level in a male-specific fashion in liver and kidney of mouse (Bell *et al* 1993). However *Cyp4a10* has been shown to exhibit no sex- specific expression in the kidney suggesting that the use of polyclonal antibodies derived from CYP4A1, and LAH activity are not sufficient tools to investigate the expression and regulation of the different members of the Cyp4a family. It is also unclear what phenomenon gives rise to differential male-specific regulation of these genes in liver and kidney in the various mouse strains.

Section 1.2.5 Role of CYP4A in fatty acid metabolism and renal function

Research has shown that P450 4A isoforms are the major mammalian arachidonic acid (AA) ω/ω-1 hydroxylases. Table 1.6 shows the metabolism of fatty acids and prostanoids by CYP4A isoforms.

All 4A proteins catalyze AA ω-oxidation and most hydroxylate both the ω and ω-1 carbons. This reaction generates hydroxyicosatetraenoic acids (HETEs), epoxyicosatetraenoic acids (EETs) and dihydroxyicosatet-raenoic acids (DHTs). CYP4A2, 4A3, and 4A8 exhibit different catalytic efficiencys for the production of 20- and 19-HETE (Wang *et al* 1996 and Nguyen *et al* 1999). CYP4A1 exhibits the highest catalytic activity for 20-HETE formation, followed by 4A2 and 4A3. Arachidonic acid is not metabolized by CYP4A8 but catalyses the ω-hydroxylation of short-chain unsaturated fatty acids, such as lauric acid.

In the mouse targeted disruption of *Cyp4a14* gene caused hypertension which was greater in males. Hypertension in *Cyp4a14* -/- mice was sensitive to testosterone and was accompanied by induction of renal *Cyp4a12*, 20-HETE formation and renal vasoconstriction (Holla *et al* 2001).

Arachidonic acids metabolites have been shown to have potent biological activities involved in renal vascular tone and platelet aggregation. For example 2-HETE has been shown to be a potent vasoconstrictor in the renal and cerebral

microcirculations and an inhibitor of vascular calcium dependent K channels, a modulator of Ca^{2+} and Cl^- fluxes and a mediator of EGF, vasopression, parathyroid hormone and norepinephrin signaling (Maier and Roman 2001, Moreno and Maier 2001).

Table 1.6 Metabolism of fatty acids and prostanoids by CYP4A isoforms (Capdevila and Falck 2001)

4A isoform	Species	Enzymatic activities
4A1	Rat	ω-oxidation of laurate and arachidonate
4A2	Rat	ω/ω-1 -oxidation of laurate and arachidonate
4A3	Rat	ω/ω-1 -oxidation of laurate and arachidonate
4A8	Rat	Unknown
4A4	Rabbit	ω-oxidation of palmitate, arachidonate and of prostaglandins A,E,D and $F_{2\alpha}$
4A5	Rabbit	ω/ω-1 -oxidation of laurate and palmitate some ω-oxidation of PGA_1 and arachidonate
4A6	Rabbit	ω/ω-1 -oxidation of laurate, palmitate, arachidonate and Low PGA_1 ω-oxidation.
4A7	Rabbit	ω/ω-1 -oxidation of laurate, palmitate, arachidonate and PGA_1. Inactive towards PGE_2
4a10	Mouse	ω-oxidation of laurate
4a12	Mouse	ω-oxidation of laurate and arachidonate
4a14	Mouse	ω-oxidation of laurate
4a11	Human	ω-oxidation of laurate and arachidonate

Section 1.2.6 Role of CYP4A in peroxisome proliferation

Peroxisome proliferation involves an induction of the enzymes responsible for peroxisomal β-oxidation, and the ω-hydroxylation of fatty acids including CYP4A1 (Bell and Elcombe 1991). The mechanisms, responsible for these changes are unclear but it has been speculated that CYP4A protein plays a pivotal role in peroxisome proliferation. Clofibrate-treatment results in lipid accumulation within the liver, similar to that seen in animals on a high-fat diet (Neat *et al* 1981). This observation supports the substrate overload hypothesis (figure 1.4) where the

fatty acid hydroxylase activity of CYP4A1 results in the production of long chain dicarboxylic acids in the cytosol. Mitochondrial β-oxidation is unable to metabolise these compounds and therefore peroxisomal β-oxidation is the preferred pathway (Kaikaus *et al* 1993). Peroxisomal β-oxidation of dicarboxylic acids results in fatty acids of shorter chain length available for mitochondrial metabolism (Lock *et al*., 1989; Sharma *et al*., 1988; Aoyama *et al*., 1990, Gibson *et al* 1991). CYP4A1 induction precedes acyl-CoA oxidase gene (Bell *et al*., 1991; Bell and Elcombe, 1991). Primary hepatocyte studies using the protein synthesis inhibitor cyclohexamide show that after PP exposure CYP4A1 induction is unaffected but acyl-CoA oxidase activity is absent suggesting that CYP4A1 is required for induction of acyl-CoA oxidase (Milton *et al* 1990).

Figure 1.4 The role of CYP4A1 induction in peroxisome proliferation by hypolipidaemic agents (from Simpson 1997).

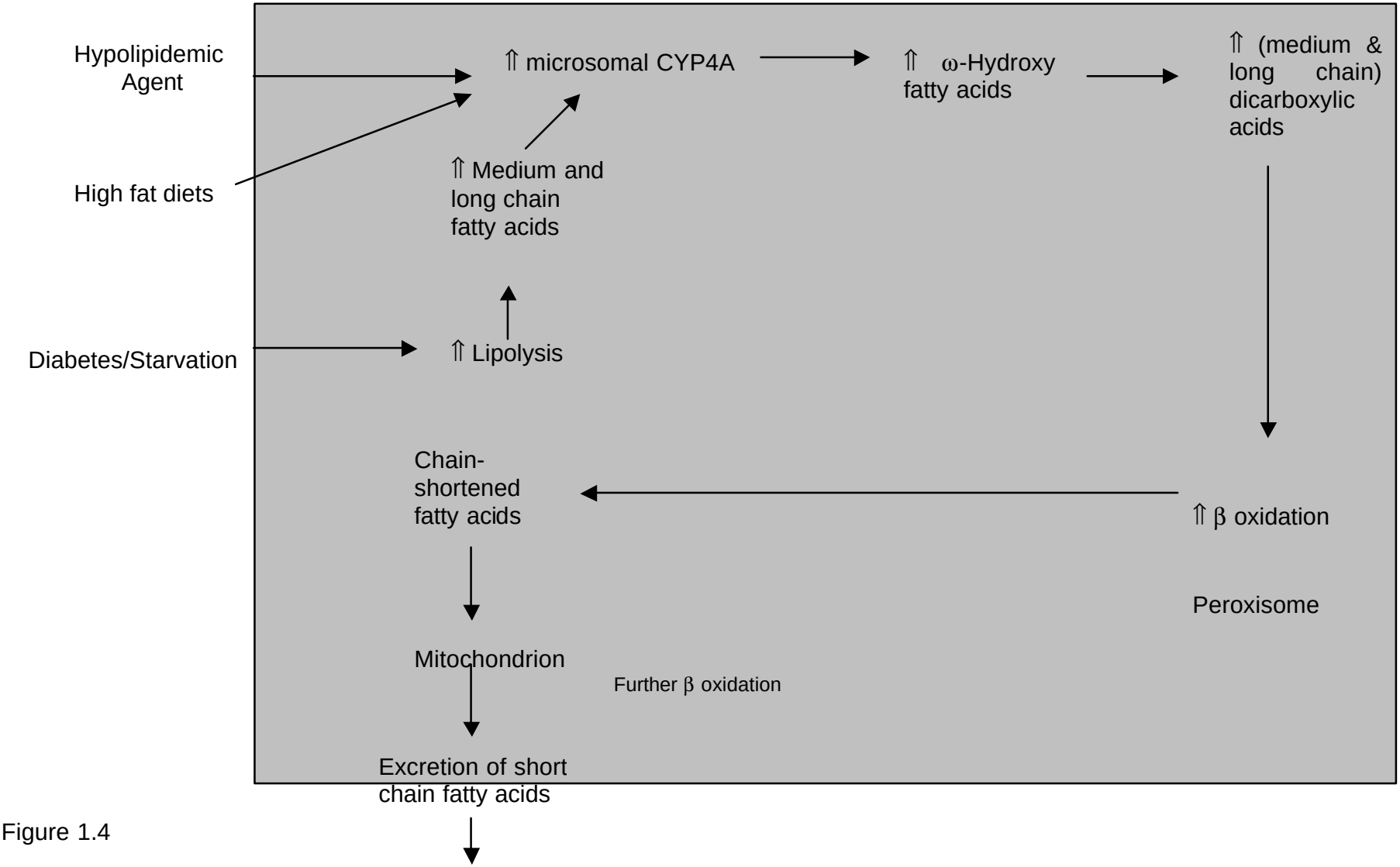

Hypolipidemic Agent
⇑ microsomal CYP4A
⇑ ω-Hydroxy fatty acids
⇑ (medium & long chain) dicarboxylic acids
High fat diets
⇑ Medium and long chain fatty acids
Diabetes/Starvation
⇑ Lipolysis
Chain-shortened fatty acids
⇑ β oxidation
Peroxisome
Mitochondrion
Further β oxidation
Excretion of short chain fatty acids

Figure 1.4

It has been proposed that this induction of the fatty acid and β-oxidation metabolizing systems is an adaptive response by the hepatocyte to maintain cellular lipid homeostasis.

CYP4A knockout mice will provide further information into their role in peroxisome proliferation and physiological systems. This has already been done in the case of *Cyp4a14* -/- mice and the regulation of hypertension by Cyp4a genes (Holla *et al* 2001).

Section 1.3 The steroid hormone receptor super family

Steroid hormone receptors (SHR) are structurally similar intra-cellular receptors that are ligand dependent transcription factors, which regulate gene expression SHRs play an important role in cell differentiation, and homeostasis. The nuclear hormone receptor super family represents the largest group of eukaryotic transcription factors and includes receptors for thyroid and steroid hormones, retinoids and vitamin D (Aranda *et al* 2001). In addition this super family includes receptors for which there is no identified ligand (e.g. NGFI-B). An example of the importance of these SHR is provided by the generation of transgenic mice which contain either mutant or are null for a specific receptor. ERα and β -/- mice provide an example of the importance of these SHR in reproductive function (Krege *et al* 1998, Lubahn *et al* 1993, Couse and Korach *et al* 1999).

SHRs exhibit a modular structure with different regions for functional domains (figure 1.5).

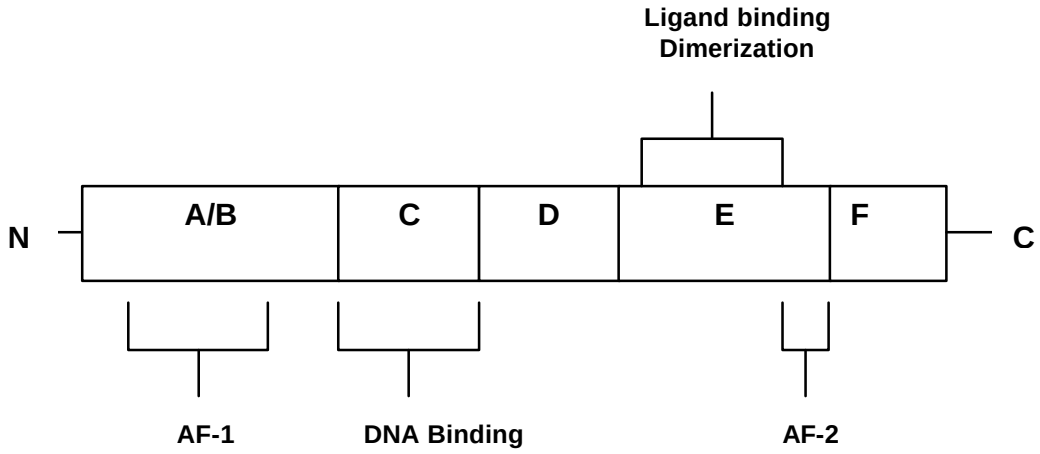


Figure 1.5 Functional domains of nuclear hormone receptors. The variable NH2-terminal region (A/B) contains the ligand-independent AF-1 transactivation domain. The C region is the conserved DNA binding domain (DBD), which is responsible for the recognition of specific DNA sequences. Region D connects the DBD to the conserved E/F region that contains the ligand-binding domain (LBD) as well as the dimerization surface. The A/B region contains the ligand-independent transcriptional activation domain, and the ligand-dependent AF-2 core transactivation domain within the COOH-terminal portion of the LBD.No specific function of the variable C-terminal F domain has been determined. (Modified from Aranda and Pascual 2001).

Section 1.3.1 Peroxisome proliferator activated receptors (PPARs)

Isseman and Green *et al* (1990) first isolated the mouse PPARα (mPPARα) gene using a probe similar to other nuclear receptors to screen a mouse cDNA library. Since then several related PPAR subtypes have been isolated in different species e.g. Xenopus (Dreyer *et al* 1992), Rat (Gottlicher *et al* 1992), Hamster (Aperlo *et al* 1995), and human (Schmidt et 1992, Sher *et al* 1993, Greene *et al* 1995). These receptors are members of the steroid hormone super family of ligand-activated transcription factors. Based on the mechanism of regulation of steroid hormone, it has been proposed that mPPARα mediates peroxisome proliferation in the mouse (Isseman and Green 1990). Full length PPARα cDNA encodes a 468 amino acid protein with a predicted molecular weight of 52kDa.

Based on the analysis of the highly conserved DNA- and ligand-binding domains, the PPARs have been assigned to a subfamily of nuclear receptors that include the retinoic acid receptors (RARs), the thyroid hormone receptors (THRs) and the revErbAα-related orpan receptors (Laudet *et al* 1992, Desvergne and Wahli 1995). Isseman and Green (1990) proposed that PPARα mediates the peroxisome proliferator after it was found that PP transcriptionally actives a reporter gene under the control of the chimeric receptor hER-PPARα which contains the putative ligand binding domain of PPARα and the N-terminal sequence and DNA-binding domain of human estrogen receptor (ER). A Vit-G-CAT reporter plasmid that contains the bacterial chloramphenicol acetyltransferase (CAT) gene was used to screen activators for PPARα which included nafenopin, MCP, clofibrate, Wy-14,643, MEHP and trichloroacetic acid.

Section 1.3.2 PPARs and tissue-specific expression

PPAR may occur in three different isotypes α, β/δ, and γ. In the adult rat and mouse *in situ* hybridization and RNase protection assays showed that PPARα is predominantly expressed in brown adipose tissue, liver, kidney, duodenum, heart, skeletal muscle. PPARγ expression is mostly expressed in brown and white adipose tissues but is also found at lower levels in the large intestine, retina, and immune system. PPARβ/δ is the most ubiquitously expressed PPAR found in greater amounts that α or γ in all tissues except adipose tissue (Jones *et al* 1995, Braissant *et al* 1996). Two distinct isoforms of PPARγ (PPARγ1 and

PPARγ2) have been identified in mouse, rat and human. Both human and rat PPARγ1 and PPARγ2 are homologues of mouse PPARγ1 and PPARγ2 respectively, and have been shown to be the products of alternative promoter usage and differential splicing. In other species PPARγ mRNA expression has also been found in adipose tissue of the rat, mouse and hamster (Kliewer *et al*., 1994 Tontonoz *et al* 1994a and 1994b, Aperlo *et al* 1995; Elbrecht *et al* 1996, Mukherjee *et al* 1997).

Differing levels of PPAR tissue expression suggests that they have different physiological roles. Adipose tissue is the major site of lipid homeostasis and the expression of PPARα and γ suggests that these receptors are involved in adipose tissue differentiation and fat storage. In addition to the involvement of PPARα in peroxisome proliferation, this receptor has been shown to be involved in fatty acid oxidation and ketone body synthesis as demonstrated in fasted PPARα knockout mice (Kersten *et al* 1999, Leone *et al* 1999). PPARγ and PPARα have also been found in atherosclerotic lesions suggesting that both these receptors are involved in the vascular disease (Tontonoz *et al* 1998, Ricote *et al* 1998, Fruchart *et al* 1999).

Section 1.3.3 PPARα activation and mechanism of gene expression

The mechanism of PPARα gene expression is thought to be via the classical pathway for nuclear hormone receptors. In the cytosol PPARα binds to ligand. PPARα form heterodimers with the 9-cids retinoid X-receptor (RXR) (Bardot *et al* 1993, Gearing *et al* 1993). Phosphorylation of the ligand-binding domain of PPARα enhances transcriptional activation (Shalev *et al* 1996, Juge-Aubry *et al* 1999). The PPARα-RXR heterodimer binds to DNA specific sequences, called peroxisome proliferator response elements (PPREs). PPRE's reside in the 5'-flanking sequence of those genes that are regulated by peroxisome proliferators (Tugwood *et al* 1992, Osumi *et al*., 1991, Green, 1992). Functional PPREs are comprised of two copies of the binding motif hexanucleotide TGA(A/C/T)CT, separated by a single nucleotide (Bardot *et al* 1993, Muerhoff *et al* 1992, Zhang *et al* 1992). This arrangement is typical of other nuclear receptor response elements, including those for the thyroid hormone, vitamin D or retinoid X receptors (Umesono *et al* 1991, Yu *et al* 1992). Activated PPARα-RXR binds to the PPRE located upstream of the peroxiosme proliferator target gene leading to an increase in the transcription rate of the gene. Target genes of PPARα include rabbit CYP4A6 (Muerhoff *et al* 1992), rat CYP4A1 (Aldridge *et al* 1995), rat HMG-CoA synthase (Rodriguez *et al* 1994), human fatty acyl-CoA oxidase (see table 1.7).

Gene	PPRE sequence	Reference
Rat CYP4A1	TCCCCT C TGACCT	Aldridge *et al*., 1995
Rabbit CYP4A6 (z-element)	TCAACT T TGCCCT	Muerhoff *et al*., 1992
Rabbit CYP4A6	TGACCC T TGCCCA	Palmer *et al*., 1994
Rat acyl-CoA oxidase	TGACCT T TGTCCT	Tugwood *et al*., 1992
Human fatty acyl-CoA	AGGTCA G CTGTCA	Varanasi *et al*., 1996
Rat acyl-CoA synthase	TGACTG A TGCCCT	Schoonjans *et al*., 1995
Rat malic enzyme	GGACCT G TGCCCT	Castelein *et al*., 1994
Rat fatty acid binding protein	TGACCT A TGGCCT	Issemann *et al*., 1992
Rat acyl CoA binding protein	TCACCT T TGCACT	Elholm *et al*., 1996
HMG-CoA synthase	AGACCT T TGGCCC	Rodriguez *et al*., 1994
Human apolipoprotein A-I	TGACCC C TGCCCT	Vu-Dac *et al*., 1994
Human apolipoprotein C-III	TGACCT T TGCCCA	Hertz *et al*., 1995
Rat apolipoprotein C-III	TGACCT T TGACCA	Hertz *et al*., 1995
Human lipoprotein lipase	TGCCCT T TCCCCC	Schoonjans *et al*., 1996

Table 1.7 PPRE sequences in PPARα responsive genes. Sequences of functional PPRE identified in the promoter region of peroxisome proliferator responsive genes.

Regulation of genes induced during peroxisome proliferation and the ω -oxidation of fatty acids is therefore by PPAR binding to specific DNA regulatory elements located in the upstream promoter region of these genes (Johnson *et al* 1996, figure 1.6). Gene regulated by PPRE's may contain sequences that are not uniform, as they may differ by five nucleotides at a maximum (Table 1.6). Work by Isseman *et al* (1993), Palmer *et al* (1994) and Chen *et al* (1995) indicates that 1-2 nucleotide mutations can reduce or abolish response to peroxisome proliferation.

PPAR-ligand complexes result in conformational changes in receptor that lead to coactivation by cofactors such as steroid receptor coactivator 1 (SRC-1, Nolte *et al* 1998). SRC-1 interacts in a ligand dependent manner and exhibits intrinsic histone acetyltransferase activity, which modifies chromatin structure and

transcription factor gene expression (Onate *et al* 1995, Spencer *et al* 1997, Krey *et al* 1997,Shibata *et al* 1998, Sterner and Berger 2000).

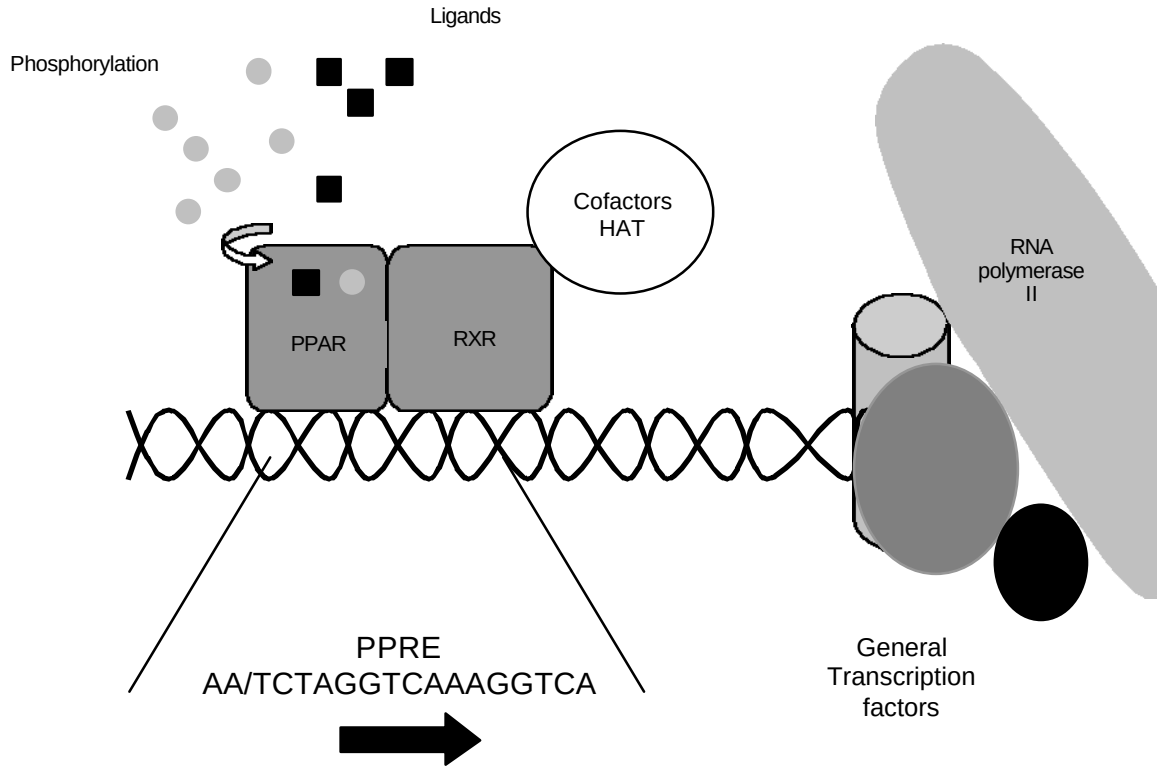


Figure 1.6. Cartoon of PPARα and its heterodimerization partner, RXRα, on a PPRE. The PPAR/RXR heterodimer binds to PPRE located in the promoter region of the PP-responsive gene through the C domain (DNA-binding domain) of PPAR and RXR. Phosphorylation of the receptor A/B and E/F domains regulates receptor activation. PPAR/RXR heterodimers with cofactors (e.g. SRC-1) containing histone acetyl-transferase activity (HAT) modify chromatin structure and transcription of transcription factors (RNA polymerase II).(Modified by Escher and Wahli 2000).

Section 1.3.4 PPARα knockout mice

The generation of PPARα null mice by Lee *et al* (1995) has done much to increase understanding of this receptor in peroxisome proliferation and lipid metabolism. The targeted disruption of the PPARα LBD in these mice results in the loss of functional PPARα protein. PPARα null mice are viable and fertile and

exhibit no detectable gross phenotypic defects but do not display the pleiotrophic effects of peroxisome proliferation, induction of any known target gene, and hepatocarcinogenesis (Gonzalez 1997,Peters *et al* 1997, Gonzalez *et al* 1998). PPARα null mice administered Wy-14,643 or clofibrate do not exhibit induction of acyl-CoA oxidase, bifunctional enzyme, CYP4A and fatty acid binding protein. PPARα null mice display a normal level of peroxisomes compared to wild types. PPARα null mice fed a high fat diet for 10 weeks show massive accumulation of lipid in their livers. Starvation for 24 hours also displayed symptoms of fatty acid metabolism inhibition. These included hypoglycemia, hypoketonia, hypothermia, and elevated plasma free fatty acid levels (Kersten *et al* 1999). This study and others demonstrate that PPARα is a vital regulator of the genes involved in hepatic fatty acid oxidation in both peroxisomes and mitochondria (Aoyama *et al* 1998; Peters *et al* 1997; Leone *et al* 1999, Kroetz *et al* 1998, Hashimoto *et al* 1999).

Bacterial endotoxin (lipopolysaccharide, LPS) increases levels of CYP4A mRNA and protein levels in Fischer 344 and Sprague-Dawley rats (Sewer *et al* 1996). Metabolism of arachidonic acid by CYP4A genes results in the formation of HETEs (McGiff *et al* 1999). These compounds have been shown to be mediators of the inflammatory response (Borish *et al* 1992, Capdevila *et al* 1992). Studies using PPARα null mice demonstrate that PPAR α is required for endotoxin-induced renal expression of the *Cyp4a* gene, and endotoxin-induced inflammation is attenuated in knock out mice (Devchand *et al* 1996 Barclay *et al*

1999). PPARα null mice offer the opportunity to determine the molecular basis of lipid metabolism, inflammation and peroxisome proliferation.

Section 1.3.5 Sex-specific gene expression

Section 1.3.5.1 Cyp2d9

Cyp2d9 is a member of the testosterone 16α-hydroxylase gene family which is 4.5-5.2 kbp in size and consists of nine exons with junctions at identical sites (Wong *et al* 1989, Yoshioka *et al* 1990, Harada and Negishi 1984). *Cyp2d9* expression is male specific and androgen dependent in mouse liver and kidney and shares the same enzymatic activity as the female specific CYP2B gene (Park *et al* 1999). *Cyp2d9* male specific expression in the liver is under the positive influence of pulsatile GH pulses. *Cyp2d9* male-specific gene expression is thought to be regulated by the association between the DNA binding factors NF2d9 and GABP and a cis-acting element designated SDI (sex difference information). This contains a CpG site that is preferentially de-methylated in males (Noshiro *et al* 1986, Yoshioka *et al* 1991, Yokomori *et al* 1995, Gonzalez and Lee 1996).

Section 1.3.5.2 Sex-limited protein (Slp)

The SDI element is shared by another sex-specific gene, Slp. Slp is a mouse serum protein encoded by a major histocampatibility complex class III gene and is thought to be involved in the EDTA-resistant complement activation pathway (Van der Berg *et al* 1992, Beurskens *et al* 1999). Slp is predominantly expressed in male liver and peritoneal macrophages but it is also present in the mammary gland, lung, spleen, and kidney. Lower levels are found in the testis, brain, heart, and submaxillary gland (Cox and Robins 1988). Slp may also be expressed in female mice who are homozygous recessive for the rsl gene (Jiang *et al* 1996). In hypophysectomised mice, Slp expression is refractory to testosterone but not growth hormone. In addition Slp expression is abolished in Tfm mice, which are absent of plasma GH. Hormonal treatment of these mice restores Slp expression independent of testosterone (Georgatsou *et al* 1993). Slp and *Cyp2d9* share the same conserved sequence TTCCGGGC in their 5'-flanking regions in DBA/2 and CD-1 mice. Male-specific gene expression is thought to be regulated by SDI containing a CpG site that is preferentially de-methylated in males and binding of STAT5 (Yokomori *et al* 1995, Varin-Blank *et al* 1998).

Section 1.3.5.3 Mouse urinary protein (MUP)

MUP is a family of α2-microglobulin-related liver secretory proteins that comprise a major protein component of mouse urine. In male mouse urine MUP protein levels are approximately 3-fold greater than in female urine. Male MUP liver mRNA is 6-fold greater than female liver (Derman 1981). Induction of MUP by pulsatile plasma GH has been demonstrated using GH-deficient *Little* mice (Norstedt and Palmiter 1984). Little mice have very low levels of MUP, and GH replacement by intermittent injection (pulsatile administration) increases MUP to normal male levels (Norstedt and Palmiter 1984). MUP and α2u-globulin have both been implicated in male specific hyaline droplet nephropathy caused by 2-sec-butyl-4,5-dihydrothiazole (Lehman-McKeeman *et al* 1998)

Section 1.3.6 Regulation of sex-specific gene expression

To fully understand the interspecies and intraspecies differences in P450 expression, the molecular mechanisms of gene expression is necessary. Several cytochrome P450's are subject to hormonal regulation and sex-dependent expression. In the rat CYP2C11 (testosterone 16α and 2α-hydroxylase exhibits a male specific expression and CYP2C12 (steroid sulfate 15β-hydroxylase) expression is female specific (Sundseth *et al* 1992,Waxman *et al* 1996, Sasaki *et al* 1999, Agrawal and Shapiro 2000).

In the mouse *Cyp2d9* (testosterone 16α-hydroxylase) and *Cyp2a4* (testosterone 15α-hydroxylase) is expressed in the male and female liver, respectively (Wong *et al* 1987, Noshiro *et al* 1986). Sexual dimorphic expression of liver cytochrome P450s is primarily determined by the differential pattern of plasma growth hormone (GH) secretion (Furukawa *et al* 1999). Growth hormone under the control of the suprachiasmatic nucleus (SCN) is excreted in a pulsatile manner in males (interpulse interval ~2.5h) and continuously in females (interpulse interval <1h).

STAT5, a member of the Signal transducers and activators of transcription family (Horvath and Darnell 1997) is activated by pulsatile GH in the rat liver. Using western blotting it has been shown that STAT5 under the influence of pulsatile GH is translocated from the cytosol to the nucleus (Waxman *et al* 1995). Electromobility shift assays (EMSA) demonstrated that pulsatile GH activates the DNA binding activity of STAT5 (Ram *et al* 1996). Two homologous STAT5 genes have been identified, STAT5a and STAT5b (~90% sequence homology; Mui *et al* 1995, Azam *et al* 1995). STAT5 is predominantly 5b isoform (Ram *et al* 1996, Ripperger *et al* 1995). Knockout mice have been generated by target disruption of the STAT5b and STAT5a genes (Udy *et al* 1997, Park *et al* 1999). The characteristic spurt in growth is absent in male STAT5b null mice that exhibited reduced growth from 3 weeks of age. Male-specific gene expression is also reduced in STAT5b mice (Park *et al* 1999). Northern blot analysis revealed that

Cyp2a4 expression was increased in STAT5b null male mice. *Cyp2d9* protein concentrations were reduced in STAT5b null male mice; this protein in wild type mice is expressed in a male-specific pattern in mouse liver under the influence of plasma GH pulses (Udy *et al* 1997, Noshiro *et al* 1986). Levels of mouse urinary protein (see section 1.3.6) were reduced to female levels demonstrating that STAT5b is required for the male specific pulsatile GH induction of MUP. These results indicate that STAT5b, and specifically the formation of STAT5b-STAT5b homodimers play an essential role in mediating sexual dimorphism of body growth rates and liver gene expression. *Cyp2d9* and MUP protein levels were not restored to male wild type levels when hypophysectomized STAT5b null mice were administered pulsatile GH further demonstrating that STAT5b is an important regulator of male specific gene expression (Davey *et al* 1999). STAT5a disruption was found to result in no loss of expression in male mice of *Cyp2d9* and no feminization of male livers (absence of CYP3A induction, Park *et al* 1999).

Work by Zhou *et al* (1999a/b) has investigated the potential cross talk between JAK-STAT (Janus Kinase-Signal Transducer and Activator of Transcription) and PPARα. Using COS-1 cells containing expression plasmids encoding growth hormone receptor (GHR), JAK2 kinase and STAT5b, and mouse PPARα, it was found that GH-activated STAT5b reduces the activation of the CYP4A6 promoter by Wy-14,643. GH was also found to inhibit PPARα induction of enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase. However the mechanism for

these observations is not clear, as no interaction between STAT5b and PPARα, was determined by EMSA. STAT5b and PPARα do not compete for coactivators including SRC-1 or p300 but STAT5b was found to inhibit the ligand independent activation of the AF-1 domain of PPARα as shown using a chimera of yeast GAL4-CAT and AF-1. (Zhou *et al* 1999).

Identification of Cytochrome P450 genes that exhibit sex-specific expression will provide model systems to further elucidate the molecular mechanisms of the GH-STAT5b pathway.

Section 1.4 Aims of the thesis

Section 1.4.1 Cyp4a gene expression

So far three members of the Cyp4a family have been identified in the mouse (Henderson *et al* 1994, Bell *et al* 1993, Heng *et al* 1997). There is conflicting evidence about the sex-specific expression of the Cyp4a protein and LAH activity. In ddY mice the hepatic level of Cyp4a related protein and LAH activity was higher in male mice than in female mice. No sex difference was observed in expression of Cyp4a related protein and LAH activity in mouse strains Balb/c and C57BL/6 (Hiratsuka *et al* 1996a). Renal Cyp4a related protein and LAH activity is greater in males than female mice although strain differences were not seen in the animals examined (ddY, Balb/c, C57BL/6, C3H/HeN and C3H/HeJ (Hiratsuka

et al 1996b and Henderson *et al* 1990). In addition Henderson & Wolf 1991 also described a female specific Cyp4a related protein detected in ‚little™ testicular feminized mice (Tfm) when administered pulsatile growth hormone. Tfm animals possess a defunct androgen receptor, producing phenotypically female, yet genotypically males. Therefore not only it is unclear which protein is expressed but is it is also unclear how there genes are regulated. Male specific expression of hepatic Cyp4a related protein in ddy mice, absence of sex-specific expression in other strains, sex specific renal expression of Cyp4a protein without strain differences and the detection of a female specific Cyp4a protein in a transgenic animals suggest that the Cyp4a gene family is regulated in a sexually differentiated manner. It has been shown previously by RNase protection assay that *Cyp4a12* is expressed at high level in a male-specific fashion in liver and kidney of mouse (Bell *et al* 1993). However *Cyp4a10* has been shown to exhibit no sex- specific expression in the kidney suggesting that the use of polyclonal antibodies derived from CYP4A1, and LAH activity are not sufficient tools to investigate the expression and regulation of the different members of the Cyp4a family. It is also unclear what phenomenon gives rise to differential male-specific regulation of these genes in liver and kidney in the various mouse strains. A more detailed knowledge of the murine Cyp4a gene family is essential for understanding the regulation and physiology of Cyp4a in mice or for establishing strategies for analysing the Cyp4a gene family in transgenic mice.

The aim of this study is to examine the expression of the *Cyp4a12* gene in several inbred mouse strains in order to describe any strain or sex differences and determine whether *Cyp4a12* is the gene, which exhibits sex and strain differences in expression

Section 1.4.2 Immediate early gene (IEG) induction and PP-induced DNA synthesis.

Several studies have shown that the PP, ciprofibrate induces DNA synthesis *in vivo*. Dietary administration of ciprofibrate to C57BL/6 mice for 2 weeks resulted in a 9 and 8-10-fold increase in peroxisomes and β-peroxisomal enzymes respectively. Chronic dietary administration of 0.025% w/w (15 months) followed by 0.0125% w/w ciprofibrate (6 months) resulted in a 57% incidence of hepatic adenomas and 21% hepatocellular carcinomas (Rao *et al* 1988). C57BL/6 and Balb/c male mice administered dietary ciprofibrate from 0.1-250ppm exhibited DNA synthesis at doses of >100ppm and 1ppm respectively over a 4-day period (Budroe *et al* 1992). Male F-344 rats (n=8) fed ciprofibrate (0.025% w/w) for 1-70 weeks exhibited a biphasic pattern on hepatocyte proliferation. Labeling indices of hepatocyte nuclei revealed by [^{3}H]-thymidine labeling, were increased by 35% (with a 170% increase in liver weight) after 1 week dosing. No such increase was seen in animals given ciprofibrate for 5 or 20 weeks. After 40 and 70 weeks labeling indices in ciprofibrate treated rats was significantly different to control animals coinciding with the appearance of preneoplastic changes (Yeldandi *et al*

1989). F-344 rats given 0.025% w/w ciprofibrate for 4 or 7 days showed a 2 and 4.4 induction of DNA synthesis respectively (Rao and Subbarao 1997). As discussed in section 1.1.5 induction of DNA synthesis by PPs coincides with an increase in the 2X2N (binucleated) hepatocyte population (here N=chromosome number, Styles *et al* 1990, Styles *et al* 1990, Styles *et al* 1991). 25mg/kg bw MCP administered by gavage at time points 0,24,48 hours to wild type Snell dwarf mice resulted in an increase of hepatocytes in S-phase which peaked at 36 hours but returned to near control levels after 72 hours (Styles *et al* 1990).

Further to studies investigating PP-induced DNA synthesis in the liver, work has since focused on changes in expression of genes or proteins involved in the regulation of the cell cycle. Ciprofibrate (0.0125% w/w) administered for 2 years resulted in tumours that contained point mutations for the H-ras and K-ras genes dissimilar to those found in tumours caused by genotoxic carcinogens as found by oligonucleotide hydridisation analysis (Hegi *et al* 1993). PPs, nafenopin, and cyproterone acetate induced hepatocyte hyperplasia was not accompanied by an increase in the expression of c-jun or c-fos genes when administered orally (200 and 60mg/kg bw respectively, Coni *et al* 1993). However a more potent PP, Wy-14, 643 increased c-fos c-jun, jun-B and jun-D mRNA expression *in vitro* 1 hour post-administration demonstrating that the route and relative peroxisome proliferation potency determines the induction of IEG (Ledwith *et al* 1993,1996). Induction of CDK1 and PCNA proteins occurred within 24 hours when male Sprague-Dawley rats were administered 45mg/kg bw/day Wy-14, 643 indicating

that PP induce proteins associated with regulation of the cell cycle (Rininger *et al* 1996). IEG expression and induction of DNA synthesis by PP was found to correlate when male F344 rats were given diethylhexyl phthalate (DEHP) and carbon tetrachloride. c-myc mRNA expression was detected 1 hour post-dose providing further evidence for the involvement of IEG in PP-induced DNA synthesis (Hasmall *et al* 1997). In F344 rats administered a single dose of 100mg/kg bw (oral) Wy-14,643, levels of cell cycle proteins CDK1 and PCNA were increased 4.2 and 3.3-fold 48 hours post dose. p53, a protein implicated in regulation of the cell cycle and detection of DNA damage was found to be induced 24-36 hours after dosing (Rininger *et al* 1997). Studies using PPARα knockout mice have demonstrated that this receptor is required for PP-induced hepatocarcinogenesis (Peters *et al* 1998). Immediate early genes and cyclin dependent kinases are candidates for initiating the proliferative response (Holden *et al* 1998) however differences are observed in gene expression, for example c-myc, *in vitro* and *in vivo* (Ledwith *et al* 1993,1996, Belury *et al* 1998).

While these studies have identified genes or proteins that may mediate hepatocyte proliferation during peroxisome proliferation the molecular mechanisms involved are still unclear. This thesis aims to establish a time course for the immediate induction of DNA synthesis by the PP, ciprofibrate and determine changes in gene expression that mediate the hepatic proliferative response.

Chapter 2 Materials and Methods

Section 2.1 Materials

Section 2.1.1 General Materials

All chemicals were obtained at the highest grade possible. [α-32P] CTP (>600 Ci/mmol) was obtained from ICN. DNase-free RNase A>30 units/mg protein was obtained from Boehringer Mannheim. Ciprofibrate (synthesised by Sanofi-Winthrop Ltd, Alnwick, Northumberland, UK.) was a kind gift of Dr.T.J.B. Gray. Oligonucleotides were synthesised by J.Keyte of the Biopolymer synthesis and analysis unit, Department of Biochemistry, University of Nottingham. UHP grade water > 13MΩ/cm was produced using a Purite Select Biosystem. DEPC water was produced by adding 1ml of diethyl pyrocarbonate (Sigma), dissolved in 5ml ethanol, to 1 litre of water. This was allowed to stand for 1 hour at 37°C and was then autoclaved for 1 hour. Sequagel stock acrylamide solution (19:1 acrylamide:bisacrylamide) up to 20% was obtained from National Diagnostics. Lithium chloride , bromophenol blue, Ethanol and formamide were obtained from BDH. Sodium acetate, glacial acetic acid, boric acid, methanol, anhydrous di-sodium hydrogen orthophosphate ammonium acetate, sodium chloride, phenol and chloroform were from Fisher Scientific International. Magnesium Chloride

was from Fisons Scientific Equipment. Dithiothreitol (DTT), 100bp ladder, 1Kb ladder, proteinase K, urea and Tris base were from Gibco Life Technologies. Isoamyl alcohol, sodium dodecyl sulphate, N,N,N',N'-tetramethylethylenediamine (TEMED), ammonium persulphate, imidazole, xylene cyanole, 1,4-piperazine-diethanesulphonic acid (PIPES), N-[2-hydroxyethyl]piperazine -N'[2—ethanesulphonic acid], ethylenediamine-tetraacetic acid (EDTA), spermidine, tetracycline, ampicillin and ethidium bromide were obtained from Sigma. Agarose, EcoRI and SuRE/Cut Buffer H were from Boehringer Mannheim. ADP, dNTP's, yeast tRNA, RNase guard, SP6 RNA polymerase (40300units/ml), RNase A (40μ/mg) and Ficoll were from Pharmacia Biotech. [α-^{32}P]CTP specific activity - 800 Ci/mmol was from ICN. Liquid scintillation counter cocktail was from Parkard. Bacto-tryptone and bacto-yeast extract were from Difco. Klenow DNA polymerase (5-10U/μl), DNase 1 (1μ/μl) and the PGEMT-Easy vector cloning systemwere from Promega.

Section 2.1.2 Plasmids

PCR fragments of *Cyp4a10* and Cyp4a1 in cloned pGEM7 (Promega) were described in Bell *et al* (1993). *Cyp4a14* was isolated as described by Heng *et al* (1997). Genomic clones were initially isolated from a genomic library using riboprobes transcribed from *Cyp4a10* and *Cyp4a12* partial cDNA clones, originally described in Bell *et al* (1993). Since the exon sequences were distinct from both *Cyp4a10* and *Cyp4a12*, this proved that l clone was part of a novel

murine gene. This gene has been designated *Cyp4a14* by consultation with the Cytochrome P450 nomenclature committee A genomic clone of *Cyp4a14* was cloned into pGEM7 in our laboratory to give the plasmid pGEM.4a14. This contains the 3' non-coding region of the gene including all of exon 11 and half of exon 12.

Section 2.1.4 Bacterial Genotypes

The *E.coli* strains below had the following genotypes and were obtained from Stratagene.

XL-1 Blue {recA1 and A1 gyrA96 thi -1 hsdR17 supE44 rel A1 lac [F' proAB lacIq20M15 Tn10(Tetr); XL-1 Blue MRA {D(mcrA) 183, D (mcrCB-hsdSMR-mrr)173,end A1, supE44, thi-1,gyrA96,relA1,lac}; XL-1 Blue Sure{e14-(mcrA-)D(mcrCB-hsdSMR-rnrr)171 and A1 supE44 thi-1 gyrA96 rel A1 lac rec B rec J sbc C unu C::Tn5 (Kar)uvr C [F' proAB lacIq2 DM15 Tn10 (Te^r)}

Section 2.1.5 Storage of Bacterial Strains

Glycerol stocks were made by placing 10% glycerol into the bacterial culture, and storing at -80°C.

Section 2.2 Methods

Section 2.2.2 Bacterial growth Media

Luria-Bertani Broth (LB) consisted of: 10g Bactotryptone, 5g Bacto yeast extract, 10g NaCl, and made up to 1 litre with Ultra High Pure (UHP) water and autoclaved. To make LB-Agar plates; 15g agar was added to 1 litre of LB and then autoclaved. Media was melted, antibiotics added and poured into 10cm petri dishes. Antibiotics were used at the following final concentrations; Tetracycline: 50ug/ml; Ampicillin 10μg/ml; Kanamycin 12.5μg/ml Chloramphenicol 4μg/ml For blue/white selection 40μl of a 20mg IPTG solution and 40μl of 20mg/ml 5-bromo-4-chloro-3-indolyl-b-D-galactoside (X-gal) solution was added per plate.

Section 2.2.2.1 Preparation of competent XL-1 blue *E.coli* cells using CaCl

Single colonies of XL-1 Blue *E.coli* were inoculated in 10ml of LB-Tet/Amp media. Cultures were grown over night in a shaking incubator at 37^{o}C. A 500ml culture of LB/tetracycline medium was inoculated with 1ml of the fresh overnight culture of bacteria and grown until an OD_{600} of 0.6-0.8 was reached (300 cycles/minute in a rotary shaker). For efficient transformation, it is essential that the number of viable cells should not exceed 10^8 cells/ml. To monitor the growth of the culture,

determine the OD_{600} every 20-30 minutes. The cultures were placed on ice for 15 minutes and then centrifuged at 4000g for 15 minutes at 4°C. The supernatant was decanted off and tubes were inverted to allow the last traces of media to drain. Pellets were then resuspended in 1ml ice cold 0.1M $CaCl_2$ and kept on ice. Tubes were then spun at 4000g at 4°C for 10 minutes. Supernatant was decanted off the pellets and the tubes were inverted to drain traces of fluid. Pellets were resuspended in 2ml of ice cold 0.1M $CaCl_2$ and dispensed into aliquots and stored at -80°C. Alternatively the cells were used immediately for transformation (section 2.2.3). The cells maintain competency under these conditions.

Section 2.2.2.2 Transformation of Calcium competent *E.coli*

500µl of competent cells were thawed on ice. 50ng in less than 10µl vol. (0.1M Tris pH 8.0) was added to 100ul of cells and placed on ice for 10 minutes. The cells were then heat shocked at 42°C for 90 seconds and then placed immediately on ice for 2 minutes. 900µl of L-Broth (-Amp) was added to the cells and the cells were cultured for 1 hour at 37°C. Dilutions of cells were then plated out on to L-Broth and Ampicillin and Tetracycline petri dishes. Plates were incubated overnight at 37°C.

Section 2.2.3 Preparation of electro-competent XL-1 blue *E.coli*

Colonies were treated as in section 2.2.2.1, except that after the first centrifugation the pelleted cells were resuspended in 50ml of ice-cold sterile UHP water. Cells were then pelleted by centrifugation at 4000g for 15 minutes at 4^{o}C and re-suspended in ice-cold sterile UHP water a further 5 times. Cells were finally re-suspended in 2ml of sterile UHP water, 20% glycerol and stored as 200μl aliquots at -80^{o}C.

Section 2.2.4 Electro-transformation of XL-1 blue *E.coli*

An aliquot of XL-1 Blue electro-competent cells was thawed and placed on ice for 5 minutes. Electroporation cuvettes and cuvette are pre-chilled on ice prior to use. 10-50ng of DNA was added to 50μl of cells and allowed to incubate on ice for a further 10 minutes. A BioRad Genepulser cuvette (1mm path length), was used to electroporate the cells at a voltage of 1.8kV. The cells were immediately resuspended in 1ml of LB broth and incubated for 1 hour at 37^{o}C. The transformed cells were selected by growing the transformed bacteria on LB agar plates, containing the appropriate selection antibiotics.

Section 2.2.5 Plasmid DNA preparation

Section 2.2.5.1 Plasmid DNA preparation by Alkaline Lysis

Bacterial clones were used to inoculate LB broth containing the appropriate selection antibiotics. The culture was incubated overnight at 37^{o}C. A modified method of the Birnboim 1983 alkaline lysis mini-prep method was used to extract plasmid DNA. 1.5ml of bacterial culture was spun at 15000rpm for 1 minute. The supernatant was discarded and the cell pellet was resuspended in 100μl of solution 1(50mM glucose, 25mM Tris-HCL pH 8.0, 10mM EDTA and RNase A 100μg/ml). 200μl of freshly made solution 2 (200mM NaOH, 1% SDS) was added to each tube and the solutions were mixed by inversion. Tubes were allowed to stand at room temperature for 5 minutes.150μl solution 3 (5M potassium acetate, 11.5% glacial acetic acid) was added and the tubes were placed on ice for 10 minutes, to allow for the precipitation of the bacterial lysate. Samples were then spun at 15000rpm for 10 minutes. Supernatant was added to a fresh eppendorf tube and extracted with phenol/chloroform. (Section 2.2.6) and isopropanol precipitated at room temperature (section 2.2.7). Pellets were washed in 75% ethanol and allowed to air dry. Pelleted plasmid DNA was resuspended in 20μl of UHP water and stored at -20^{o}C.

Section 2.2.5.2 Purification of plasmid DNA using Qiagen columns

Section 2.2.5.3 Mini-preps

Sequencing grade plasmid DNA was prepared using a Qiagen-tip 20 plasmid mini-kit. 3ml of an overnight culture was pelleted by centrifugation at 15000g for one minute. The pellet was resuspended in 0.3 ml of buffer P1 (50mM Tris-HCl pH 8.0, 10mM EDTA and 100ug/ml RNase A). A further 0.3ml of buffer P2 (200mM NaOH, 1%SDS) was added and the solutions mixed thoroughly and incubated at room temperature for 5 minutes. 0.3ml of chilled Buffer P3 (3M potassium acetate pH 5.5) was then added, mixed by inversion and incubated on ice for 10 minutes. The sample was then centrifuged for 15 minutes at 15000 rpm. A Qiagen tip-20 was equilibrated by the addition of 1ml buffer QBT (750mM NaCl, 50mM MOPS pH 7.0, 15% ethanol, and 0.15% Triton X-100). After Buffer QBT was allowed to drain through the column the supernatant resulting from the centrifugation step was placed on to the Qiagen tip-20 column and allowed to drain. Using 1ml of buffer QC (1.0M NaCl, 50mM MOPS pH7.0 and 15% ethanol) the column was then washed four times. Plasmid DNA was eluted from the column using 0.8ml of buffer QF (1.25M NaCl, 50mM Tris-HCl pH 8.5 and 15% ethanol) and collected into a fresh microcentrifuge tube. Plasmid DNA was then precipitated by adding 0.7 volumes of isopropanol at room temperature for 20 minutes. The solution was then centrifuged for 30 minutes at 15000rpm, washed in ice cold 70% ethanol and re-pelleted. The pellet was then air-dried and

resuspended in 25μl of UHP water. Plasmid DNA obtained from this method was of adequate quality to be sequenced by an ABI 373A fluorescent sequencer.

Section 2.2.5.4 Maxi-preps

To prepare larger quantities of sequencing grade plasmid DNA a Qiagen maxi-prep column was used. Typically 500ml of bacterial culture was used and a similar protocol to the above for mini-preps was performed according to the manufacturers instructions. The DNA pellet was re-solubilised in 500μl of UHP water and stored at -20°C.

Section 2.2.5.5 Ligation of DNA

This reaction is catalysed by the presence of T4 ligase, which requires ATP to be present. A balance between the ratios of vector DNA to insert DNA must be found when performing a ligation reaction. The simplest ligation is formed when a 1:1 ratio of vector DNA to insert DNA is formed. Here the reaction volume takes place in 10ml and consists of 4ml of vector DNA, 4ml of insert DNA, 1ml of T4 ligase (1U/μl) and 1ml 5x T4 ligase buffer (250mM Tris-HCl (pH 7.6), 50mM $MgCl_2$, 5mM ATP, 5mM DTT and 25% (w/v) polyethylene glycol-8000) made up with UHP water. The tube was vortexed and then centrifuged for 10 seconds. The solution was incubated overnight at 4◦C. This solution can then either be

used directly for transforming calcium competent cells or ethanol precipitated and resuspended in 10ml UHP water if using electro-competent cells.

Section 2.2.6 Phenol/Chloroform treatment of nucleic acids.

Phenol and Chloroform in equal volumes was added to the nucleic acid sample and vortexed into an emulsion. Organic and aqueous phases are separated by centrifugation at 15000 rpm for 5 minutes. Contaminant proteins partition at the interface between the organic and aqueous phases. The aqueous phase is kept for ethanol precipitation of nucleic acid.

Section 2.2.7 Ethanol and Isopropanol precipitation of nucleic acids.

Two volumes of ethanol or 1 volume isopropanol are added to the nucleic acid sample. In addition ¼ volume ammonium acetate was added to the sample, which was when kept at -20°C to precipitate the nucleic acid. Nucleic acids were pelleted by centrifugation at 15000rpm for 25 minutes and subsequently washed in 75% ethanol. Nucleic acid pellets were air-dried.

Section 2.2.8 Restriction Digest

An analytical restriction enzyme reaction was usually performed in a volume of approximately 20µl on 0.2-1.5µg of substrate DNA using a 2-to 10-fold excess of

enzyme over DNA, based on unit definition. Use of an unusually large volume of DNA or enzyme may give aberrant results. Caution should be exercised to prevent higher than normal concentrations of EDTA and glycerol. The following is an example of a typical analytical single restriction enzyme digestion:

1.Under sterile conditions the following components, in the order stated, was added to a sterile micro-centrifuge tube.

Sterile, nuclease-free water	14µl
Restriction enzyme 10X buffer	2µl
BSA, Acetylated (1mg/ml)	2µl
DNA sample 0.2-1µg, in water or TE buffer	1µl
Restriction enzyme, 2-10U	1µl
Final volume	20µl

2.Mix gently by pipetting. Centrifuge briefly at 15000rpm in a micro-centrifuge to collect the contents at the bottom of the tube.

3.Incubate at the optimum temperature for 1-4 hours.

4.Add 4µl DNA loading buffer, and proceed to gel analysis.

Larger scale restriction enzyme digestions can be accomplished by scaling this basic reaction proportionately. The reaction was then stopped by adding a quarter volume of 7M ammonium acetate (12.5µl) and then 2 volumes of ethanol

(125μl) and placed on ice for 30 minutes. This precipitates the DNA. The tube was then centrifuged at 15000rpm for 5 minutes and the supernatant removed by gentle pipetting. The pellet was then washed with 1ml cold 70% ethanol and centrifuged again for 2 minutes. The ethanol was then removed and the pelleted DNA was then air-dried and re-suspended in 5μl of clean filter sterilised UHP water.

Section 2.2.8.1 Automated DNA sequencing

DNA was purified using a Qiagen mini-prep kit (see section 2.2.5.3) before sequencing could be carried out. J. Keyte, Biochemistry Dept. Nottingham University performed automated sequencing, using the Perkin Elmer ABI 373A Fluorescent Sequencer and an ABI Cycle sequencing Kit. DNA was sequenced on both strands, unless otherwise stated. Sequencing was performed using the forward and reverse primers, T7 and SP6 as well as custom made primersfor oligonucleotide directed sequencing.

Section 2.2.9 Mouse genomic DNA extraction

This method is derived from a protocol by Laird *et al* (1991). 0.5g of liver or 5mm of mouse tail was obtained at necropsy The tissue sample was added to 500μl of extraction buffer (10 mM Tris, pH 7.5, 400 mM NaCl, 100 mM EDTA, 0.6% SDS)

and 35μl of proteinase K (20mg/ml). The tissue was then incubated overnight at 56^{o}C. Equilibrated phenol (Tris pH 8.0) was then added and the mixture was vortexed and centrifuged at 15000g for two minutes at room temperature. The aqueous phase was removed to a new tube and an equal volume of chloroform/isoamyl alcohol (49:1) was added. After mixing vigorously the sample was spun at room temperature at 15000g for two minutes. The aqueous phase was placed in a new tube. 250μl of 7.5M ammonium acetate and 750μl isopropanol was added and the tube was mixed by inversion. Samples were then centrifuged at 15000g for five minutes at room temperature. Pellets were washed in 70% ethanol, centrifuged and air dried. DNA pellets were then resuspended in 400μl UHP water at stored at -20^{o}C. DNA was visualised by 1% TBE-Agarose and quantified by measuring the optical density at 260nm (section 2.3)

Section 2.3 Spectrophotometric Quantification of DNA and RNA

The spectrophotometer was zeroed at 260nm using water as a blank. 1μl of DNA or RNA samples was diluted 1:1000 in water and pipetted into a quartz cuvette. Absorbance readings were taken at 260nm and the concentration of nucleotides was calculated as shown below.

Nucleic acid concentration (μg/ml) = (Absorbance $^{(260nm)}$ X 50) X dilution factor

As $1A_{260nm}$ = 50μg/ml double-stranded DNA

And $1A_{260nm}$ = 40μg/ml single-stranded RNA

Protein and salt contamination can affect the absorbance at 260nm. To examine the effect this could have on determination of nucleic acid concentration a measurement was taken at 280nm. A 260:280nm ratio was then calculated. Values below 1.7 or above 2.0 indicate a sample is contaminated with protein or salt. If this was the case the samples was purified by phenol:chloroform extraction(section 2.2.6) and re-quantified.

Section 2.3.1 Electrophoresis

Section 2.3.2 Agarose electrophoresis of nucleic acids

Electrophoresis in agarose or polyacrylamide gels is the most usual way to separate DNA molecules according to size. DNA and RNA agarose electrophoresis was performed using a submarine Pharmacia GNA-100 gel electrophoresis kit or a Stratagene gel kit. 0.7-2.0% (w/v) agarose gels were prepared by dissolving the appropriate amount of agarose in 1XTBE buffer (45mM Tris HCl, 44mM Boric acid and 1mM EDTA). If a denaturing agarose gel was used to determine the integrity of RNA, 0.1% SDS (w/v) was added after to microwaving. Agarose was dissolved into 1XTBE by microwave heating of the

solution. To allow visualisation of the nucleic acid in the gel ethidium bromide was added to the agarose to a final concentration of 0.5μg/ml. The gel mixture was then poured into the gel casting mould and a comb fitted before setting. After cooling the gel was placed into the gel tank and immersed in 1XTBE. DNA samples were prepared in a 10% solution of 10X loading buffer (30% glycerol, 0.25% bromophenol blue and 0.25% xylene cyanol FF) and loaded into the gel wells. RNA samples were loaded in the same way but with a denaturing 10% loading buffer (90% formamide (v/v), 0.25% bromophenol blue and 0.25% xylene cyanol FF and 1XTBE). At a constant voltage nucleic acid samples were run typically at 6V/cm, until the desired migration had been reached. Gels were visualised by illumination with an UV trans-illuminator.

Section 2.3.3 Polyacrylamide gel electrophoresis

Polyacrylamide gels are used to separate DNA fragments between 2000-6 base pairs. Polyacrylamide gels were made so that they contained a percentage of between 5-20% acrylamide depending upon the size resolution required. Acrylamide gels were run on Mini Biorad Protean II vertical electrophoresis kits. Acrylamide monomer is copolymerised with N,N'methylenebisacrylamide in the presence of a catalyst accelerator-chain initiator mixture. This mixture consists of freshly prepared ammonium persulphate as catalyst (0.1 to 0.3% w/v) together with an equal amount of N,N,N',N'-tetramethylenediamine (TEMED) as initiator.

This mixture is then pipetted into the gel casting kit according to the manufacturers instructions. One hour was allowed for polymerisation after which the comb was removed and the resulting wells flushed with 1XTBE buffer. After loading of the samples the gel was run at 15V/cm. Polyacrylamide gels were post-stained with ethidium bromide (10mg/ml stock) in 100ml of UHP water to give a final concentration of 0.5μg/ml for 15 minutes. The gel was then rinsed in UHP water and visualised by illumination with an UV trans-illuminator.

Section 2.4.1 Genotyping Transgenic Mice by Polymerase Chain Reaction (PCR)

To gain an insight into the function of peroxisome proliferator activated receptor (PPAR) isoforms in rodents, Lee *et al* (1995) have disrupted the ligand-binding domain of the α isoform of mouse PPAR (PPARα) by homologous recombination (HR). This technique introduces HR constructs into embryonic stem cells. The HR construct in this case was the phophoribosyltransferase II gene conferring neomycin resistance To generate mice lacking an mPPARα protein, exons 7 and 8 of the gene which encodes the ligand binding domain of the protein the *neo* gene was inserted and replaced 83bp of the coding region of exon 8. This work produced mice derived from the Sv129 strain (Lee *et al* 1995).

Section 2.4.2 Primer Design for geno-typing of PPARα wild type and null mice.

Primers specific to the exon 8 and *neo* insert were used to amplify PCR products of either 111bp or 180bp respectively. For PPAR α wild type mice the forward primer (P1) has the sequence 5'-GTC TTA ACC GGC CCA ATG TT-3' and the reverse primer's (P2) sequence is, 5'-GTG GCA GGA AGG GAA CAG AC-3'. This reaction amplifies the region between 511-622bp of exon 8. These primers were designed using the EMBL database entry accession number X75294 (*M.musculus* gene for peroxisome proliferator activated receptor alpha exon 8). For PPAR α null mice containing the *neo* insert, the forward primer (N1) had the sequence, 5'-ATG TTT CCA CCC AAT GTC GAG CAA ACC CCG CCC AGC GTC-3'. The reverse primer (N2) has the sequence 5'-CCA TCT TGT TCA ATG GCC GAT CCC ATA TTG GCT GCA GGG TCG C-3'. This reaction amplifies the region between 578-758bp of the *neo* insert. These primers were designed using the EMBL database entry accession number U43612 (cloning vector pMC1neo-polyA, complete sequence).

Section 2.4.4 Optimisation of PCR geno-typing of mPPARα transgenic mice

Optimising the PCR geno-typing of the transgenic mice involved altering the Mg^{2+} concentration of the reaction mix. Reactions were set up between 0.5mM-5mM and the reactions were then run at varying Tm. from 50-64^{o}C. The following reaction cocktail was used for PCR geno-typing of PPAR wild type mice; Taq DNA Polymerase: 1μl (5U, Sigma), 25mM $MgCl_2$ 1-20μl, 10X reaction buffer (without $MgCl_2$, 100mM Tris HCl pH8.3, 500mM KCl) 5μl, Genomic mouse DNA: 1μg, UHP water: up to 50μl, 10mM dNTPs: 1μl, 5'-Primer: 1μl (70pmol), 3'-Primer: 1μl (70pmol). The PCR reaction was overlaid with 50μl of mineral oil to prevent evaporation. PCR was performed on a Perkin Elmer DNA Thermal Cycler 480.It was determined that the optimal conditions for PCR of exon 8 in PPARα wild-type mice were

2 minutes denaturing	95^{o}C	
1 minute annealing	56^{o}C	} 20 cycles
1 minute extending	56^{o}C	
1 minute denaturing	95^{o}C	
5 minutes extending	56^{o}C	

For PPARα null mice a different method was used to amplify the neo insert present. ABgene PCR master mixes, which contain all the components for PCR with the exception of template and primers, were employed. Each master mix contained Thermoprine Plus polymerase (0.025U/μl), Tris HCL pH 8.8 (75mM), $(NH_4)SO_4$ (20mM). Tween 20 (0.01% v/v), dNTPs (200uM). On addition of 1μl of

forward and reverse primer (70pmol) and 25μl master mix the following conditions were used:

2 minutes denaturing	94°C	
1 minute annealing	72°C	} 30 cycles
1 minute extending	72°C	
1 minute denaturing	94°C	
5 minutes extending	72°C	

Samples were stored at -20°C or loaded on to a 12% or 15% acrylamide and post-stained in ethidium bromide (0.5μg/ml). Negative controls of UHP water and C.elegans DNA which does not contain PPARα was included in all PCR reactions.

Section 2.4.3 Sub-Cloning of mouse Slp by PCR

The sequence of the mouse Slp gene is known (Nakayama *et al* 1987). Primers homologous to sequence in exon 3 were designed based on this sequence (figure 2.1, EMBL accession number M12974). The forward primer (Slp1) has the sequence 5'-TTG CTC CTG TTT TCC CCT TCT G-3' and the reverse primer's (Slp2) sequence is, 5'-CTC AAG GCT GAG CAG CAC AAA G -3'. PCR was

performed on genomic DNA from s129 PPARα wild-type mice using the following reaction

2 minutes denaturing	95°C	
1 minute annealing	59.6°C	} 25 cycles
1 minute extending	56°C	
1 minute denaturing	95°C	
5 minutes extending	56°C	

PCR is predicted to generate a 192bp fragment (180-371b.p). The PCR product was cloned into PGEM-T and its identity was confirmed by sequence analysis (not shown).

```
1   GGATCCTCCA GCCATGCGGC TCCTCTGGGG GCTGGCTTGG GTGTTCAGCT TCTGTGCCTC
61  ATCCCTGCAG AAGCCCAGGT CCCGGGGAAG ACACTGGCGT TTGCGTGGGC CAGGGGTGAG
121 AGAGAGTGGG GTGGTGGGGT TCGGCTGGGT CTGAAGATGT ACCTCACTGT TCTCCTAGGT
Slp1
181 TGCTCCTGTT TTCCCCTTCT GTGGTTAATT TGGGGACTCC CCTGTCAGTG GGGGTACAGC
241 TCCTGGATGC CCCTCCAGGA CAGGAGGTAA AAGGATCAGT GTTCCTCAGA AACCCAAAGG
301 GTGGTTCCTG CTCCCCAAAG AAGGACTTTA AGCTGAGCTT GGGAGATGAC TTTGTGCTGC
Slp2
361 TCAGCCTTGA GGTAACCAGT ACCAGCACCC CTGCTGTTTC TCAGGTCTCT CTGCTGCACC
421 CCATTGTCTG CTTATTTAGC AGCAGACAGG AGCTTGTTAT GTGACATAGG CTGGCTTTGA
481 ACTCACAGCT GTCCTCTTGC CTCAGCCCCC AAAGTGCTAA GATTTACAAG TACACCTGGT
541 TTGCCTGTTG CCTTTTGTTT TATTGTTTAG TTTTGCTTTT GAGACGGGAT TTCAGGGTGT
601 AACCTAGATT GATTGAAATT GCTCAGTGAC CCTACTGTGG GCCTCTCAAG TGTTGGGATT
661 ACAGCCGGGA GCCATTGTGC CTGGCACTTC TGTTGCCTTT TCCATCCCTG TCCCCCTCAA
721 TGACTTCCTA CCTCTCTCTC CTCTGGCACA ATGGCCTTGC TGTCCCTACC TTAGGCTCTG
781 TATTGTGTTC CTTTTGCTCT GTTTGATAGT CTCCTTCTGT CTTGATAGGT CCCACTGGAA
841 GATGTGAGGA GCTGTGGCCT CTTTGACCTG CGCAGAGCCC CCTACATCCA GCTGGTAGCT
901 CAGTCTCCGT GGCTAAGGAA CACAGCTTTC AAAGCCACAG AGACTCAGGG TGTC
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Figure 2.1. PCR of the Slp gene. Nucleotides in bold represent the position of primers (Slp1 and Slp2) designed to amplify the DNA segment during PCR.

Section 2.5 RNA Manipulation and Diethylpyrocarbonate treatment of solutions

To prevent degradation of RNA all solutions must be RNase free. Solutions and glassware used in RNA-orientated work were treated with diethylpyrocarbonate (DEPC) to inactivate any RNase by ethoxyformylation. Plasticware can be treated in a 2-3% hydrogen peroxide solution to remove any RNase contamination. DEPC in solution at a concentration of 0.1% v/v is a potent inhibitor of RNase activity. During all work involving RNA DEPC treated water and solutions were used. DEPC stock solution (1:10 in 100% ethanol) was added to give a final concentration of 0.1% (v/v) and incubated at 37^{o}C for 1 hour or at room temperature for 24 hours. DEPC treated solutions were then autoclaved for 20 minutes at 120^{o}C.

Section 2.5.1 Phenol Preparation

Prior to use phenol must be equilibrated to remove contaminant substances and to adjust the pH. At a pH less than 7.8 liquefied phenol's acidic nature prevents efficient separation of proteins from nucleic acids. 500g of crystalline phenol was melted at 68^{o}C and 0.1% (w/v) hydroxyquinoline was added. An equal volume of 0.5M Tris HCl (pH 8.0) was added to the melted phenol and this was stirred for 15 minutes. The phenol was then allowed to separate and the aqueous phase was discarded. 0.1M Tris HCl (pH 8.0) was added to the phenolic phase stirred for 15 minutes. The mixture was allowed to separate again and the pH was

determined. This final equilibration step was repeated until the phenolic phase was greater than 7.8. After equilibration and the final aqueous phase discarded 0.1 volumes of 0.1M Tris HCl (pH 8.0) was added. The Phenol solution was stored under Tris HCl (pH 8.0) in the dark at 4^{o}C for up to one month.

Section 2.6 Isolation of Total RNA from Mammalian Tissue.

The method used to extract total RNA from mammalian tissue is derived from a method first devised by Green *et al* (1976) and modified by Auffray and Rougeon (1980). At necropsy tissue was removed from the animals and flashed frozen with liquid nitrogen. Tissue samples were then stored at -80^{o}C to prevent RNA degradation. Alternatively tissue was placed in a RNase-free poly-propylene tube containing 15ml lysis buffer (6M Urea, 3M lithium chloride, 3M sodium acetate pH 5.2, and 10% SDS). Using a Silverston homogeniser the tissue was homogenised in bursts of 30 seconds to prevent over heating which would lead to denaturing. Homogenate was transferred to a clean RNase-free tube and stored at 4^{o}C overnight. The homogenate was spun for 20 minutes at 11000g at 4^{o}C. Resulting supernatant was decanted off and the clear white pellet re-suspended in 5ml aqueous buffer (DEPC water, 10mM Tris HCl pH 7.4, 0.2% SDS). To this 2.5ml Tris-buffered phenol pH 8.0 and 2.5ml chloroform: isoamyl alcohol (IAA) 24:1 was added and the solution was shaken vigorously on a flask shaker for 15 minutes. The mixture was then spun for 20 minutes at 11000g for 20 minutes at 4^{o}C, and the upper aqueous phase removed to a new tube. Again

2.5ml Tris-buffered phenol pH 8.0 and 2.5ml chloroform:(IAA) was added to the tube and this was mixed and spun as before. The consequent aqueous phase was chloroform extracted with 5ml of chloroform:IAA (24:1), mixed, spun for 10 minutes at 11000g at 4ºC. Supernatant was ethanol precipitated (with two volumes absolute ethanol and ¼ volume sodium acetate pH 5.2) overnight at -20ºC. This was spun at 11000g for 30 minutes at 4ºC and the RNA pellet washed with 70% ethanol. The resulting pellet was air-dried and resuspended in 250µl DEPC treated water. RNA was then stored at -20ºC until required.

Section 2.7 DNA template preparation

Anti-sense transcripts for *Cyp4a10, Cyp4a12, Cyp4a14* was synthesised by *in vitro* transcription of DNA templates. These templates were first prepared by linearising the cloning vector containing the target sequence by restriction digest. pGEM7-4a10 and pGEM74a12 were both linearised using a Eco RI restriction digest. pGEM74a14 was linearised using BamHI restriction digest. The probe lengths for *Cyp4a12* and *10* were 480b.p. and 190b.p. respectively. The protected fragments were 401b.p. and 157b.p respectively. The probe length for *Cyp4a14* is 237.b.p and the protected fragment is 157b.p. A Qiagen maxi-prep 30µg aliquot of template DNA was linearised with 4U of the appropriate restriction enzyme, 5ul of restriction digest buffer and made up to 30ul with DEPC treated water. Visualisation of the linearised template was achieved by running an 1% TBE-agarose gel. Template DNA was then purified by first inactivating the

restriction enzyme by the addition of 100ug/ml of proteinase K and 0.1% SDS (w/v) and incubating at 37ºC for 1 hour. Phenol:chloroform extraction (1:1 v/v) removed protein contamination and the sample was then centrifuged at 15000g at 4ºC for one minute. Purified linear template was precipitated from the aqueous phase by addition of absolute ethanol (two volumes) and ¼ volume of 3M sodium acetate (pH 5.2) for 25 minutes at -20ºC. The sample was then centrifuged at 15000rpm for 25 minutes at 4ºC. The resulting pellet was resuspended in 15ul of DEPC treated water.

Section 2.7.1 *In vitro* transcription of DNA templates

In vitro transcription was performed by adding 4μl 5x TCS (200mM Tris-HCl pH 7.9, 30mM $MgCl_2$, 10mM spermidine, 50mM NaCl), 1μl 100mM DTT, 1μl RNAguard (Pharmacia), 2ul NTP mix (5mM ATP, GTP, UTP), 1μl 100uM CTP, 13μl DEPC treated water, 5μl [α-^{32}P] CTP (12.5μM [α-^{32}P] CTP, specific activity 600Ci/mmol), 1.5ul (1-2μg) template DNA, and 1μl SP6 or T7 RNA polymerase. The transcription mix was vortexed and pulse-spun in a micro-centrifuge and then incubated at either 37ºC or 42ºC for SP6 or T7 RNA polymerases respectively, for 1 hour. To stop the reaction 1μl of DNase I was added and the sample was incubated for a further 25 minutes at 37ºC. The radio-labelled probe was then phenol/chloroform (one volume) extracted and ethanol precipitated. The pellet was washed in 70% ethanol, and after air drying resuspended in 15μl of

deionised formamide. Probes were stored at -20^{o}C. Incorporation of radioactive CTP into the probe and specific activity was estimated using the DE81 test.

Section 2.7.2 Determination of percent incorporation

DE81 ion-exchange filters have a high affinity for polynucleotides and a low affinity for single nucleotides. 1μl of synthesised probe was pipetted to each half of an Whatman DE81 filter and allowed to dry. One half of the filter was washed in 10ml 0.5M Na_2HPO_4 for minute, this was repeated five times each time the solution being replaced. The filter was then washed for one minute, twice in DEPC treated water and twice in absolute ethanol. After air drying both filters were placed into scintillation vials containing 3ml of scintillation fluid and the radioactivity measured for 1 minute on a ^{32}P program using a Packard 1900 TR liquid scintillation analyser. The percent incorporation was measured by calculating the equation below:

$$\frac{\text{cpm washed filter}}{\text{cpm unwashed filter}} \times 100\% = \text{incorporation (\%)}$$

Incorporation efficiency of 5% or greater was acceptable, but was routinely over 40%.

Section 2.7.3 Probe Specific Activity

The specific activity of a probe in a transcription reaction is independent of the yield of RNA. The specific activity of a probe in a transcription reaction is determined solely by the ratio of [^{32}P]-NTP to unlabelled NTP added and is, therefore, independent the yield of RNA.

The actual yield of RNA must be determined experimentally by establishing how much [^{32}P]-CTP was incorporated into transcript product (the ratio of labelled to unlabelled CTP incorporated is assumed to reflect the ratio of labelled to unlabelled CTP available in the reaction). The amount of ^{32}P incorporated into RNA can be determined by the DE81 test (section 2.7.2).

A 30μl transcription reaction contains 5ul of [^{32}P]-C (800Ci/mM, 10mCi/ml) and 1μl of 100μm unlabelled CTP. At the end of the synthesis reaction, 1μl of DNase I, is added, after incubation a DE81 test is performed and the percentage incorporation is calculated.

Section 2.7.4 RNase Protection Assay

This assay involves the preparation of radiolabeled anti-sense RNA probes, hybridisation of these probes to total RNA in solution, and subsequent removal of

un-hybridized RNA by RNase digestion. RNase protection assays were based on a method described by Melton *et al* (1984). The RNase Protection Assay used was obtained from Pharmingen Ltd, commercially available as. Riboquant^{R} An overview of the assay protocol is illustrated in figure 2.2.

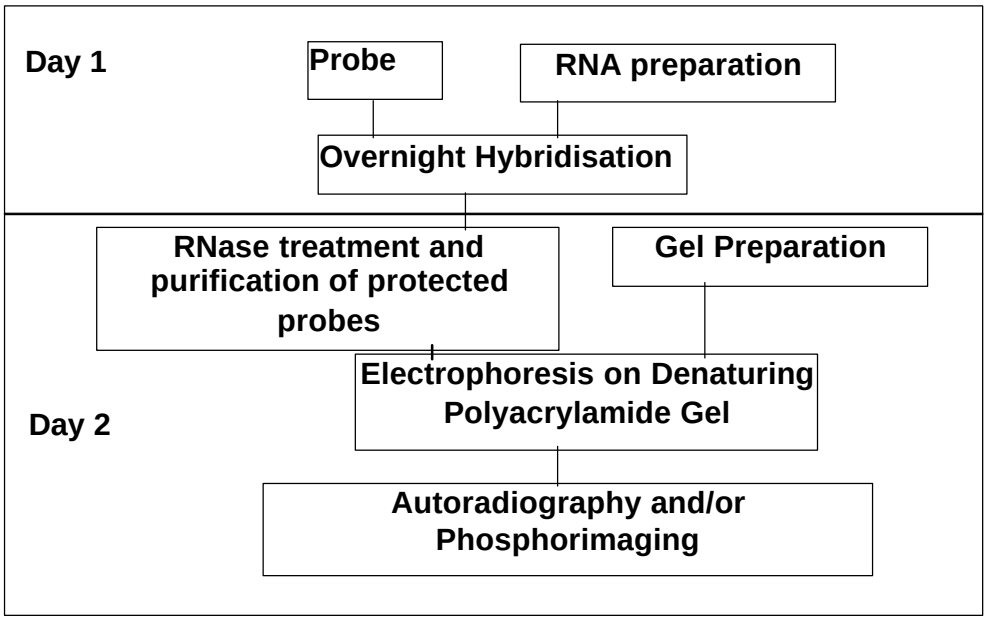

Figure 2.2. Overview of an RNase protection assay

Standard precautions were applied to avoid RNase contamination and exposure to radioactivity. The Riboquant assay system contained the following reagents; 1X hybridisation buffer, 1X RNase buffer, RNase A+T1 (80ng/ml and 250U/µl), 1X Proteinase K buffer, Proteinase K (10mg/ml), yeast tRNA (2mg/ml), 4M ammonium acetate, 1X loading buffer.30µg of total RNA was ethanol precipitated (100µl absolute ethanol, 50µl DEPC treated water, and 15µl 7M ammonium acetate) and the pellets solubilised by vortexing in 8µl hybridisation buffer. Riboprobe was diluted 100-fold in hybridisation buffer and 2µl of this solution was

added to each sample. Samples were topped with mineral oil and pulse spun in a microcentrifuge. Hybridisation was started by placing samples in a water bath at 90^{o}Cfor 3 minutes. Samples were then transferred to another water bath at 56^{o}C and incubated overnight. Controls included in the assay were two 30µg yeast tRNA samples. Following hybridisation 100µl of RNase cocktail (2.5ml RNase buffer, 6ul RNase A+T1) was pipetted beneath the oil layer to each aqueous sample. Tubes were pulse spun for ten seconds and incubated at 30^{o}C for 45 minutes. To a new tube 18µl of Proteinase K cocktail (390µl Proteinase K, 30µl Proteinase K, 30µl yeast tRNA) was added. From the RNase digested samples the aqueous layer was pipetted off and added to the fresh tubes for proteinase K treatment. After addition the samples were vortexed and centrifuged for 10 seconds, before incubation at 37^{o}C for 15 minutes. Each protected sample was then treated with 65µl of Tris-saturated phenol and 65ul of chloroform:isoamyl alcohol (50:1). The samples were vortexed into an emulsion and spun at 15000g for 5 minute at room temperature. The aqueous layer from each sample was pipetted off and added to a new tube. 650µl ice-cold absolute ethanol and 120µl 4M ammonium acetate was added and the samples were mixed by inversion. Samples were then incubated at -80^{o}C for 30 minutes. Samples were then centrifuged at 15000g for 15 minutes at room temperature and the supernatant carefully removed. Pellets were washed with 100µl ice-cold 90% ethanol and centrifuged for 5 minutes at 15000g. Supernatant was removed from the samples and allowed to air dry completely. 5µl of 1X loading buffer was added and the pellets solubilised by vortexing for 2-3 minutes. Tubes were then pulse spun and

heated at 90^{o}C before being placed immediately on ice prior to loading on to a denaturing polyacrylamide gel (section 2.7.5). Also loaded onto the gel was a labelled DNA ladder (section 2.7.7).

Section 2.7.5 Denaturing Polyacrylamide electrophoresis Gel

To separate RNA on absolute size and not secondary structures denaturing acrylamide electrophoresis is used in RNase protection assays. Using a BioRad Protean II vertical electrophoresis kit gels were cast between plates cleaned with detergent to remove any dirt or acrylamide residue 6% gels were prepared by adding sequagel concentrate (23.7g acrylamide, 12.5g methylene bisacrylamide, and 450g urea (7.5M) per litre) and Sequagel diluent (450g urea (7.5M) per litre) The following equation was ueed to calculate the amounts of concentrate and diluent to be used in order to make a fixed percentage gel.

(% acrylamide gel required) x (volume of gel) / 25 = volume of concentrate needed

volume of gel - (1/10th volume of gel +volume of concentrate) = volume of diluent

For a 6% gel 2.88ml of concentrate, 7.88ml of diluent, 1.2ml 10XTBE, 120µl APS, and 7.2µl of TEMED was added and mixed. A 25-well gel was then cast by pipetting the acrylamide mix in between the glass plates and allowed to polymerise for approximately an hour. After setting, the gel was placed in the gel tank and 1XTBE was used to fill the anode and cathode compartments. Prior to

loading samples, wells were flushed with 1XTBE using a needle and syringe. Gels were run at 120V for approximately 90 minutes according to the size of the protected fragments. Gels were then fixed by placing them in 10% (v/v) methanol, 10% (v/v) acetic acid solution for 25 minutes. Fixed gels were then placed on to 3M Whatman paper and dried for 2 hours at 85^{o}C in a Bio Rad 583 flatbed gel dryer under a vacuum.

Section 2.7.6 Autoradiography and Phosphor imaging

Gels were placed in a intensifying screen and exposed to Kodak BioMax MS film for between 12-24 hours. Intensifying screens were stored at -80^{o}C and then film was developed using 1X Kodak LX24 X-ray developer for 3 minutes. Film was then plunged into tap water before fixing in 1X rapid fixer from Ilford Hypam for 3 minutes. The film was again rinsed in tap water and allowed to dry. Films were also placed under a blanked phosphor screen for approximately 12 hours or when samples were within the linear range of the screen. After exposure the phosphor screen was scanned using the GS-250 molecular imager at 100μm intervals. Volume analysis was performed using Bio Rad software version 1.0. Volume analysis is expressed as average radioactive density/mm^{2}. The density of protected fragments was normalised to the density of the negative control at the same mobility. Raw data was exported directly from the software into other data analysis software.

Section 2.7.7 Synthesis of [α-^{32}P] dCTP labelled 100bp DNA ladder

100bp ladder (Gibco BRL, Life Technologies) was labelled with [α-^{32}P] dCTP by a Klenow reaction. 3μl 5X labelling buffer (250mM NaCl, 150mM Tris HCl (pH 7.5), 50mM $MgCl_2$), 3.5μl nuclease free water, 1.5μl 10mM dA, dG, dTTP, 5ul [α-^{32}P] dCTP (3.3μM, specific activity 600Ci/mmol), 5μg 100bp ladder and 1μl Klenow DNA polymerase. The reaction mix was vortexed and briefly spun before incubating at room temperature for 2 hours. After which a DE81 incorporation test was performed (section 2.7.2). 100bp DNA ladder was diluted 1/10 in UHP water and 1μl of this diluted in 10μl of denaturing loading buffer. This sample was heated at 85°C for 3 minutes before loading 5ul to the denaturing polyacrylamide gel.

Section 2.8 Preparation of Microsomal fractions from whole tissue

Throughout the whole procedure the tissue and tissue fractions were kept cold (0-4^{o}C) by using pre-cooled buffers and centrifuge rotors, and by keeping tissue homogenizers, beakers and centrifuge tubes in crushed ice. Fresh tissue (liver or kidney) was homogenised to generate a 25% (w/v) homogenate. The homogenisation buffer contained 0.05M KPO_4 (pH 7.4), 0.15M KCl, 0.6mM EDTA, 0.2mM PMSF (phenylmethylsulfonyl fluoride). 0.05M Potassium hydrogen phosphate was made by adding 80.2ml of 1M K_2HPO to 19.8ml of 1M KH_2PO_4.

Homogenisation took place on ice using a 10ml Potter-Elvejem homogeniser. The pestle was mounted on a drill motor (15/2 Inch Drill Press), which can be rotated between 1000 and 2500 rpm. Homogenate was transferred to Beckman ultracentrifuge tubes and centrifuged at 11000g at 4^{o}C for 20 minutes. Pellets were discarded and supernatant was ultra-centrifuged at 100,000rpm for 1 hour at 4^{o}C. Microsomal pellets were transferred to a 2ml Dounce homogenisation tube and gently resuspended in 1ml of storage buffer (0.01M KPO_4 (pH 7.4), 0.1mM EDTA, 20% glycerol, 0.1mM DTT). Microsomal fractions were stored at -80^{o}C

Section 2.8.1 Spectral Determination of microsomal Cytochrome P450 integrity

The purpose of this protocol is to describe the method for rapid determination of the total Cytochrome P450 (i.e., the sum of the various isoenzymes) in microsomal fractions using a spectrophotometric technique. The principle of the spectral determination of Cytochrome P450 is that the reduced haemoprotein combines with carbon monoxide to give a characteristic absorption spectrum with a maximum at 450nm. Microsomal samples were split into microcentrifuge tubes in 200ul aliquots. Samples were diluted down by addition of 1.8ml UHP water. A few granules (3-4mg) of sodium dithionate crystals were added to each sample and scanned from 500nm to 400nm in a dual-beam spectrophotometer to

establish a baseline. To one sample carbon monoxide was bubbled into the microsomal dilution for 10 seconds. Gassing of the test cuvette with carbon monoxide was carried out soon after dithionite addition, as the reduced ferrous form of Cytochrome P450 is relatively unstable. The samples were then re-scanned at 400-500nm. Samples were assayed on three occasions to have a total of three replicates for each microsomal preparation.

Section 2.8.2 Bradford Assay for determining protein concentration

To determine the concentration of protein samples the Coomassie blue method of Bradford (1967) was employed. Bradford reagent is prepared from 100mg Serva blue G dissolved in 100ml 85% phosphoric acid and 50ml of 95% ethanol, made up to 1 litre. The spectrophotmeter was warmed for 15 minutes before use. 30μl of protein sample was added to 50μl of 1M NaOH and to this 950μl Bradford reagent is added and mixed immediately. Absorbance was measured at 590nm. The absorbance maximum of the dye in an acidic solution shifts from 465nm to 595nm after adding protein due to stabilisation of the anionic form of the dye by both hydrophobic and ionic interactions. The dye principally reacts with arginine residues and to a lesser extent with histidine, lysine, tyrosine, tryptophan, and phenylalanine residues. A standard curve was constructed using bovine serum albumin (BSA) at concentrations between 0-30μg/ml. All assay samples and standards were measured in triplicate and the means determined. A standard curve of BSA concentrations against Absorbance at 590nm produces a linear plot

on what a linear regression can be calculated. R^2 values greater than 0.95 were used and sample concentrations were based on the linear regression.

Section 2.8.5 Measurement of lauric acid hydroxylase (LAH) activity

This assay was performed by using a HPLC method developed by Dr.D.A Barratt and Ms.M,Bruce at the School of Pharmaceutical Sciences, University of Nottingham, University Park, NG7 2RD.

Section 2.8.6 Measurement of DNA synthesis *in vivo* in transgenic mice.

In order to measure the level of S-phase in mouse liver *in vivo*, cells that underwent replicative DNA synthesis were labelled through the administration of a base analogue intra-peritoneally. The method used here involved the base analogue bromodeoxyuridine (BrdU). S129 PPAR α wild type and knockout mice were used to study the role of PPARα in non-genotoxic carcinogenesis. These mice were a kind gift from F.Gonzalez from the National Institute of Health, USA Crystalline BrdU was stored as a stock at -20^{o}C. For dosing intra-peritoneally BrdU was used at 100mg/kg in phosphate buffer and injected into the lower abdomen. BrdU was always dosed 2 hours before sacrifice to allow the BrdU to circulate and incorporate into the tissue.

Section 2.8.7 Dosing of Animals

The peroxisome-proliferator Ciprofibrate was used to dose mice at doses ranging from 12.5-75mg/kg in phosphate buffer. To determine the time course of induction ciprofibrate was administrated over varying time points from 0-48 hours. Another peroxisome proliferator Methylclofenapate was used as a positive control in some experiments at a dose of 25mg/kg in phosphate buffer. Both these drugs were administered intra-peritoneally. During these experiments animals were kept on a 12-hour light cycle at 23^{o}C± 2^{o}C 40-70% humidity. Animals were fed and watered ad libitum. Animals were sacrificed by spinal dislocation and tissue was removed, and fixed in 10% neutral buffered formalin for 2 weeks at 4^{o}C. As a positive control to ensure that the BrdU had circulated into high proliferating tissue, duodenum tissues were also collected.

Section 2.8.8 Histology and Immunohistochemistry of tissue

Fixation with 10% neutral buffered formalin results in the formation of methylene bridges with side chain amino groups of proteins. Processing to histological wax block was carried out according to routine histological procedures. Sections (2um thickness) were mounted on polysine or similar adhesive coated slides and dried at 37^{o}C before staining. Immunohistology for BrdU was performed by dewaxing the sections in xylene for 30 minutes. The section was then taken to eater and treated with freshly made 0.1% trypsin at 40^{o}C for 10 minutes. After rinsing in

water for 5 minutes sections were treated with 1M HCl at 40°C for 30-45* minutes. Sections were then washed twice in water for 5 minutes each and then treated with 3% H_2O_2 (1:10 dilution) for 10 minutes. Sections were rinsed in water for 5 minutes and then in phosphate buffered saline (PBS) pH 7.3 for a further 5 minutes. Slides were treated with normal rabbit serum (1:20 dilution in PBS) for 10 minutes and washed briefly in PBS. Primary antisera, Mouse-anti-Human BrdU (1:100 dilution in PBS) was added for 1 hour and the sections were then rinsed in water twice for 5 minutes each. Secondary Rat-Anti-Mouse immunoglobulins (1:50 dilution in PBS) was added for 20 minutes and again the slides were rinsed in water twice for 5 minutes each. Sections and tertiary antisera, Mouse PAP (1:50 dilution in PBS) was incubated for 20 minutes and then rinsed in PBS twice for 5 minutes each. A Biogenex liquid DAB kit was used to develop the stain for 5-10 minutes£. A counterstain of Harris's haematoxylin was used and applied for 1 minute. Sections were then dehydrated, cleared and mounted.

*Hydrolysis times may vary between mouse tissues, i.e. Pancreas and Duodenum: 30 minutes and liver, kidney and thyroid 45 minutes.

£ The above times changed following microscopical examination of control slides.

In addition to the study slides being stained, positive and negative (exclusion of primary antisera) were included to ensure confidence of the results. To monitor

consistency within each run, a positive slide was included as the first and last slide.

Section 2.9 Quantification of DNA synthesis

In order to prevent reader bias, the printed slide label was printed with a randomised animal number. Slides were viewed under x200 magnification. Nuclei that showed very faint staining were counted as negative. Nuclei that were positive had a blue appearance. In total 1000 nuclei were counted and the number of labelled to unlabelled were calculated as a percentage and designated the labelling index. visualisation under light microscopy (magnification x10).

Chapter 3 Results

Section 3.1 Total RNA extraction *in vivo*

Total RNA was isolated from untreated mouse liver and kidney using the method of Green *et al* (1976) as described in methods. The approximate yield of total RNA from 1g of liver and kidney was 1.2 and 0.5mg, respectively. Figure 3.1 shows the integrity of RNA as analysed by agarose gel electrophoresis.

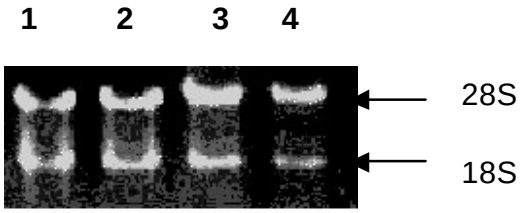

Figure 3.1. Agarose gel electrophoresis of mouse liver RNA. RNA was extracted from 1g of tissue using the method of Green *et al* as described in methods, and re-suspended in 500µl DEPC treated water. 1µl of sample from four different liver samples was run on a 1.0% agarose gel (containing 0.1% SDS and 0.5µg/ml ethidium bromide) at 70V for 2 hours using 1xTBE. The arrow shows intact 28S and 18S rRNA bands. The gel was photographed under UV transillumination.

Section 3.1.1 Extraction of Genomic DNA from mouse tissue

DNA was extracted from mouse livers using a modified technique described by Laird *et al* (1991). This method uses a salt solution to lyse cells with subsequent isopropanol precipitation (figure 3.2), and yields adequate DNA for further analysis.

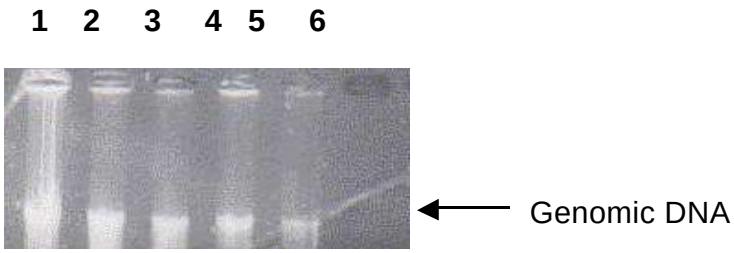

Figure 3.2. Agarose gel electrophoresis of mouse liver genomic DNA. DNA was extracted from 1g of tissue using a technique described by Laird *et al*. (Laird, P. W., A. Zijderveld, K. Linders, M. A. Rudnicki, R. Jaenisch, and A. Berns) and re-suspended in 1µl UHP water.. 1µl of sample from each preparation (samples 1-5 are from five different liver samples) was run on a 1.0% agarose gel containing 0.5µg/ml ethidium bromide at 110V for 2 hours using 1xTBE. The gel was photographed under UV trans-illumination.

Section 3.1.2 PCR genotyping of PPARα mice null and wild-type

Mice were genotyped by polymerase chain reaction (PCR), using two assays designed to discriminate between PPARα wild type and null mice. Genomic DNA was isolated from individual 7-8 week old Sv/129 and mPPARα -/- mice. (Lee *et al* 19975). 20mer primers were designed to amplify a segment of exon 8 of the PPARα gene, which is absent from PPARα -/- mice (Lee *et al* 1996, figure 3.3). Primers specific to 511-622 b.p exon 8 of the mouse PPAR-α gene (Gearing *et al* 1993) were used to amplify a PCR product of 111b.p from wild-type mice.

```
          TCCCCACATC CTTTCTGAAT GGGCACTTCT AAGACTACCT GCTACCGAAA   450
          TGGGGGTGAT CGGAGGCTAA TAGGATTCAG ACAGTGACAG ACAATGGGAG   500
P1        CCCCAGTCTG GTCTTAACCG GCCCAATGTT AATCAATGCA CAGCACTCTA   550
          CGTTGCGTTT ATAATTCGCC ATTAATTAAC GGGTAACCTC GAAGTCTGAG   600
P2        CGGTCTGTTC CCTTCCTGCC ACCCTTCTGG ATATGTGCAC TCTCTTAAAT   650
          CCCTGAAAAC TAATCTGCAC TTTTTAACCT TTGAAAACCT ACAAGTCAAG   700
          GTGTGGCCCA AGGTTAGCCA TTTAAATGTG GCAAAAAAAA AAAAATATGT   750
```

Figure 3.3. PCR of mouse PPARα gene. Nucleotides in bold represent the position of primers P1 and P2 designed to amplify DNA from wild-type mice. Exon 8 in PPARα null mice has been substituted by the neo^r gene.

```
          AAAAAGCCTC TCCACCCAGG CCTGGAATGT TTCCACCCAA TGTCGAGCAG TGTGGTTTTG 540
N1        CAAGAGGAAG CAAAAAGCCT CTCCACCCAG GCCTGGAATG TTTCCACCCA ATGTCGAGCA 600
          AACCCCGCCC AGCGTCTTGT CATTGGCGAA TTCGAACACG CAGATGCAGT CGGGGCGGCG 660
          CGGTCCCAGG TCCACTTCGC ATATTAAGGT GACGCGTGTG GCCTCGAACA CCGAGCGACC 720
N         CTGCAGCCAA TATGGGATCG GCCATTGAAC AAGATGGATT GCACGCAGGT TCTCCGGCCG 780
          CTTGGGTGGA GAGGCTATTC GGCTATGACT GGGCACAACA GACAATCGGC TGCTCTGATG 840
          CCGCCGTGTT CCGGCTGTCA GCGCAGGGGC GCCCGGTTCT TTTTGTCAAG ACCGACCTGT 900
```

Figure 3.4. PCR of neor gene Nucleotides in bold represent the position of primers (N1 and N2) designed to amplify the DNA segment during PCR.

The presence of the PPARα null allele was determined by PCR using 43mer primers complementary to the neo^r gene inserted into exon 8 of the mouse PPARα gene. Primers specific to neo^r were complementary to 578-758b.p of the neo^r gene sequence to amplify a PCR product of 180bp. (Figure 3.4).

In order to obtain a fragment in the wild-type mice optimisation of the PCR was required. Magnesium concentration is a crucial factor affecting the performance of Taq DNA polymerase (Hu *et al* 1992). Reaction components, including template DNA, chelating agents present in the sample (e.g. EDTA or citrate), dNTPs and proteins, all affect the amount of free magnesium. In the absence of adequate free magnesium, Taq DNA Polymerase is inactive. Conversely, excess free magnesium reduces enzyme fidelity (Eckert *et al* 1990.) and may increase the level of non-specific amplification (Williams *et al* 1989, Ellsworth *et al* 1993). For these reasons, it is important to empirically determine the optimal $MgCl_2$ concentration for each reaction. There is no detectable amplication from *C.elegans* DNA (lane 6) demonstrating that the primers used are specific to PPARα from mouse. No PCR product in the reaction lacking any input DNA (lane 7) demonstrates that there is no contamination of the PCR mixture with mouse PPARα gene. A magnesium concentration of 2.0mM was found to be optimal for amplification of the PCR product when inputting 1μg of genomic DNA (figure 3.5). 2.0mM Mg^{2+} was used in subsequent PCR with these primers.

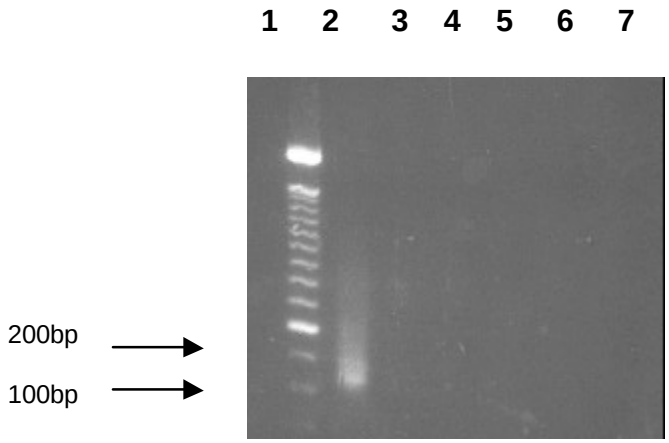

Figure 3.5. Optimization of PCR for PPARα gene. Primers specific to exon 8 of mouse PPAR-α was used to amplify a PCR product of 111bp from S129 mouse genomic DNA. Lane 1 is a 1KB Plus DNA ladder. 1µl of sample from each preparation (samples 2-6) was run on a 1.0% agarose gel at 110V for 2 hours using 1xTBE. The gel was photographed under UV trans-illumination. Lanes: 1 100bp ladder, 2: 2.0mM, 3: 2.5mM, 4: 5.0mM, 5:10mM, 6: *C.elegans* genomic DNA, 7:negative control: H_2O.

A region (511-622b.p) of exon 8 of the mouse PPARα gene was amplified from mouse genomic DNA. Figure 3.6 shows that these primers amplify the sequence to generate a 111b.p. product which is the size expected from the sequence (Gearing *et al* 1994). No amplification product is present in lane 2, which contained genomic DNA from a PPARα null homozygous mouse. PCR genotyping for the PPARα wild- type gene was thus able to discriminate between wild type and knockout mice.

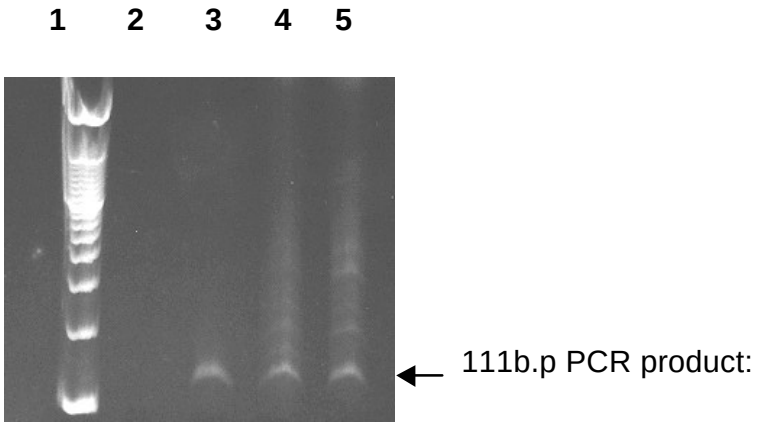

Figure 3.6 PCR genotyping for mPPAR-α+/+ mice. Primers specific to exon 8 of mouse PPAR-α was used to amplify a PCR product of 111bp. Lane 1 is a 100bp DNA ladder.PCR reactions were carried out on a PPARα -/- mouse (lane 2). Lanes 3-5 are samples from Sv/129 mPPAR-α +/+ mice. 1µl of sample from each preparation (samples 1-5) was run on a 15% polyacrylamide gel at 110V for 2 hours using 1xTBE.

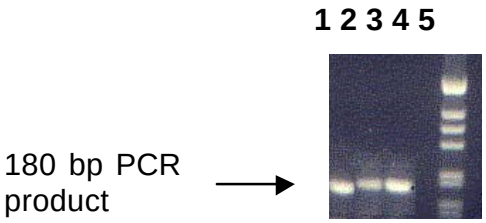

Figure 3.7. PCR genotyping for neo^r gene. Primers specific for the *neo* gene was used to amplify a PCR product of 180bp Lane 5 is a 1KB Plus DNA ladder. Lane 1-3 are genomic DNA samples from Sv/129 mPPAR-α-/- mice. Lane 4 is a sample from a Sv/129 mPPAR-α +/+ mouse. 1µl of sample (samples 1-5) was run on a 15% polyacrylamide gel at 110V for 2 hours using 1xTBE and post-stained with 0.5µg/ml ethidium bromide. The gel was photographed under UV trans-illumination.

PPAR-α -/- mice homozygotes have had the mPPAR-α gene, disrupted by replacement of exon 8 of the ligand-binding domain by phosphoribosyltransferase II gene conferring neomycin resistance (Neo; derived

from plasmid pMC1NeoPolyA; Stratagene) inserted in the opposite direction of transcription of the genomic clone. (Lee *et al* 1996) Thus primers were designed to amplify the phosphoribosyltransferase II gene. PCR yielded a product of 180b.p (figure 3.7 lanes 1-3.) as expected from the sequence of the neor gene (Lee *et al* 1996). Lane 4 is the product of the PCR of PPARα wild type mice; no DNA product is visible demonstrating that wild type mice do not contain the neo^r gene and confirming the specificity of the primers. Genotyping by PCR was completed by inclusion of negative controls including a genomic DNA from *C.elegans* which does not contain the homologous sequence and DEPC-treated water which should not contain DNA. PCR of both these negative controls did not result in a DNA product for both PPAR-α and neo^r.(data not shown).

Primers were designed to amplify mouse PPAR-α exon 8 and the phosphoribosyltransferase II gene, and it was shown that these were specific for wild-type and knockout mice respectively. It was possible to determine that the colony is composed of pure homozygous PPARα +/+ and -/- mice. In order to confirm the integrity of the colony, breeding pairs were genotyped after a litter and their offspring were used for subsequent experiments.

Section 3.2 Constitutive expression of murine Cyp4a genes.

It has previously been shown that there is male-specific hepatic expression of a Cyp4a protein (Hiratsuka *et al* 1996) in ddY mice, although data from a variety of

sources appears conflicting (see introduction pg 23). The aim of this study was to examine the expression of the *Cyp4a12* gene in several inbred mouse strains in order to describe any strain or sex differences and determine whether *Cyp4a12* is the gene that exhibits sex and strain differences in expression.

Section 3.2.1 Template preparation and *in vitro* transcription

Probes were designed from 3' half of the gene (nucleotides 1474-1711 for *Cyp4a14*, and 958-1380 for *Cyp4a12* and *Cyp4a10*), have previously been shown to be specific for their respective genes (Bell *et al* 1993, Heng *et al* 1997. *Cyp4a10* and 12 anti-sense probes were transcribed from the SP6 promoter of EcoRI digested templates, which gave 190bp and 480bp full-length probe, and 157bp and 401bp protected fragments, respectively. The anti-sense probe for *Cyp4a14* was transcribed from the T7 promoter, producing a full-length transcript of 237bp and a protected fragment of 157bp. *Cyp4a10*, 12 and 14 clones were in the pGEM7 vector (Figure 3.8).

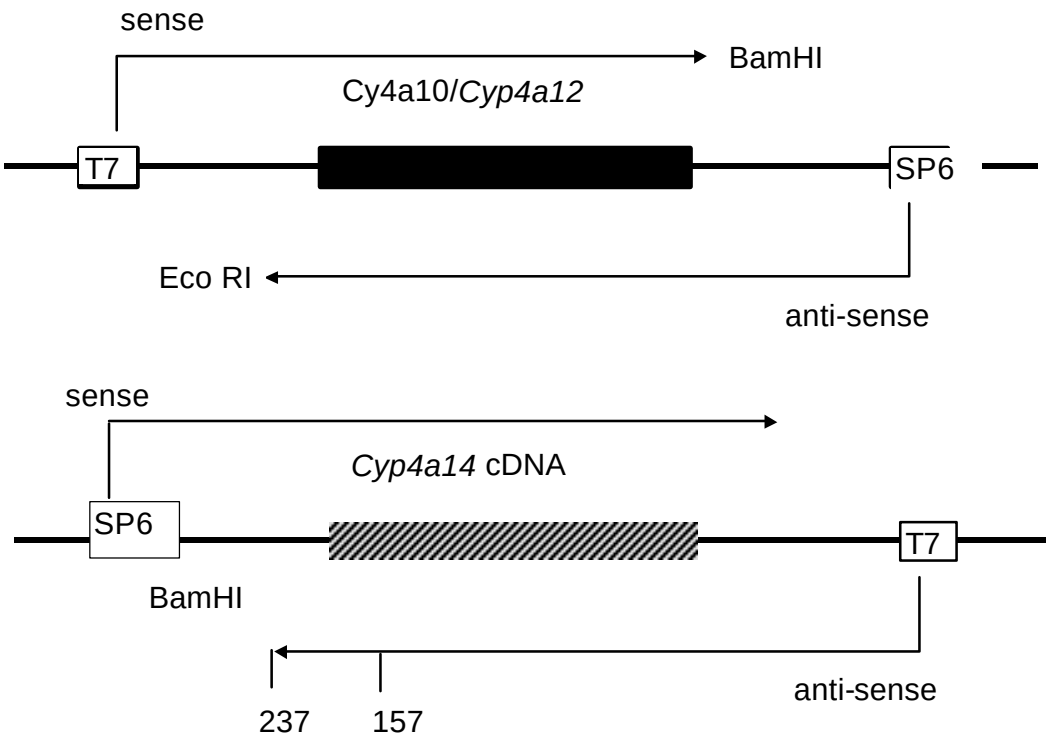

Figure 3.8 Cyp4a gene templates for *in vitro* transcription. Fragment of the 3' end of the Cyp4a was cloned into the polylinker of the pGEM7 cloning vector. This vector contains bacteriophage polymerase promoters, which allow the synthesis of "run-off" transcripts. *In vitro* transcription of anti-sense riboprobe of *Cyp4a10* is generated from EcoRI digested template by SP6 polymerase yielding a 190bp full-length probe of which 157bp corresponds to the cDNA insert. *In vitro* transcription of anti-sense riboprobe of *Cyp4a12* is generated from EcoRI digested template by SP6 polymerase yielding a 480bp full-length probe of which 401bp corresponds to the cDNA insert. Anti-sense riboprobe of *Cyp4a14* is generated byT7 polymerase *in vitro* transcription of BamHI cut template, resulting in a 237bp probe length and a 157bp insert.

Section 3.2.2 *Cyp4a12* in inbred mouse strains

In order to analyse the strain and sex differences of *Cyp4a12* RNA expression in the liver and kidney of eight inbred mouse strains, RNase protection assays were

performed (figure 4.0). Two strains were assayed at a time, and the same riboprobe was used in all four experiments and prior to contemporaneous data acquisition. Expression of *Cyp4a12* RNA was compared between sexes by calculating the ratio of male versus female from phosphor image analysis (figure 4.1)

The *Cyp4a12* probe was shown to be substantially full-length (RNase –ve track), and to be present in excess over the signal in test samples (figure 3.9). The RNase positive track demonstrates an absence of signal when hybridised to yeast tRNA, demonstrating the specificity of the signal in samples. Finally, the size of the protected fragment in mouse samples was substantially shorter than the full-length probe; in agreement with RNase removal of 79bp of unprotected probe this provides additional information of the specificity of the assay.

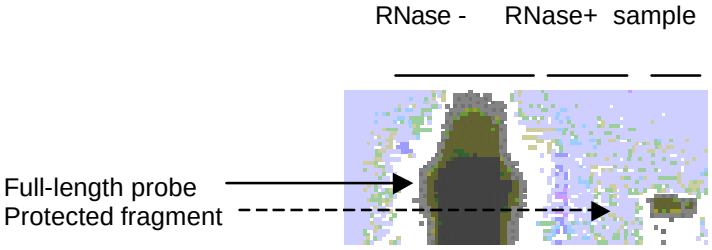

Figure 3.9. RNase protection assay specificity. Riboprobe was hybridisation to yeast tRNA and treated with RNase A/T1. No detectable signal was observed after yeast tRNA hybridisation with probe and RNase A/T1 treatment verifying that the radioactive signal observed in test samples was due to specific hydridisation of probe to RNA. Yeast tRNA was also hybridised to probe without RNase treatment to confirm that the input probe was in excess, that the probe was substantially intact, and that the reagents were RNase free. The size of protected fragment in the mouse sample (30µg total RNA) was substantially shorter than the full-length probe in agreement with RNase removal of 79bp of unprotected probe.

The specificity of the assay was shown by hybridisation of the riboprobe to yeast tRNA. No detectable signal was observed after yeast tRNA hybridisation with probe and RNase A/T1 treatment verifying that the radioactive signal observed in test samples was due to specific hydridisation of probe to RNA. Yeast tRNA was also hybridised to probe without RNase treatment to confirm that the input probe was in excess, that the probe was substantially intact, and that the reagents were RNase free.

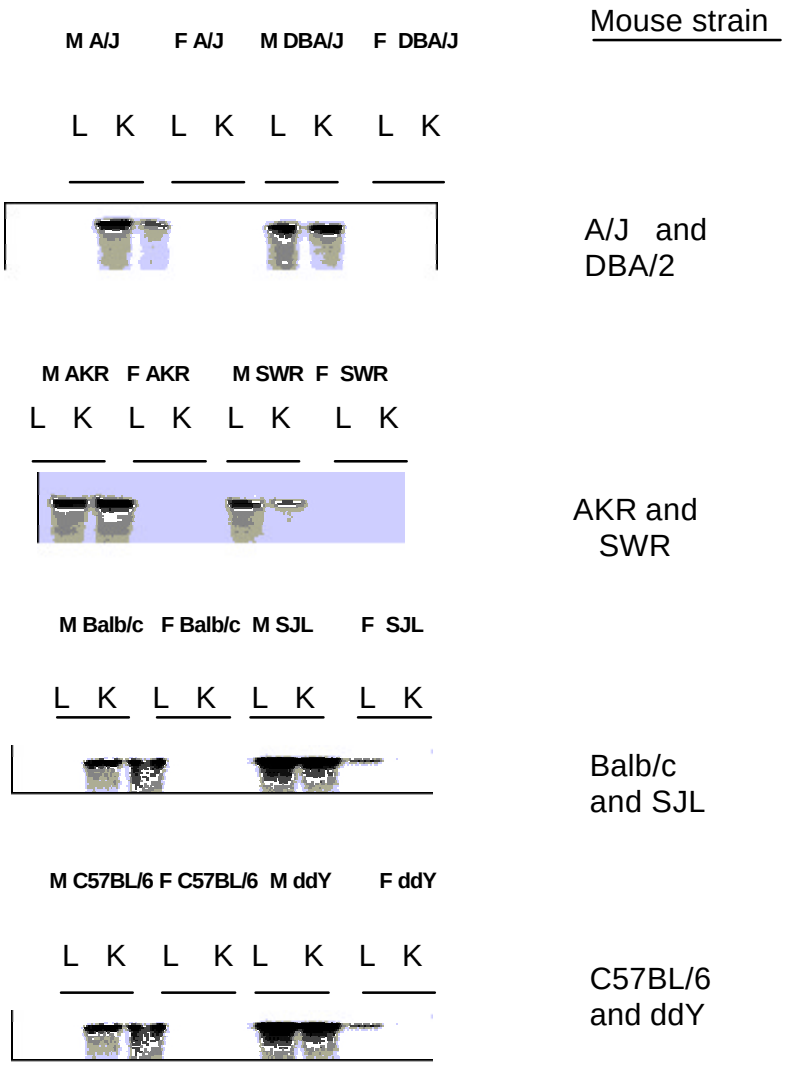

Figure 4.0 Expression of *Cyp4a12* in mouse liver and kidney. RNase protection assay was performed on 30µg total RNA of untreated mouse liver (L) and kidney (K) (M) denotes male and (F) female tissue samples, as described in the methods (pg 72). A 480bp length probe was generated which gave a 401bp protected fragment representing the *Cyp4a12* sequence. Two control yeast tRNA samples were hybridised to the probe, without or with RNase A/T1 (-veor +ve). All RNase protection assays were analysed contemporaneously. This data is representative of 2 assays performed on 2 independent mice.

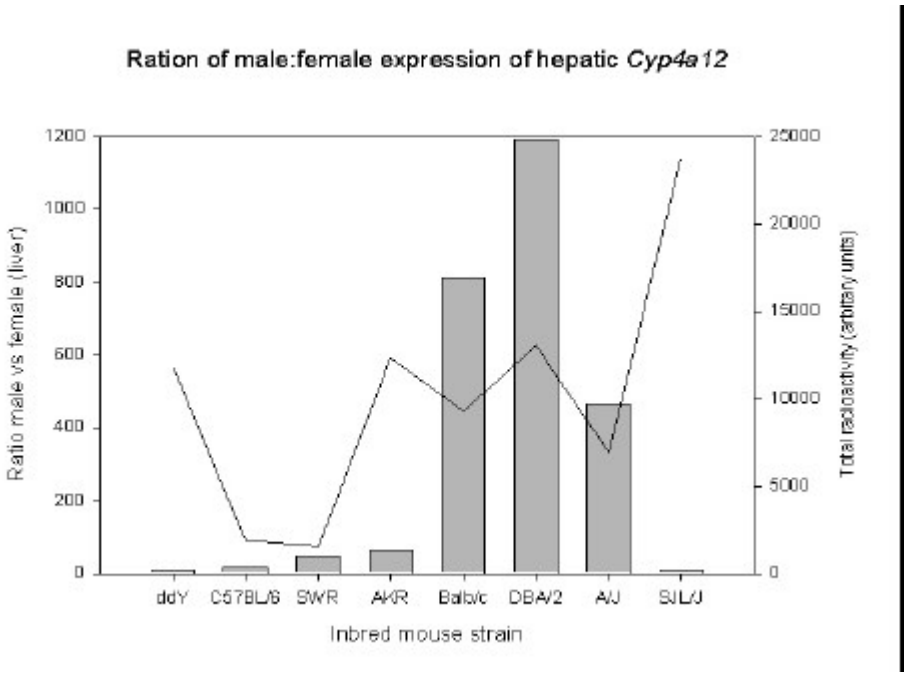

Figure 4.1 Ratio of male: female expression of hepatic *Cyp4a12*. Radioactive signal from each strain is obtained by phosphorimage analysis onf the gel from figure 3.8. Data points are adjusted for tRNA background and are from equal areas on the gel. Ratios (left Y-axis and bars) are calculated by dividing total radioactivity (right Y-axis) in the male with total radioactivity in the female. This provides an indication of how male expression compares with female expression of *Cyp4a12*. The line plot is the male level of *Cyp4a12*.

Sex differences in hepatic *Cyp4a12* RNA were examined by calculating the ratios of male to female expression (figure 4.1). DBA/2 mice exhibited the greatest ratio of 1190:1 (male vs female). Balb/c displayed a ratio of 810 (male vs female). A/J male mice had *Cyp4a12* levels 465 times greater than females. The remaining strains also exhibited sex-specific expression of *Cyp4a12* with ratio of 7.5, 16, 48, 63, 9.6 for ddY, C57BL/6, SWR, AKR, and SJL/J respectively. Therefore there is sex specific expression of *Cyp4a12* RNA in all strains examined, but the ratio between male and female expression showed variation between the strains.

Strain variations in male specific expression were observed Balb/c mice had a 5-fold greater level of *Cyp4a12* than C57BL/6 and DBA/2. Also SJL/J mice had a 15-fold greater *Cyp4a12* level than SWR mice. ddY mice exhibited 6- and 1.2-fold greater *Cyp4a12* expression than C57BL/6 and Balb/c mice respectively so ddY mice have greater levels of hepatic *Cyp4a12* RNA than C57BL/6 and Balb/c mice. Another conclusion is that the ratio of male:female is not simply a reflection of the absolute level of *Cyp4a12* in male liver.

Cyp4a related protein and LAH activity had been reported to be male and strain specific, with hepatic expression in ddY but not C57BL/6 and Balb/c mice (Hirasuka *et al* 1996a). The data in figure 3.8, obtained using a specific RNase protection assay shows that *Cyp4a12* RNA is not a strain specific P450, but that it is male specific in the liver of the eight inbred mouse strains examined (Bell *et al* 1993).

Cyp4a12 RNA hepatic and renal expression in female was very low compared to males. The female liver mouse RNA was intact and lacked RNase activity (data not shown); further, these samples gave a signal with other probes (e.g. *Cyp4a10*, *14*). Therefore, the low signal in female livers in figure 4.0 is not due to an artefact of degraded RNA, but reflects the level of *Cyp4a12* in these samples.

This data with a gene specific probe for *Cyp4a12* demonstrates that *Cyp4a12* is male specific in mouse liver. This is in contrast to other reports of strain

differences in expression of mouse hepatic Cyp4a proteins (Hiratuska *et al* 1996). My original hypothesis, that reported strain and sex specific differences in expression of mouse Cyp4a were due to expression of *Cyp4a12*, is not supported by the data.

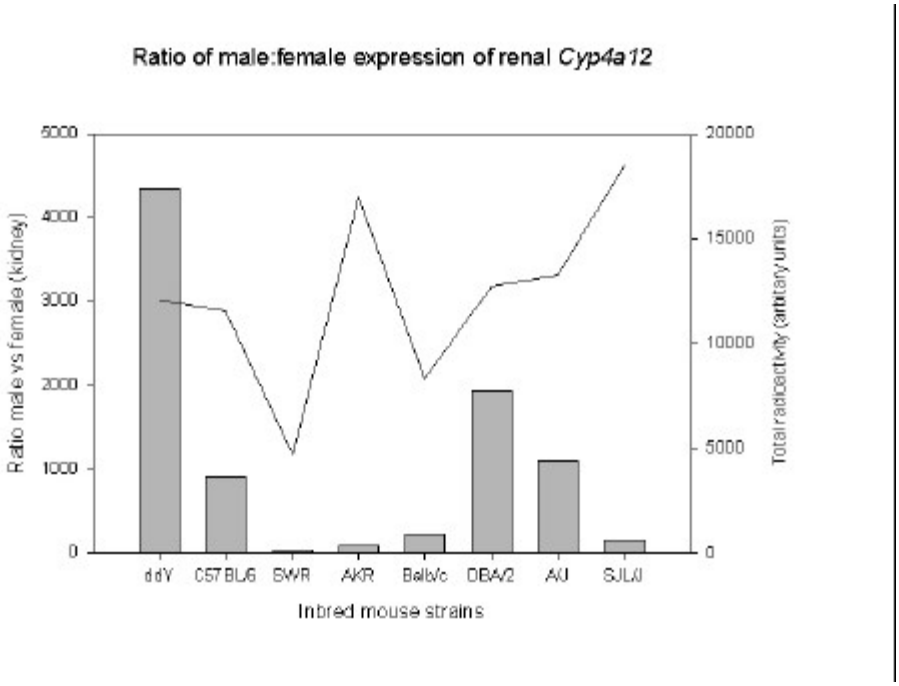

Figure 4.2 Female expression of renal *Cyp4a12*. Radioactive signal from each strain is obtained by phosphor image analysis on the autoradiograph in figure 3.8. Data points are adjusted for tRNA background and are from equal areas on the gel. Ratios (left Y-axis and bars) are calculated by dividing total radioactivity (right Y-axis) in the male with total radioactivity in the female. This provides an indication of how male expression compares with female expression of *Cyp4a12*. The line plot is the male level of *Cyp4a12*.

Sexes differences in renal *Cyp4a12* RNA were examined by calculating the ratios of male to female expression (figure 4.2). ddY mice exhibited the greatest ratio of 4335:1 (male vs female). A/J male mice had *Cyp4a12* levels 1098 times greater than females DBA/2 male mice had *Cyp4a12* levels 1930 times greater than females. Balb/c displayed a ratio of 220:1 (male vs female). The remaining

strains also exhibited sex-specific expression of *Cyp4a12* with ratio of 911, 26, 83, 148 for C57BL/6, SWR, AKR, and SJL/J respectively.

These strain differences in *Cyp4a12* RNA expression may explain reported sexual differences in the renal microsomal lauric acid metabolism; I propose that the putative LAH activity of *Cyp4a12* would account for these sex differences (Henderson *et al* 1990, Henderson *et al* 1994 and Hirasuka *et a* 1996a). Renal Cyp4a related protein and LAH activity has been reported to be male specific in ddY, C57BL/6, Balb/c, C3H/HeN and C3H/HeJ mice (Hirasuka *et al* 1996a) and C3H, DBA. C57L and AKR (Henderon *et a* 1990). Figure 4.0 shows that *Cyp4a12* RNA is a sex-specific P450 in the kidney of the mouse strains examined. However it is anomalous that the *Cyp4a12* expression profile is consistent with published reports of renal LAH and Cyp4a expression, but not with reports of hepartic LAH and Cyp4a expression.

Hirasuka *et al* (1990b) found that Cyp4a protein and LAH activity to be sex-specific in the kidney of ddY, Balb/c, C57BL/6, C3H/Hen and C3H/HeJ mice. Therefore these results confirm reports of a male specific Cyp4a (Henderson *et al* 1994, Hiratsuka *et al* 1996) and extend the data by showing that *Cyp4a12* is a principal sex-regulated renal Cyp4a/LAH.

Section 3.2.3 Tissue expression of male *Cyp4a12*

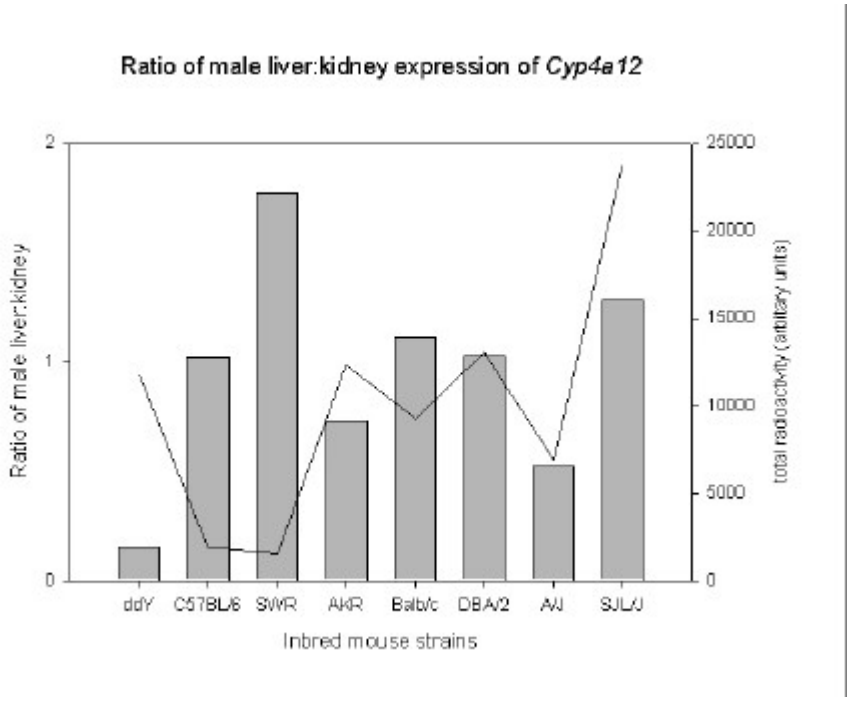

Figure 4.3 Ratio of male liver: kidney *Cyp4a12* expression. Radioactive signal from each strain is obtained by phosphor image analysis on the autoradiograph in figure 3.8. Data points are adjusted for tRNA background and are from equal areas on the gel. Ratios (left Y-axis and bars) are calculated by dividing total radioactivity (right Y-axis) in the male with total radioactivity in the female. This provides an indication of how liver *Cyp4a12* expression compares with kidney in male animals. The line plot is the male level of *Cyp4a12*.

Tissue expression of *Cyp4a12* was compared by calculating the ratio of liver versus kidney (figure 4.3). Mouse strains C57BL/6, Balb/c, DBA/2 and SJL/J are expressed *Cyp4a12* at comparable levels in the liver and kidney (ratios of 1.01, 1.1,1.02, 1.2). Hepatic *Cyp4a12* expression is less than renal in ddY (0.15), A/J (0.5) and AKR (0.7) strains. SWR mice had a higher level of *Cyp4a12* in the kidney than liver with a ratio of 1.7.

Section 3.2.4 Tissue expression of female *Cyp4a12*

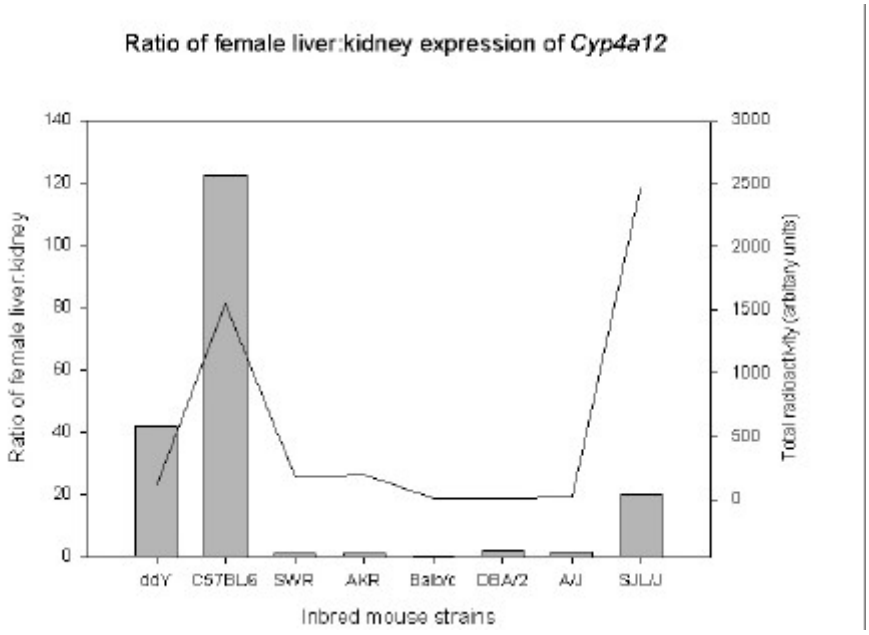

Figure 4.4 Ratio of female: liver: kidney expression of *Cyp4a12*. Radioactive signal from each strain is obtained by phosphor image analysis on the autoradiograph in figure 3.8. Data points are adjusted for tRNA background and are from equal areas on the gel. Ratios (left Y-axis and bars) are calculated by dividing total radioactivity (right Y-axis) in the male with total radioactivity in the female. This provides an indication of how liver *Cyp4a12* expression compares with kidney in female animals. The line plot is the female level of *Cyp4a12*.

In female liver (DBA/2) the absolute levels of *Cyp4a12* (figure 4.4) is approximately 2400-fold lower compared with those of the male (SJL/L) (figure 4.4). Female mice of ddY and C57BL/6 strains have liver: kidney ratios of 41 and 122 respectively, which shows that levels of *Cyp4a12* in the liver are greater than in the kidney. This is also the case, but not the same degree of magnitude for DBA/2, A/J, SJL/L female mice which have ratios of 1.65, 1.23 and 20 respectively. SWR, AKR and Balb/c mice have equivalent or less liver *Cyp4a12* than kidney indicated by their ratios (0.99,0.95, 0.30 respectively). Strains SWR, AKR, DBA/2, AJ all exhibit equal levels of *Cyp4a12.*

Data here shows that *Cyp4a12* expression is male specific in the liver and kidney. Females have lower levels of *Cyp4a12* in agreement with the lower Cyp4a protein and LAH activity but in 3/8 of the mouse strains measured *Cyp4a12* expression was similar in liver and kidney.

Section 3.2.5 Expression of Cyp4a in ddY mouse strains

Data here shows that *Cyp4a12* is a male specific gene found in the liver and kidney of several inbred mouse strains (figures 4.0). However Hiratsuka *et al* (1996) report a strain –specific hepatic Cyp4a protein, suggesting that there are genetic variations in the expression of Cyp4a genes. Data here shows that this cannot be solely due to *Cyp4a12*.

Possible explanations for this include the existence of another Cyp4a gene that is expressed in male ddY mice. Investigation in the genetic variations of Cyp4a expression was conducted using ddY mouse tissue.

Cyp4a10 and *Cyp4a14* are the other two members of the Cyp4a mouse family. RNase protection assay were used to determine the expression pattern of these two genes due to assay's ability to discriminate between the individual genes.

Section 3.2.6 Expression of *Cyp4a10* in ddy *mice*

Constitutive expression of *Cyp4a10* was studied in the liver and kidney of ddY mice. RNase protections were performed on total RNA using a riboprobe for *Cyp4a10* (figure 4.5). Hybridisation of riboprobe to total tissue RNA resulted in a protected fragment, which is detected in a denaturing urea/polyacrylamide gel. The specificity of the assay was shown by hybridisation of the riboprobe provided in excess with yeast tRNA; No detectable signal was observed after RNase A/T1 treatment verifying that the radioactive signal observed in test samples was due to specific hydridisation of probe to target RNA. Yeast tRNA alone was also used without RNase treatment to confirm input probe integrity

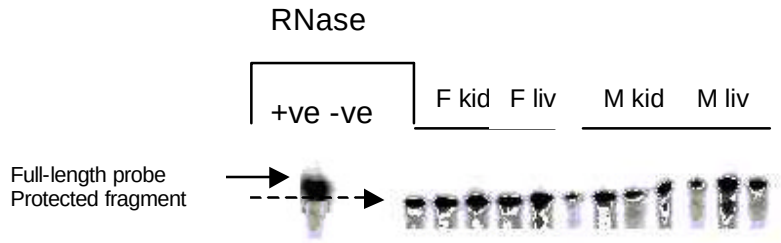

Figure 4.5 Expression of *Cyp4a10* in ddy mice. A RNase protection assay was performed on 30µg total RNA of untreated male (M) and (F) mouse liver (liv) and kidney (kid). The position of the full length, 190bp length probe is shown by a solid arrow and the 157bp protected by a dashed arrow. RNase protection assays for *Cyp4a10* were performed as described in methods (pgX.) and visualised on a phosphorimager. The specificity of the probe was determined by inclusion of two control yeast tRNA samples and hybridised to the probe. One sample was treated without RNase A/T1 (-ve), while the other was treated with RNase A /T1 (+ve). Each experiment was repeated twice using different tissue samples.

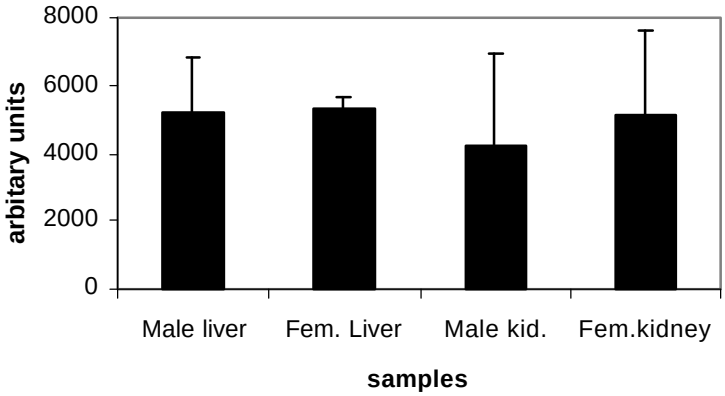

Figure 4.6. Expression of *Cyp4a10* gene in ddY mice. Radioactive signal from each strain was obtained by phosphor image analysis on the gel from figure 4.3. Data points are adjusted for tRNA background and are from equal areas of the gel. Protected bands were quantified using a phosphorimager and the results are expressed as the mean total radioactive signal (n=3 per data point). Data points are adjusted for tRNA background and are from equal areas on the gel. Each experiment was repeated twice using different tissue samples.

Figure 4.5 shows that the liver and kidney samples have a specific protected fragment of 157bp, corresponding to the expected size of the *Cyp4a10* RNA message.

In ddY mice *Cyp4a10* is expressed constitutively in both male and female liver and kidney (figure 4.6). Levels of *Cyp4a10* in ddY mice do not show a sex specific expression as calculated by a bonferroni post-hoc, multiple comparisons test ($P>0.05$). This data is similar to other reports that show *Cyp4a10* RNA and protein expression is not sex specific in C57BL/6 and NMC mice (Bell *et al* 1993, Heng *et al* 1997).

Section 3.2.7 *Cyp4a10* polymorphism between ddY and C57BL/6 mice

The *Cyp4a10* probe detects an unambiguous signal in ddY (figure 4.5). However it is possible that an allelic variant might explain the discrepancy between the RNAse protection data and the proteina and enzyme data of Hiratuka *et al* (1996). A polymorphism of the *Cyp4a10* gene has been reported in ddY. Examining the cDNA sequence on *Cyp4a10* in the two strains there is a 1b.p difference in 5'end of the Bell *et al* (1993) probe (figure 4.7). It is unlikely to effect the probe specificity and no polymorphism exists between ddy *Cyp4a10* and C57BL/6 *Cyp4a10* where the probe is disparate.

Figure 4.7. *Cyp4a10* polymorphism between ddY and C57BL/6 mice. Alignment of *Cyp4a10* genes in ddY (ab018421), C57BL/6 (x69296) and NMC (x71478) mice was aligned using the programme gcg, and displayed using Genedoc, as described in methods(pg X). Highlighted sequence represents the 5' end of the cDNA sequence and shows the 1bp difference between the strains.

Figure 4.7 Polymorphism in *Cyp4a10* between C57BL/6 and ddY mice

```
                      *        920          *        940          *        960          *        980
x71478    : ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ACACTCATGT :   10
ab018421  : GATATCCTTTTATTTGCCAGAATGGAGAATGGGGACAGCATGTCTGACAAGGACCTACGTGCTGAGGTGGACACATTCATGT :  984
x69296    : GATATCCTTTTATTTGCCAGAATGGAGAATGGGGACAGCATGTCTGACAAGGACCTACGTGCTGAGGTGGACACATTCATGT :  964
            gatatccttttatttgccagaatggagaatggggacagcatgtctgacaaggacctacgtgctgaggtggacACAtTCATGT

                    *       1000          *       1020          *       1040          *       1060
x71478    : TCGAGGGCCATGACACCACAGCCAGTGGAGTCTCCTGGATCTTCTATGCTCTGGCCACACACCCTGATCACCAACAGAGATG :   92
ab018421  : TCGAGGGCCATGACACCACAGCCAGTGGAGTCTCCTGGATCTTCTATGCTCTGGCCACACACCCTGATCACCAACAGAGATG : 1066
x69296    : TCGAGGGCCATGACACCACAGCCAGTGGAGTCTCCTGGATCTTCTATGCTCTGGCCACACACCCTGATCACCAACAGAGATG : 1046
            TCGAGGGCCATGACACCACAGCCAGTGGAGTCTCCTGGATCTTCTATGCTCTGGCCACACACCCTGATCACCAACAGAGATG

                  *       1080          *       1100          *       1120          *       1140
x71478    : CAGAGAGGAAGTTCAGAGCCTCCTGGGGGATGGATCC~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ :  129
ab018421  : CAGAGAGGAAGTTCAGAGCCTCCTGGGGGATGGATCCTCTATCACCTGGGATCACCTAGACCAGATTCCCTATACCACAATG : 1148
x69296    : CAGAGAGGAAGTTCAGAGCCTCCTGGGGGATGGATCCTCTATCACCTGGGATCACCTAGACCAGATTCCCTATACCACAATG : 1128
            CAGAGAGGAAGTTCAGAGCCTCCTGGGGGATGGATCCtctatcacctgggatcacctagaccagattccctataccacaatg

                *       1160          *       1180          *       1200          *       1220          *
x71478    : ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ :    -
ab018421  : TGCATCAAGGAGGCCCTAAGGCTCTACCCACCTGTCCCAGGCATTGTCAGAGAACTCAGTACATCTGTCACCTTCCCTGATG : 1230
x69296    : TGCATCAAGGAGGCCCTAAGGCTCTACCCACCTGTCCCAGGCATTGTCAGAGAACTCAGTACATCTGTCACCTTCCCTGATG : 1210
            tgcatcaaggaggccctaaggctctacccacctgtcccaggcattgtcagagaactcagtacatctgtcaccttccctgatg
```

Section 3.2.8 Expression of *Cyp4a14* in ddy mice

Constitutive expression of *Cyp4a14* was studied in the liver and kidney of ddY mice (figure 4.8). RNase protections for *Cyp4a14* was performed as described in methods (pg 74). The position of the full-length, 237bp probe is shown by a solid arrow, and the 157bp protected fragment by a dashed arrow. No detectable signal was observed RNase A/T1 treatment verifying that the radioactive signal observed in test samples was due to specific hybridisation of probe to target RNA. Yeast tRNA alone without RNase treatment to confirm input probe integrity. Figure 4.8 presents an RNA protection assay for *Cyp4a14* in ddY mice. An autoradiograph of this gel was produced by an extended exposure time (72hr) due to the low constitutive expression of *Cyp4a14*.

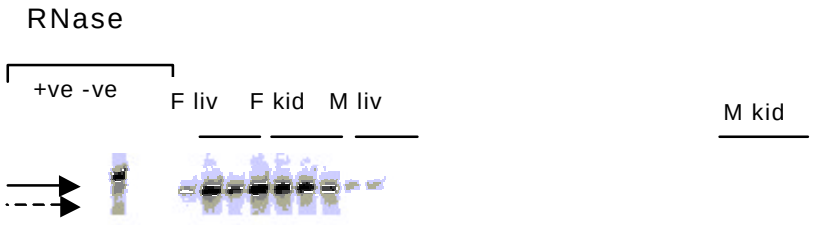

Figure 4.8 Expression of *Cyp4a14* in ddy mice. A RNase protection assay was performed on 30µg total RNA of untreated male (M) and female (F) mouse liver (liv)and kidney(kid). The position of a 237bp length probe is shown by a solid arrow, and the 157bp protected fragment by a dashed arrow. RNase protections for *Cyp4a14* were performed as described in methods (pg X). Two control yeast tRNA samples were hybridised to the probe, with (+ve) and without (-ve) RNase A /T1. Each experiment was repeated twice using different tissue samples.

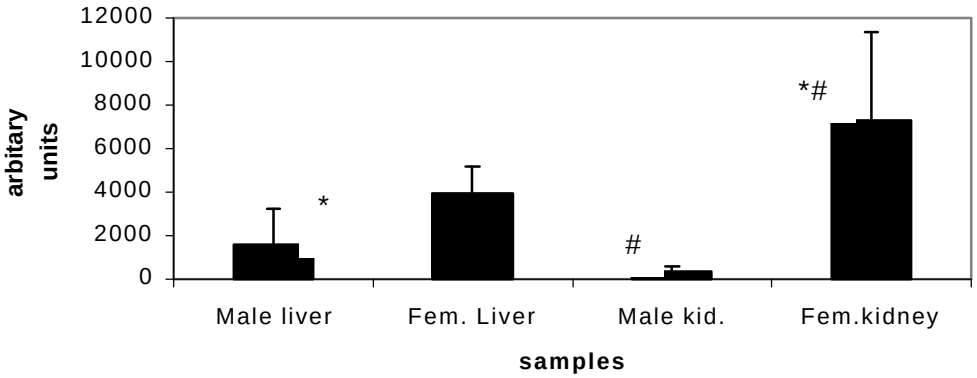

Figure 4.9 Expression of *Cyp4a14* gene in ddY liver. Radioactive signal from each strain was obtained by phosphor image analysis on the autoradiograph in figure 4.4. Data points are adjusted for tRNA background and are from equal areas of the gel. Protected bands were quantified using a phosphorimager and the results are expressed as the mean total radioactive signal (n=3 per data point). Data points are adjusted for tRNA background and are from equal areas on the gel. Each experiment was repeated twice using different tissue samples. Renal *Cyp4a14* is significantly higher in female mice compared to male mouse liver (* P<0.05) and kidney (# P<0.01). *Cyp4a14* expression between male liver and female liver is not significant

Male hepatic *Cyp4a14* is approximately 5-fold greater than male kidney (figure 4.9). Hepatic *Cyp4a14* in the female is 2.5-fold greater than in the male. Female kidney *Cyp4a14* expression is approximately 2-fold higher than female liver. Renal *Cyp4a14* is significantly higher in female mice compared to male mouse liver (P<0.05) and kidney (P<0.01). The difference in *Cyp4a14* expression between male liver and female liver is not significant. These data show that *Cyp4a14* in ddY mice exhibit a female specific pattern. In NMC mice *Cyp4a14* expression (using the same riboprobe) was found to be expressed at low levels in control liver of both male and female mice (Heng *et al* 1997) suggesting that this gene is subject to strain variability. Northern blot analysis of kidney Cyp4a isoform expression showed that *Cyp4a14* is expressed, albeit at low levels in both sexes (Holla *et al* 2001).

The female-specific expression of *Cyp4a14* fails to explain the strain-specific and male-specific hepatic expression of LAH and Cyp4a protein reported by Hirasuka *et al* (1996).

Section 3.2.9 Hepatic LAH activity for C57BL/6 and ddY mice

Data here demonstrates the male-specific expression of *Cyp4a12* in mouse liver. However LAH activity is strain and sex-dependent (Henderson *et al* 1990, Henderson *et al* 1994 and Hirasuka *et a* 1996a). In order to determine if the putative LAH activity of *Cyp4a12* accounts for these sex differences, LAH activity was measured in ddY and C57BL/6 mice. LAH activity was determined as described in the methods (section 2.8.5). Results are presented in table 3.1 below.

Sample	mean (nmol/ml/min 11H)	mean (nmol/ml/min 12H)
C57BL/6 male	1.765	1.225
C57BL/6 female	2.325	1.32
DdY male	0.645	0.69
Ddy female	0.45	0.305

Table 3.1 Lauric acid hydroxylase activity in the liver of C57BL/6 and ddY mice (n=2). Assay was performed as described in the methods (section 2.8.5).

There is no difference in hepatic LAH 11H and 12H activity between male and female C57BL/6 mice. Hepatic LAH-12H activity in ddY mice is greater activity

in male mice compared to female. Male LAH12 activity is approximately 2.2-fold greater than in female mice. Therefore in C57BL/6 mice there is no sex difference in LAH activity. ddy mice exhibit a sex difference with respect to LAH-12H activity. This sex difference in LAH activity therefore supports data by Hiratsuka *et al* (1996a) by confirming the presence of a strain and sex-specific lauric acid hydroxylase.

Section 3.3 *Cyp4a12* gene in S129 PPARα wild type and null mice

PPARα is known to regulate the induction of Cyp4a genes (Bell *et al* 1993). The effect of PPARα on expression of male-specific genes was therefore investigated. RNase protection assays for *Cyp4a12* were performed as described in the methods (pg 74).

Wild type male mice expressed 2.4-fold greater hepatic *Cyp4a12* than in the kidney ($P<0.05$). In PPARα knockout mice *Cyp4a12* was also expressed in male liver and kidney (figure 5.0). *Cyp4a12* showed a 12-fold difference wild type and knockout in the kidney ($P<0.01$). In knock out mice *Cyp4a12* was found to be 1.9-fold greater in the liver than the kidney of male mice ($P<0.05$).

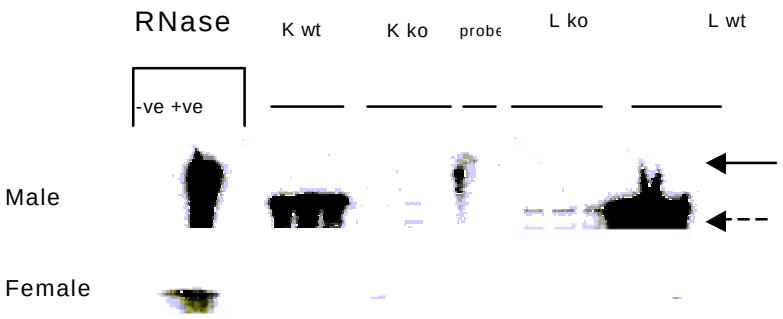

Figure 5.0 Expression of *Cyp4a12* in male and female S129 PPARα wild-type (wt) and null (ko) mice RNase protection assay was performed using 30µg total RNA of untreated male mouse liver (L) and kidney (K). as described in methods (pg X). The position of the full-length, 480bp length probe is shown by a solid arrow, and the 401bp protected fragment by dashed arrow. Two control yeast tRNA samples, treated with (+ve) and without (-ve) RNase A/T1 demonstrates probe integrity. Each experiment was repeated twice using different tissue samples.

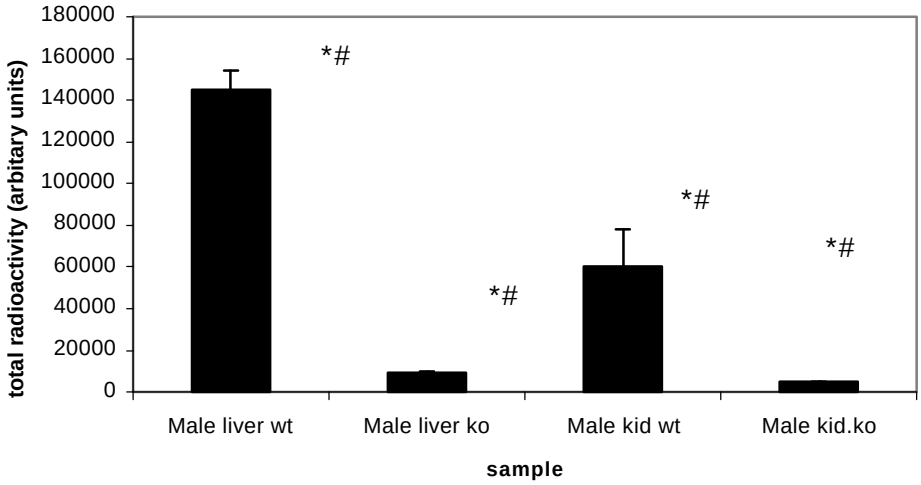

Figure 5.1. Expression of *Cyp4a12* gene in S129 PPARα wild type and null mice. Radioactive signal from each strain was obtained by phosphor image analysis from the gel in figure 4.6. Data points are adjusted for tRNA background and are from equal areas of the gel. Protected bands were quantified using a phosphorimager and the results are expressed as the mean total radioactive signal (n=3 per data point).. Each experiment was repeated twice using different tissue samples. *: Indicates significant difference (male liv wt vs male liv ko and male kid wt vs male kid ko P<0.01). # Indicates significant difference (male liv wt vs male kid wt and male liv ko vs male kid ko P<0.05).

Constitutive expression of *Cyp4a12* RNA was found to be high in the male liver of PPARα wild type Sv/129 mice, provided by F.J.Gonzalez. *Cyp4a12*

was undetectable in female wild type and knockout mice. In PPARα knockout mice hepatic *Cyp4a12* levels were 15-fold less than that found in wild type mice (figure 5.1, P<0.01). These findings confirm the previous report where *Cyp4a12* has been shown to be regulated differently to other Cyp4a isoforms (Bell *et al* 1993). That study demonstrated that *Cyp4a12* is expressed constitutively at high levels in male liver and kidney. By contrast *Cyp4a12* was expressed at low levels in liver of female mice (figure 5.1). The observed low expression of murine *Cyp4a12* in PPARα knock out mice demonstrates that PPARα plays a significant role in the constitutive expression of this gene.

Section 3.3.1 Total P450 concentration in S129 PPARα wild type and knockout mice

Cyp4a12 mRNA in PPARα wild type mice is 15-fold greater than in knockout mice (figure4.6). To determine if the absence of PPARα results in the reduction of total cytochrome P450 protein concentration a spectral determination of total P450 protein concentration was performed (table 3.2, see methods section 2.8.1)

Sample	nmols P450/mg protein
Male wt	1.04±0.27
Male ko	0.77±0.06
Female wt	0.73±0.12
Female ko	0.76±0.17

Table 3.2. Total P450 concentration in S129 PPARα wild type (n=5) and null mice (n=3). Protein and P450 concentration was determined as previously described in the methods (section 2.8.1).

Data here demonstrates that there is a significant difference in total P450 concentration between male and female wild type mice and no sex difference in knockout animals. There is no significant difference between wild type and knockout mice in total P450 concentration.

Section 3.3.2 Hepatic LAH activity in PPARα wild type and knockout mice

Greater P450 protein concentration in male liver and the male specific expression of *Cyp4a12* suggests that this gene constituents the predominant P450 in male liver. To confirm that male specific *Cyp4a12* expression corresponds to lauric acid hydroxylase activity, liver microsomes were prepared from S129 PPARα wild type and knockout mice. LAH activity was determined by the method described in section 2.8.5 and is presented in table 3.3.

	S129 PPARα wild type mice		S129 PPARα knock out mice	
Enzyme	Male	Female	Male	Female
LA11	0.59 ± 0.06	0.57±0.06	0.51±0.01#	0.46±0.00#
LA12	0.71±0.09*	0.60±0.04*	0.57±0.03	0.58±0.05

Table 3.3. Hepatic LAH activity in S129 PPARα wild type (n=5)and null mice (n=3). Assay was performed as described in the methods (section 2.8.5).* indicates a statistical difference between male and female wild type mice (P=0.038). # indicates a statistical difference between male and female knock out mice (P=0.0004).

Data in table 3.3.1.3 demonstrates that there is a significant sex difference in the LAH (12-OH) activity of hepatic microsomes from S129 PPARα wild type mice but not knockout mice. No statistically differences were observed for LAH (11-OH) activity. These findings support the hypothesis that the sex specific expression of *Cyp4a12* corresponds to the increased LAH activity and greater total P450 protein concentrations in male S129 PPARα wild type. It is not possible to compare LAH activity between wild type and knock out due to unequivalent group sizes.

Section 3.3.3 *Cyp4a14* expression in S129 PPARα wild type and null mice

Since absence of PPARα decreases male-specific hepatic expression of *Cyp4a12*, it was of interest to determine if this effect of PPARα was specific to *Cyp4a12* or was common to all Cyp4a genes. The expression of *Cyp4a14* was investigated (figure 5.2).

Cyp4a14 RNA expression in S129 PPAR PPARα male wild type and null mice was characterised by RNAse protection assay as described in methods (pg 74)

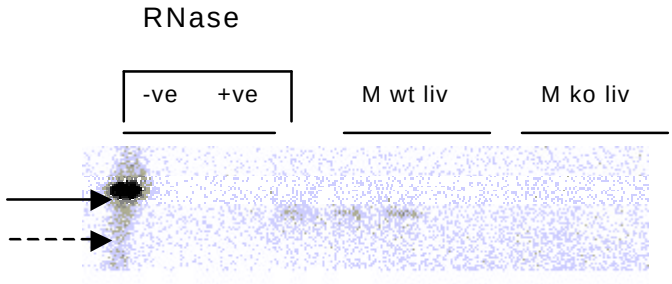

Figure 5.2 Expression of *Cyp4a14* in S129 PPARα wild type (wt) and null mice (ko) A RNase protection assay was performed on 30µg total RNA of untreated male (M) mouse liver (liv). The position of the full-length, 237bp length probe is shown by a solid arrow, and the 157 bp protected fragment by dashed arrow. Two control yeast tRNA samples, treated with (+ve) and without (-ve) RNase A/T1 demonstrates probe integrity. Each experiment was repeated twice using different tissue samples.

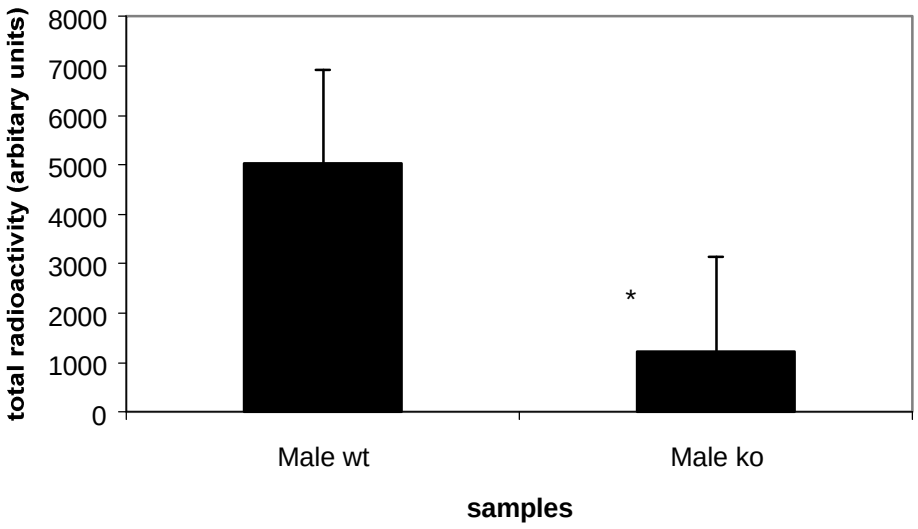

Figure 5.3. Expression of hepatic *Cyp4a14* gene in S129 PPARα wild type and null mice. Radioactive signal from each strain was obtained by phosphor image analysis on the autoradiograph in figure 4.8. Data points are adjusted for tRNA background and are from equal areas of the gel. Protected bands were quantified using a phosphorimager and the results are expressed as the mean total radioactive signal (n=3 per data point). Data points are adjusted for tRNA background and are from equal areas on the gel. Each experiment was repeated twice using different tissue samples. *: Indicates significant difference (P<0.01).

Constitutive hepatic *Cyp4a14* in S129 PPARα null mice is 4-fold less than in wild type (figure 5.3, P<0.01). As with *Cyp4a12*, *Cyp4a12* is reduced in the

liver of male mice lacking PPARα. Using riboprobes specific for *Cyp4a12* and *Cyp4a14*, this data demonstrates that PPARα is a regulator of male Cyp4a RNA expression.

Section 3.4 Expression of male specific genes in S129 PPARα mice.

In order to establish if PPARα regulates all male specific genes or to determine that sexually dimorphic regulation is confined to *Cyp4a12* and *Cyp4a14*, riboprobes were prepared for three other known male specific genes. *Cyp2d9* is expressed in a sex-specific manner in mouse liver and kidney and encodes the male specific activity of testosterone 16α-hydroxylase (Harada and Negishi 1984a,b, 1988.)

Mouse urinary protein (MUPI) belongs to a family of homologous proteins that form the major urinary proteins excreted by adult male mice and believed to assist in the transport of pheromones to the urine (Lehman-McKeeman *et al* 1998).

Section 3.4.1 Preparation of *Cyp2d9* and MUPI Image clones

Clones for *Cyp2d9* (clone number 931222) and MUP (931184) were obtained from the UK HGMP resource centre, Cambridge and supplied in E.coli XL1-blue in the vector pBluescript SK. As described in the methods section clones

were grown overnight at 37°C. Crude mini-preps and restriction digests were used to identify the presence of a correctly sized insert in the vector (figure 5.4). The identity of the DNA clones was confirmed by sequence analysis (not shown). Multiple alternative IMAGE clones for each gene were also analysed.

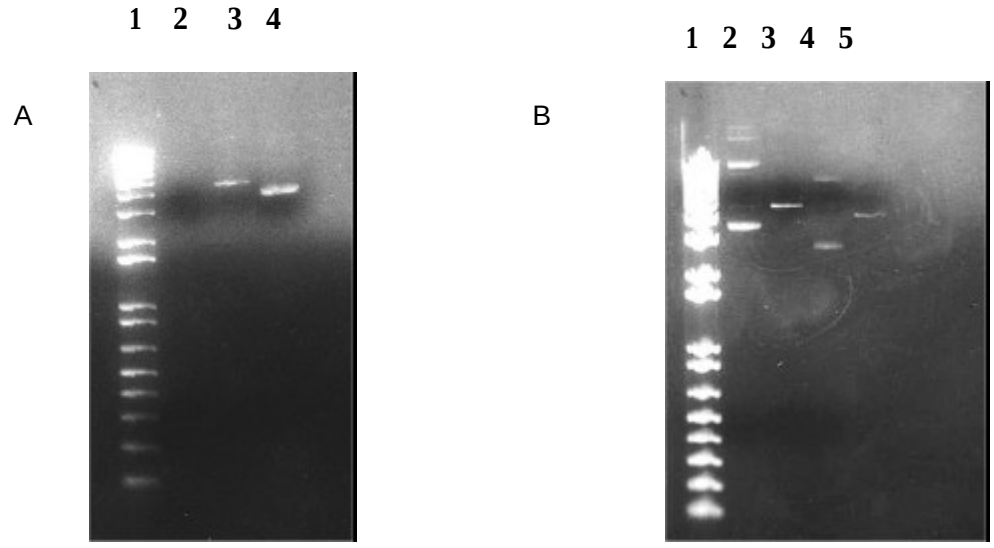

Figure 5.4. Agarose gel electrophoresis of of pBK-/- MUPI and *Cyp2d9* clones and pGEMT-Slp. Gel A : Lane 1: 1KB Plus DNA ladder, Lane 2: space, Lane 3: Uncut pGEMT-Slp Lane 4: pGEMT-Slp EcoRI digest. Gel B : Lane 1: 1KB Plus DNA ladder, Lane 2: uncut pBKMUPI, Lane 3 pBKMUPI EcoRI digest, Lane 4:uncut pBK*Cyp2d9*, Lane 5: pBK*Cyp2d9* digest EcoRI. 1µl of sample was run on a 1.0% agarose gel (0.5µg/ml ethidium bromide) at 110V for 2 hours using 1xTBE. The gel was photographed under UV trans-illumination. Linearised templates were subsequently used for *in vitro* transcription.

Section 3.4.2 Sub-Cloning of mouse Slp by PCR

The sequence of the mouse Slp gene is known (Nakayama *et al* 1987). Primers homologous to sequence in exon 3 were designed (figure 5.5) and PCR was performed on genomic DNA from s129 PPARα wild-type mice (see methods section2.4.3). PCR generated a 192bp fragment (180-371b.p), which

was mouse specific, as evidenced by the failure to amplify a product from *C.elegans* DNA (figure 5.5 lane 6). The PCR product was cloned into PGEM-T and its identity was confirmed by sequence analysis (not shown).

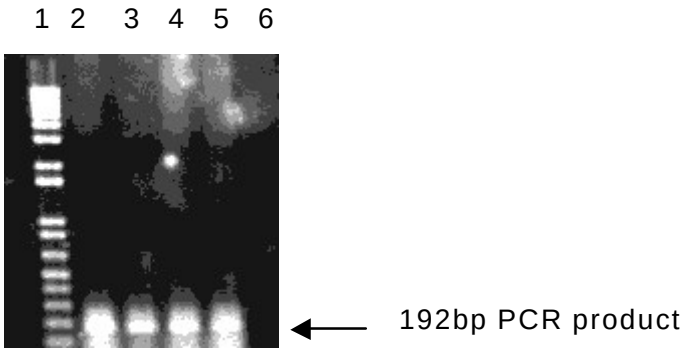

Figure 5.5. Agarose gel electrophoresis PCR of genomic DNA using primers for exon 3 of Slp Lane 1: 1KB Plus DNA ladder, Lane 2-5: 192bp PCR products, Lane 6:Negative control (C.elegans DNA). 5µl of sample from was run on a 1.0% agarose gel (0.5µg/ml ethidium bromide) at 110V for 2 hours using 1xTBE. The gel was photographed under UV trans-illumination and is the PCR product of 25 cycles.

Section 3.4.2 Template preparation and *in vitro* transcription of *Cyp2d9*, MUP and Slp.

The riboprobe for MUP was designed between the T7 promoter region of the pBluescript vector (657-1191bp) and the EcoRI in the multiple cloning site. The riboprobe for *Cyp2d9* was designed between the T7 promoter region of the pBluescript vector (657-1167bp). MUP and *Cyp2d9* anti-sense probes were transcribed from T7 promoter of EcoRI digested templates, which gave 781 bp and 800bp full-length probe, and 706 bp and 790bp protected fragments, respectively. The anti-sense probe for *Slp* was transcribed from

the T7 promoter, producing a full-length transcript of 310 bp and a protected fragment of 192 bp. *Cyp2d9* and MUP were provided in the pBluescript SK vector. Slp was ligated into the pGEMT-Easy vector (figure 5.6).

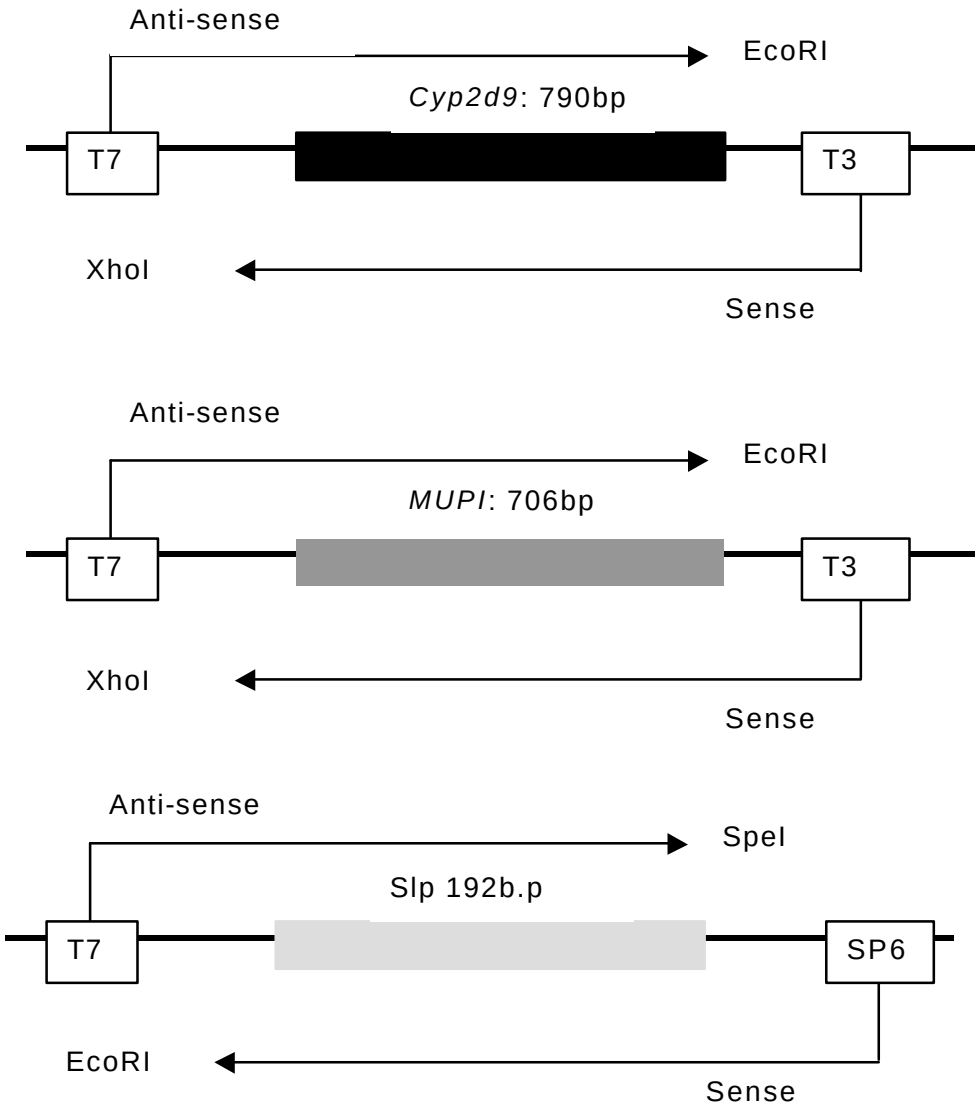

Figure 5.6 Male-specific gene templates for *in vitro* transcription. *Cyp2d9* and MUP cDNAs were provided in the vector pBluescript SK. Slp PCR product was ligated into the PGEMT-Easy vector from Promega. *In vitro* transcription of anti-sense riboprobe of *Cyp2d9* is generated from EcoRI digested template by SP6 polymerase yielding a 521bp full-length probe of which 510bp corresponds to the cDNA insert. *In vitro* transcription of anti-sense riboprobe of MUPI is generated from EcoRI digested template by SP6 polymerase yielding a 545bp full-length probe of which 534 bp corresponds to the cDNA insert. Anti-sense riboprobe of Slp is generated byT7

polymerase *in vitro* transcription of BamHI cut template, resulting in a 310bp probe length and a 192bp insert.

Section 3.4.3 Expression of mouse male specific *Cyp2d9*

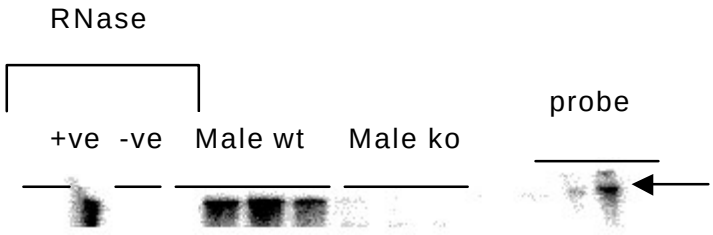

Figure 5.7 Expression of *Cyp2d9* gene in mouse liver. A RNase protection assay was performed on 30µg total RNA of untreated male mouse liver from s129 PPARα wild type and null mice. RNase protection assays were performed as described in methods (pg X). The position of the full-length, 800bp length probe is shown by a solid arrow,. Two control yeast tRNA samples, treated with (+ve) and without (-ve) RNase A/T1 demonstrates probe integrity. Each experiment was repeated twice using different tissue samples.

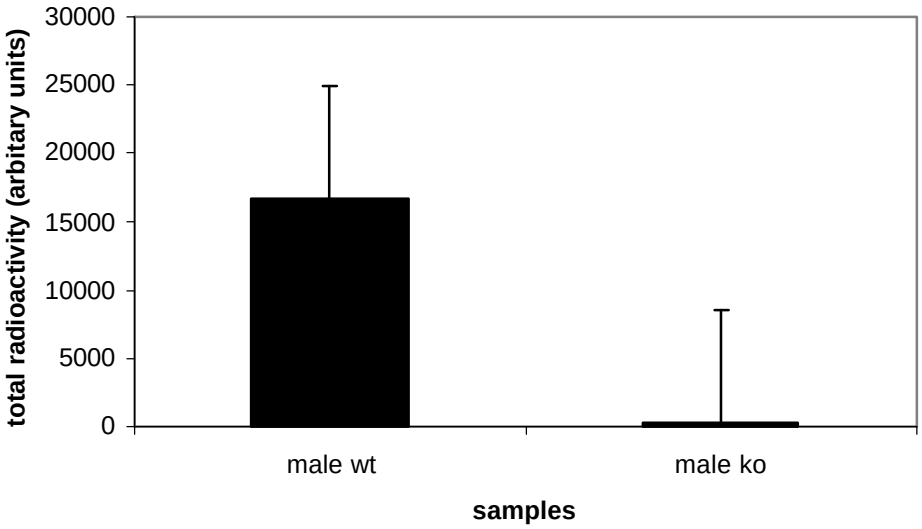

Figure 5.8 Expression of *Cyp2d9* in S129 PPAR male wild type and knockout mice. Radioactive signal from Protected bands each strain was obtained by phosphor image analysis on the autoradiograph in figure 5.4. Data points are adjusted for tRNA background and are from equal areas of the gel were quantified using a phosphorimager and the results are expressed as the mean total radioactive signal (n=3 per data point). Data points are adjusted for tRNA background and are from equal

areas on the gel. Each experiment was repeated twice using different tissue samples. *: Indicates significant difference (P<0.05).

Due to the length of the probe, it is not possible to distinguish between full length and protected fragments. Constitutive expression of *Cyp2d9* was studied in the liver of S129 PPARα wild-type and knock out male mice (figure 5.7). RNase protections were performed as described in methods (pg 74). In the liver of male knockout PPARα mice constitutive expression of *Cyp2d9* is approximately 65-fold less than in wild type mice (figure 5.8, P<0.05). This data shows that *Cyp2d9* RNA expression is reduced in PPARα knockout mice suggesting a possible role of PPARα in regulation of male specific gene expression.

Section 3.4.4 Expression of mouse hepatic MUP I

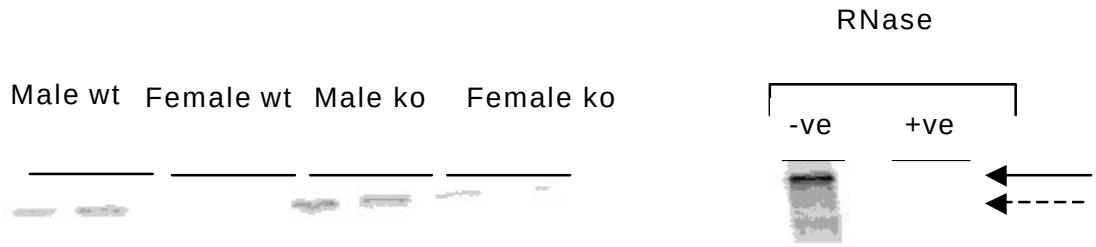

Figure 5.9 Constitutive expression of MUPI gene in mouse liver. RNase protection assay was performed on 30µg total RNA of untreated male and female mouse liver from s129 PPARα wild type and null mice. RNase protection assays were performed as described in methods (pg X). A 545 bp length probe (solid arrow) was generated which gave a 534bp protected fragment (dashed arrow) representing the *Cyp2d9* sequence.. One sample was treated without RNase A/T1 (-ve), while the other was treated with RNase A /T1 (+ve). All RNase protection assays were performed in twice with tissues from different animals.

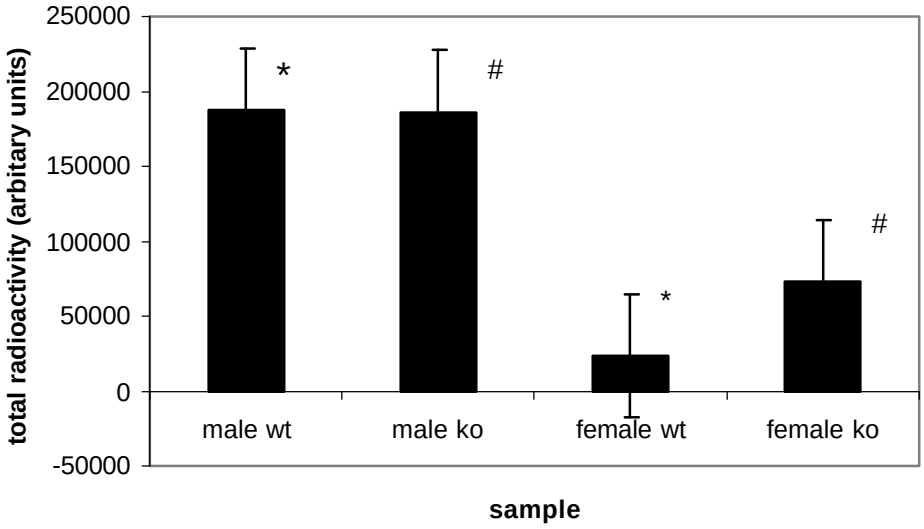

Figure 6.0. Expression of MUPI in S129 PPAR wild type and knock out mice. Radioactive signal from each strain was obtained by phosphor image analysis on the autoradiograph in figure 5.5. Data points are adjusted for tRNA background and are from equal areas of the gel. Protected bands were quantified using a phosphorimager and the results are expressed as the mean total radioactive signal (n=2 per data point). Data points are adjusted for tRNA background and are from equal areas on the gel. Each experiment was repeated twice using different tissue samples. * and # indicates significant difference for male and female wild type and male and female knockout mice respectively (P<0.05).

Expression of MUPI was studied in the liver of S129 PPARα wild-type and knock out male and female mice (figure 5.9). RNase protections were performed on total RNA using a riboprobe for *MUPI*.

Expression of constitutive liver MUPI RNA is male specific as reported in the literature (Derman *et al* 1981) with male wild type MUPI 8-fold greater than female wild type (figure 6.0, P<0.05). No significant difference in MUPI expression was observed between wild type and knock out mice male and female animals. Between S129 PPARα knock out mice, males exhibited a 2.5-fold greater expression of MUPI than females. Therefore the non-Cyp

P450 gene, MUPI is a male specific gene found in mouse liver whose expression is unaffected by the abolition of the PPARα.

Section 3.4.5 Expression of mouse male specific Slp

Sex-limited protein (Slp) expression was determined by RNase protection assay to collaborate the MUP data and further investigate the role of PPARα in male specific gene regulation.

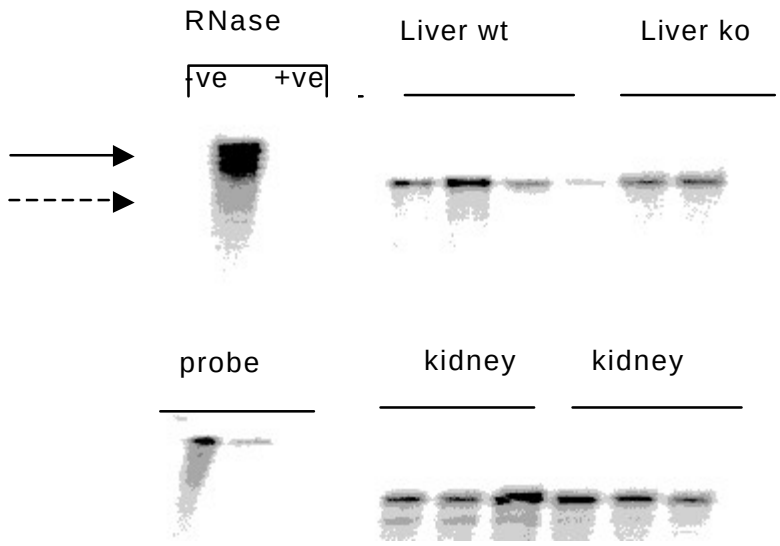

Figure 6.1. Expression of Slp in S129 PPARα wild type and knockout mice. A RNase protection assay was performed on 30µg total RNA of untreated mouse liver and kidney. A 310bp length probe (solid arrow) was generated which gave a 192bp protected fragment (dashed arrow) representing the *Slp* sequence.. One sample was treated without RNase A/T1 (-ve).

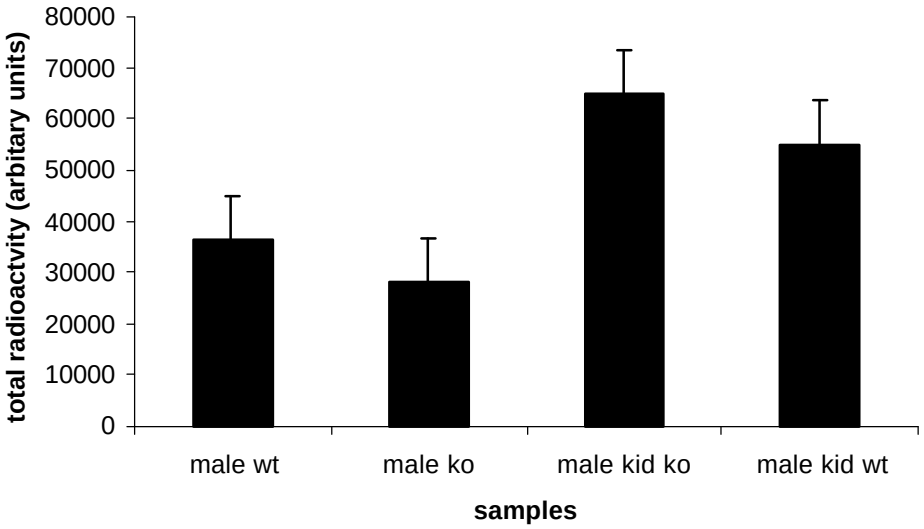

Figure 6.2 Expression of Slp in S129 PPARα wild type mice. A RNase protection assay was performed on 30µg total RNA of untreated mouse liver and kidney.. A 274bp length probe (solid arrow) was generated which gave a 210bp protected fragment (dashed arrow) representing the *Slp* sequence. One sample was treated without RNase A/T1 (-ve), while the other was treated with RNase A /T1 (+ve).. (n=2 per data point). Data points are adjusted for tRNA background and are from equal areas on the gel.

Expression of Slp was studied in the liver of S129 PPARα wild type and knock out male (figure 6.1). RNase protections were performed on total RNA using a riboprobe for *Slp*. Female animals were not examined due to time constraints but their inclusion would have been used to confirm Slp male specific expression.

Expression of hepatic Slp RNA is male specific (Georgatsou *et al* 1993). No statistically significant differences were determined for Slp expression between wild type and knock out mice male animals. Renal Slp expression in is between 2-3-fold greater than that in the liver of wild type and knockout

male mice. Slp expression has been reported to be predominantly in the liver (Georgatsou *et al* 1993) although levels of Slp have been found in other tissues including the kidney (Cox *et al* 1988). Figure 6.1 and 6.2 show that Slp RNA levels in S129 mice are greater in the kidney than the liver but are not affected by the absence or presence of PPARα in either liver or kidney.

Section 3.5 Induction of hepatocyte DNA synthesis

In order to characterise changes in gene expression associated with PP-induced cell proliferation, experiments were designed to establish the optimal dose and time period over which S-phase was initiated in hepatocytes.

Section 3.5.1 Time-course Experiment

Based on the literature (Styles *et al* 1987,1991,1990) a dose of 25mg/kg bw i.p. ciprofibrate was administered to S129 wild type mice with 100mg/kg bw i.p. BrdU dosed 1 hour before sacrifice (n=8 per group, Styles *et al* 1988). A control group of mice (n=4) was administered 500µl corn oil (vehicle). At time points 0.5, 1, 2, 3, 24 hours animals were sacrificed and 2-4mm longitudinal mid-sections of left, median, and right anterior hepatic lobes were removed and tissues were fixed in 10% neutral buffered saline. Detection of BrdU-labelled hepatocytes was performed as described in methods (section 2.8.6).

Duodenum samples were also collected and used as a positive control given the high replicative nature of this tissue.

Over the time points examined dosed animals did not exhibit any increase in BrdU labelled cells (figure 6.3). These results show that DNA synthesis has not been increased by ciprofibrate at the dose used (25mg/kg bw i.p.). Presence of BrdU in the systemic circulation is demonstrated by the labelling of nuclei of cells in the duodenum.

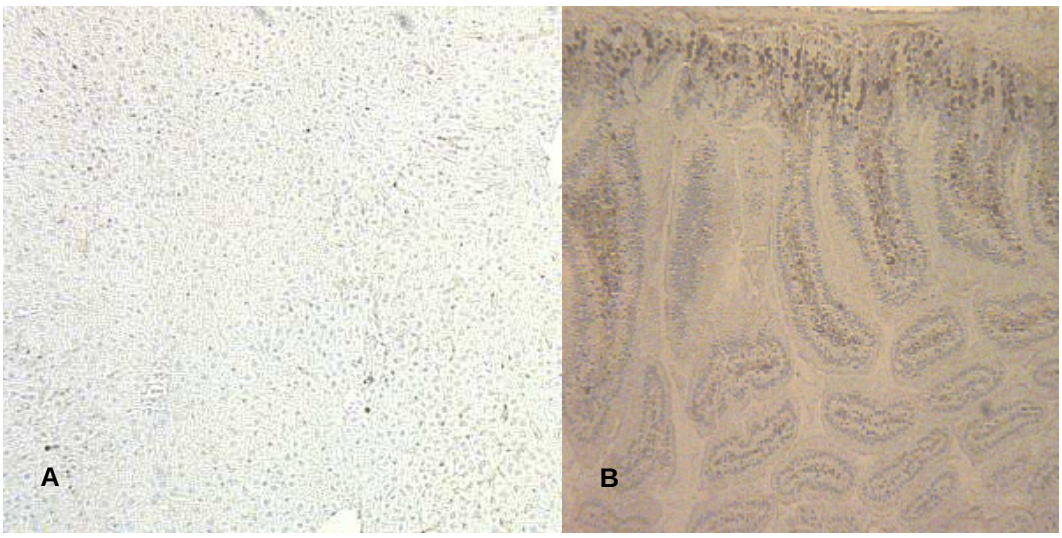

Figure 6.3. Time course experiment: S129 wild type mice were administered 25mg/kg bw i.p. and liver and duodenum tissues were collected at time points 0.5,1,2,3,24 hr. A: This is a represent slide of a treated animal. Ciprofibrate did not increase the number of hepatocytes in S-phase compared to control animals. It is estimated that there was less than 1% difference between treated and control animals. B: Representative slide demonstrating that BrdU entered the systemic circulation and incorporated into the duodenum of treated animals.

Section 3.5.2 Factors effecting induction of DNA synthesis

A dose of 25mg/kg i.p. ciprofibrate did not induce DNA synthesis over a 24 hour period (figure 5.9A). Therefore it was necessary to investigate the effects of other variables on the induction of DNA synthesis by peroxisome proliferators.

Section 3.5.2.1 Effect of Circadian Rhythms

Hepatocyte replication has been shown to follow circadian rhythm (Smaaland 1996).A study aimed to measure DNA synthesis over an 18-hour time point to investigate the effect of circadian rhythms on control and ciprofibrate treated animals. These animals were administered 100mg/kg bw i.p. BrdU as previously described (table 3.4).

Dosed				
Timepoint	6am	12pm	6pm	12am
1	0	0	0.4	0
2	0	0	0	0
3	0	0	0	0
mean	0	0	0.133333	0
Control	0	0	0	0

Table 3.4. Effect of circadian rhythm on DNA synthesis. S129 wild type mice administered 50mg/kg bw i.p. ciprofibrate and 100mg/kg bw i.p BrdU (n=3). Tissues were collected at the time points and labelled nuclei were detected by immunohistochemistry. After 18 hours DNA synthesis was not observed in livers of either control (n=1) or treated animals except at the 6pm timepoint.

Table 3.4 demonstrates that the circadian rhythm of cell division is not detectable in the livers of animals used in this experiment. The level of DNA synthesis detected in the livers of control or dosed animals was very low.

Section 3.5.2.2. Time course study

A time course study was conducted in order to further characterise the early proliferative response by ciprofibrate after 18,24,30,36 and 42 hours. S129 wild type mice (n=3) were administered 50mg/kg bw i.p. ciprofibrate and 100mg/kg bw i.p. BrdU (figure 6.4).

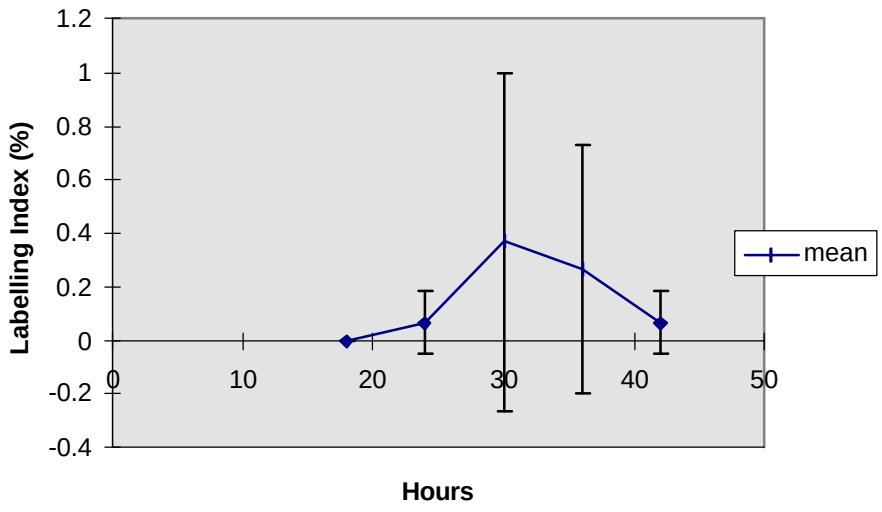

Figure 6.4.Time course study: S129 wild type mice administered 50mg/kg bw i.p. ciprofibrate and 100mg/kg bw i.p BrdU (n=3). Ciprofibrate was re-administered after 36hr at a dose of 50mg/kg bw i.p. in order to maintain a constant plasma concentration (t1/2=36hours). Tissues were collected at the time points and BrdU labelled nuclei were detected by immunohistochemistry. An increase in DNA synthesis was observed over the 42hour period with the peak LI occurring at 30hr. However DNA synthesis is subject to inter-animal variation.

At 50mg/kg bw i.p. ciprofibrate increased the number of hepatocytes in S-phase. DNA synthesis is greatest at 30-hour after which it returns to control level. However this result is not statistically significant (P>0.05). Labelling indices between animals exhibited a large degree of variability suggesting that the dosing regime and small group numbers are insufficient for detecting the low occurrence of labelled hepatocytes.

Section 3.5.2.3 Oral administration of BrdU

The low induction of DNA synthesis in mouse liver is in contrast to the literature (Styles *et al* 1990, Barrass *et al* 1993). The high variability of data obtained from the LI values suggested that the BrdU labelling schedule was inadequate. Administration of BrdU in the drinking water of animals has been used to increase the sensitivity of the assay. This technique has been shown to be a measure of the cumulative number of hepatocytes in S-phase rather than the labelling cells at one time point only (Ton *et al* 1997). S129 wild type mice were administered 50mg/kg bw i.p. ciprofibrate and provided BrdU ad libitum in the drinking water at a concentration of 0.8mg/ml (figure 6.5).

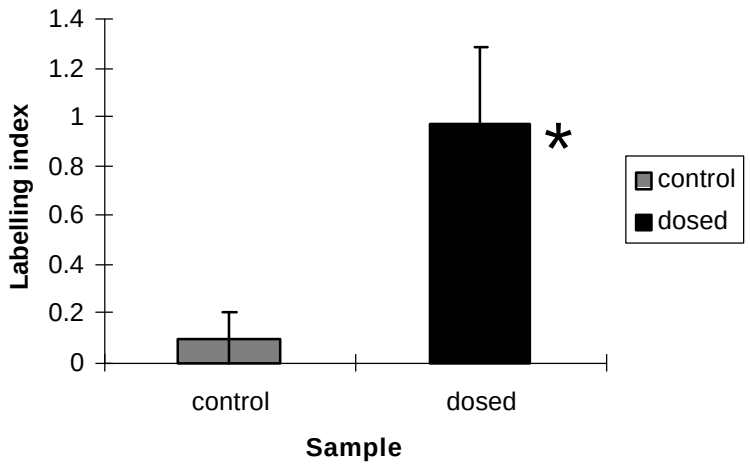

Figure 6.5. Oral administration of BrdU:BrdU (0.8mg/ml) was added to the drinking water of S129 wild type mice administered 50mg/kg bw i.p. ciprofibrate (n=3) After 24 hours dosed animals showed a significant increase in DNA synthesis compared to controls (P<0.05).

Figure 6.5 shows that including BrdU in the drinking water of 50mg/kg bw i.p. ciprofibrate dosed mice enables the detection of hepatocytes in S-phase. There is a 9-fold difference between control and dosed groups after 24 hours. This data shows that there is much lower variability and more accurate measurement of DNA synthesis when BrdU is given orally in drinking water, compared to a single i.p. injection (figure 6.5)

Section 3.5.3 Repeat dose ranging and time course studies

The detection of ciprofibrate-induced DNA synthesis after oral administration of BrdU (figure 6.5) prompted a repeat of time course and dose ranging studies. Although a significant 9-fold induction was observed at 50mg/kg i.p.

bw ciprofibrate this is level of induction is conflicting with previous work. An oral ciprofibrate dose of 250mg/kg bw was required to increase DNA synthesis by 15-fold in C57BL/6 mice after four days (Budroe *et al* 1992). Therefore the effect of administering ciprofibrate via the oral route was also investigated.

Section 3.5.3.1 Time course study

A time course over 48 hours was used to determine if the induction of S-phase by ciprofibrate occurs over a greater duration than 24 hours (section 3.5.3). C57BL/6 male mice (n=6 per group) were administered 50mg/kg i.p. bw ciprofibrate and provided free access to BrdU in the drinking water (0.8mg/ml).Control mice were injected i.p. with 200µl corn oil. Mice were sacrificed after 24, 36 and 48 hours and liver and duodenum tissues were collected as previously described. Labelling indices (LI) were calculated by dividing labelled nuclei by unlabelled nuclei and multiplying by 100. 1000 nuclei were calculated per slide.

Examining the time course of ciprofibrate induced DNA-synthesis (figure 6.6A), a significant difference was observed between control and treated groups over 48 hours (n=6, P=0.0021).

Section 3.5.3.2 Route of Administration

The route of administration was also examined by dosing male C57BL/6 mice by gavage 50mg/kg ciprofibrate i.p. bw (n=3, figure 6.4B). No significant difference was observed between control or dosed groups administered by gavage (n=3, P>0.05).

Section 3.5.3.3 Dose response study

C57BL/6 mice were administered 10,25,50,75mg/kg i.p. ciprofibrate (n=5) and BrdU in the drinking water (0.8mg/ml). One group of mice was dosed MCP (25mg/kg bw i.p n=5.) as a positive control due to the known proliferative effects described in the literature (Styles *et al* 1990). However no increase in DNA synthesis was observed (data not shown). A 2-fold induction with 50mg/kg i.p. was observed in dosed animals (n=5 P=0.017) indicating the start of a proliferative response to ciprofibrate. Statistical differences were observed at ciprofibrate doses of 75mg/kg (*: P=0.025) and 100mg/kg (*: P=0.008).

Data from figure 6.6C (and figure 6.5) indicates that oral administration of BrdU exhibits sufficient sensitivity to detect ciprofibrate induced DNA synthesis in the liver of C57BL/6 and S129 mice. However the high variability of data presented in figure 6.6A and B indicates that any increase of cells in

S-phase is small and the group numbers are not large enough to provide statistically sufficient results.

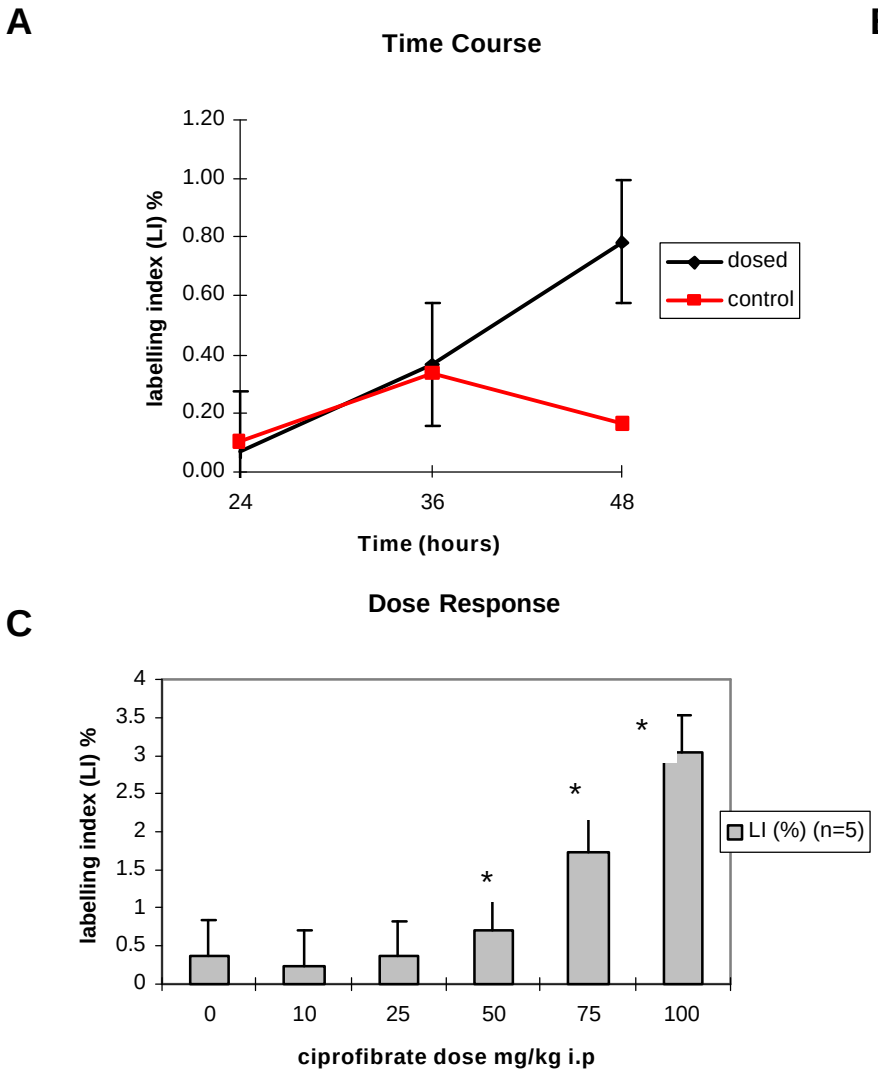

Figure 6.6. Ciprofibrate was administered to C57BL/6 either i.p. (A&C) or by gavage (B). BrdU (0.8mg/ml in drinking water) immunohistochemistry was used to determine the LI of treated animal livers in experiments A an. 1000 nuclei were counted per slide. Experiments B and C were conducted over a 24hr period. A: Time course of ciprofibrate induced DNA-synthesis. No significant difference was observed between control and treated groups over 48 hours (n=6, P>0.05). B: No significant difference was observed between control or dosed groups administered by gavage (n=3, P>0.05). C: A 2-fold induction with 50mg/kg i.p. was observed in dosed animals (n=5 *: P=0.017) indicating the start of a proliferative response to ciprofibrate. Statistical differences were observed at 75mg/kg (*: P=0.025) and 100mg/kg (*: P=0.008).

Chapter 4 Discussion

Section 4.0 Constitutive expression of murine Cyp4a genes

Previous reports of strain and sex-dependent expression of the Cytochrome P450 4a subfamily in the mouse warranted further characterisation of Cyp4a gene expression. Strain differences in Cyp4a expression observed by Hiratsuka *et al* (1996) and Henderson & Wolf (1991) were determined by the use of non-specific immunoblotting of Cyp4a-related protein using a polyclonal antibody to rat CYP4A1. Determination of sex and strain differences in the expression of a cytochrome P450 will provide a useful model for the study of the regulation of sex-specific gene regulation.

Section 4.1 Hepatic *Cyp4a12* expression in inbred mice strains

Constitutive expression of the *Cyp4a* gene family has been determined in 8 inbred mouse strains using male and female liver and kidney RNA. Strains studied were A/J, DBA/2, AKR, SWR, Balb/c, SJL, C57BL/6 and ddY mice. Since members of *Cyp4a* subfamily show high sequence similarity to each other, a RNase protection assay was employed due to its high specificity (Myers *et al*., 1985) and ability to discriminate between the *Cyp4a10*, *12* and *14* genes (Bell *et al* 1993, Heng *et al* 1997). Probes used for the protection of

4a10 and *12* have been shown to be specific for their respective genes (Bell *et al* 1993). RNAse protection assays allow the quantification of Cyp4a gene expression by incorporation of [^{32}P] CTP in the transcription reaction and subsequent phosphor imaging.

Male constitutive expression of *Cyp4a12* was detectable in the liver RNA of all the strains examined (Figure 3.8). In the female liver *Cyp4a12* expression was very low compared to males. While *Cyp4a12* expression was shown to be sex-specific determined from the absolute levels of *Cyp4a12* expression. The ratio between male and female expression showed variation between strains. Balb/c mice had a 5-fold greater level of *Cyp4a12* than C57BL/6 and DBA/2. SJL/J had a 15-fold greater *Cyp4a12* level than SWR mice. ddY mice exhibited 6- and 1.2-fold greater *Cyp4a12* expression than C57BL/6 and Balb/c mice respectively. Therefore strain variations seen between the ratio of male:female *Cyp4a12* and the absolute levels indicate a clear sex-difference and fail to account for the sex-specific expression of *Cyp4a12*.

Thus in the liver *Cyp4a12* is not a strain specific cytochrome P450 but is male specific in the eight mouse strains examined.

The male specific expression of hepatic *Cyp4a12* RNA demonstrated here is in disagreement with data from Hiratsuka *et al* (1996a). Their findings present no sex difference in Balb/c and C57BL/6 expression of Cyp4a proteins. Thus it appears that there is still a discrepancy between the *Cyp4a12* hepatic

expression pattern presented here and that of the enzyme and protein determined by Hiratsuka *et al* (1996a). I find a sex difference in expression of hepatic *Cyp4a12* in all strains examined. While my data is consistent with the male-specific expression of LAH and a CYP4A protein in ddY mice, it does not explain the lack of sex-specificity seen in other strains (e.g. C57BL/6).

Several other P450 isozymes have been found to be catalysts of the hydroxylation of medium- and long-chain saturated and unsaturated fatty acids (Orrenius *et al* 1969, Prough *et al* 1978). For example CYP2E1 in the rat and human is not only inducible by ethanol but is also involved in the microsomal (ω-1) hydroxylation of lauric acid and several other fatty acids (Fukuda *et al* 1994, Amet *et al* 1994, Adas *et al* 1999, Leclercq *et al* 2000). At present the expression and activity of Cyp2e1 related LAH has yet to be investigated to see if this is a possible candidate for the strain and sex-dependent LAH activity observed in the mouse.

Section 4.2 Renal *Cyp4a12* expression in inbred mice strains

Renal *Cyp4a12* was also sex-specific as demonstrated by the male:female ratios of *Cyp4a12* expression (figure 4.0). ddY mice exhibited the greatest ratio between male and female (4335:1). Mouse strains Balb/c and C57BL/6 exhibited ratios of 220:1 and 911:1 respectively. The remaining five strains also exhibited male-specific expression of *Cyp4a12*. These strain dependent

differences in the levels of expression may explain the reported sex differences in the renal microsomal lauric acid metabolism reported by Hiratsuka *et al* (1996b) and Henderson *et al* (1990,1991,1994).

Data presented in figure 3.8 supports previous reports in the kidney of ddY, Balb/c and C57BL/6 mice, male specific LAH enzyme activity and Cyp4a protein. *Cyp4a12* expression has also been shown to exhibit a male-specific pattern in the kidneys of S129 mice (Holla *et al* 2001).

Sex differences in the expression of murine renal *Cyp4a* proteins and lauric acid hydroxylase activity have been described (Henderson *et al* 1990, Henderson and Wolf, 1992), as well as in the expression of CYP4A2 in rat that shows sex specific regulation similar to *Cyp4a12* (Sundseth and Waxman, 1992). However it has been shown that rat CYP4A2 is the homologue of mouse *Cyp4a14*.

This data supports the hypothesis that the putative LAH activity of *Cyp4a12* would account for the sex-specific LAH activity and expression of *Cyp4a*-related protein. However the data is also anomalous in that the *Cyp4a12* expression profile is consistent with published reports of renal LAH and *Cyp4a* expression, but not with reports of hepatic LAH and *Cyp4a* expression.

Section 4.3 Expression of other Cyp4a genes in ddY mice

Further work proceeded to determine if the anomalous reports of a sex and strain dependent expression of a *Cyp4a* in male ddY mice was due to the presence of another *Cyp4a* gene.

RNase protection assays were used to determine the levels of expression of *Cyp4a10* and *Cyp4a14* in ddY mice. This assay was used as it has been shown previously (Heng *et al* 1997) that the use of specific probes distinguishes between genes that share high sequence identity.

Constitutive expression of *Cyp4a10* and *14* mRNA was determined in the liver and kidney of male and female ddY mice. *Cyp4a10* mRNA is expressed in both sexes in the liver and kidney (figure 4.3). *Cyp4a10* has been shown to be expressed in the kidney of male and female S129 mice supporting this data. This data agrees with previous reports that *Cyp4a10* does not exhibit a sex-specific expression pattern despite a polymorphism in the 5'end of the probe used (figure 4.4; Bell *et al* 1993, Heng *et al* 1997).

Hepatic and renal *Cyp4a14* expression has previously been shown to be low in both sexes of NMC mice. In the kidney no sex-specific pattern of expression was detected in ddY (Heng *et al* 1997). Prolonged exposure of the phosphor image was required in order to detect levels of *Cyp4a14* (Figures 4.4). Figure 4.5 shows that in ddY mice renal *Cyp4a14* is significantly higher

in female than male liver and kidney ($P<0.05$ and $P<0.01$ respectively). In males, hepatic *Cyp4a14* is greater than kidney expression (5-fold). This data demonstrates that *Cyp4a14* exhibits a predominantly female expression pattern in ddY. Similarly northern blot analysis of kidney Cyp4a isoform expression showed that *Cyp4a14* is expressed, albeit at low levels in both sexes (Holla *et al* 2001). Dissimilar expression between observed between ddY and NMC maybe as a result of different autoradiography exposure times. Another possibility is that *Cyp4a14* is subject to genetic variation between strains as seen for *Cyp4a12*. Evidence for this originates from northern blot analysis of S129 mice, which shows low levels of *Cyp4a14* RNA in male kidney (Holla *et al* 2001). However it is unclear how specific the northern blot technique is for detecting members of the same P450 subfamily.

Reports of a strain and sex-dependent *Cyp4a* in ddY were investigated using the gene specific RNase protection assay. In ddY mice no sex-dependent expression of *Cyp4a10* was observed and no statistically significant differences between male and female animals was determined. In ddy mice while in the male *Cyp4a14* is expressed at higher levels than the kidney, *Cyp4a14* expression was found to be predominantly female in both liver and kidney samples.

RNAse protection analysis of members of the Cyp4a gene family demonstrates clear differences in expression pattern. *Cyp4a12* is male specific found at high levels in the liver and kidney of inbred mice, and

exhibits genetic variation between strains. The male specific hepatic expression of *Cyp4a12* in ddY and 7 other inbred mouse strains and the characterisation of *Cyp4a10* and *Cyp4a14* expression exclude these genes as possible candidates for a sexually dimorphic, strain dependent *Cyp4a*.

Section 4.4 Hepatic lauric acid hydroxylase activity

Data here demonstrates the male-specific expression of *Cyp4a12* in mouse liver. However LAH activity is strain and sex-dependent (Henderson *et al* 1990, Henderson *et al* 1994 and Hirasuka *et a* 1996a/b). In order to determine if the putative LAH activity of *Cyp4a12* accounts for these sex differences, LAH activity was measured in ddY and C57BL/6 mice. Lauric acid hydroxylase activities involve the catalysis of two different reactions. LA11H (ω-1 hydroxylase) and LA12H (ω-hydroxylase) catalyse the hydroxylation of a carbon atom at either the ω-1 or ω position of lauric acid.

C57BL/6 exhibited no sexually dimorphic activity of hepatic LAH11 and LAH12 Hepatic LAH12H activity in ddY mice is greater activity in male mice compared to female. Male LAH12 activity is approximately 2.2-fold greater than in female mice. Therefore in C57BL/6 mice there is no sex difference in LAH activity. ddy mice exhibit a sex difference with respect to LAH-12H activity. This sex difference in LAH activity therefore supports data by

Hiratsuka *et al* (1996a) by confirming the presence of a strain and sex-specific lauric acid hydroxylase.

Hepatic LAH activity in ddY and C57BL/7 mice support previous analysis by Hiratsuka *et al* (1996a). However the sex and strain- dependent differences observed in LAH activity do not match the constitutive expression of *Cyp4a12*. Male-specific expression of *Cyp4a12* was found in all the strains examined. ddY mice did not exhibit strain-specific expression of *Cyp4a12*, indicating that *Cyp4a12* is not responsible for the sex and strain dependent LAH activity seen in these animals. Expression of *Cyp4a10* in ddY mice does not exhibit sexual dimorphism, but adduced evidence shows that *Cyp4a10* mRNA is predominant in the livers of female mice (Henderson *et al* 1994). However it is not clear if a full-length probe was used in the northern blot analysis of *Cyp4a10*.

Data demonstrating high level male specific expression of *Cyp4a12*, with strain-dependent sexual dimorphic LAH activity and female expression of *Cyp4a10* suggest that although *Cyp4a10* mRNA expression is found in females, the LAH activity suggests that the message is not translated into the protein or the protein has a diminished activity. An alternative possibility is that there may be a contribution to hepatic LAH from other P450s such as Cyp2e1 (Adas *et al* 1999)

Further investigation using specific antibodies for *Cyp4a10* is required to confirm the association between *Cyp4a* gene expression and the presence of a corresponding protein.

Section 4.5 *Cyp4a12* expression in PPARα wild type and knock-out mice

PPARα knock out mice do not express any level of PPARα (Lee *et al* 1996). These transgenic mice provide a model for investigation of the regulation of the *Cyp4a* gene family. Peroxisome proliferators induce *Cyp4a* gene expression; this indicates that these genes are regulated by PPARα. The absence of PPARα in knockout mice allows the study of the regulation of constitutive *Cyp4a* gene expression by this receptor, and may indicate their physiological function.

Constitutive expression of *Cyp4a12* was found to be high in the male liver of both PPARα wild type and knock out mice (Figure 4.7). However compared to wild-type control mice, a 15-fold reduction of *Cyp4a12* was observed in knock out mice. Total P450 concentrations were determined to show if high level expression of *Cyp4a12* in male liver and reduced levels in knock out mice resulted in a reduced protein expression (section 3.3.1).

The significant difference in total P450 concentration between male and female wild type mice, observed in PPARα mice indicates *Cyp4a12* represents a major P450 expressed in the male mouse liver (table 3.2).

Absence of PPARα diminishes the expression of *Cyp4a12* but may also result in the reduction of other genes or proteins under its regulation. Decreased *Cyp4a12* expression and total P450 protein concentration also suggests that the reduced mRNA expression of *Cyp4a12* also results reduced protein expression. However to demonstrate this definitively, characterisation of protein levels would need to be conducted using an antibody specific to the *Cyp4a12* gene. Alternatively the LAH activity in PPARα wild type and knockout mice was investigated to examine the effect of this receptor in the regulation of LAH enzyme activity.

Sexual dimorphism was observed in the LAH12 activity of hepatic microsomes from S129 PPARα wild type mice but not knockout mice.

In summary, *Cyp4a12* is expressed in a sexual dimorphic pattern in the mouse liver of the inbred mice examined. Strain differences in the male specific expression of *Cyp4a12* were observed although reports of a strain and sex-specific Cyp4a were not confirmed. PPARα transgenic knock out mice were used to investigate the possible regulation of *Cyp4a12* expression by this steroid nuclear receptor. In PPARα male knock out mice *Cyp4a12* mRNA expression is reduced compared to wild type animals. Lower LAH activity in PPARα knockout male mice and lower *Cyp4a12* mRNA expression indicates that PPARα is involved in the regulation of P450 genes encoding for LAH enzymes. Supporting this is the reduced total P450 protein concentration

in knock out mice, suggesting that PPARα is involved in the regulation of *Cyp4a12* and possibly other cytochrome P450 genes in the male liver. This is confirmed by the expression pattern of *Cyp4a14* in PPARα knockout mice. Constitutive expression of *Cyp4a14* is comparatively low compared to *Cyp4a14* and is not subject to sexual dimorphic expression in mouse liver (Heng *et al* 1997). In male PPARα knockout mice, *Cyp4a14* expression is 4-fold less compared to wild type (figure 4.9). Expression of *Cyp4a12* and *Cyp4a14* is reduced in the liver of male S129 PPARα indicating that PPARα is a key regulator of Cyp4a RNA expression.

Other researchers have found that expression of *Cyp4a* genes in S129 PPARα. Mice are not affected by deletion of this gene (Lee *et al* 1996 and Gonzalez *et al* 1998). However, this maybe due to the lack of sensitivity in detecting specific members of the *Cyp4a* gene family. Northern analysis using a probe for rat CYP4A1 and CYP4A3 determined that there was no reduced in *Cyp4a* RNA expression in the livers of PPARα knock out mice (Lee *et al* 1997, Kroetz *et al* 1998, Leone *et al* 1999, Gueraud *et al* 1999). Northern blot analysis using probes for *Cyp4a10* and *Cyp4a14* also found no difference in *Cyp4a* expression in control livers of S129 PPARα mice. The absence of any observable reduction in Cyp4a expression in these studies may be due to the low very level of expression and different exposure times for phosphor imaging (Barclay *et al* 1999).

These findings confirm previous reports where *Cyp4a12* has been shown to be regulated differently than other *Cyp4a* isoform (Bell *et al* 1993). That study demonstrated that *Cyp4a12* is expressed constitutively at high levels in male liver and kidney and was non-inducible by MCP. By contrast, the *Cyp4a12* gene was expressed at low levels in both liver and kidney of female mice. Treatment of these animals with MCP MCP induced *Cyp4a12* mRNA to levels comparable with those in the male (M. Choudhury PhD thesis 2000). As mentioned sexual dimorphic expression of murine renal *Cyp4a* proteins and lauric acid hydroxylase activity have been described (Henderson *et al*., 1990; Henderson and Wolf, 1992), as well as in the expression of CYP4A2 in rat that shows sex specific regulation similar to *Cyp4a12* (Sundseth and Waxman, 1992).

Low expression of *Cyp4a12* and 4a14 genes in PPARα knock out mice suggest that PPARα may play a significant role the constitutive expression of these genes. Previous work has demonstrated a differential induction of *Cyp4a12* and *14* by PPs in wild type mice (M. Choudhury PhD thesis 2000). *Cyp4a14* is high induced by PPs, while *Cyp4a12* induction by PPs is comparatively low. The high inducibility of both *Cyp4a10* and *14* by PPs and their low constitutive expression tend to suggest that they may play an important role in peroxisome proliferation in the mouse liver. In contrast, the lack of inducibility of *Cyp4a12* in the liver and its high constitutive level suggest that *Cyp4a12* may have house keeping function and is less important in peroxisome proliferation in the liver. In addition the reduced constitutive

expression and lack of inducibility in knock out mice demonstrate that PPARα is involved in the transcriptional regulation of the constitutive and inducible expression these two Cyp4a genes.

The reduced expression of *Cyp4a12* and *14* seen in the liver of male knock out mice and their induction by PPs (Barclay *et al* 1999) suggest that some involvement of PPARα in the constitutive expression of these two genes in the male liver of this mouse strain.

Other studies investigating the effect of PPARα on cytochrome p450 expression are limited to the levels of cholesterol 7 α-hydroxylase (Cyp7a1). This gene does not exhibit sex-specific expression and is not reduced in PPARα knockout mice (Patel *et al* 2000).

Although PPARα regulates induction by peroxisome proliferators, it is surprising that it regulates the male-specific constitutive expression of *Cyp4a12*. The male-specific expression of *Cyp4a12* is unlikely to be due to an endogenous peroxisome proliferator inducer (e.g. a lipid), since there is no concomitant induction of *Cyp4a10* and *Cyp4a14* in control males. The effect of PPARα on male-specific expression is therefore paradoxical.

Section 4.6 Regulation of cytochrome P450 expression by PPARα

To further investigate the role of PPARα in the regulation of the expression of male specific genes in the mouse male liver, three genes were chosen due to well-documented sex-specific expression pattern.

Mouse urinary protein I (MUPI), *Cyp2d9* and sex-limited protein (Slp) exhibit expression, which is limited to the male liver (section 3.4). Riboprobes were designed to be gene specific using clones acquired either from the IMAGE clone consortium (MUPI and *Cyp2d9*) or sub-cloned by PCR (Slp).

Sequence analysis of all clones were performed to confirm the authenticity of each gene to be examined (data not shown). Sub-cloning using PCR was successful in amplifying an 192bp fragment of exon 3 of the Slp gene (figure 5.1) The PCR product was subsequently ligated into PGEM-T and sequence analysis was performed to confirm the identity of the amplified sequence.

As for members of the mouse Cyp4a gene family, a RNase protection assay was used to detect and quantify the levels of the three male specific genes in mouse liver of S129 PPARα wild type and knock out mice.

Male wild type MUPI was 8-fold greater than female wild type MUPI confirming the sexual dimorphic expression of this gene (figure 5.6). This is

similar to previous work reporting that the expression of MUPI mRNA in male adult mice is 5-6-fold higher than in females (Hastie *et al* 1979, Derman *et al* 1981). MUPI is transcriptionally regulated by androgen regulation similar to the homologous rat α2u-globulin (Kurtz and Feigelson 1977, Hastie *et al* 1979) and *Cyp4a12* (Heng *et al* 1997). Administration of testosterone to female mice results in an increase in *Cyp4a12* mRNA expression in liver and to a greater extent kidney (Heng *et al* 1997). However no statistical difference was observed in MUPI expression between PPARα wild type and knock out mice (figure 5.6).

A RNase protection assay used a riboprobe specific to a fraction of exon 3 of the Slp gene (figure 5.7). Northern blot analysis of Slp RNA expression has previously determined that this gene is male specific in the male livers of Balb/c mice (Georgatsou *et al* 1993). No significant differences were observed in Slp expression between wild type and knockout male animals (figure 5.8).

Expression of Slp in the kidney was found to be 2-3-fold higher than in male wild type and knockout mice indicating that renal expression is transcriptionally regulated in a different to that in the liver. Georgatsou *et al* (1993) demonstrated that hepatic Slp expression is transcriptionally regulated by growth hormone and although Slp has been detected with high levels in the kidney (Cox *et al* 1988), its regulation has yet to be investigated.

Therefore expression of the non-P450 genes MUPI and Slp, were found to be unaffected by the absence or presence of PPARα in male liver or kidney.

Cyp2d9 gene encodes for the testosterone 16α-hydroxlyase enzyme (Harada and Negishi 1984). *Cyp2d9* sex-specific expression is regulated by growth hormone (Sueyoshi *et al* 1999) via ERα as demonstrated in female ERα deficient mice. In males *Cyp2d9* expression was unaffected by the absence of ERα indicating that ERα is not required for the constitutive expression of *Cyp2d9*.

Further more in growth hormone deficient mice *Cyp2d9* expression is induced by growth hormone and recently the cellular mechanism mediating the GH-regulated sex-specific cytochrome P450 expression has begun to become apparent (Udy *et al* 1997, Teglund *et al* 1998 and Sueyoshi *et al* 1999).

In PPARα knock out mice *Cyp2d9* is 65-fold less than in wild type male liver (figure 5.4). This data demonstrates that regulation by PPARα of another male-specific cytochrome P450. Table 3.4 illustrates the various mechanisms of regulation of *Cyp4a12* and *Cyp2d9*. The male specific expression of *Cyp4a12* is regulated in a similar manner to the rat CYP4A2 gene

Mouse P450	*Cyp4a12*	*Cyp2d9*
Liver	⇑ by MCP in	⇑ by ER in (ovx) ⇓ by GH in ⇑ by GH in
Kidney	⇓ by test in ⇑ by test in	n.d

Table 3.5. Regulation of *Cyp4a12* and *Cyp2d9*. Administration of MCP to female but not male mice results in the induction of *Cyp4a12*, In the kidney *Cyp4a12* expression is reduced in males but increased in females by testosterone (test, Heng *et al* 1997). Constitutive expression of *Cyp2d9* is regulated by growth hormone (GH) in the male liver (Noshiro and Negishi 1986, Sueyoshi *et al* 1999). Estrogen treatment increases *Cyp2d9* in ovariectomised (ovx) female mice implicating the role of ERα in the regulation of *Cyp2d9*.

Expression of CYP4A2 was found to be regulated in a sex specific manner (Waxman *et al*., 1991). This apparent sex specific expression is regulated by the different growth hormone profiles between the sexes (Waxman *et al* 1991). Another member of the mouse Cyp4a family, has been reported to be regulated by growth hormone (Henderson *et al* 1994). Expression of *Cyp4a10* is predominantly female, but following hypophysectomy it is also found in males.

Sex-specific expression of *Cyp4a12* and *Cyp2d9* and reduction in PPARα knock out mice indicate that the expression of Cyp4a is regulated by distinct mechanisms in male and female animals. The involvement of growth hormone and PPARα in the regulation of these P450s is of interest because previous work has investigated the mechanisms by which these pathways regulate sex-specific P450 expression (section 1.3.6). Park *et al* (1999) report that STAT5b is involved in the regulation of GH-regulated expression of genes in

the liver. GH pulse induced expression of male-specific Cyp genes require STAT5b-DNA interactions (Park *et al* 1999)

Potential cross-talk between JAK-STAT (janus kinase-signal Transducer and activator of tran-scription) and PPARα has been described by Zhou and Waxman (1999). Inhibition of clofibrate-induced peroxisomal β–oxidation by growth hormone (GH) has been described in rats (Yamada *et al*., 1994; Sato *et al*., 1995). This inhibition is thought to occur following binding of the GH to its membrane-bound receptor, leading to receptor dimerisation. This activity eventually leads to the activation of a cytoplasmic transcription factor STAT through JAK2 kinase mediated phosphorylation. GH inhibition of PPARα activity was dependent upon the presence of active STAT5b and GH receptor. Since STAT5b was unable to bind HD-PPRE, the inhibitory effect of GH may be due to competition for an essential PPARα coactivator or synthesis of a more proximal PPARα inhibitor.

Alternatively there may be a factor specific to *Cyp4a12* and *Cyp2d9*, and different to MUPI or Slp, which is preventing male-specific transcription. This may be due to both genes being P450s. For example, if a male-specific part of the haem synthesis pathway is switched off, then that may cause transcriptional repression of P450s such as that in porphobilinogen deaminase-deficient mice (Jover *et al* 2000), and animals treated with lead acetate (Jover *et al* 1996). However, this is less likely, since there is no decrease in the absolute amount of P450 (table 3.2)

The identification of male-specific genes in mouse liver such as *Cyp4a12* and *Cyp2d9* provide model systems, which may be used to further investigate the pathways GH-activated signaling pathways that activate members of the cytochrome P450 family and to establish the precise roles that STAT5a and STAT5b play in their expression.

Section 4.7 Induction of hepatocyte DNA synthesis *in vivo*

It has been demonstrated that PPs result in a hyperplastic response involving liver enlargement due to an increase in transient or sustained stimulation of cell replication as measured by DNA synthesis, in a dose-dependent manner (Lock *et al* 1988, Styles *et al* 1988). A 16-fold induction in DNA synthesis has been observed *in vivo* (Price *et al* 1992). In order to study the initial changes in gene expression mediated by PPARα, replicative DNA synthesis is a useful marker of peroxisome proliferation. With the aim of determining genomic changes in PPARα +/+ and -/- mouse liver after acute exposure to a PP, it was necessary to detect the earliest time point of S-phase induction.

25mg/kg bw i.p ciprofibrate was administered to S129 PPARα wildtype mice and induction of S-phase was assessed using BrdU immunohistochemistry (Eldridge *et al* 1990). Over the time points examined (0.5,1,2,3,24hr) dosed animals did not exhibit any increase in BrdU labelled (figure 6.3A) despite

evidence that BrdU was present in the systemic circulation via intraperitoneal administration as demonstrated by labelling of intestinal sections (figure 6.3B).

These results are anomalous to more detailed studies, which have established time, and dose-dependent changes seen after acute exposure to peroxisome proliferators. In Snell dwarf mice dosed 25/mg/kg MCP (oral) the incidence of hepatocytes in S-phase (by BrdU pulse labelling) peaked at 36 hours in heterozygote animals (Styles *et al* 1990). The number of binucleated hepatocytes also decreased in response to exposure to MCP indicating a susceptible sub-population of hepatocytes to acute hyperplasia. This initial hyperplastic response is due to the conversion of binucleated cells to mononucleated tetraploids by amitiotic cytokinesis following S-phase (Styles *et al* 1987,1988, 1990a/b).

Therefore it was necessary to determine if other factors were responsible for the failure to detect or induce S-phase in the livers of ciprofibrate treated mice. Circadian variations of liver cell proliferation have been described including hepatocyte DNA synthesis and mitotic activity (reviewed in Smaaland *et al* 1996). Over an 18-hour period, hepatocyte replication was assessed by in control and ciprofibrate treated animals. Animals were administered 100mg/kg bw i.p. BrdU as previously described (table 3.4). In both treatment groups the level of DNA synthesis was very low and the experiment was unable to demonstrate a circadian rhythm in hepatocyte DNA synthesis (table 3.4).

After 25mg/kg bw i.p. ciprofibrate was administered to S129 PPARα mice DNA synthesis was not observed after 24 hours. Previous studies have reported a peak activity after 36 hours (Styles *et al* 1990). Therefore the time course over which DNA synthesis was extended up to 42 hours and ciprofibrate was administered at 50mg/kg bw i.p (figure 6.4). A non-statistically significant increase in BrdU-labelled nuclei was observed at the 30hour time point. Labelling indices between animals exhibited a large degree of variability suggesting that the dosing regime and small group numbers (n=3) were insufficient for detecting the low occurrence of labelled hepatocytes.

BrdU may be administered as a pulse dose by intraperitoneal injection or by continuous dosing via a osmotic mini-pump (Eldridge *et al* 1990) or via the drinking water (Ton *et al* 1997). A pulse dose of BrdU permits the evaluation of replicative DNA synthesis during a relatively narrow time course and is most useful in tissues with a fast turnover rate such as the gastrointestinal or integumentary systems (Goldsworthy *et al* 1993). Because detectable blood plasma levels of BrdU decline after several hours following a pulse dose (Kitchin and Brown 1995), such a discontinuous method of BrdU administration may not detect the long term fluctuations in cell proliferation following treatment with a peroxisome proliferator.

Data in figures 6.3 and 6.4 indicate that in order to evaluate the replicative DNA synthesis in the liver a continuous method of BrdU dosing may provide

increased sensitivity. The use of osmotic mini-pumps present technical difficulties in terms of transplantation, cost, and time. Therefore the administration of BrdU in the drinking water was chosen due to its easier application, faster experimental implementation and avoidance of surgery-associated stress to the animals.

S129 wild type mice were administered 50mg/kg bw i.p. ciprofibrate and provided BrdU *ad libitum* in the drinking water at a concentration of 0.8mg/ml (figure 6.5). A BrdU dose of 0.8mg/ml was used based on the previous literature demonstrating that water consumption was unaffected and the absence of hepatoxicity by histological analysis (Ton *et al* 1997). Limitations of this method of delivery are acknowledged however as the BrdU dosage is entirely reliant on the drinking behaviour of the animals under treatment and the pharmacokinetics of target tissue concentrations of BrdU are unknown. A 9-fold difference between control and dosed groups in labelling indices was observed 24 hours after ciprofibrate administration. In addition the data exhibits reduced inter-animal variability, excluding the necessity to dramatically increase group sizes.

Time course and dose ranging studies were repeated using oral administration of BrdU as previously described. Although a significant 9-fold induction was observed at 50mg/kg i.p. bw ciprofibrate this level of induction is conflicting with previous work. An oral ciprofibrate dose of 250mg/kg bw was required to increase DNA synthesis by 15-fold in C57BL/6 mice after four

days (Budroe *et al* 1992). Therefore the effect of administering ciprofibrate via the oral route was also investigated in the C57BL/6 strain of mouse. No significant difference was observed between control or dosed groups (figure 6.4B).

Further to the results from the previous time course study 50mg/kg bw i.p. ciprofibrate was administered to male C57BL/6 mice with free access to BrdU in the drinking water. A significant difference between control and treatment groups were determined after 48 hours demonstrating that this dosing protocol was capable of detecting the induction of hepatocyte S-phase in PPARα +/+ mice.

A dose-response relationship was determined in order to establish a lowest observable effect level (LOEL) for the induction of DNA synthesis by ciprofibrate.

One group of mice was dosed MCP (25mg/kg bw i.p n=5.) as a positive control due to the known proliferative effects described in the literature (Styles *et al* 1990). However no increase in DNA synthesis was observed (data not shown). This is anomalous to previous reports and may be explained by the very low levels of induction occurring in the liver, which are not statistically significant when using small group numbers.

A 2-fold induction with 50mg/kg i.p. was observed in dosed animals (n=5 P=0.017) indicating the start of a proliferative response to ciprofibrate. Statistical differences were observed at ciprofibrate doses of 75mg/kg and 100mg/kg. Data from figure 6.6C (and figure 6.5) indicates that oral administration of BrdU exhibits sufficient sensitivity to detect ciprofibrate induced DNA synthesis in the liver of C57BL/6 and S129 mice. However the high variability of data presented in figure 6.6A and B indicates that any increase of cells in S-phase is small and the group numbers are not large enough to provide statistically sufficient results.

C57BL/6 mice administered 50mg/kg bw i.p. ciprofibrate exhibited a 2-fold increase in BrdU labelled nuclei. This induction also appears not to have reached it maximal induction after 24 hours. Studies using 25mg/kg bw i.p MCP have reported maximal induction 20-30 hours after administration (Styles *et al* 1988,1990a). Data here demonstrates that ciprofibrate is different to MCP in its ability to induce S-phase after acute administration *in vivo*. Such quantitative differences in induction, dose, route, strain and labeling technique make comparison of studies difficult. Experiments using greater size numbers and a similar route of administration are more favorable in order to characterise generic changes seen in liver cytology and associated gene expression.

However in order to characterise the possible early changes seen in gene expression after PP administration, a dose response relationship is required

that involves the induction S-phase at the earliest possible stage of the hyperplastic response. Therefore intraperitoneal administration is the obvious choice for delivery of xenobiotic directly into the systemic circulation. High inter-animal variability in LIs suggests that any small changes seen after PP administration will not be detected if that response is transient and is determined using small group sizes. This data demonstrates that pulse-labelling of BrdU is not the best method of detection of hepatocytes in S-phase. Continuous labeling of BrdU increases the sensitivity of the experiment as it provides a measure of the magnitude of induction over the duration of the assay as well as ensuring that BrdU reaches the systemic circulation.

Failure of ciprofibrate to induce DNA synthesis to levels previously reported for MCP may reflect differences in potency between the two compounds. Another possible explanation are strain differences as reported between C57BL/6 and Balb/c mice. When administration orally at doses ranging from 0.1 to 250ppm increased LI were observed at 1.0ppm in Balb/c mice but not C57BL/6 animals after 3 days (Budroe *et al* 1992). C57BL/6 mice require doses of 100 and 250ppm before significant increases in LI were detected. In this group of studies S129 or C57BL/6 mice were used, however the previous studies of Styles *et al* have used predominantly rats and heterozygous and homozygous Snell dwarf mice. Studies investigating the mechanisms of hepatocarcinogenesis by PPs should consider strain difference in responsiveness to PPs when producing experimental designs from previous literature.

Chapter 5: References

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