THE ISOLATION AND CHARACTERISATION OF CYTOCHROME P4504A FAMILY GENES FROM THE RAT / 1994

A THESIS PRESENTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

BY

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ABSTRACT

The phenomenon of induced peroxisome proliferation in rodents has invariably been associated with upregulation of member(s) of the CYP4A subfamily of heme-proteins. However, regulation of the members of the rat CYP4A family has not been fully examined at the molecular level. Despite the publication of the rat CYP4A1 and CYP4A2 genomic sequences (Kimura et al., 1989a), no functional assays have been performed to determine the mechanism of peroxisome proliferator modulated transcriptional activation of these genes. The objective of this study was to isolate members of the rat CYP4A subfamily of genes and investigate their inducibility by peroxisome proliferators at the molecular level. In this thesis I present results to demonstrate:-

1) the isolation and partial characterisation of recombinant DNA clones corresponding to 3 different rat genomic sequences, showing a degree of similarity to the rat CYP4A1 cDNA,

2) identification, by sequencing, of one of the groups of clones (group C) to correspond to CYP4A2 and another (group B) to be, possibly, the genomic sequence of CYP4A3. The identification of the remaining group (group D) remains unknown, but most likely represents a novel member of the rat CYP4A subfamily,

3) the apparent absence of a peroxisome proliferator response element (PPRE) in the 5' flanking DNA sequence of the clones, as determined by hybridisation to the rat acyl-CoA oxidase PPRE oligonucleotide (GTOX285),

4) a lack of a response of a -5.8kb upstream sequence of the newly-isolated human CYP4A11 gene in a co-transfection assay with the mouse PPAR and in the presence of the peroxisome proliferator Wy 14643.
The results further extend knowledge on the multiplicity of CYP4A genes in the rat and provide data on the transcriptional regulation of CYP4A genes in rodents and man.
DEDICATED
TO MY MUM AND DAD
ACKNOWLEDGEMENTS

I would like to express my gratitude to my joint supervisors Professor G.G. Gibson and Dr P.S. Goldfarb for their guidance throughout the course of this work.

I would also like to thank my industrial supervisor Dr C.R. Elcombe and his colleagues at ICI for allowing me to work in their laboratory and generally clutter the place up with my "molecular biology stuff". Thanks are also due to Dr S. Green and colleagues (ICI) for their help and advice and use of equipment during my time there.

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Finally, a great debt is owed to my parents for their constant encouragement and support, without which this thesis would still be a figment of my imagination, thank you.
ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>A</td>
<td>Adenine</td>
</tr>
<tr>
<td>Ah</td>
<td>Aromatic Hydrocarbons</td>
</tr>
<tr>
<td>Amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>ARNT</td>
<td>Ah receptor nuclear translocation factor</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>C</td>
<td>Cytosine</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic Adenosine monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complimentary DNA</td>
</tr>
<tr>
<td>CIAP</td>
<td>Calf intestinal alkaline phosphatase</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>CRE</td>
<td>Cyclic AMP responsive element</td>
</tr>
<tr>
<td>dATP</td>
<td>Deoxyadenosine triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>Deoxycytidine triphosphate</td>
</tr>
<tr>
<td>dGTP</td>
<td>Deoxyguanosine Triphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleotide triphosphates</td>
</tr>
<tr>
<td>DRE</td>
<td>Drug response element</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>dTTP</td>
<td>Deoxythymidine Triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>Oestrogen receptor</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>G</td>
<td>Guanine</td>
</tr>
<tr>
<td>3H</td>
<td>Tritium</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-thiogalactosidase</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilobase</td>
</tr>
<tr>
<td>Kdal</td>
<td>Kilodaltons</td>
</tr>
<tr>
<td>KoAc</td>
<td>Potassium acetate</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani media</td>
</tr>
<tr>
<td>LCFA</td>
<td>Long chain fatty acid</td>
</tr>
<tr>
<td>LMP</td>
<td>Low melting point</td>
</tr>
<tr>
<td>mA</td>
<td>Milliamperes</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
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<td>--------------</td>
<td>------------</td>
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<tr>
<td>NAD(P)</td>
<td>Nicotinamide adenine dinucleotide (phosphate)</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>pfu</td>
<td>Plaque forming units</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>$^{32}$p</td>
<td>Phosphorous 32</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator activated receptor</td>
</tr>
<tr>
<td>PPRE</td>
<td>Peroxisome proliferator responsive element</td>
</tr>
<tr>
<td>RAR</td>
<td>Retinoic acid receptor</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X receptor</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulphate</td>
</tr>
<tr>
<td>SSC</td>
<td>Standard saline citrate buffer</td>
</tr>
<tr>
<td>$^{35}$S</td>
<td>Sulphur 35</td>
</tr>
<tr>
<td>T</td>
<td>Thymine</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris/borate EDTA buffer</td>
</tr>
<tr>
<td>TE</td>
<td>Tris/EDTA buffer</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N,N'-tetramethylethylenediamine</td>
</tr>
<tr>
<td>TR</td>
<td>Thyroid hormone receptor</td>
</tr>
<tr>
<td>Tris</td>
<td>2-amino-2-hydroxymethylpropane-1,3-diol</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
<tr>
<td>VDR</td>
<td>Vitamin D3 receptor</td>
</tr>
<tr>
<td>X Gal</td>
<td>5-bromo-4-chloro-3-indoly-β-D-galactosidase</td>
</tr>
<tr>
<td>XRE</td>
<td>Xenobiotic response element</td>
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INTRODUCTION

1.1 THE CYTOCHROME P450 GENE SUPERFAMILY

The cytochrome P450 gene superfamily (EC 1.14.14.1) comprise a family of b-type haemoproteins with an identical prosthetic group and mechanism of catalysis, but widely different substrate specificities.

The first cytochrome P450 was identified independently by two groups in the same year (Klingenberg, 1958, and Garfinkel, 1958) as a reduced pigment that had an absorption band with a soret maximum at 450nm after binding to carbon monoxide. However it was not until 1964 that the haemoprotein nature of the pigment was documented by Omura and Sato (classifying it as a b type) and provisionally gave it the name cytochrome P450. The role of cytochrome P450 in the microsomal mixed function monoxygenase system was also established at this time (Cooper et al., 1965).

Since this time it has become evident that multiple forms of cytochrome P450 exist with a ubiquitous distribution in nature. Cytochrome P450s are found in prokaryotes, unicellular eukaryotes, plants, fowl, insects, fish, invertebrates and mammals (Nelson et al., 1993 and references therein). Whilst the prokaryotic enzyme cytochrome P450cam (CYP101), is a soluble haemoprotein, those of higher organisms are membrane bound. Mammalian cytochrome P450s may be divided into two major groups based on their intracellular location and the enzyme from which they receive electrons (Gonzalez, 1990). The mitochondrial P450s, such as side chain cleavage enzyme (CYP11A1) and steroid 11β-hydroxylase (CYP11B1), are found in the mitochondria of the adrenal cortex and are involved in steroid synthesis. These enzymes are synthesised by cytoplasmic polyribosomes (Nabi et al., 1983). However the great majority of P450s are found primarily in the endoplasmic reticulum and are
synthesised on membrane-bound polyribosomes (Gonzalez and Kasper, 1980).

1.1.1 Nomenclature

Omura and Sato (1964) had intended that the name "P450" would be provisional, until more knowledge about the substance had been elucidated. However as more cytochrome P450s were discovered, primarily through protein purification research, it became clear that many cytochrome P450 forms had broad overlapping substrate specificities which precluded establishing a nomenclature system based upon unique catalytic functions. However, within the last decade the isolation and sequencing of P450 proteins, genes and cDNAs (Table 1.1) have facilitated the development of a P450 classification system based on primary amino acid sequence alignment data (Nebert et al., 1987).

This system of nomenclature is constantly evolving as more P450s are isolated and characterised (Nebert et al., 1989a, 1991, and Nelson et al., 1993). According to this classification system, those cytochrome P450 proteins with 40% or greater sequence similarity are included in the same family (designated by an Arabic number), and those with greater than 55% similarity are included in the same subfamily (designated by a capital letter). The individual genes are arbitrarily assigned numbers in order of their discovery. For all P450 genes and cDNAs the italicised root symbol "CYP" for human ("Cyp" for mouse), representing "cytochrome P450," is used, followed by the family number, subfamily letter and then the individual gene number. The mRNAs and enzymes in all species (including mouse) are written with all capital letters, without italics.

1.1.2 Evolution of the Cytochrome P450 Superfamily

Cytochrome P450 evolution is believed to have originated with the emergence of a common ancestral gene 3.5 billion years ago. There are presently 221 P450 genes and
Table 1.1 Total number of cytochrome P450 genes and cDNA's that have been reported in the last seven years in two year intervals.

<table>
<thead>
<tr>
<th>YEAR</th>
<th>NUMBER OF P450's genes</th>
<th>NUMBER OF P450's pseudogenes</th>
<th>NUMBER OF GENE FAMILIES</th>
<th>REFERENCE</th>
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<tr>
<td>1987</td>
<td>65</td>
<td>-</td>
<td>10</td>
<td>Nebert et al., 1987</td>
</tr>
<tr>
<td>1989</td>
<td>71</td>
<td>4</td>
<td>14</td>
<td>Nebert et al., 1989a</td>
</tr>
<tr>
<td>1991</td>
<td>154</td>
<td>7</td>
<td>27</td>
<td>Nebert et al., 1991</td>
</tr>
<tr>
<td>1993</td>
<td>221</td>
<td>12</td>
<td>36</td>
<td>Nelson et al., 1993</td>
</tr>
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12 putative pseudogenes that have been described in 31 eukaryotes (including 11 mammalian and 3 plant species) and in 11 prokaryotes (Nelson et al., 1993). Of the 36 gene families so far described, 12 families exist in all mammals comprising 22 subfamilies of which 17 have been mapped to the human genome.

This wealth of sequence information has provided important insights into cytochrome P450 evolution (Nelson and Strobel, 1987, Gonzalez and Nebert, 1990, Nebert et al., 1991). Since all cytochrome P450 enzymes studied to date metabolise endogenous substrates, it is perhaps not unreasonable to assume that they originated early in evolution to provide the organism with oxidative and peroxidative metabolism of important endogenous molecules, as well as the breakdown of environmental chemicals utilised for energy. Subsequently, the P450 enzymes in eukaryotes probably diversified more recently, in response to the adverse effects of dietary pressures, plant/animal warfare and other environmental chemicals (Gonzalez and Nebert, 1990).

Those mammalian cytochrome P450 isozymes involved with steroid and bile acid synthesis are highly conserved across species and are among the oldest forms. It has been proposed that these earliest P450s evolved to maintain membrane integrity through the metabolism of lipids and steroids (Gonzalez, 1990). The highly conserved nature implies that any mutation in these genes proves fatal and is therefore not carried through evolution (Gonzalez, 1990). The majority of microsomal cytochrome P450s act on xenobiotics and constitute part of a detoxification system. These xenobiotic metabolising enzymes, present in CYP families 1 to 3, are thought to have evolved due to the need to remove toxins ingested in the diet. Their diversity has been the result of gene duplication and mutational events which have conferred an evolutionary advantage on the animals concerned.

One consequence of human cytochrome P450 gene evolution is the polymorphism of drug metabolism, leading to marked differences in the response of individuals to the
toxic and carcinogenic effects of drugs and other environmental chemicals. The differences between human individuals, races or ethnic groups, are either at the level of expression of some forms (extrahepatic CYP1A1 after induction, hepatic CYP1A2, 2B7, 2D6, 2E1), or at the absence of a particular cytochrome P450 form, as compared to rodents (e.g. forms CYP2A3 and 3A5), and may become clinically manifest in quantitative differences in the metabolism of certain drugs resulting in "poor" or "extensive" metabolizers eg. the 4-hydroxylation of debrisoquine by CYP2D6 (Guengerich, 1989, Gonzalez et al., 1991).

1.1.3 Cytochrome P450 Catalysed Reactions

As well as the widespread distribution throughout the animal and plant kingdom, one also finds within the mammalian population a marked tissue specificity of enzyme synthesis. It was initially thought that cytochrome P450s occurred only in the liver, however, although in mammalian species they are most abundant in this latter tissue, they also occur in extra-hepatic tissues, including the small intestine, colon, kidney and lung (DeWaziers et al., 1990).

Cytochrome P450s are of importance in the oxidative, peroxidative, and reductive metabolism of numerous endogenous compounds such as steroids, bile acids, fatty acids, prostaglandins, leukotrienes and biogenic amines (Gonzalez 1989, 1990). Many of the P450 enzymes can also metabolise a wide range of foreign chemicals (xenobiotics) including drugs, environmental pollutants, natural plant products and alcohols. By introducing a polar hydroxyl group into these xenobiotic molecules the P450 enzyme allows for phase II conjugation enzymes to introduce additional polar groups thereby facilitating the elimination of these xenobiotics from the body. However, in some cases the oxidation of these xenobiotics can generate biologically reactive electrophiles that are detrimental to the host and may cause toxicity or cancer (Guengerich, 1988).
The extensive range of reactions catalysed by cytochrome P450, including hydroxylation of aromatic, aliphatic and cyclic compounds, oxidation, dealkylation and deamination, is made possible by its ability to cleave molecular oxygen to active oxygen species, one atom of which is incorporated into the substrate and the other is reduced to the level of water according to the following equation (where RH represents the substrate).

\[ \text{RH} + \text{O}_2 + \text{NADPH} + \text{H}^+ \rightarrow \text{ROH} + \text{H}_2\text{O} + \text{NADP}^+ \]

The essential stages of this reaction are as follows. The initial step involves substrate binding to the oxidised form of the iron atom in the haem group of cytochrome P450. The substrate-enzyme complex is then reduced by the transfer of an electron from NADPH, which is catalysed by NADPH-cytochrome P450 reductase. The reduced enzyme-substrate complex then binds with molecular oxygen and further reduction of this complex occurs by the transfer of an electron either from NADPH-cytochrome P450 reductase or NADH via NADH-cytochrome b5 reductase and cytochrome b5. Finally, the substrate-enzyme-oxygen complex splits into water, oxidised substrate and the oxidised form of the enzyme (for a review see Porter and Coon, 1991).

1.1.4 Mechanisms of Induction and Regulation of the Cytochrome P450 Superfamily

Consonant with the multiplicity of cytochrome P450s is the considerable diversity in the mechanisms of regulation of these enzymes, as depicted schematically in Figure 1.1. Not surprisingly, the most common means of regulation is transcriptional. Post-transcriptional mechanisms include mRNA stabilisation and protein stabilisation or degradation that may be mediated through changes in the phosphorylation state of the enzyme. Moreover, many cytochrome P450s are subject to tissue-specific patterns of expression, with resulting differences in isoform compositions and activities in various tissues eg. the CYP4A subfamily (for review see Okey, 1990).
Fig 1.1 Multiplicity in the regulation of cytochrome P-450 expression. P-450 designations (shown at the bottom of the figure) are as described in Nebert et al. (1991) and are based on selected examples from various mammalian species. (Taken from Porter and Coon, 1991).
1.1.4.1 Transcriptional Regulation

All eukaryotic genes are under control of transcription factors that bind to DNA sequences in their 5' flanking regions, which act as promoters, enhancers and suppressors. These transcription factors are contained within four families of DNA-binding proteins that are characterised by the presence of various sequence motifs including zinc fingers, leucine zippers and homeodomains (Mitchell and Tjian, 1989, Schwabe and Rhodes, 1991). Cis-regulatory elements can also occur downstream of the promoter eg introns.

The most extensively characterised cytochrome P450 with regard to regulation is CYP1A1 (Nebert and Jones, 1989). The CYP1A1 gene is transcriptionally activated by a number of chemicals including benzo(a)pyrene and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) that exert their effect by binding to the Ah receptor and converting it into a form capable of interacting with the cis-acting elements (variously referred to as drug regulatory elements, DRE, xenobiotic regulatory elements, XRE, and Ah regulatory elements, AhRE) located upstream of the CYP1A1 coding gene. From three to five AhREs are found in the mouse, rat and human CYP1A1 genes. The gene and cDNA encoding a second protein, named the Ah receptor nuclear translocator (Arnt) have been cloned (Hoffman et al., 1991), and identified as being necessary for Ah receptor function, the two proteins forming a heterodimeric structure (Hankinson, 1993). It should be noted, however, that the complete functional Ah receptor activity has not been reconstituted from cDNA-expressed subunits. Another factor involved in CYP1A1 regulation is the presence of a negative regulatory element that inhibits transcription. A repressor protein has been identified and appears to associate with the Ah receptor inhibiting its binding to the AhRE (Watson et al., 1992). The heterodimer motif seems to be a reoccurring theme in the control of transcription as demonstrated by the steroid hormone receptors and the proto-oncogenes fos and jun (Green, 1992a).

A number of other P450s are transcriptionally activated by xenobiotics (i.e. induced). For example, phenobarbital induces a number of cytochrome P450s in the CYP2B,
CYP2C and CYP3A subfamilies (reviewed in Waxman and Azaroff, 1992).

Many xenobiotic-metabolising cytochrome P450s are constitutively expressed in the absence of inducers. Two P450 genes, \textit{CYP2E1} and \textit{CYP2C6}, have been studied and found to be under the control of known hepatocyte-enriched transcription factors. The factor HNF-1\(\alpha\) (Hepatocyte Nuclear Factor) was found to control \textit{in vitro} expression of the \textit{CYP2E1} gene by binding to an upstream \textit{cis}-acting element (Ueno and Gonzalez, 1990). The hepatic \textit{CYP2C6} gene becomes maximally transcriptionally activated when male and female rats reach puberty. The timing of this activation is coincident with expression of a protein designated DBP (albumin gene D region Binding Protein), and a DBP response element upstream from the \textit{CYP2C6} transcription start has been found, which activates its transcription (Yano et al., 1992).

In rodents, but less so in man, the expression of a number of cytochrome P450s is sexually determined, by neonatal imprinting and by hormonal regulation in mature animals. The \textit{CYP2C11} is a male specific P450 whereas \textit{CYP2C12} is a female specific P450, both expressed in rat liver under the influence of pituitary-dependent hormones (Waxman, 1988). The effect of sex hormone exposure appears to be related to the subsequent pattern of growth hormone secretions which is pulsatile in male and constant in female rats.

The bovine \textit{CYP11A}, \textit{CYP11B}, \textit{CYP17} and \textit{CYP21A1} genes have been shown to be transcriptionally activated in cultured adrenocortical cells by ACTH which acts via cyclic AMP (John et al., 1986). It has been postulated that cAMP does not stimulate transcription directly but rather that cAMP activates a gene encoding a hypothetical steroid hydroxylase inducing protein (SHIP) (Nebert and Gonzalez, 1987), although this SHIP protein remains to be isolated and fully characterised.
1.1.4.2 mRNA Processing and Stabilisation

Another point of control in cytochrome P450 regulation is the efficiency of removing introns from the pre-mRNA and the subsequent splicing of exons to form a mature mRNA. Aberrant splicing (due to a G to A base pair transition at the intron 3/exon 4 boundary) is one of the mechanisms (others being frame shift mutations and deletions) which underlies the observed polymorphism in the human CYP2D subfamily (Gough et al., 1990). Alternative mRNA splicing has also been observed for CYP2A3, which results in the production of both functional and non-functional enzyme (Okino et al., 1987).

Messenger RNA stabilisation as a mechanism of induction is demonstrated by several cytochrome P450s, including rat CYP3A1 which is up-regulated as a result of administering the macrolide antibiotic triacetyloeandomycin (TAO) (Watkins et al., 1986), and CYP2E1 which is elevated 10-fold as a consequence of diabetes (Song et al., 1987). It is interesting to note that a 10-fold increase in CYP2E1 mRNA is also seen following fasting. This, however, results from an increase in gene transcription (Johansson et al., 1990).

1.1.4.3 Enzyme Stabilisation and Protein Phosphorylation

In addition to the post-transcriptional effects described above, cytochromes P450 have also been shown to be controlled at post-translational levels. Obviously the availability of the haem prosthetic group is an important factor as described by Padmanaban et al. (1989), but more specific mechanisms of post-translational control have been demonstrated to be the protection of CYP2E1 apoprotein by ethanol, acetone or pyrazole (Song et al., 1989) and the protection of CYP3A apoproteins by TAO against degradation (Watkins et al., 1986). The role of phosphorylation by cAMP dependent protein kinases in the regulation of cytochrome P450 activity is less clear but appears to lead to inactivation of several forms, namely CYP2B1, CYP2C2 and CYP2E1 (Goldfarb, 1990).
1.2 CYTOCHROME P450 FAMILY 4 (CYP4)

The first member of the CYP4 family to be identified was a clofibrate inducible, lauric acid ω-hydroxylase, first purified to electrophoretic homogeneity in 1982 (Gibson et al., 1982). This new form of cytochrome P450 had a characteristic absorbance maximum at 452nm and was therefore named P452 (Tamburini et al., 1984). Since this time, a number of complementary DNAs of the CYP4 family have been isolated from cDNA libraries made from liver, kidney, prostate and lung of various species including the rat, mouse, guinea pig, rabbit, human and insects (Nelson et al., 1993), Table 1.2. To date there are 22 gene members of the CYP4 family, arranged into 6 subfamilies (A-F).

The CYP4 gene family encodes a group of cytochrome P450 enzymes that hydroxylate the terminal ω carbon and, to a lesser extent, the ω-1 position of saturated and unsaturated fatty acids (Bjorkhem and Hamberg, 1971). ω-Hydroxylation is an important step in the metabolism of saturated fatty acids, arachidonic acid, and the pharmacologically active eicosanoids, prostaglandins (PG), hydroxyeicosatetraenoic acid (HETE), and leukotrienes (LT) (Ellin et al., 1972, Powell, 1984, Sumimoto et al., 1984). For straight-chain fatty acids, ω-oxidation is a minor pathway except during ketotic states such as starvation and diabetes. ω-Hydroxylation represents a major pathway for the metabolism of branched-chain or substituted fatty acids (Yamakawa, 1950). These hydroxylated fatty acids are further oxidised to dicarboxylic acids by alcohol and aldehyde dehydrogenase, subsequently shortened by β-oxidation, and excreted as C6-C10 dicarboxylic acids.

The cDNA for the first isozyme identified in the CYP4A family, P452, was cloned and characterised from the livers of clofibrate-treated rats (Hardwick et al., 1987, Earnshaw et al., 1988) and was designated CYP4A1 in accordance with the system of nomenclature (Nelson et al., 1993). The genomic clone of CYP4A1 has
Table 1.2 The CYP4 gene family. Adapted from Nelson et al. (1993) and references therein. Per. com. indicates unpublished data.

<table>
<thead>
<tr>
<th>Gene/Protein Designation</th>
<th>Trivial Names</th>
<th>Species</th>
<th>Tissue Expression</th>
<th>Representative Substrate</th>
<th>Induction</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP4A1</td>
<td>LA0</td>
<td>Rat</td>
<td>Liver, kidney</td>
<td>Fatty acids</td>
<td>Induced by clofibrate in liver and kidney</td>
<td>Hardwick et al. 1987 (cDNA) Earnshaw et al. 1988 (cDNA) Kimura et al. 1989a (genomic)</td>
</tr>
<tr>
<td></td>
<td>P452</td>
<td></td>
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<tr>
<td>CYP4A2</td>
<td>IVA2</td>
<td>Rat</td>
<td>Liver, kidney</td>
<td>Fatty acids and prostaglandins</td>
<td>High constitutive expression in kidney, induced by clofibrate in liver. Induced by starvation and diabetes in liver and kidney.</td>
<td>Kimura et al. 1989a (genomic) Imaoka et al. 1990a (protein)</td>
</tr>
<tr>
<td></td>
<td>k-5</td>
<td></td>
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<td>Fatty acids</td>
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<td></td>
<td>k-2</td>
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<tr>
<td>CYP4A3</td>
<td>DM-2</td>
<td>Rat</td>
<td>Liver, kidney</td>
<td>Fatty acids</td>
<td>Induced by diabetes</td>
<td>Imaoka et al. 1988a (protein) Imaoka et al. 1990b Kimura et al. 1989b (cDNA)</td>
</tr>
<tr>
<td></td>
<td>IVA3</td>
<td></td>
<td></td>
<td></td>
<td>Induced by clofibrate in liver and kidney</td>
<td></td>
</tr>
<tr>
<td>CYP4A4</td>
<td>p-2</td>
<td>Rabbit</td>
<td>Lung, kidney, liver, uterus, placenta</td>
<td>Fatty acids and prostaglandins (PGE1, PGE2, PGD2, PGF2α)</td>
<td>Induced in lung, liver and uteruses by progesterone treatment or during pregnancy</td>
<td>Matsubara et al. 1987 (cDNA) Kikuta et al. 1989 (protein) Palmer et al. 1993a (genomic)</td>
</tr>
<tr>
<td>CYP4A5</td>
<td>KDB3</td>
<td>Rabbit</td>
<td>Kidney, liver, small intestine</td>
<td>Fatty acids</td>
<td>Induced by clofibrate in liver</td>
<td>Johnson et al. 1990 (cDNA) Yokotani et al. 1989 (protein)</td>
</tr>
<tr>
<td></td>
<td>kd</td>
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<tr>
<td>CYP4A6</td>
<td>ka-1</td>
<td>Rabbit</td>
<td>Kidney, liver</td>
<td>Fatty acids and prostaglandins (PGA₁, PGA₂)</td>
<td>Induced in liver and kidney by clofibrate</td>
<td>Yokotani et al. 1989 (protein)</td>
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<tr>
<td>CYP4A7</td>
<td>ka-2</td>
<td>Rabbit</td>
<td>Liver, kidney, small intestine</td>
<td>Fatty acids and prostaglandins (PGA₁, PGA₂)</td>
<td>Induced in liver only by clofibrate</td>
<td>Yokotani et al. 1989 (protein)</td>
</tr>
<tr>
<td></td>
<td>R4</td>
<td></td>
<td></td>
<td></td>
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<td>Johnson et al. 1990 (cDNA)</td>
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<td></td>
<td>KDB18</td>
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<td></td>
<td></td>
<td></td>
<td>Kikuta et al. 1990 (protein)</td>
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<tr>
<td></td>
<td>LPGo-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Muehoff et al. 1992a (genomic)</td>
</tr>
<tr>
<td>CYP4A8</td>
<td>PP1, KPI</td>
<td>Rat</td>
<td>prostate, kidney</td>
<td>?</td>
<td>Induced by androgens</td>
<td>Stromstedt et al. 1990 (cDNA)</td>
</tr>
<tr>
<td>CYP4A9</td>
<td>HL14Acon</td>
<td>Human</td>
<td>Liver</td>
<td>?</td>
<td></td>
<td>Imaoka et al. 1990a (protein)</td>
</tr>
<tr>
<td>Cyp4a-10</td>
<td>A14</td>
<td>Mouse</td>
<td>Liver, kidney</td>
<td>?</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>clone 1</td>
<td></td>
<td></td>
<td>Induced in liver and kidney by methylclofenapate</td>
<td></td>
<td>Hencerson et al. 1992 (cDNA)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Bell et al., 1993 (cDNA)</td>
</tr>
<tr>
<td>CYP4A11</td>
<td>HK₁₀</td>
<td>Human</td>
<td>Liver, kidney</td>
<td>Fatty acids</td>
<td>?</td>
<td>Kawashima et al. 1992 (protein)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Palmer et al. 1993b (cDNA)</td>
</tr>
<tr>
<td>Cyp4a-12</td>
<td>clone 2</td>
<td>Mouse</td>
<td>Liver, kidney</td>
<td>?</td>
<td>High constitutive expression in male, low in female liver and kidney. Induced by methylclofenapate.</td>
<td>Bell et al., 1993 (cDNA)</td>
</tr>
<tr>
<td>CYP4A13</td>
<td></td>
<td>Guinea pig</td>
<td>Liver</td>
<td>?</td>
<td>Not induced by methylclofenapate.</td>
<td>Bell et al., 1993 (cDNA)</td>
</tr>
</tbody>
</table>
Table 1.2 (cont. 2.) The CYP4 gene family.

<table>
<thead>
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</tr>
</thead>
<tbody>
<tr>
<td>CYP4C1</td>
<td>Cockroach</td>
<td>Fat body</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td>Bradfield et al. 1991 (cDNA)</td>
</tr>
<tr>
<td>CYP4F1</td>
<td>A3</td>
<td>Rat</td>
<td>Hepatic tumours</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td>Chen and Hardwick 1992 (cDNA)</td>
</tr>
</tbody>
</table>
since been identified along with a closely related gene, designated \textit{CYP4A2}, as well as the cDNA of a third member of this rat \textit{CYP4A} family, \textit{CYP4A3} (Kimura \textit{et al.}, 1989a, 1989b). The mRNAs for these three genes have been shown to be coordinately regulated in the liver by hypolipidemic drugs (Kimura \textit{et al.}, 1989a, 1989b). \textit{CYP4A2} is a constitutive isozyme in the kidney which is weakly induced by clofibrate, whereas both \textit{CYP4A4} and \textit{CYP4A3} are dramatically induced in the kidney at a lower dosage of clofibrate than \textit{CYP4A2} (Kimura \textit{et al.}, 1989b).

A prostaglandin \(\omega\)-hydroxylase has been isolated from lungs of pregnant rabbits (Williams \textit{et al.}, 1984) and rabbits treated with progesterone (Yamamoto \textit{et al.}, 1984). The cDNA for this enzyme, designated \textit{CYP4A4} (formerly P450p-2), has been isolated and sequenced (Matsubara \textit{et al.}, 1987) and more recently the genomic clone of \textit{CYP4A4} has been isolated (Palmer \textit{et al.}, 1993a). A similar or possibly identical protein to \textit{CYP4A4} has also been isolated from the livers of pregnant rabbits (Kikuta \textit{et al.}, 1989). The observation that preparations of \textit{CYP4A4} exhibited negligible lauric acid \(\omega\)-hydroxylase activity (Yamamoto \textit{et al.}, 1984) suggested that members of the \textit{CYP4A} subfamily, distinct from \textit{CYP4A4}, may be expressed in the rabbit. Using \textit{CYP4A4} as a hybridisation probe, two different groups have isolated cDNAs for \textit{CYP4A4}-related forms from rabbit kidney cDNA libraries. Yokatani \textit{et al.} (1989) have reported the isolation of two different cDNAs encoding lauric acid \(\omega\)-hydroxylases. P450ka-1 mRNA was expressed in liver and kidney and was induced by the treatment of rabbits with clofibrate, while P450ka-2 mRNA was constitutively expressed in liver, kidney and small intestine, but only induced in the liver by clofibrate. Johnson \textit{et al.} (1990) have isolated cDNAs for three rabbit kidney lauric acid \(\omega\)-hydroxylases denoted \textit{CYP4A5-7}. \textit{CYP4A6} and \textit{CYP4A7} are identical to P450ka-1 and P450ka-2, respectively. More recently the genomic sequence for \textit{CYP4A6} has been isolated (Muerhoff \textit{et al.}, 1992a).

A fourth member of the rat \textit{CYP4A} family was isolated from a rat prostate cDNA
library and shares 76% amino acid similarity with CYP4A1 (Stromstedt et al., 1990). This cytochrome P450 isoform, designated CYP4A8, was found to have high constitutive expression in the kidney, but no detectable signal was obtained with RNA from liver. This finding led to the isolation of a rat kidney cDNA, differing from CYP4A8 in only three nucleotide positions.

The first report of a human member of the CYP4A subfamily (designated CYP4A9) was in 1991, in the form of a personal communication by Hardwick et al. (Nebert et al., 1991). However, details of this human liver P450 isoform have yet to be published.

The first murine member of the CYP4A subfamily was identified by Henderson et al. (1992) and was designated Cyp4a-10. This isoform shared 92% sequence similarity with the rat CYP4A1 and Western Blot analysis revealed three and four mRNA species present in mouse liver and kidney respectively.

A 52kDa protein, which exhibits lauric acid ω-hydroxylase activity, has been isolated from human kidney microsomes (Kawashima et al., 1992). Amino acid sequencing of the NH₂ terminal end of the protein showed it to possess between 75-80% similarity with other members of the CYP4A family. Subsequently, the use of the cDNA for CYP4A4 as a hybridisation probe, identified a full length cDNA (designated CYP4A11) from a human kidney cDNA library, (Palmer et al., 1993b) that may or may not correspond to the 52kda protein identified by Kawashima et al. (1992). The CYP4A11 cDNA was expressed in E. coli, and a solubilised preparation of the enzyme reconstituted with cytochrome P450 reductase was shown to catalyse the ω-hydroxylation of lauric acid, palmitic acid and arachidonic acid. CYP4A11 was found to be expressed at high levels in the kidney, with the liver showing the next highest abundance. Concurrently, results in our laboratory demonstrated the presence of a fatty acid ω-hydroxylase activity in human liver microsomes (Chinje, personal communication), confirming the existence of an in vivo expressed human member of
the CYP4A subfamily. The genomic clone of \textit{CYP4A11} has recently been isolated and partially sequenced in our laboratory (Hood \textit{et al.}, 1992). The exons so far sequenced, 1, 8, 9 and 10 have identical DNA sequence to the \textit{CYP4A11} cDNA, and exhibit 80\% amino acid similarity to the four rabbit genes (\textit{CYP4A4-7}) and 73\% amino acid similarity to the three rat genes (\textit{CYP4A1-3}).

More recently a second member of the murine \textit{Cyp4a} family was identified and designated \textit{Cyp4a-12} (Bell \textit{et al.}, 1993). This isoform shows the highest degree of similarity to the rat \textit{CYP4A8} gene (93\% amino acid similarity). Unlike the rat \textit{CYP4A8}, this isoform shows high constitutive expression in both male kidney and liver (as opposed to just kidney), however low expression of \textit{Cyp4a-12} in female kidney and liver was observed, although this could be greatly induced in both organs by methylclofenapate. The sex specific regulation of the rat \textit{CYP4A8} gene has not been investigated. This same group (Bell \textit{et al.}, 1993) identified the first guinea pig \textit{CYP4A} gene and designated it \textit{CYP4A13}. This isoform was found to be expressed in liver, but was not inducible by the administration of methylclofenapate confirming the earlier findings in this laboratory (Gibson, 1989). The guinea pig \textit{CYP4A13} gene is most similar to the rat \textit{CYP4A1}, sharing 83\% amino acid similarity.

The \textit{CYP4B} subfamily has three orthologous cDNA members isolated from rat and rabbit (Gasser and Philpot, 1989), and from man (Nhamburo \textit{et al.}, 1989). The \textit{CYP4B} subfamily proteins mediate mainly the pulmonary N-oxidation of aromatic amines such as the promutagen 2-aminofluorene. Unlike the orthologous rabbit form, human \textit{CYP4B1} was unable to metabolise this latter compound despite the presence of \textit{CYP4B1} mRNA in three out of four lungs analysed (Nhamburo \textit{et al.}, 1989). In contrast to its expression in lung, \textit{CYP4B1} mRNA was undetectable in livers obtained from 14 individuals, including those from which the lungs were derived. Similarly, \textit{CYP4B1}-related mRNA was also expressed in rat lung but was undetected in untreated rat liver. Expression of \textit{CYP4B1} in rabbit but not rat or human liver
suggests an interesting species difference in tissue-specific expression of this gene. The gene for human CYP4B1 has also been isolated (Yokotani et al., 1990).

In 1991, a new gene member of the CYP4 family, sharing 35% sequence similarity with CYP4A1, was identified in the cockroach (Blaberus discoidalis) (Bradfield et al., 1991). Despite this gene not satisfying the 40% rule for classification into an existing family, the gene was found to contain a 13 base-pair sequence characteristic of the CYP4 family (Nhamburo et al., 1989) and was therefore designated CYP4C1. The physiological role for CYP4C1 is highly speculative, but the mRNA level is known to be elevated by both the hypertrehalosemic hormone (HTH) and starvation. A principal function of HTH is stimulation of fat body glycogen to trehalose, the main circulating carbohydrate in insects. Bradfield and co-workers (1991) concluded from their studies that CYP4C1 may be hormonally regulated in association with energy substrate mobilisation. However, the protein catalytic activity and regulation of the corresponding CYP4C1 gene by HTH still remains to be ascertained. CYP4 genes have also been identified in Drosophila melanogaster and have been designated CYP4D1 and CYP4E1 (Gandhi et al., 1992, Nelson et al., 1993).

The last subfamily to be identified (to date), as a member of the CYP4 family, has been designated CYP4F. Chen and Hardwick (1992) constructed a cDNA library from a 2-acetylaminofluorene-induced transplantable rat hepatic tumour and screened it with a CYP4 family probe. A cDNA was isolated which exhibited 44-45% amino acid identity to rat CYP4A members, 43% to human CYP4B1, and 35 and 32% to insect CYP4C1 and CYP4D1, respectively. This new P450 was thus named CYP4F1. This form is expressed at a high level in hepatic tumours which is in contrast to other rat CYP4A members whose expression is reduced in most cases, thus implying that the CYP4A and CYP4F1 genes are differentially regulated (Chen and Hardwick, 1992).
1.2.1 Regulation of Expression of the CYP4A Subfamily

*CYP4A1*, *CYP4A2* and *CYP4A3* have been shown to be coordinately induced in the rat liver by hypolipidemic drugs (Kimura *et al.*, 1989a,b). *CYP4A2* is a constitutive isozyme in the kidney where it is weakly induced by clofibrate, whereas both *CYP4A1* and *CYP4A3* are dramatically induced in the kidney at a lower dosage of clofibrate than *CYP4A2*. The *CYP4A1*, *CYP4A2* and *CYP4A3* genes are coordinately regulated by clofibrate in the liver and are induced transcriptionally by clofibrate, starvation and drug-induced diabetes (Kimura *et al.*, 1989b, Kusunose *et al.*, 1981).

*CYP4A* expression has been shown to be controlled at the level of transcription (Hardwick *et al.*, 1987) and it is also thought that mRNA processing or stabilisation may play a part in the control of these genes (Earnshaw *et al.*, 1988). As well as being transcriptionally activated by peroxisome proliferators, hepatic CYP4A mRNA has also been shown to be suppressed by 49% from a maximally induced level, by the administration of polyinosinic acid.polycytidylic acid (poly IC), an alpha/beta interferon inducer (Knickle *et al.*, 1992, D. Bulsara, personal communication). How interferon (IFN) mediates this suppression is unknown. Protein kinase C (PKC) has been shown to be involved in the signal transduction pathway induced by IFNα (Reich and Pfeffe, 1990). It could be that phosphorylation of a transcription factor(s) (such as members of the steroid hormone receptor superfamily), involved in maintaining the basal level of the *CYP4A* genes or upregulating them, would cause the factor to be inactivated, thereby causing downregulation of the *CYP4A* genes. Alternatively phosphorylation by PKC might cause the activation of an unidentified negative transcriptional regulation factor. This activated factor might compete directly with the peroxisome proliferator activated transcription factor for its DNA binding site, or compete indirectly by binding to the factor itself preventing it from binding to the DNA and enhancing the transcription of the *CYP4A* genes.

Sex differences have been observed in the responsiveness of rat liver to several
peroxisome proliferators. Female rats are less susceptible to clofibrate-induced peroxisome proliferation than males (Reddy and Kumar, 1979, Kawashima et al., 1989). Sundseth and Waxman (1992) demonstrated by Northern blot analysis that in the liver, CYP4A1 and CYP4A3 mRNAs were induced to a much greater extent in male as compared to female rats following clofibrate treatment, whereas CYP4A2 mRNA was absent from female rat liver. They also observed male-specific expression of CYP4A2 in the kidney. If indeed, CYP4A induction is an early and perhaps obligatory step in the peroxisome proliferation pathway, then sex differences in the constitutive expression and/or inducibility of these genes might contribute to the sex-dependent effects of clofibrate on peroxisome proliferation, however the underlying mechanistic basis for the lower responsiveness of female rats to clofibrate-inducible CYP4A expression is unknown.

The CYP4A1 and CYP4A2 genes have been isolated and sequenced and found to possess 13 and 12 exons respectively (Kimura et al., 1989a). Interestingly the CYP4A1 and CYP4A2 genes lack the TATA box upstream of the transcriptional start site. Genes that lack the TATA box are believed to have multiple transcriptional start sites, however, both the CYP4A1 and CYP4A2 have a single start site.

The CYP4A6 gene has been isolated and sequenced and found to contain 12 exons (Muerhoff et al., 1992a). Intron/exon junctions within the coding region of CYP4A6 are conserved relative to the rat CYP4A1 and CYP4A2 genes and primer extension analysis indicates the presence of a single transcriptional start site. The CYP4A6 promoter region, like that of the rat CYP4A1 and CYP4A2 genes, does not contain a consensus TATA box.

The other CYP4A gene that has been sequenced is that of CYP4A4 (Palmer et al., 1993a). This gene is not induced by clofibrate but is elevated in the lung, liver and kidney during pregnancy. Treatment of male rabbits with dexamethasone also
increases the levels of CYP4A4 mRNA in the lung and liver, but the levels are eightfold less than those seen for pregnant rabbits. The intron/exon structure is highly conserved in relation to the clofibrate inducible genes rat CYP4A1, CYP4A2 and rabbit CYP4A6 and like these genes the CYP4A4 promoter does not contain a TATA consensus sequence.

The mechanism of regulation of CYP4A has been studied intensely by this laboratory and others. The proposed role of the PPAR receptor in this regulation is discussed in detail in section 1.5.2.

1.2.2 The Physiological Role of the CYP4A Subfamily

The CYP4A gene subfamily encodes a group of cytochrome P450 enzymes that hydroxylate the terminal ω carbon and, to a lesser extent, the ω-1 position of saturated and unsaturated fatty acids. ω-Hydroxylation is an important step in the metabolism of saturated fatty acids, arachidonic acid, and the pharmacologically active eicosanoids, prostaglandins (PG), hydroxyeicosatetraenoic acid (HETE), and leukotrienes (LT) (Ellin et al., 1972, Powell, 1984, Sumimoto et al., 1984). For straight-chain fatty acids, ω-oxidation is a minor pathway except during ketotic states such as starvation and diabetes (Orton and Parker, 1982). It has been proposed that the (ω-1)-hydroxylating activity is catalysed by several cytochrome P450 isoenzymes and is therefore non-specific. By contrast the ω-hydroxylase is highly specific for fatty acids (Kufper, 1982) and represents a major pathway for the metabolism of branched-chain or substituted fatty acids. These hydroxylated fatty acids are further oxidised to dicarboxylic acids by alcohol and aldehyde dehydrogenase, subsequently shortened from both ends by β-oxidation, and excreted as C₆-C₁₀ dicarboxylic acids.

The substrate specificities of the members of the CYP4A subfamily can be seen in Table 1.2. CYP4A1, CYP4A3, CYP4A5, CYP4A6 and CYP4A7 actively catalyse the ω-hydroxylation of fatty acids and have low or negligible prostaglandin ω-hydroxylase
activity, whereas the reverse substrate specificity has been reported for CYP4A4. Although the specific substrate for CYP4A2 has not been determined, work in spontaneous hypertensive rats by Iwai and Inagami (1991) strongly suggests that CYP4A2 has some role in water and electrolyte metabolism in the kidney. These workers demonstrated that expression of CYP4A2 mRNA in the kidney was positively modulated by low sodium chloride diet feeding, indicating that this enzyme may have some role in reducing sodium excretion from the kidney.

The use of oligonucleotide probes, specific for CYP4A1, CYP4A2 and CYP4A3, in \textit{in situ} hybridisation (Hardwick, 1991), has demonstrated that in untreated rats, CYP4A1 and CYP4A3 mRNA's are distributed throughout the liver, however most of the mRNA is found in the periportal region. In kidney, CYP4A1 and CYP4A3 mRNA's are localised within the proximal tubule cell, whereas CYP4A2 is found within the renomedullary region. When rats are administered clofibrate, the level of CYP4A1 and CYP4A3 mRNA increases in the proximal tubules, whereas CYP4A2 remains unchanged in the cells of the renomedullary region (Hardwick, 1991).

Arachidonic acid serves as an excellent substrate for the clofibrate-induced CYP4A hydroxylase system in the rat liver and kidney (Bains et al., 1985, Sharma et al., 1989). Pretreatment of rats with ciprofibrate resulted in an 8-fold stimulation of \( \omega \)- and \( \omega-1 \) oxidation of arachidonic acid with a simultaneous net decrease in the formation of the other oxygenated metabolites (Capdevila et al., 1984).

The major metabolite in both rat hepatic and renal arachidonate metabolism is the 20-hydroxyarachidonic acid (20 HETE), however, in both control and clofibrate-induced states, the kidney remains more efficient at metabolising arachidonic acid to the 20-hydroxy metabolite (Sharma et al., 1989). It has been demonstrated that \( \omega \)- and \( \omega-1 \) metabolites of 20-HETE are a potent vasoconstrictor in rat aortic rings and a stimulator of the Na\(^+\), K\(^+\)-ATPase pump respectively (Escalante et al., 1989,
Ma and co-workers (1993) have demonstrated that 20-HETE constricted canine renal arcuate arteries and was associated with a significant depolarization of vascular smooth muscle cells. In contrast, these workers found that 19(R)-HETE was a vasodilator in renal arteries. These results are consistent with previous findings indicating that myogenic activation of renal arteries is potentiated by arachidonic acid and attenuated by inhibitors of cytochrome P450 (Kauser et al., 1991).

Arachidonic acid is also metabolised in the kidney by a cytochrome P450 epoxygenase located in the thick ascending loop of Henle (TALH). This cytochrome P450-dependent epoxygenase produces epoxyeicosatrienoic acid (EET) which inhibits the Na\textsuperscript{+}, K\textsuperscript{+}-ATPase pump, resulting in increased hypertension (Schwartzman et al., 1986). As mentioned above, specific ω- and ω-1-HETE products produced by the kidney prove to be potent vasoconstrictors and a stimulator of the Na\textsuperscript{+}, K\textsuperscript{+}-ATPase pump respectively. Since the CYP4A1 and CYP4A3 enzymes are localised in the proximal tubules of the kidney, induction of these enzymes would increase the conversion of arachidonic acid to ω/ω-1 products and therefore prevent the oxidation of arachidonic acid by the P450 epoxygenase in the TALH cells.

1.3 PLEIOTROPIC RESPONSE TO THE ADMINISTRATION OF PEROXISOME PROLIFERATORS

The CYP4A gene family is unique in that its members are induced by a group of structurally diverse compounds including hypolipidaemic drugs, phthalate esters, and halogenated hydrocarbon solvents. Administration of these compounds to susceptible species such as rodents and more particularly the mouse and rat, induces the proliferation of peroxisomes in hepatocytes (Gibson et al., 1982) and hence such compounds are called peroxisome proliferators. These compounds have been
extensively studied and the results have been surveyed by the European Chemical Industry Ecology and Toxicology Centre (ECETOC, 1992). The degree of peroxisome proliferation varies markedly depending upon the substance inducing the change. Examples of some peroxisome proliferators are shown in Figure 1.2. The proliferation may be accompanied by changes in the morphology of the peroxisomes, and also results in characteristic changes in the morphology and biochemistry of the liver. Peroxisome proliferators have been used clinically, in man, to lower the risk of heart disease.

1.3.1 Peroxisomes

Peroxisomes are cytoplasmic organelles widely distributed in animal and plant cells (DeDuve, 1969) and are particularly abundant in cells which are involved in lipid metabolism and they are often associated with lipid droplets within the cell (Gorgas, 1987). Peroxisomes are single membrane bound organelles that stain positive for peroxidase activity (due to the presence of catalase). Their shape and size varies greatly depending on the cell type investigated. In general, they appear to be round and contain an electron-dense crystallloid core, which contains the enzyme urate oxidase. Human hepatic peroxisomes are generally smaller than their rodent counterparts and lack the crystallloid core.

Peroxisomes contain a large number of enzymes many of which are intimately involved in lipid metabolism, e.g. breakdown of lipid (i.e. the fatty acid $\beta$-oxidation system), the synthesis of plasmalogens (ether lipids), the synthesis of cholesterol and of long chain fatty esters which are found in waxes and pheromones. Most of these functions are also performed by other subcellular organelles, e.g. microsomes or mitochondria, and it is often difficult to assess the contribution of the peroxisomal enzymes to the total cellular reactions.

Although the reactions involved during peroxisomal $\beta$-oxidation are very similar to those of mitochondrial $\beta$-oxidation, the enzymes differ from their mitochondrial
Hypolipidaemic Agents

\[
\text{Clofibrac acid}
\]

\[
\text{Wy 14,643}
\]

\[
\text{Nafenopin}
\]

Plasticizers

DEHP [di-(2-ethylhexyl)phthalate]

Surfactants / Flame Retardants

\[
\text{PFDA (perfluoro-n-decanoic acid)}
\]

\[
\text{PFOA (perfluoro-n-octanoic acid)}
\]

**Figure 1.2** Examples of peroxisome proliferators and some of their uses
counterparts with respect to their catalytic and molecular properties. It has been shown that unlike the carnitine-dependent, cyanide-sensitive mitochondrial system, peroxisomal β-oxidation enzymes are cyanide-insensitive in vitro and do not require carnitine (Lazarow, 1982). Peroxisomal β-oxidation generates reduced FAD which is reoxidised by molecular oxygen (Figure 1.3). NADH/H⁺ produced by the oxidation of beta-hydroxy-fatty acyl CoA's is reoxidised by glycerol-3-phosphate dehydrogenase in a peroxisomal shunt for NADH/H⁺. The first enzyme in the peroxisomal β-oxidation pathway, acyl CoA oxidase, is rate limiting and results in the generation of FADH₂. The reduced flavin units are not available for ATP generation via the mitochondrial electron transport chain, but are oxidised by molecular oxygen with the resultant formation of hydrogen peroxide. This results in an overall waste of energy in comparison to the mitochondrial beta-oxidation pathway. The hydrogen peroxide can also be generated within the peroxisome via urate oxidase, D-amino acid oxidase and glycolate oxidase (Lock et al., 1989, Reddy and Lalwani, 1983).

Many theories of peroxisomal biogenesis have been proposed. The classical model suggests that peroxisomal proteins are synthesised within the lumen of the endoplasmic reticulum and then form vesicles which detach from the endoplasmic reticulum to form peroxisomes (De Duve and Baudhuin, 1966). However, the polypeptide composition of the peroxisomal membrane and the endoplasmic reticulum have been shown to be markedly dissimilar (Fujiki et al., 1982). The current model of peroxisome biogenesis suggests that peroxisomes are formed by fission from pre-existing organelles (Yamamoto and Fahimi, 1987, Fujiki et al., 1984) and that the peroxisomal enzymes, synthesised on free polysomes, enter these organelles. Lazarow et al., (1982), showed that peroxisomal proteins are made as mature proteins i.e. are not processed during uptake into pre-existing peroxisomes.

A great deal of speculation as to the metabolic role of the peroxisomes has been made. In the case of fatty acid β-oxidation it would appear that very long fatty acids which
Figure 1.3 Peroxisomal β-oxidation
are poorly oxidised by the mitochondrial system are good substrates for the peroxisomal system so in this manner the two systems may complement each other (Boeck et al., 1980, Fahimi and Sies, 1987). In this respect it is of interest that children suffering from Zellweger's syndrome (a hereditary disease characterised by a lack of peroxisomes), or adrenoleukodystrophy (a disorder characterised by defective transport of enzymes into peroxisomes), show accumulation of very long chain fatty acids (C25 and C26) in adrenals as well as the central nervous system (Singh et al., 1981).

1.3.2 Hepatomegaly
Within a few days of administration of peroxisome proliferators to rats or mice, a marked hepatomegaly is observed (Hess et al., 1965), measured as an increase in liver to body weight ratio, which is characterised by hyperplasia (increase in cell number and content of DNA) and hypertrophy (increase in cell size). The increased number of peroxisomes and the hepatomegaly start to appear within 24 hours of the first dose and this initial growth period lasts 3-4 days. The earliest observable change is an accumulation of small lipid droplets similar to those found in hyperphagia which are possibly associated with alterations in fatty acid metabolism (Mitchell et al., 1986). However, within a few hours an increase in messenger RNAs for c-myc, c-fos, CYP4A1 and for the peroxisomal enzymes can be observed (Bentley et al., 1987, Milton et al., 1990). This is accompanied by increased hepatocyte ³H-thymidine incorporation (a measurement of DNA synthesis) reaching a peak at the third day which subsequently reverts to normal levels for most, but not all, peroxisome proliferators. Liver growth continues and reaches a steady state within 14 days of treatment and is sustained as long as the agent is administered. Rao and Reddy (1987) reported continuation of this steady state level up to 25 months of treatment with ciprofibrate. Peroxisomal number may increase 5-7 fold as, for example, after treatment with high doses of clofibrate and ciprofibrate (Cohen and Grasso, 1981). Liver cells revert to a normal phenotype between 2-3 weeks following the withdrawal
of the peroxisome proliferator (Svoboda and Azarnoff, 1966). However, if administration is continued, the liver remains enlarged (Mitchell et al., 1985b) and other aspects of the peroxisome proliferation phenomenon become apparent. These include the deposition of lipofuscin and eventually the formation of pre-neoplastic foci, liver nodules and ultimately tumours (Rao and Reddy, 1987). The interval between the early changes and the eventual appearance of tumours is measured in terms of several months. The rates of appearance of liver nodules and tumours are related and differ from compound to compound (reviewed in ECETOC, 1992).

1.3.3 Peroxisomal and Mitochondrial Changes

Peroxisomes produced after the administration of peroxisome proliferators to rats are heterogeneous, some may contain fibrillar striations, cavitations and double walled tubules, while others may be devoid of their crystalloid cores (Schulte-Hermann, 1979). The increase in peroxisomal number is accompanied by a very marked increase in the 'marker' enzymes for this organelle. The β-oxidation enzymes are induced up to 30-fold, as measured by cyanide-insensitive palmitoyl CoA oxidation (Lazarow and De Duve, 1976, Osumi and Hashimoto, 1976). The activity of enoyl CoA hydratase is increased 30-50 fold and the 80kD peroxisome proliferation-associated polypeptide (PPA-80) which is identical to the multi-functional peroxisome protein, is selectively induced by peroxisome proliferators. In contrast, catalase activity is only slightly induced, often not more than 2-fold (Chatterjee et al., 1983).

Other peroxisomal enzymes are also induced. Short chain carnitine acyl-CoA transferase, which is important for the removal of acetyl CoA from the peroxisome, is induced 20 to 160 fold in parallel to peroxisome proliferation (Stott, 1988). Likewise, carnitine octanoyltransferase can be induced over 40-fold by DEHP (Miyazawa et al., 1983).

Increases in the activity of acyl-CoA synthetase, carnitine acetyltransferase, carnitine
palmitoyltransferase and acyl-CoA hydrolase have been observed in hepatic mitochondria isolated from rats treated with peroxisome proliferators (Leighton et al., 1982, Berge et al., 1983). A slight induction in the mitochondrial β-oxidation of short-chain acyl-carnitines has been demonstrated in clofibrate-treated rats (Christiansen et al., 1978). Clofibrate administration has also been shown to inhibit branched-chain amino acid metabolism in the mitochondria (Wagenmakers et al., 1985).

The administration of peroxisome proliferators to rats also induces certain cytosolic proteins, namely palmitoyl-CoA hydrolase, cytosolic epoxide hydrolase and fatty-acid binding protein (Berge et al., 1987, Moody et al., 1991). The proportion of catalase in rat liver cytosol is also increased and may reflect an altered fluidity of the peroxisomal membrane (Crane et al., 1985). Inhibition of cytosolic glutathione peroxidase, glutathione transferase and superoxide dismutase have also been observed when rats have been treated with a number of peroxisome proliferators for a period of 30 days or more (Lake et al., 1987, 1989).

The smooth endoplasmic reticulum proliferation seemingly parallels the proliferation of peroxisomes i.e. it is apparent by 2-3 days and persists at 14 and 30 days. Immunochemical methods have shown an increase in CYP4A1 from its constitutive levels of 5% of the total cytochrome P450 population, to 45% after clofibrate treatment in the rat (Sharma et al., 1988). CYP4A1 induction precedes peroxisome proliferation, and a close correlation between this induction and peroxisomal β-oxidation has been observed (Sharma et al., 1988).

Peroxisome proliferation appears to be a tissue and species specific phenomenon. In rats the liver is the most susceptible organ, though the kidney, heart, intestinal mucosa and skeletal muscle all respond depending on the dose and duration of exposure to peroxisome proliferators (Stott, 1988). The induction of mitochondrial and
peroxisomal enzymes involved in the β-oxidation of fatty acids and the induction of phase II metabolising enzymes such as the microsomal UDP-glucuronosyl transferase (Fournel et al., 1985), in conjunction with other known cellular responses, points to a common mechanism involved in their hypolipidaemic effect.

1.4 SPECIES DIFFERENCES IN RESPONSE TO PEROXISOME PROLIFERATORS

It is now well established that in rats and mice hypolipidaemic agents and other chemicals such as DEHP induce hepatic peroxisome proliferation and peroxisomal and CYP4A enzyme induction (Hawkins et al., 1987). Hamster liver is also responsive to these compounds, although to a lesser extent (Reddy et al., 1982, Lake et al., 1984, Orton et al., 1984). Other species that have been examined for their response after challenge by numerous peroxisome proliferators, include various rat strains, dogs, rhesus monkeys, guinea pigs, mice, ferrets, rabbits, marmosets and humans (Grey and De La Iglesia, 1984, Orton et al., 1984, Reddy et al., 1984a, Gariot et al., 1983, Watanabe et al., 1989, Makowska et al., 1991). The results obtained so far revealed that higher mammalian species are considerably less sensitive than rats and mice, or totally insensitive to the effects of known peroxisome proliferative agents at tolerated or therapeutic doses. For instance, Lake et al. (1989) demonstrated the insensitivity of marmosets to nafenopin, only a small (2-fold) increase in β-oxidation being seen after 250mg/kg/day for 3 weeks. A clear no effect level of 50mg/kg/day in the marmoset was noted; in contrast, this dose given to rats, resulted in a 10-fold increase in peroxisomal β-oxidation. Similar differences between rats, mice and higher mammalian species have been reported for DEHP and trichloroacetic acid (Elcombe et al., 1985, Elcombe and Mitchell, 1986).

A number of studies have been conducted on patients who had been treated with hypolipidaemic agents for several months to several years, in an attempt to elucidate
the effect, if any, of peroxisome proliferators in the human liver. The only substantial report of a chemically induced increase in the number of peroxisomes in humans was made by Hanefeld et al. (1983). They compared the number of peroxisomes found in biopsy specimens from 16 patients on hypolipidaemic therapy before and after receiving treatment with clofibrate for three to four months, and found that there was a 50% increase in the mean number of peroxisomes but that the volume density was increased by only 23%. Statistically, the mean increase in the number of organelles was significant whereas the increase in volume density was not. In contrast Gariot et al. (1987) compared liver biopsies from 12 hypolipidaemic patients receiving a mean daily dose of 317mg fenofibrate and from 15 patients receiving a low fat diet. There was no difference in the volume density or in the number of peroxisomes between test and control groups. In another earlier study by De La Iglesia et al. (1982), liver biopsies from 9 patients on long term (17-25 months) treatment with gemfibrozil were examined. It was found that the content of hepatic peroxisomes in the treated patients varied widely from cell to cell according to the location in the hepatic lobule but peroxisome proliferation as observed in rodents does not occur. A study on the prolonged use of clofibrate (the average length of the trial was 5.8 years) on 15,745 males was carried out by WHO (Oliver et al., 1978). However, the results were inconclusive and have since restricted the use of clofibrate, reflecting doubts about the effectiveness of its therapeutic action and the possibility of latent side-effects, probably unrelated to the phenomenon of peroxisome proliferation.

Studies designed to evaluate species differences in vivo are frequently confounded by variations in administered dose, target organ dose, or differences in routes and rates of biotransformation. Hence several laboratories have attempted to eliminate these confounding factors by using in vitro hepatocyte culture systems (Gray et al., 1982, 1983, Mitchell et al., 1984, Foxworthy et al., 1986), and cultured hepatocytes from a number of species have been used to assess their response to peroxisome proliferators (Mitchell et al., 1985a, Elcombe and Styles, 1989, Foxworthy et al., 1990). These
latter studies have shown a marked species difference in response to the proximate peroxisome proliferators: mouse and rat hepatocytes respond, while cultured guinea pig, and human hepatocytes revealed little effect upon peroxisomes or related enzyme activities, such as peroxisomal β-oxidation or microsomal ω-hydroxylation of lauric acid.

In general, the sensitivity of species to peroxisome proliferators follows the trend: rats, mice >> hamster > rabbit > guinea pig > dog, primates (including man). On the basis of limited studies in humans, it has been argued by some researchers that humans are at a limited risk from the carcinogenic effects of peroxisome proliferators. This may be a reasonable argument, but to be convincing, peroxisome proliferation must be proven as the cause of the carcinogenic response.

1.5 MECHANISMS OF PEROXISOME PROLIFERATION

The exact underlying cellular mechanism responsible for the induction of peroxisome proliferation and associated enzyme activities, remains to be fully ascertained. Two mechanisms have been proposed which remain to be fully explored.

1.5.1 Perturbation of Lipid Metabolism, Substrate Overload Mechanism

The ability of structurally diverse chemicals to elicit peroxisome proliferation has led to the proposal that perturbation in lipid metabolism is the event which initiates the process (Bremmer and Norman, 1982, Elcombe and Mitchell, 1986, Berge et al., 1987). This concept has gained support from the observation that high fat diets induce an increase in peroxisomal β-oxidation (Osmundsen, 1982) and in the microsomal cytochrome P-450 hydroxylase system (Ishii et al., 1980); however, the exact way in which this occurs is still not clear.
The accumulation of hepatic lipid can occur in a number of ways, and the structurally diverse chemicals that produce peroxisome proliferation may act at many different loci to perturb lipid metabolism. In the substrate overload theory (Sharma et al., 1988), it is thought that the hypolipidaemic agent or peroxisome proliferator is taken up by the hepatocyte followed by, initially, inhibition of fatty acid oxidation by the dual mechanism of inhibition of carnitine acyl transferase in the mitochondrion (Lock et al., 1989) or sequestration of essential CoA by the peroxisome proliferator itself (Bronfman et al., 1989). Inhibition of mitochondrial β-oxidation due to sequestration of CoA would lead to the accumulation of medium (C₆-C₁₀) and long chain (C₁₂-C₂₀) fatty acids or their CoA esters in the cell. Induction of CYP4A synthesis then occurs as an early event along with peroxisome proliferation in order to maintain lipid homeostasis (Figure 1.4).

In support of this hypothesis, it has been shown that medium chain (C₆-C₈) fatty acids induce CYP4A in cultured hepatocytes. It is also known that CYP4A preferentially metabolises long chain fatty acids (Orton and McCormick, 1982). Hence, the accumulated long chain fatty acids could be converted to long chain dicarboxylic acids (Robbins, 1968) by the newly induced CYP4A followed by the action of cytosolic alcohol and aldehyde dehydrogenases. It is then proposed that these dicarboxylic acids are then taken up by the peroxisome, thus presenting the organelle with a substrate overload of one of its preferred substrates (Singh et al., 1984). These long chain dicarboxylic acids are also able to stimulate ³H-thymidine incorporation into DNA in isolated rat hepatocytes in culture (Lock et al., 1989) thus contributing to the hyperplasia seen following the administration of peroxisome proliferators in vivo.

The proposed mechanism in Figure 1.4 requires the induction of CYP4A prior to the induction of peroxisomal enzymes such as acyl CoA oxidase. Several studies (Bell et al., 1991, Bell and Elcombe, 1991 and Bieri et al., 1991) suggest that CYP4A is indeed induced prior to acyl CoA oxidase, both in vivo and in primary rat hepatocyte
Figure 1.4 Possible inter-relationship between CYP4A induction and peroxisome proliferation. MCFA and LCFA indicate medium chain and long chain fatty acid respectively. (Derived from Sharma et al., 1988 and Lock et al., 1989).
cultures. Furthermore, inhibition of protein synthesis by cyclohexamide did not prevent the transcription of CYP4A mRNA but inhibited the transcription of acyl CoA oxidase mRNA (Milton et al., 1990, Bieri et al., 1991). This suggests the requirement of a protein, possibly CYP4A, for the subsequent induction of acyl CoA oxidase.

It may well be possible that the above sequential mechanism put forward by Sharma and co-workers, may not be dependent on CYP4A induction of peroxisomal β-oxidation indicating a casual relationship between the two phenomena. An alternative mechanism is possible, whereby the commonality is related to structurally similar regulatory elements in the 5' upstream flanking regions of the CYP4A and β-oxidation genes. This would imply the peroxisome proliferator may directly interact with the common regulatory sequences or indirectly modulate gene expression by lipids derived as a direct result of inhibition of mitochondrial fatty acid β-oxidation described above, or from lipids displaced from fatty acid binding proteins (Cannon and Eacho, 1991). This alternative mechanism may provide an explanation as to why several structurally diverse inducers of CYP4A and the peroxisomal β-oxidation enzymes exist, in that the peroxisome proliferators may not all directly interact with the inducible genes but indirectly act by influencing lipid disposition or a common perturbation of lipid biotransformation.

1.5.2 Receptor Mediated Mechanism

Lalwani et al (1983) described a saturable pool of binding sites for nafenopin in the cytosol of rat liver. This demonstration of a reversible, specific, albeit weak binding of nafenopin to a cytosolic protein, led to the proposal by Reddy and co-workers (Reddy and Lalwani, 1983, Rao and Reddy, 1987), that peroxisome proliferators evoke their action possibly by a receptor-mediated mechanism. There is a good deal of evidence to support this suggestion including: (a) the tissue-specific biological response in transplanted hepatocytes (Reddy et al., 1984b, Rao et al., 1986); (b) the inducibility of peroxisome proliferation in hepatocytes in primary culture (Gray et al., 1982, Mitchell
et al., 1984); (c) the induction of a similar changes in protein composition in the livers of rats exposed to structurally dissimilar peroxisome proliferators (Sharma et al., 1988); and (d) the rapid and significant increase in the rate of synthesis of mRNAs for peroxisomal \( \beta \)-oxidation and microsomal \( \omega \)-hydroxylation enzymes (CYP4A) in liver (Reddy et al., 1986, Hardwick et al., 1987) and the rapidity of the transcriptional response of these genes.

A 70kD protein that binds \(^3\)H-nafenopin has been isolated from rat liver and partially characterised (Lalwani et al., 1987). When clofibric acid and ciprofibrate were used as affinity ligands, the same 70kD protein was isolated. However, other workers have failed to detect any specific binding of \(^3\)H-nafenopin or \(^3\)H-ciprofibrate to hepatic microsomal or cytosolic fractions (Milton et al., 1988) thereby raising doubts about the existence of such a receptor, as described by Reddy (Reddy and Lalwani, 1983). More recently, Alvares et al. (1990) have further characterised this postulated receptor, and have been able to show that the protein is homologous with the 'Heat shock' protein HS P70 family. Additional work is required to ascertain if binding occurs \textit{in vivo} and if this plays a role in transcriptional activation.

The diverse nature of the chemical structures producing peroxisome proliferation suggested that agents do not interact with a single type of binding protein with a single recognition site. Hence it was suggested (Reddy and Rao, 1986) that a binding protein with multiple recognition sites exists or, alternatively, that several binding proteins with different ligand binding properties are involved. Issemann and Green (1990) have reported the cloning and characterisation (from a mouse liver cDNA library) of a member of the nuclear hormone receptor family that can be activated by structurally diverse peroxisome proliferators, and hence, have called it the 'Peroxisome Proliferator Activated Receptor' (PPAR). These workers constructed chimeric receptors containing the putative ligand binding domain of PPAR and the N-terminal sequence and DNA binding domains of both the human oestrogen and human glucocorticoid
receptors. They demonstrated that these chimeric receptors were able to activate an oestrogen/glucocorticoid responsive gene in the presence of peroxisome proliferators. However, they were unable to demonstrate ligand binding. When the oestrogen chimeric receptor was tested using several different peroxisome proliferators (Wy-14,643, nafenopin, MEHP, clofibrate, and trichloroacetic acid) a good correlation was observed between their ability to activate the chimeric receptor and their potency either as peroxisome proliferators or as rat liver carcinogens. The pattern of expression of PPAR mirrors the tissue specific effects of peroxisome proliferators, being highly expressed in liver, kidney and heart and to a lesser extent in brain, brown adipose tissue and testis.

The data above suggests that PPAR mediates some of the biological effects of peroxisome proliferators. The classical scheme of steroid hormone receptor action is shown in Figure 1.5. The receptor-ligand complex is thought to translocate to the nucleus and bind to response elements in target genes causing an increase or decrease in their transcription. In 1991, Osumi et al., identified an activating and inhibitory cis-acting regulatory sequence upstream of the rat Acyl CoA-oxidase gene. Tugwood et al. (1992) demonstrated that PPAR could bind to a region in the 5′ sequence of rat acyl CoA-oxidase gene and could activate transcription of a CAT reporter construct in the presence of the peroxisome proliferator, Wy-14,643. This region was identical to the activation region identified previously (Osumi et al 1991). The sequence that PPAR binds to is termed a 'Peroxisome Proliferator Response Element' (PPRE), and can be seen below:

-572bp
5′-ACG TGACCT T GTCCT GG-3′

Rat Acyl CoA-oxidase PPRE

PPRE's have since been identified in rat 3-ketoacyl CoA-thiolase (Kliewer et al., 1992b), rat enoyl CoA hydratase/3-hydroxyacyl CoA dehydrogenase (Zhang et al., 1992a), rat fatty acid binding protein (Issemann et al., 1992) and in the rabbit CYP4A6.
Fig 1.5 The classical model of steroid hormone action. Steroids enter target cells by passive diffusion where they bind to and induce a conformational change in their cognate receptor proteins. The active steroid-receptor complex translocates to the nucleus where it interacts with target genes by binding to steroid response elements (SREs) in the flanking regions of these genes. This leads to mRNA production, protein synthesis and altered cellular function. (Taken from Power et al., 1992).
gene (Muerhoff et al., 1992b). Other PPRE's will undoubtedly be discovered, but to date none have been identified in the rat CYP4A gene family. It will be important to determine whether all of the effects of peroxisome proliferators are receptor-mediated.

Despite the apparent conflict between the receptor hypothesis and the substrate overload hypothesis of peroxisome proliferation the two mechanisms are not necessarily mutually exclusive. It has been demonstrated by a number of groups (Gottlicher et al., 1992, Issemann et al., 1992 and Keller et al., 1993) that long chain polyunsaturated fatty acids can activate PPAR, and thus may serve as the endogenous ligand for PPAR, although no direct ligand binding has been shown. Figure 1.6 shows a possible strategy for the induction of peroxisome proliferation.

1.6 THE PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR (PPAR)

The PPAR is a member of the nuclear receptor superfamily and is structurally related to the subfamily of receptors that includes the thyroid hormone receptor (TR), retinoic acid receptor (RAR) and vitamin D3 receptor (VDR). The steroid hormone receptors are intracellular proteins that bind their cognate ligand with high affinity and specificity. Additionally they are DNA binding proteins that recognise short DNA motifs generally termed Hormone Response Elements (HREs). Such HREs are usually located upstream of the target genes and behave as transcriptional enhancers or in some cases as transcriptional silencers, which appears to be dependent upon the context of the HRE within the promoter of each target gene.

The cloning of the cDNAs for the steroid hormone receptors has revealed that they all share a common primary organisation with a highly conserved DNA and ligand binding domain. Indeed, it was the fact that the DNA binding domain of these receptors is so highly conserved that was utilised by Issemann and Green (1990) in the isolation of the
Inhibition of Fatty Acid Oxidation, High Fat Diets etc

Metabolic Perturbation
- stimulation
- inhibition

Accumulation of Intermediate (lipid?; acyl CoA?)

Peroxisome Proliferator

CoA Derivative

Inactive Receptor(s)

Activated/ Dimerised Receptor(s)

Genome

Fig 1.6 Possible unifying receptor-mediated mechanism for peroxisome proliferation. (Taken from ECETOC, 1992).
mouse PPAR (mPPAR).

Since the isolation of the mPPAR, a number of other PPARs have been isolated and characterised from different species including *Xenopus laevis* PPAR (xPPAR) α, β and γ, sharing 77%, 55% and 50% amino acid similarity to mPPAR, respectively (Dreyer *et al.*, 1992), rat PPAR (rPPAR) sharing 97% amino acid similarity to mPPAR (Gottlicher *et al.*, 1992), and more recently human PPAR (hPPAR) sharing 91% amino acid similarity to mPPAR (Sher *et al.*, 1993). A PPAR-like receptor isolated from a human osteoblastoma cell line termed NUC-1 has been isolated and found to have a 64% amino acid similarity to mPPAR (Schmidt *et al.*, 1992). This may indicate the presence of another family of related PPAR receptors.

The PPARs have been shown to be activated *in vitro* by peroxisome proliferators (Issemann and Green, 1990) and by fatty acids (Gottlicher *et al.*, 1992, Issemann *et al.*, 1992 and Keller *et al.*, 1993), but, as of yet no direct ligand binding has been demonstrated.

The known peroxisome proliferator response elements (PPREs), found in rat acyl-CoA oxidase (Tugwood *et al.*, 19912), rat 3-ketothiolase (Kliwer *et al.*, 1992b), rat enoyl CoA hydratase/3-hydroxyacyl CoA dehydrogenase (Zhang *et al.*, 1992a), rabbit CYP4A6 (Muerhoff *et al.*, 1992a) and rat liver fatty acid binding protein (Issemann *et al.*, 1992), contain a DNA binding motif consisting of a direct repeat (DR) of a hexanucleotide sequence [5' - TGA (A/C/T) CT -3'] separated by a single nucleotide (DR1). This kind of DR sequence is also found in a number of other nuclear receptor response elements, eg., the TR, RAR, VDR, COUP (chicken ovalbumin upstream promoter) transcription factor and retinoid X receptor (RXR). Although these receptors can all recognise the same half-site motif, they discriminate between target elements through the arrangement and spacing of the half-site motifs.
Recently, it has been demonstrated that receptors such as the Drosophila ecdysone receptor (EcR), RAR, TR and VDR do not efficiently bind to their cognate response elements as homodimers, but rather require additional factors present in cell nuclear extracts to achieve high affinity DNA binding. This additional factor has been identified as the RXR (Yao et al., 1992, Leid et al., 1992, Zhang et al., 1992b, Kliewer et al., 1992a, and Carlberg et al., 1993).

The observation that the acyl-CoA oxidase gene could be induced by retinoic acid in cultured rat hepatocytes (Yu et al., 1991 and Hertz and Bar-Tana, 1992) and that heterodimerisation of RXR occurred with other members of the nuclear hormone receptor superfamily, indicated a possible coupling between PPARs and RXRs. This has since been demonstrated by a number of groups (Kliewer et al., 1992b, Issemann et al., 1993, Gearing et al., 1993 and Keller et al., 1993), who showed that PPAR and RXR formed a heterodimer that activated the rat acyl-CoA oxidase PPRE in response to either peroxisome proliferators or the RXR ligand, 9-cis retinoic acid; simultaneous exposure to both activators resulted in a synergistic induction of gene expression. The implications of these results in relation to the possible involvement of PPAR in the regulation of the rat and human CYP4A genes will be discussed in more detail in Chapter 7.

1.7 MECHANISMS OF HEPATOCARCINOGENICITY OF PEROxisOME PROLIFERATORS

Several studies have now unequivocally established the hepatocarcinogenicity of peroxisome proliferators in rats and mice (Reddy et al., 1980). A strong correlation exists between the incidence of liver cancer and the effectiveness of the administered dose in inducing peroxisomes (Reddy and Lalwani, 1983). However, the basic mechanism(s) by which peroxisome proliferators induce tumours in rodents is unknown.
The development of cancer is generally accepted to be a multistage process. Operationally, these stages have been defined as initiation, promotion, progression and metastasis (Figure 1.7). Initiation appears to represent damage to key genes involved in the regulation of cell growth. By definition, genotoxic chemicals produce DNA damage and therefore are believed to contribute to initiation. The clonal expansion of these initiated cells requires cellular hyperplasia and both growth factors and hormones could contribute to promotion. Numerous studies have indicated that peroxisome proliferators (or their metabolites) are not mutagenic and do not interact directly with nor damage DNA (Butterworth et al., 1984, Glauert et al., 1984, Warren et al., 1980), i.e. they are non-genotoxic or epigenetic agents. Since cellular hyperplasia is a common feature associated with the action of many non-genotoxic carcinogens then these may act as tumour promoters.

A variety of peroxisome proliferators have given negative results in the Ames Salmonella typhimurium reversion assay, DNA repair assays and also the $^{32}\text{p}$ postlabelling assay (Gupta et al., 1985). The findings from such experiments strongly suggest that direct DNA damage is not a prerequisite for the carcinogenicity of peroxisome proliferators. The observation that many carcinogenic peroxisome proliferators were not mutagenic led Reddy et al. (1980) to postulate that such compounds formed a unique class of carcinogens and that there was a causal link between peroxisome proliferation and carcinogenicity. Various mechanisms have been suggested to explain this phenomenon as described below and as depicted in Figure 1.8.

1.7.1 Oxidative Stress Hypothesis
This hypothesis was proposed (Reddy et al., 1980) based on the finding that the enzyme pattern in induced peroxisomes differed from that of control peroxisomes. Certain oxidases, in particular fatty acyl CoA oxidase (the first step of peroxisomal β-oxidation) are markedly induced whilst catalase activity is generally only slightly
Fig 1.7 Multi-stage carcinogenesis. Listed are some of the factors associated with the development of cancer at each of the different stages. (Taken from Green, 1991).
Figure 1.8 Mechanisms of tumour induction by peroxisome proliferators (taken from ECETOC, 1992)
increased or even repressed (Reddy and Lalwani, 1983, Hawkins et al., 1987, Lake et al., 1989, Nemali et al., 1989). Reddy et al. (1980) postulated that this imbalance between hydrogen peroxide production (fatty acyl CoA oxidase) and degradation (catalase, glutathione peroxidase) could lead to increased formation of oxygen radicals which could eventually lead to oxidative DNA damage.

This model has gained support from a number of observations. Conway et al. (1989) demonstrated a correlation between the potency of weak (DEHP) and potent (Wy 14,643) carcinogens and the level of accumulated hepatic lipofuscin used as an indicator of oxidative damage. Fahl et al. (1984) have shown that, in the presence of palmitoyl CoA, peroxisomes from ciprofibrate-pretreated animals catalysed the formation of single strand breaks in SV-40 DNA in vitro. The authors suggested that hydrogen peroxide production formed by the induced peroxisomes was inducing the DNA damage. Oxidative damage may also be assessed by the presence of oxidised DNA bases, particularly 8-hydroxydeoxyguanosine and thymidine glycol (Kasai et al., 1986). Kasai et al. (1989) have investigated the formation of 8-hydroxydeoxyguanosine in liver DNA during treatment of rats with ciprofibrate for up to 40 weeks. A persistent increase in the formation of 8-hydroxydeoxyguanosine was observed for 16 to 20 weeks. Takagi et al. (1990) showed a small (less than 2-fold, but statistically significant) increase in the level of 8-hydroxydeoxyguanosine in rats treated with DHEP and DHEA for 1 and 2 weeks respectively. However, at present, too little is known about variations in endogenous 8-hydroxydeoxyguanosine levels to decide whether a 2-fold increase above the control level is biologically significant.

1.7.2 Promotion of Spontaneous Preneoplastic Lesions
Schulte-Hermann et al. (1981, 1983) have proposed that selective promotion of "spontaneously" initiated cells may explain the production of tumours following long term exposure of rats to non-genotoxic compounds. This was supported by the observations that nafenopin will stimulate DNA synthesis preferentially in spontaneous
and nitrosamine induced enzyme-altered foci and will also stimulate tumour formation in old rats much more rapidly than in young rats (Kraup-Grasl, 1990).

The increase in size of preneoplastic lesions is a reflection of the difference between the rates of cell division and cell death. Tumour promoters, e.g. phenobarbitone, inhibit programmed cell death (apoptosis) in a manner which is at present poorly understood (ECETOC, 1992). Cessation of promoter treatment results in a rapid increase in the number of apoptotic bodies within the liver and reapplication of the promoter during the regression inhibits this process. Peroxisome proliferators have also been shown to inhibit apoptosis (Bursch et al., 1984). Consequently, peroxisome proliferators may both stimulate the growth of preneoplastic lesions and inhibit cell turnover (apoptosis) in these lesions.

1.7.3 Sustained Growth Stimulation
As discussed above, most peroxisome proliferators will also induce DNA synthesis and mitotic activity within the liver with subsequent liver enlargement. The induction of DNA synthesis can be reproduced in vitro in primary cultures of adult rat hepatocytes and is, therefore, a direct property of the compounds (Bieri et al., 1984, 1990, Butterworth et al., 1987).

The actual mechanism by which peroxisome proliferators induce cell growth is unclear. Thompson et al. (1986) have shown that liver regeneration is controlled by the ordered sequential expression of hepatic cellular protooncogenes. Bentley et al. (1987) also showed that upon nafenopin administration, induction of the c-fos and c-myc nuclear oncogenes occurred within a few hours of initiation of treatment and continued throughout the treatment. These findings suggest that the mechanism which control cell growth induced by peroxisome proliferators and normal regenerative cell division are the same.
Preliminary observations (Ochsner et al., 1990) indicate that one of the earliest events after addition of nafenopin to hepatocyte suspensions is an increase in the cytoplasmic calcium concentration. Indeed, several recent papers have suggested a role for calcium in peroxisome proliferation. The first reports indicated that the dihydropyridine calcium antagonists, nicardipine, nifedipine and diltiazem suppress peroxisome proliferation and CYP4A induction (Watanabe et al., 1988, 1991, Itoga et al., 1990). These authors suggested that peroxisome proliferators increase intracellular calcium concentrations by opening plasma membrane calcium channels. Ochsner et al. (1990) have shown that nafenopin, clofibrate and ciprofibrate increase intracellular calcium concentrations in rat hepatocytes and suggested that this increase was due to release of calcium from the endoplasmic reticulum. Bennett et al. (1992) have found that ciprofibrate inhibited the endoplasmic reticulum Ca\(^{2+}\)-ATPase and this resulted in impaired sequestration of calcium in microsomes, which could lead to an increase in cytoplasmic calcium as the calcium diffuses out of the endoplasmic reticulum.

There is no doubt that peroxisome proliferators modulate calcium homeostasis; however the mechanism by which this occurs and its consequences are not clear. The importance of cell signalling in peroxisome proliferation is beginning to become apparent. Protein kinase C (Ca\(^{2+}\)-activated, phospholipid-dependent protein kinase) has been implicated in tumorigenesis by the observation that this protein represents the major cellular receptor for tumour-promoting phorbol esters (Nishizuka, 1984). It has been suggested that the carcinogenic effects of many peroxisome proliferators may be due to their corresponding CoA thioesters and that the latter (but not the free acids) are able to stimulate protein kinase C (possibly by increasing intracellular calcium, since protein kinase C activity depends on the presence of calcium), which may result in the phosphorylation of regulatory proteins (Bronfman et al., 1986, 1989). However, the significance of this process is not known.
1.8 OBJECTIVES OF THE PRESENT INVESTIGATION

The use of hypolipidaemic drugs and various industrial chemicals which have been shown to induce peroxisome proliferation in rodents has caused growing concern with respect to the long term exposure of man to these chemicals. This concern is based on the large amount of evidence that these compounds constitute a class of hepatic non-genotoxic carcinogens, in rodents at least. Since only limited biochemical data is available for human or primates, it is important to understand the molecular basis of peroxisome proliferation and hepatocarcinogenesis in the rodent at the molecular level in order to compare and explain the observed species differences.

The phenomenon of peroxisome proliferation has invariably been associated with induction of members of the cytochrome P450 4A subfamily of proteins, a relationship which is not well characterised at present. At the start of my PhD I set out to isolate the regulatory 5' flanking DNA sequences of members of the rat CYP4A gene subfamily by screening a rat genomic library using the 2.1kb full length cDNA encoding rat CYP4A1 (Earnshaw et al., 1988), the only published member of the CYP4A family at that time. During the characterisation of the genomic clones I had isolated, Kimura et al., (1989a and b) published the genomic sequences of CYP4A1, CYP4A2 and the cDNA for CYP4A3. It was therefore possible to compare my DNA sequences with those published, to search for familiar regulatory sequences, and, using functional in vitro assays to investigate the possible role of the recently identified PPAR in the regulation of those members of the rat CYP4A genes I had isolated and also that of the recently isolated human CYP4A11 gene (Hood et al., 1992).
CHAPTER 2

MATERIALS AND METHODS
CHAPTER 2
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2.1 MATERIALS

2.1.1 Chemicals
All chemicals purchased were of the highest purity available and unless otherwise stated were supplied by BDH Chemicals Ltd.

Bacto-Agar: Difco Laboratories.

N,N,N',N'-tetramethylethylenediamine (Temed), T4 DNA ligase, agarose, low melting point (LMP) agarose, 5-Bromo-4-chloro-3-indolyl-\(\beta\)-D-galactosidase (X-gal), urea and all mammalian cell culture media: Gibco BRL.

2% N,N'Methylene bisacrylamide solution and 30% acrylamide solution: National Diagnostics.

Bacto-tryptone and yeast extract: Oxoid Ltd.

Citric acid (trisodium salt), sodium chloride, EDTA, ethidium bromide, bromophenol blue, xylene cyanol, spermidine trihydrochloride, activated charcoal, acetyl co-enzyme A, o-nitrophenol-\(\beta\)-D-galactosidase, polyvinylpyrrolidone, lysozyme, ampicillin, DMSO (ACS reagent), bovine serum albumin (BSA) fraction V and isopropyl-\(\beta\)-D-thiogalactosidase (IPTG): Sigma Chemical Company.

Calf intestinal alkaline phosphatase (CIAP), Klenow fragment of DNA polymerase, T4 polynucleotide kinase, deoxynucleotide triphosphates and Taq polymerase: Promega.
DMSO (Analar reagent): *Fisons*.

Ficoll 400 and dextran T70: *Pharmacia*.

Proteinase K, DNase I and RNase A: *Boehringer Mannheim*.

The sources for other materials and kits are given in the text.

### 2.1.2 Radiochemicals

i) Deoxycytidine 5'-[α\(^{32}\)P] triphosphate, triethylammonium salt. Specific activity approximately 3,000Ci/mmol. 10mCi/ml. (*ICN Flow*).

ii) Deoxyadenosine 5'-[α\(^{35}\)S] thiotriphosphate, triethylammonium salt. Specific activity 1,200Ci/mmol. (*Amersham*).

iii) Adenosine 5'-[γ\(^{32}\)P] triphosphate, triethylammonium salt. Specific activity approximately 5,000Ci/mmol. (*Amersham*).

iv) \([^{14}\text{C}]\) Chloramphenicol in 0.25M, pH 7.5 tris.HCl buffer. Specific activity 50-60mCi/mmol. (*Amersham*).

### 2.1.3 Mammalian cells, Bacteria, Bacteriophage and Plasmids

i) Mammalian cell lines

Hepa1c1c7: Mouse hepatoma cell line, obtained from Stephen Green, ICI.

ii) Bacterial strains, Bacteriophage and Plasmids

The *E. coli* strains used in this work are listed in Table 2.1 along with their genotype and use. The source of bacteriophage libraries and plasmid vectors are given in the text. Figures 2.1, 2.2 and 2.3 show the bacteriophage vector λCh4A and the plasmid vectors pGEM7Zf(+) and pG.CAT respectively.
<table>
<thead>
<tr>
<th>STRAIN</th>
<th>USE OF STRAIN</th>
<th>GENOTYPE</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM109</td>
<td>Host for pGEM7Zf(+)</td>
<td>endA1, recA1, gyrA96, thi, hsdR17, (rK-,mK+), relA1 pE44, l-, D(lac-proAB), [F', traD36, proAB, lacZDM15]</td>
<td>Yanisch-Perron et al., 1985</td>
</tr>
<tr>
<td>TB1</td>
<td>Host for pUC9</td>
<td>ara, hsdR, thi, strA, Dlac-pro, rK+, f80lacZDM15</td>
<td>BRL Inc., 1987</td>
</tr>
<tr>
<td>XL1-blue</td>
<td>Host for the following plasmids: pBluescript, pSG5, pCH110, pG.CAT, pSG5.PPAR, pACO(-1273/-471)G.CAT</td>
<td>supE44, hsdR17, recA1, endA1, gyrA46, thi, relA1, lac-F[proAB]+, laq9, lacZDM15, Tn10(tetr)</td>
<td>Bullock et al., 1987</td>
</tr>
<tr>
<td>LE392</td>
<td>Host for l Charon 4A</td>
<td>F', hsdR514, rK+, mK+, supE44, supF58, galK2, D(lacZY)6, galT22, medB1, trpR55, D+</td>
<td>Murray et al., 1977</td>
</tr>
</tbody>
</table>

Table 2.1  *E.coli*  Strains and their Genotypes
Figure 2.1 Charon 4A bacteriophage vector (taken from Sambrook et al., 1989, Molecular Cloning, a Laboratory Manual, second edition).
Figure 2.2  pGEM7Zf(+) vector circle map (taken from Promega protocols and applications guide, 1991, second edition).
Figure 2.3 pG.CAT vector circle map. G represents the rabbit βglobin promoter, CAT represents the coding sequence for chloramphenicol acetyl transferase, SV40 3' represents the SV40 splice acceptor site and polyadenylation signals and pEMBL8+ represents the plasmid vector backbone (Issemann and Green, 1990).
2.2 GENERAL NUCLEIC ACID PROCEDURES

For all nucleic acid procedures, solutions, glassware and plasticware were sterilised for 20 min at 120°C in a Rodwell series 32, automatic autoclave to destroy nucleases.

2.2.1 Extraction of Solutions of Nucleic Acid with Phenol-chloroform

Phenol was redistilled prior to use to remove impurities. Phenol-chloroform was prepared as a 1:1 (v:v) mixture, saturated with TE (10mM Tris.HCl pH 8.0, 1mM EDTA). An equal volume of phenol-chloroform was added to the aqueous solution containing nucleic acid and emulsified by mixing. The aqueous and organic phases were separated by centrifugation at 2,500g (10 min) and the aqueous phase was transferred to a fresh tube. The extraction procedure was repeated until the interface between the phases became clear. A final extraction was performed using an equal volume of chloroform to remove residual phenol.

2.2.2 Ethanol Precipitation of Nucleic Acid

Nucleic acids were precipitated from aqueous solution using one of the following methods:

Method A: Addition of 0.1 volume of 3M sodium acetate pH 5.2 followed by 2.5-3.0 volumes of 100% ethanol.

Method B: Addition of 0.5 volume of 7.5M ammonium acetate followed by 2.5-3.0 volumes of 100% ethanol.

After the addition of ethanol the solutions were mixed and were placed at -70°C for 15 min or -20°C for 30 min or longer. The precipitated nucleic acids were pelleted by centrifugation at 12,000g (4°C, 15 min) and then washed in 70% (v/v) ethanol and dried under vacuum (Univap centrifugal evaporator, Uniscience Ltd.) prior to resuspension in an appropriate volume of water or TE.
2.2.3 Quantitation of Nucleic Acids in Aqueous Solution

The concentration and purity of nucleic acids in aqueous solution was estimated by measuring the UV absorbance of the solution over the wavelength range of 220-300nm. Pure solutions of DNA and RNA have an absorbance peak at 260nm, and a 260nm/280nm ratio of 2.0 and 1.8 respectively (Sambrook et al., 1989). The concentration of nucleic acid was calculated using an absorbance value of 1 at 260nm to be equivalent to 50μg/ml for double stranded DNA and 40μg/ml for single stranded nucleic acid. When estimating the concentration of oligonucleotides synthesised in the Applied Biosystems DNA synthesiser (section 2.7.6), a value of 33 μg/ml per absorbance unit was used to calculate DNA concentration as recommended by the manufacturers. Before the UV absorbance for the nucleic acids was determined the spectrophotometer was automatically set to zero with the use of an appropriate blank, namely TE buffer.

2.2.4 Agarose Gel Electrophoresis of DNA

The standard protocol as described by Maniatis et al., (1982) was used routinely. Gels were electrophoresed at 150mA for a time which depended on the gel dimensions and the agarose concentration at room temperature. Preparative 1.0% (w/v) low melting point (LMP) agarose gels were run in the cold room at 100mA. Gels were cast and run in 1x TAE (40mM Tris-acetate, 1mM EDTA pH 8.0) with samples loaded in 1x loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol in 40% (w/v) sucrose). After electrophoresis, gels were stained with ethidium bromide (0.5μg/ml) for 20-30min, destained in water for 10min and the nucleic acid bands visualised by UV-irradiation (Sharp et al., 1973). 300nm irradiation absorbed by the bound dye is re-emitted at 590nm in the red/orange region of the visible spectrum.

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2.2.5 Photography and Autoradiography

A permanent record of ethidium bromide stained gels was made by photographing the UV-irradiated gel with a polaroid land speed MP-4 camera and type 665 positive/negative film. The film was exposed for approximately 14 sec with an aperture of f4.5 and an orange filter.

 Autoradiography was used to visualise radioactive molecules hybridised to nylon filters, present in gels and attached to thin layer chromatography (TLC) plates. For all procedures Kodak X-Omat AR5 high speed film was used. For Southern blots where \(^{32}P\) was the incorporated radionucleotide, filters were wrapped in Saran wrap and placed in cassettes containing Cronex lightening plus intensifying screens. The films were exposed at -70°C and developed automatically in a Fuji RGII Medical Film Processor. The RG developer and RG fixer were purchased from Wardray products Ltd. For \(^{35}S\) sequencing gels and TLC plates containing \(^{14}C\) no intensifying screen was used and the film exposed overnight at room temperature.

2.2.6 Purification of DNA Fragments from Agarose Gels

Agarose gel electrophoresis was performed on restriction enzyme digested DNA and the gel stained with ethidium bromide as described in section 2.2.4. The fragment(s) of interest were recovered from the gels by one of two methods depending on the intended use of the DNA. DNA to be used as a radiolabelled probe was purified using the GeneCleen™ II (Stratech Scientific) method, whereas DNA to be used in ligations was purified by cold phenol extraction.

2.2.6.1 GeneCleen™ Purification

The method is based on that of Vogelstein and Gillespie (1979). The DNA sample was electrophoresed through TAE agarose gels as previously described and a gel slice containing the required DNA fragment was excised from the gel and weighed. Sodium iodide (2-3 volumes) was added and then incubated at 55°C until the agarose
gel slice had melted. "Glassmilk" suspension was then added to the DNA solution (5 μl per 5 μg DNA), mixed thoroughly and incubated on ice for 5 minutes to allow binding of the DNA to the silica matrix. The silica matrix was pelleted by centrifugation (10000g, 5 seconds) and the sodium iodide supernatant discarded. The pellet was washed by resuspension in 400 μl ice cold "NEW" buffer and the silica matrix sedimented by centrifugation (10000g, 5 seconds). The supernatant was discarded and the washing procedure repeated twice more before resuspending the pellet in 5-10 μl of TE buffer. The suspension was incubated at 55°C for 3-4 minutes and pelleted by centrifugation (10000g, 10 seconds). The supernatant containing the DNA was recovered. The recovered DNA was stored at -20°C.

2.2.6.2 Cold Phenol Extraction

DNA was electrophoresed as described in section 2.2.4 in low melting point (LMP) agarose, after which the gel was stained with ethidium bromide and the DNA bands of interest excised under UV light. The agarose was placed in a preweighed Falcon tube, its weight determined and an equal volume of water was added. The gel was melted at 90°C in the minimum amount of time and allowed to cool to 37°C. The DNA was extracted by the addition of ice cold phenol and phases separated as described in section 2.2.1. Extraction was repeated 2-3 times before ethanol precipitation of the DNA (section 2.2.2 method A). The volume was reduced to approximately 100 μl by butan-2-ol extraction as described by Maniatis et al., (1982).

2.2.7 In Vitro Enzyme Reactions

2.2.7.1 Restriction Enzyme Digestion of DNA

Restriction enzymes were obtained from BRL and NBL and digests performed according to the manufacturer's instructions. Small scale analysis was performed using approximately 1 μg of DNA and 1-5 units of enzyme in a volume of 10-20 μl. When analysing small DNA fragments, RNase A (0.1 mg/ml) was included in the digestion buffer.
2.2.7.2 Dephosphorylation of DNA 5' Ends

Dephosphorylation of vector DNA was performed using calf intestinal alkaline phosphatase (CIAP) and was performed after vector DNA had been digested with a single restriction enzyme. The reaction was carried out at 37°C for 60min in the presence of 1x CIAP buffer (50mM Tris-HCl, pH 9.0, 1mM MgCl₂, 0.1mM ZnCl₂, 1mM spermidine) and with the addition of 1 Unit of enzyme per nmol of 5' ends. The reaction was stopped with the addition of EDTA (1mM final concentration) and the CIAP extracted using phenol/chloroform (section 2.2.1). The dephosphorylated vector was then recovered by ethanol precipitation (section 2.2.2, method B).

2.2.7.3 Filling 3' Recessed Ends of Double Stranded DNA

The 3' recessed ends of restriction enzyme digested DNA were filled in using Klenow fragment of DNA polymerase. The DNA was incubated at room temperature for 45min with 2mM dNTPs and 6U of enzyme in 1x Klenow buffer (40mM Tris-HCl, pH 7.2, 10mM MgSO₄, 0.1mM DTT). The DNA was then purified by phenol/chloroform extraction (section 2.2.1) followed by ethanol precipitation (section 2.2.2, method A).

2.2.7.4 Phosphorylation of DNA

The following reaction was set up in a total volume of 40μl:-

- dephosphorylated DNA
- 4μl kinase buffer, 10x (700mM Tris-HCl, pH 7.6, 100mM MgCl₂, 50mM DTT)
- 2μl ATP (2mM)
- 1μl T4 polynucleotide kinase (10U)

The reaction was incubated at 37°C for 60min and then stopped by the addition of 0.5M EDTA (2μl). The DNA was extracted with phenol/chloroform (section 2.2.1) and precipitated with ethanol (section 2.2.2, method A).
2.3 PROCEDURES WITH BACTERIOPHAGE RECOMBINANTS

There are two distinct life cycles in the growth of lambda phage, lytic and lysogenic. Briefly, lysogenic growth is established when λ repressor (CI gene product) binds the operators (Ol and OR) which control the two early promoters PL and PR and the phage DNA is inserted into the host genome. In the absence of this repression the phage enters the lytic cycle in which it reproduces until approximately 100 progeny are formed and the host cell is lysed. λ phage vectors used in recombinant DNA procedures are defective in lysogeny and can only perform the lytic cycle.

2.3.1 Preparation of Host Cells

An individual colony of a freshly grown host was picked from an LB agar plate (10g tryptone, 5g NaCl, 5g yeast extract and 15g bacto agar per litre of H2O, pH 7.0) and used to inoculate 50ml LB media (10g tryptone, 5gNaCl, 5g yeast extract per litre of H2O, pH 7.0, supplemented with 0.2% (w/v) maltose and 10mM MgSO4). The culture was grown overnight and the cells collected by centrifugation (3000g, 4°C, 10min). The cells were resuspended in 10mM MgSO4 (approximately 15ml) to an optical density (OD) at 600nm of 0.5 and placed on ice.

2.3.2 Titration of Lambda Phage (Libraries and Recombinants)

Phage was titred by making serial dilutions in SM buffer (0.1M NaCl, 0.1M MgSO4.7H2O, 0.05M Tris.HCl pH 7.5, 0.01% (w/v) gelatin). 10ml of each dilution was incubated for 15-30min at 37°C with 200µl of phage plating cells (section 2.3.1) and plated on prewarmed (37°C) LB-agar plates (petri dish, 90mm diameter) by adding 4ml of top agarose (7g/L agarose in LB media, kept at 47°C) containing 10mM MgSO4. The plates were allowed to harden at room temperature for 10min and then incubated, inverted, at 37°C overnight. The titre was calculated by counting the plaques and expressed as plaque forming units per ml (pfu/ml) of the undiluted phage suspension.
2.3.3 Plating Out of The Recombinant Library

The library was plated on LB-agar plates (20cm x 20cm) using the appropriate number of phage to give 200,000 plaques per plate. The procedure was essentially a large scale method (x10) of that used to titrate the library (section 2.3.2). In total $1 \times 10^6$ recombinant phage were screened per experiment.

2.3.4 Plaque Lifts

Plates were incubated at 4°C for 2hr, to harden the agarose top layer, before replica filters of plaques were obtained by direct contact with the plate surface. The first nylon filter (Hybond-N™, Amersham) was placed in contact with the plate for 2min and its position marked by making pin-holes into the agarose with a syringe dipped in bromophenol blue solution. The filter was carefully removed, inverted, and the attached phage lysed by placing on a pad of Whatman 3MM filter paper soaked in 0.1M NaOH for 1min. The released phage DNA was denatured by transferring the filter to a fresh pad soaked in 1.5M NaCl for 1min. Filters were neutralised by treatment with 0.2M Tris.HCl for 5min and 2x SSPE (17.5g NaCl, 2.7g NaH$_2$PO$_4$.H$_2$O, 0.7g EDTA per litre of H$_2$O pH 7.4) for 5min as before. The filters were blotted dry and the DNA fixed by UV crosslinking for 3min. The second filter was treated in the same way except it was left in contact with the agar plate for 5min.

2.3.5 Plaque Purification

Positive plaques were identified following hybridisation of the replica filters (section 2.6.2) and excised from the plate by removing a plug of agar in the region of the positive signal using the wide end of a sterile pasteur pipette. The plug was placed in 1ml of SM buffer containing 1 drop of chloroform and left overnight at 4°C to enable the phage to diffuse from the agar. The phage from this stock was then replated at lower density, rescreened, and positive plaques picked. Further purification was achieved by repeated rounds of replating and rescreening until individual positive plaques could be picked.
2.3.6 Isolation of Recombinant Phage DNA

Recombinant phage DNA was prepared in bulk using a method adapted from that of Kaslow, (1986). A cleared lysate of phage was prepared by mixing 10ml of an overnight culture of the appropriate strain of E. coli (section 2.1.3) with 100µl of high (10^8 pfu/ml) titre phage at 37°C for 10min. This was added to 450ml of LB (containing 10mM MgSO4) and the culture was incubated with shaking at 42°C for 4-7hr (until lysis had occurred). A few drops of chloroform were added to kill any remaining bacteria and the culture was cooled on ice for 5min. Debris was removed by centrifugation at 10,000g for 10min. DNase I was added to the supernatant, to a final concentration of 10µg/ml and incubated at 37°C for 30min, to degrade bacterial chromosomal DNA. 150ml of 1.5% (w/v) SDS, 0.3M Tris.HCl pH 9.0, 0.15M EDTA was then added and the mixture heated to 70°C to dissociate the phage particles and inactivate the DNase I. E.coli debris and phage proteins, E.coli DNA fragments, and SDS were precipitated from this mixture after addition of 110ml of 8M potassium acetate and incubation on ice (15-30min). The precipitate was removed by centrifugation at 6000g for 15min at 4°C, and phage DNA precipitated from the supernatant by addition of a 0.6 volume of isopropanol. After standing for 15min at room temperature the DNA was pelleted by centrifugation at 13,800g for 20min, and then resuspended in 1-2ml of sterile water. RNase A was added to a concentration of 50µg/ml, and the mixture was incubated at 37°C for 30min to degrade any residual bacterial RNA. This was followed by incubation with Proteinase K (200µg/ml) under the same conditions as above. Phenol extraction was then performed from the aqueous phase (section 2.2.1) and the DNA precipitated with ethanol (section 2.2.2, method A). The final DNA pellet was resuspended in 200µl of water and its concentration determined (section 2.2.3).
2.4 SUBCLONING INTO PLASMID VECTORS

2.4.1 Small Scale Plasmid DNA Preparation

A simple extraction of plasmid DNA from bacterial cells was used in a rapid procedure to screen recombinant subclones. The protocol applied was based on the original procedure of Holmes and Quigley (1981). 1.4ml of a 10ml overnight culture grown in LB/AMP (LB medium containing 150µg/ml ampicillin) was microcentrifuged at 10,000g for 15-30sec. The supernatant was discarded and the cell pellet resuspended in 100µl of STET (0.1M sucrose, 10mM Tris.HCl pH 8.0, 1mM EDTA pH 8.0, 5% (v/v) Triton X-100). 10µl of freshly prepared lysozyme solution (10mg/ml) was added to this and the tube left on ice for 10min. The tube was then boiled (90sec) and centrifuged at 10000g for 15min at 4°C in a microfuge. The supernatant (50-100µl) was carefully removed and the plasmid DNA purified using Geneclean (section 2.2.6.1). (Geneclean was preferred over phenol in this case since the DNA was obtained free of contaminating RNA and salt).

2.4.2 Large Scale Plasmid DNA Preparation

2.4.2.1 Ethidium Bromide/Cesium Chloride Density Gradient DNA Purification

This method was adapted from that reported by Maniatis et al., (1982). 500ml of the appropriate medium plus antibiotic was inoculated with 1ml of a fresh overnight culture of the cells containing the plasmid required. The culture was incubated, shaking overnight at 37°C and the cells harvested by centrifugation for 10min at 2,500g at 4°C for 30min. The supernatant was discarded and the cells resuspended in 17ml of Tris-sucrose solution (25% (w/v) sucrose in 0.05M Tris.HCl, pH 8.0). The suspension was transferred to two 50ml centrifuge tubes and 1.3ml of freshly prepared lysozyme solution (20mg/ml in 0.25M EDTA, pH 8.0) was added to each. The tubes were kept on ice and the solution swirled intermittently for 5min, followed by the addition of 8.6ml of 0.25M EDTA (pH 8.0) to each and a further 5min swirling on ice. 13ml of Brij/Doc solution (1% (w/v) Brij 58, 0.4% (w/v) sodium...
deoxycholate in TE buffer (10mM Tris.HCl, 1mM EDTA), pH 8.0) was added rapidly and mixed. After a further 30min of incubation on ice, the cell debris and most of the chromosomal DNA was pelleted by centrifugation for 45min, 12,500g, 4°C. The supernatant was decanted, taking care not to disturb the pellet, and 0.2ml of 20mg/ml ethidium bromide added. Cesium chloride was added to 0.95g/ml and the concentration confirmed by measurement of the refractive index (1.38-1.39). The solution was transferred to Beckman polyallomer quickseal tubes, balanced to within 0.05g and sealed. The tubes were centrifuged for 60hr at 40,000rpm, at 18°C (60Ti rotor for 35ml tubes, 70Ti rotor for 14ml tubes, Beckman L7-65 Ultracentrifuge). The upper plasmid band was extracted by side puncture and the ethidium bromide removed by extraction with equal volumes of cesium chloride saturated isopropanol (1:1 mixture of isopropanol and 0.95g/ml cesium chloride in TE buffer) until the top layer remained clear. The DNA was then precipitated by the addition of 0.25 volume of 3M sodium acetate, 1.25 volume of distilled water and 2.5 volumes of isopropanol. This mixture was left at room temperature for 30min and the DNA pelleted by centrifugation at 12,500g for 15min, at 4°C. The pellet was dried under vacuum, resuspended in 300ml of distilled water, and the concentration determined spectrophotometrically (section 2.2.3).

2.4.2.2 Qiagen Column DNA Purification
Plasmid DNA was prepared in bulk using a Qiagen™ plasmid kit (Hybaid Ltd.). A 150ml bacterial culture was routinely grown in LB media (containing 50μg/ml ampicillin) overnight at 37°C. The cells were harvested at 4000g and the bacterial pellet was resuspended in 4ml of buffer P1 (100μg/ml RNase A, 50mM Tris-HCl, 10mM EDTA, pH 8.0). To this 4ml of buffer P2 (200mM NaOH, 1% (w/v) SDS) was added, mixed gently, and incubated at room temperature for 5min, after which time 4ml of buffer P3 (2.55M potassium acetate, pH 4.8) was added, mixed and centrifuged at 15000g, 4°C, for 30min. The supernatant was removed promptly and centrifuged again at 15000g, 4°C, for 10min to obtain a particle-free clear lysate. A
Qiagen-tip 100 (an anion-exchange silica gel column) was equilibrated with 3ml of buffer QBT (750mM NaCl, 50mM MOPS, 15% (v/v) ethanol, pH 7.0, 0.15% (v/v) Triton X-100) and allowed to empty by gravity flow. The particle-free clear lysate was then applied to the column and allowed to enter by gravity flow. The column was then washed with 10ml of buffer QC (1.0M NaCl, 50mM MOPS, 15% (v/v) ethanol, pH 7.0) and the DNA eluted with 5ml of buffer QF (1.25M NaCl, 50mM MOPS, 15% (v/v) ethanol, pH 8.2). The DNA was precipitated with 0.7 volumes of isopropanol, previously equilibrated to room temperature, allowed to stand at room temperature for 30min and then centrifuged at 12,000g for 30min. The DNA pellet was washed with 70% (v/v) ethanol, dried, and resuspended in 100μl of sterile water.

2.4.2.3 pZ523 Column DNA Purification
A 500ml bacterial culture was routinely grown in LB media (containing 50μg/ml ampicillin) overnight at 37°C. The cells were harvested at 4,000g, 4°C, and the bacterial pellet resuspended in 10ml of solution A (50mM glucose, 25mM Tris.HCl, pH 8.0, 1mM EDTA, pH 8.0, 1.0M NaCl) containing 5mg/ml lysozyme. The solution was incubated at room temperature for 15min. After this time, 20ml of solution B (0.2N NaOH, 1% (w/v) SDS) was added, mixed gently, incubated on ice for 10min and then 15ml of ice-cold solution C (3.5M potassium acetate, pH 4.8) added. This was mixed and incubated on ice for a further 10min and then centrifuged at 16,000g for 60min at 4°C. The supernatant was recovered and 0.6 volumes of isopropanol added. The solution was mixed gently by inversion, incubated for 30min at room temperature, and centrifuged at 16,000g for 30min at 20°C. The pellet was washed with 20ml of cold 70% (v/v) ethanol, centrifuged at 16,000g for 5min, and then briefly air dried. The pellet was then resuspended in 5ml of TE buffer, pH 8.0 and incubated with DNase-free RNase A (10μg/ml final concentration) for 60min at 37°C. The DNA was extracted from the solution using an equal volume of phenol/chloroform (section 2.2.1). The aqueous phase was recovered and the DNA precipitated by the addition of 10M ammonium acetate to a final concentartion of 69
2.0M and 0.6 volume of isopropanol. The solution was mixed and incubated for 30min at room temperature and the DNA collected by centrifugation at 16,000g, 30min at 20°C. The pellet was washed with 70% (v/v) ethanol, briefly air-dried and then resuspended in 1.8ml of buffer D (10mM Tris.HCl, 1mM EDTA, 1.0M NaCl, pH 8.0). The sample was loaded onto a pZ523 column (NBL Ltd) that had been previously centrifuged for 1min at 1100g in a swinging bucket rotor. The column was centrifuged for 12min at 1100g in a swinging bucket rotor and the effluent collected. 0.6 volume of isopropanol was added to the plasmid effluent, mixed and incubated at room temperature for 30min. The plasmid DNA was collected by centrifugation at 12,000g for 30min at 20°C and the pellet washed in 70% (v/v) ethanol. The pellet was then briefly air-dried, resuspended in 100µl of distilled water and the DNA concentration determined spectrophotometrically (section 2.2.3).

2.4.2.4 NACS Column DNA Purification

10ml of a bacterial culture was grown overnight in LB media (containing 50µg/ml) at 37°C with constant shaking. The cells were harvested by centrifugation at 9,000g for 15min and the bacterial pellet resuspended in 0.1ml of 25mM Tris.HCl (pH 8.0), 50mM Na2 EDTA, 1% (w/v) glucose. 0.2ml of 0.2M NaOH, 1% (w/v) SDS was added, mixed gently and incubated for 10min at 4°C. 0.15ml of cold (4°C) 5M potassium acetate, pH 4.8, was added, mixed gently and then incubated for 5min at 4°C. The bacterial debris was removed by centrifugation at 12,500g for 30min and the supernatant collected. 2 volumes of cold (-20°C) 95% (v/v) ethanol was added, incubated for 20min at 4°C and centrifuged at 12,500g for 15min. The pellet was resuspended in 0.2ml of TE buffer, 0.1ml cold (4°C) 7.5M ammonium acetate added and incubated for 20min at 4°C. The solution was then centrifuged at 12,500g for 15min and the supernatant recovered to a fresh tube. 0.6ml of cold (-20°C) 95% (v/v) ethanol was added to the supernatant, incubated at 4°C for 20min, centrifuged at 12,500g for 15min and the resulting pellet was briefly air-dried. The pellet was resuspended in 0.4ml 0.05M NaCl in TE buffer. 20 units of RNAse T1 was added to
the solution and incubated at 37°C for 15min. 0.12ml of 2M NaCl in TE buffer was added, and this was loaded onto a PREPAC NACS column (BRL Ltd) previously equilibrated in 0.5M NaCl in TE buffer. The bound plasmid DNA was washed with 10ml of 0.5M NaCl in TE buffer to remove small RNA molecules, any remaining proteins and other contaminating molecules. The bound plasmid DNA was eluted twice with 0.25ml of 0.7M NaCl in TE buffer, and the DNA precipitated by the addition of 1.0ml cold (-20°C) 95% (v/v) ethanol and incubated at -70°C for 10min. The DNA was collected by centrifugation at 12,500g for 15min and the pellet briefly air-dried. The pellet was resuspended in 25μl of TE buffer and the DNA concentration determined spectrophotometrically (section 2.2.3).

2.4.3 Subcloning of DNA Fragments
The plasmid cloning vector pGEM7Zf(+) (figure 2.2) was chosen for subcloning because it contains a large number of restriction enzyme sites in the multiple cloning site and transformants are selectable by ampicillin resistance. An additional advantage is that the cloning site is situated within the plasmid β-galactosidase gene. Thus β-gal- E.coli transformed with native plasmid give blue, β-gal+, colonies on X-gal plates, whereas recombinant plasmids give white, β-gal+, colonies. In addition sequencing can be performed directly in pGEM from both ends of the DNA insert, as the SP6 and T7 primer-binding regions are present at either side of the multiple cloning site.

2.4.3.1 Isolation of DNA inserts
To subclone phage DNA inserts the recombinant bacteriophage were cut with the appropriate restriction enzymes (see section 2.2.7.1). This released rat DNA fragments of various size and the two arms of the bacteriophage. The mixture was separated on a 1% (w/v) low melting point gel, and the rat DNA fragments recovered by phenol/chloroform extraction as described in section 2.2.1.
2.4.3.2 Preparation of the Plasmid Vector

DNA from the plasmid vector pGEM7Zf(+) was prepared as described in section 2.4.2. 5μg of the DNA was routinely digested with an appropriate restriction enzyme to linearise. This was confirmed by electrophoresis of a 100ng sample on a 1% agarose gel. The 5' ends of the plasmid DNA were then dephosphorylated with calf intestinal alkaline phosphatase as described in section 2.2.7.2.

2.4.3.3 Ligation of Rat DNA into Plasmid Vectors

DNA ligations were performed using T4 DNA ligase according to the manufacturer's instructions. Generally, ligations were carried out using 100ng of a dephosphorylated vector and an insert:vector molar ends ratio of 1:1 and 3:1. Cohesive end ligations were incubated at 16°C and blunt end ligations at 10°C overnight. Ligation products were either diluted or used directly to transform competent cells.

2.4.4 Transformation of Host Bacteria with Recombinant Plasmids

2.4.4.1 Preparation of Competent Cells

5ml of an overnight culture of bacteria was diluted 1:100 in prewarmed LB media and grown with vigorous shaking at 37°C until the optical density of the culture at 600nm reached 0.45-0.55. The cells were cooled on ice water for 2 hours prior to centrifugation (2500g, 4°C, 20min) in a precooled rotor. The cells were resuspended in 20ml of ice-cold tituration buffer (100mM CaCl2, 70mM MgCl2, 40mM sodium acetate, pH 5.5, filter sterilised) and then diluted to 500ml with the same solution. The cells were incubated on ice for 45min, collected by centrifugation (1800g, 10min, 4°C) and gently resuspended in 50ml of ice-cold tituration buffer. To this suspension 80% glycerol was added dropwise with gentle swirling to give a final concentration of 15% (v/v) glycerol. The cell suspension was divided into 1ml portions, frozen on dry ice, and stored at -70°C.
2.4.4.2 Transformation of Competent Cells
Plasmid DNA (10-20ng) in 10μl of water was added to 0.2ml of thawed competent cells in the presence of 3μl of DMSO and incubated on ice for 30min. The cells were then heat shocked at 42°C for 90 seconds and then returned to ice for 1min. 2ml of LB medium was added and the cells were shaken at 37°C for 60min before dilutions of the transformation mix were spread on LB agar plates containing ampicillin (100μg/ml). When the blue/white screening was used, IPTG (0.5mM) and X-gal (40μg/ml) were also included in the agar. The plates were incubated at 37°C overnight.

2.4.4.3 Selection of Transformed Cells
Blue/White Colour Screening: The plasmid pGEM7ZF(+) contains a sequence coding for the α-peptide of β-galactosidase, interrupted by a multiple cloning site. Non-recombinant plasmids produce a functional α-peptide which complements the defective product of the host cell lacZΔM15 gene and results in the appearance of functional β-galactosidase activity. Bacterial colonies harbouring the lacZΔM15 gene on an F' episome and also containing the plasmid pGEM7ZF(+) produce a blue colour when plated on indicator media containing IPTG and X-gal. However, when the lac α-peptide is disrupted by cloning of a DNA fragment into the pGEM7ZF(+) multiple cloning region complementation does not occur and no β-galactosidase activity is produced. Therefore bacterial colonies harbouring recombinant pGEM7Z vector constructs are white on IPTG and X-gal plates.

Ampicillin Selection: Detection of bacteria transformed by plasmids which did not contain the β-galactosidase gene was performed by selection on ampicillin plates. To screen ampicillin resistant colonies for those containing recombinants, it was necessary to estimate the size of the plasmid DNA using the rapid cracking procedure.
Cracking Procedure: Individual bacterial colonies were picked from plates using sterile toothpicks and smeared near the bottom of a microfuge tube containing 50μl of 10mM EDTA, pH 8.0. 50μl of freshly prepared 2x cracking buffer (200mM NaOH, 0.5% (w/v) SDS, 200g/l sucrose) was added and the cells resuspended by vortexing. The sample was incubated at 70°C for 5min, allowed to cool to room temperature and then 1.5μl of 4M KCl and 0.5μl of 0.4% (w/v) bromophenol blue were added and mixed. The sample was placed on ice for 5min, microfuged for 3min at 4°C and 30μl of the supernatant was run on a 0.7% agarose gel alongside a sample of the supercoiled plasmid used as a marker. Recombinant plasmid DNA migrated more slowly in the gel and could be readily identified.

2.5 STORAGE OF RECOMBINANT BACTERIOPHAGE AND TRANSFORMED BACTERIA

2.5.1 Storage of Bacteriophage
A single plaque was picked from an LB agar plate into sterile SM buffer using a sterile glass pasteur pipette. Phage were stored, over chloroform, at 4°C.

2.5.2 Preparation of Bacterial Stocks
Bacterial cultures were maintained on LB-agar plates, with antibiotics if appropriate, and restreaked every 6 to 8 weeks. For long term storage, bacteria were frozen at -70°C in LB-broth containing 15% (v/v) glycerol.
2.6 HYBRIDISATION PROTOCOLS

All hybridisation experiments were performed using Hybond-N™ nylon membrane (Amersham).

2.6.1 Southern Transfer of DNA

Southern transfer was performed according to the method of Southern, (1975), as described in Sambrook et al., (1989). DNA agarose gel electrophoresis (section 2.2.4) was followed by shaking the gel gently in 0.25M HCl for 10min, to depurinate the DNA (to aid transfer of large DNA fragments), soaking the gel (2 x 20min) in several volumes of denaturing solution (1.5M NaCl, 0.5M NaOH) and in neutralising solution (3M NaCl, 0.5M Tris-HCl pH 7.5) for 30min. The DNA was transferred from the gel to the membrane by capillary action using 20 x SS (3M NaCl, 300mM sodium citrate) and blotted overnight. After blotting the membrane was dried briefly (2-3min) in an 80°C oven and then UV crosslinked for 3min.

2.6.2 DNA Hybridisation

Hybridisations (and prehybridisations) were carried out in Hybaid™ bottles at 42°C. Prehybridisations were performed in hybridisation solution (50% (v/v) deionised formamide, 5 x SSC, 5 x Denhardt's [1g Ficoll, 1g polyvinylpyrolidone, 1g bovine serum albumin fraction V per 100ml], 2.5mM sodium phosphate and just prior to use, sonicated salmon sperm DNA to give a final concentration of 100μg/ml) at 42°C for a minimum of 6 hours. After prehybridisation, denatured, radiolabelled probe DNA (section 2.6.4) was added directly to the hybridisation solution and hybridisation continued at 42°C for 16-24 hours. The membranes were washed in SSC, in the presence of 0.1% (w/v) SDS, with the concentration of SSC and the washing temperature depending on the specificity of the probe DNA for its target. After washing, the membrane was wrapped in Saran-wrap™ and autoradiographed as described in section 2.2.5.
2.6.3 Oligonucleotide Hybridisation

Oligonucleotide prehybridisations and hybridisations were carried out as described in section 2.6.2 except that, the hybridisation solution contained 6 x SSC, 5 x Denhardts solution, 1% (w/v) SDS and 0.1mg/ml sonicated salmon sperm DNA only, and the temperature of hybridisation was calculated from the formula \(2(A+T) + 4(G+C) = TM\), where TM is the melting temperature of the DNA and A, T, G and C are the number of adenine, thymine, guanine and cytosine bases present in the DNA (Itakura et al., 1984). Hybridisations were carried out at 5-10°C below TM.

2.6.4 Preparation of \(^{32}\text{P}\) labelled DNA Probes

2.6.4.1 Multiprime Labelling of DNA

Radiolabelling of DNA probes was carried out using an Amersham Multiprime DNA labelling system, based on the method of Feinberg and Vogelstein (1983). Probe DNA was isolated from agarose gels using Geneclean™ (section 2.2.6.1). The amount of DNA used per labelling reaction was estimated by visual inspection after gel electrophoresis and ethidium bromide staining. Single stranded template was prepared by boiling the probe for 5min followed by rapid cooling on ice. The denatured DNA was used directly in the labelling reaction as described by the manufacturers, the label used being \([\alpha-^{32}\text{P}]\text{-dCTP}\) (section 2.1.2). Unincorporated nucleotides were removed using NICK™ columns (Pharmacia LKB) according to the manufacturer's instructions.

2.6.4.2 5' End Labelling of DNA

Oligonucleotide probes were 5' end labelled according to the method of Maxam and Gilbert, (1980), whereby free 5' hydroxyl groups in DNA are phosphorylated with \(^{32}\text{P}\) by T4 polynucleotide kinase, using \(\gamma-^{32}\text{P}\) ATP. The following reaction mix was set up in a total volume of 25\(\mu\)l:

\[
\begin{align*}
15 & \text{ pmoles oligonucleotide} \\
2.5 & \mu l \quad \text{10x kinase buffer (500mM Tris-HCl, pH 8.0, 100mM MgCl}_2, 1mM
\end{align*}
\]
EDTA, 50mM DTT, 1mM spermidine)

5.0μl $\gamma^{32}$P ATP (50μCi)
1.0μl T4 polynucleotide kinase (10 units)

The reaction mixture was incubated at 37°C for 45min and then stopped by heating to 65°C for 10min.

2.6.5 Stripping of Probes from Filters

Radiolabelled DNA probes were removed from filters by agitating in a boiling solution of 0.1% (w/v) SDS for 2 x 15min followed by rinsing in 2 x SSC. The filters were autoradiographed before reprobing.

2.7 SEQUENCING OF DNA

Inserts were sequenced using a modification of the dideoxy sequencing strategy of Sanger et al. (1977) as described by Chen and Seeburg, (1985). The Sequenase™ version 2.0 sequenase kit (United States Biochemical Corporation) contains all the buffers, solutions and enzyme required for the addition of deoxynucleotides (and their dideoxy derivatives) to the 3' end of a growing DNA chain and was used according to the manufacturers instructions.

2.7.1 Apparatus

The sequencing apparatus for gel electrophoresis was manufactured in the departmental workshop at the University of Surrey and routinely accommodated glass plates with the dimensions of 58 x 20 x 0.5cm. The plates were cleaned with Decon™ and hot water, rinsed with distilled water and allowed to dry. After every 4-5 runs, the gel side of the front plate was resiliconised with 10ml of dimethyl dichlorosilane. The gel spacers (0.4mm) were put in place and the plates sealed using SYGLAS™ waterproof tape.
2.7.2 Template Preparation

Plasmid DNA, prepared as described in section 2.4.2, was denatured by alkali essentially as described by Zhang et al., (1988). 2-3μg of DNA was denatured for 5min at room temperature with 0.2M NaOH and 0.2mM EDTA, in a total volume of 20μl. The mixture was neutralised by the addition of 2μl of 2M ammonium acetate (pH 4.6) and the DNA precipitated with ethanol as described in section 2.2.2, method A.

2.7.3 Electrophoresis

Urea (31.5g) was dissolved with acrylamide (11.25ml of 40% (w/v) acrylamide [19:1] bisacrylamide) and 10x TBE (7.5ml of 0.89M Tris-HCl, 0.89M Borate, 0.02M EDTA) in a total volume of 75ml to give a 6% (w/v) polyacrylamide gel. Ammonium persulphate (420μl, 10% (w/v)) and TEMED (75μl) were then added and the gel was mixed before pouring between the glass plates. When not used immediately, the polymerised gels were wrapped in Cling film and stored at 4°C overnight. The gel was pre-electrophoresed for 30min and run at 50W constant power in 1x TBE. Just prior to loading the samples (3μl) on to the gel, they were heated to 75°C for 2min. Sharkstooth combs with 5.7mm point to point spacing (BRL Ltd) were routinely used and the samples were loaded with a Drummond sequencing pipette (Drummond Scientific Co.).

2.7.4 Autoradiography

After electrophoresis, the DNA was fixed in the gel by immersion in 10% (v/v) methanol, 10% (v/v) glacial acetic acid for 20min, and then rinsed in water for 5min to remove the urea. The gel was peeled off the glass plate onto a wet piece of Whatman 3MM paper and dried at 80°C for 60min in a gel dryer (slab gel dryer, Biorad, model 4823). Autoradiography was performed as described in section 2.2.5.
2.7.5 Oligonucleotide Synthesis

Oligonucleotides were chemically synthesized using an "Applied Biosystems" DNA synthesizer model 381A, according to the manufacturer's instructions. The oligonucleotides were extracted from their column supports using 18M ammonium hydroxide, and deprotected at 55°C for 12 hours in 18M ammonium hydroxide. The oligonucleotides were ethanol precipitated (section 2.2.2, method B), resuspended in TE buffer and their concentration determined spectrophotometrically using the equation A_{260} = 33μg/ml.

2.8 POLYMERASE CHAIN REACTION (PCR)

DNA (300ng) was amplified using Taq polymerase (2.5U) by cycling through 15 rounds of denaturing at 94°C (1min), annealing at 55°C (2min) and extending at 72°C (3min), the final cycle concluding with an additional 4min at 72°C, in 50mM KCl, 10mM Tris-HCl pH 8.3, 1.5M MgCl₂, 0.01% (w/v) gelatine and 1.25mM dNTPs in a total volume of 50μl. The specific oligonucleotides used to prime amplification were added before denaturation. The reaction was carried out under a layer of paraffin oil to prevent evaporation of the reaction mix. The products of the PCR reaction were then electrophoresed on an agarose gel as described (section 2.2.4).

2.9 ANIMAL CELL CULTURE TECHNIQUES

2.9.1 Cell Culture

Hepalc1c7 cells were grown as monolayers in tissue culture flasks in phenol red-free Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% (v/v) foetal calf serum (FCS) at 37°C (5% (v/v) CO₂). Cells were subcultured when the growth was confluent by treating with trypsin and diluting the cells to a suitable density. The media was aspirated off and the cells were washed with sterile PBS (8g NaCl, 0.2g KCl, 1.44g Na₂HPO₄, 0.24g KH₂PO₄, per litre of H₂O, pH 7.4). The cells were
then incubated with trypsin /EDTA solution at 37°C until the cells had detached from the surface. The cells were then diluted in pre-warmed media and seeded into 90mm Petri dishes to give $3 \times 10^5$ cells.

### 2.9.2 Frozen Cell Stocks
Growing anchorage dependent cells were trypsinised, centrifuged at 80g, 5min, and resuspended in growth medium containing 20% FCS (v/v) and 10% DMSO (v/v) to a final concentration of $1 \times 10^7$ cells. The cells were dispensed into 1ml aliquots and frozen overnight at -70°C before removing to liquid nitrogen for storage. To recover cells from liquid nitrogen storage, an aliquot was thawed at 37°C and the cells were diluted with 10ml growth medium. Cells were sedimented by centrifugation (80g, 5min) and the supernatant discarded. The cell pellet was resuspended in 10ml of fresh growth medium (containing 10% (v/v) FCS), transferred to a tissue culture flask, and incubated at 37°C with an overlay atmosphere of 5% (v/v) CO₂.

### 2.9.3 Cell Counting and Viability
Cell counts were determined by diluting aliquots of cells in culture medium, adding trypan blue (0.4% (w/v) in saline) and counting using a Hawksley cristalite Neubauer haemocytometer. Exclusion of the dye by those cells with an intact cell membrane indicated viability. Those cells with a 'leaky' cell membrane incorporated the dye and were considered to be non-viable. The % viability was calculated from the following equation:

$$\% \text{ Viability} = \frac{V}{T} \times 100$$

where $V = \text{viable cells/ml}$

$T = \text{total cells/ml}$

Cells of greater than 98% viability were used.
2.10 TRANSIENT COTRANSFECTION ASSAY

Hepalclc7 cells (3 x 10^5) were seeded into 9cm Petri dishes (section 2.9.1) in phenol red-free DMEM supplemented with 10% (v/v) FCS three days prior to transfection. Cells were then washed in serum-free medium and transfected by mixing 10μg of Qiagen prepared plasmid DNA (section 2.4.2.2) with 100μg N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) and 5ml serum-free medium which was applied to the cells. Unless otherwise stated, each plate was transfected with 1μg CAT (chloramphenicol acetyl transferase) reporter plasmid, 1μg expression vector (pSG5 or pSG5.PPAR), 3μg of the β-galactosidase expression vector, pCH110 (Pharmacia), and 5μg pBluescript (Stratagene) as carrier. Each transfection was performed in triplicate. After 5hr, 5ml DMEM containing 20% (w/v) dextran coated charcoal (DCC) treated FCS was added, along with either dimethyl sulphoxide (DMSO) or ligand (eg. Wy 14643, Chemsyn Science Laboratories, Lenexa, KS) prepared in DMSO. After 20 hours the medium was replaced with fresh DMEM containing 10% (w/v) DCC-FCS and ligand where appropriate. After 48 hours the cells were harvested and cytosol extracts (300μl in 0.25M Tris-HCl, pH 7.8) prepared by three cycles of freeze-thawing and assayed for β-galactosidase activity, used to normalise the CAT assay.

2.10.1 β-Galactosidase Assay

Extracts were normalized for β-galactosidase activity by incubating 100μl of extract with 0.28mg o-nitrophenyl-β-D-galactosidase (ONPG) in 50mM phosphate buffer pH 7.0, 10mM KCl, 1mM MgCl2 for 1 hour at 37°C. The reaction was stopped by the addition of 400μl of 1M Na2CO3. The absorbance was then read at 420nm and the following equation used to determine β-galactosidase activity:

\[
1U/\mu l.h = \frac{1000 \times \text{OD}_{420nm}}{\mu l \text{cytosol extract} \times \text{hours of incubation at 37°C}}
\]
2.10.2 CAT Assay

After normalization for β-galactosidase activity, CAT enzyme activity was determined by co-incubation of cell extracts with 0.1 μCi [14C]chloramphenicol (Amersham) and 20mM acetyl coenzyme A (Sigma) for 1 hour at 37°C. Radiolabelled products were extracted with ethyl acetate and separated by TLC using 95% (v/v) chloroform/ 5% (v/v) methanol as the solvent. The TLC plate was autoradiographed as described in section 2.2.5 and quantification of the CAT assay data was performed by scanning the TLC plates directly, using the Berthold LB2842 scanning TLC linear analyser.

2.11 SAFE HANDLING OF HAZARDOUS CHEMICALS

All chemicals were handled, and procedures carried out according to COSH regulations.

Ethidium Bromide: when handling this substance, gloves were worn at all times. The solid was weighed in a class 3 cabinet and the stock solution (10mg/ml) was kept in a double walled container. Gels were stained in a designated ethidium bromide area, and once photographed, the gel was disposed of in a biohazard bag. The liquid ethidium bromide waste was kept in a contained area and disposed of according to Maniatis.

Dichlorosilane: when coating the sequencing plates with this chemical gloves were worn and this procedure was carried out in the fume hood. The plates were only removed from the fume hood once dry.
CHAPTER 3

ISOLATION AND PRELIMINARY ANALYSIS OF RAT CYP4A GENES
CHAPTER 3
ISOLATION AND PRELIMINARY ANALYSIS OF RAT CYP4A GENES

3.1 INTRODUCTION

The following results detail experiments to isolate rat genomic DNA fragments with sequence similarity to the rat CYP4A1 cDNA from a recombinant phage library in λ Ch4A (a kind gift from Dr. J. Bonner). In particular, the objective was to isolate the 5' end of this gene, so that its regulation could be investigated.

3.2 PREPARATION OF CYP4A1 cDNA FOR USE AS A HYBRIDISATION PROBE

The cDNA probe used for screening the rat recombinant phage library in λCh4A was the rat full length 2.1Kb cDNA coding for CYP4A1, previously isolated in this laboratory (Earnshaw et.al., 1988). The 2.1Kb cDNA had been inserted into the Eco RI site of the plasmid pUC9 (Figure 3.1). A large scale preparation of plasmid DNA was performed according to the method in section 2.4.2.1 and the 2.1Kb cDNA insert was excised using the restriction enzyme Eco RI. This large scale digest of the plasmid was electrophoresed on a 1% low melting point agarose gel (Figure 3.2). The 2.1Kb cDNA fragment was excised from the gel and the DNA purified by the cold phenol method (section 2.2.6.2).

In order to identify later which clones contained possible 5' sequences, it was decided to digest the 2.1Kb CYP4A1 cDNA with Eco RI and Bgl I to produce a 600bp fragment containing the first 5 exons of the cDNA (Figure 3.1). The 600bp fragment was prepared in the same way as the full length cDNA probe. Figure 3.3 shows the low melting point agarose gel, the 600bp fragment being indicated. 20ng of each
Figure 3.1 Restriction map of the plasmid pUC9 containing the full length, 2.1Kb cDNA coding for CYP4A1. The restriction sites for Eco RI and Bgl I are shown and the position of the 600bp probe indicated.
Figure 3.2 Low melting point agarose gel (1%) of pUC9 containing the 2.1Kb cDNA coding for CYP4A1 digested with the restriction enzyme Eco RI. The marker lane contains a Hind III digest of λ phage DNA, the sizes of which can be seen in Figure 3.3.

Figure 3.3 Low melting point agarose gel (1%) of pUC9 containing the 2.1Kb cDNA coding for CYP4A1 digested with the restriction enzymes Eco RI and Bgl I. The marker lane is as Figure 3.2.
pure probe was electrophoresed on a 1% agarose gel to confirm that they were of the appropriate size (Figure 3.4).

3.3 EVALUATION OF COLUMN METHODS IN THE PREPARATION OF PLASMID DNA

New methods for purifying plasmid DNA from bacterial host cell DNA are being developed constantly as although the ethidium bromide/cesium chloride ultraspin preparation (section 2.4.2.1) is very effective, it is quite dangerous in respect to the chemicals used in the process and takes a long time to prepare (3 days). The three column methods evaluated were; Qiagen columns (Hybaid Ltd), pZ523 column (NBL Ltd), and NACS column (BRL Ltd). The Qiagen columns are available in three different sizes according to the starting volume of bacterial culture. The plasmid to be purified was pUC9 containing the rat full length 2.1kb cDNA encoding \textit{CYP4A1} grown in the host \textit{E. coli} strain TB1 in LB media containing ampicillin (150\(\mu\)g/ml).

Each column was tested with the manufacturer's recommended starting volume and the bacterial cell number in this volume assessed from the absorbance at 600nm:

\[
1 \text{ OD}_{600} = 8 \times 10^8 \text{ cells}
\]

The concentration of the DNA from each column was determined spectrophotometrically (section 2.2.3) and is shown with the DNA yield in Table 3.1. It can be seen that the Q500 column gave the highest DNA concentration, however the yield of DNA per cell, in order of greatest yield was as follows:-

\[
Q20 > Q100 > Q500 > pZ523 > \text{NACS}
\]
Figure 3.4 Agarose gel (1%) of the purified 2.1Kb and 600bp probes.
Lane 1&4: λ Hind III markers (see Fig. 3.3 for sizes)
Lane 2: 2.1Kb DNA
Lane 3: 600bp DNA
<table>
<thead>
<tr>
<th>COLUMN</th>
<th>VOLUME ELUTED IN</th>
<th>$\lambda_{260}$</th>
<th>$\lambda_{280}$</th>
<th>$\lambda_{260}/\lambda_{280}$ RATIO</th>
<th>DNA CONCENTRATION</th>
<th>TOTAL DNA (µg)</th>
<th>TOTAL NO. CELLS</th>
<th>DNA YIELD (µg/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q20 (5.0ml)</td>
<td>50µl</td>
<td>0.054</td>
<td>0.033</td>
<td>1.64</td>
<td>0.27µg/ml</td>
<td>13.5</td>
<td>3.66x10^9</td>
<td>3.69x10^-9</td>
</tr>
<tr>
<td>Q100 (150ml)</td>
<td>200µl</td>
<td>0.202</td>
<td>0.102</td>
<td>1.98</td>
<td>1.01µg/ml</td>
<td>202</td>
<td>2.0x10^11</td>
<td>1.01x10^-9</td>
</tr>
<tr>
<td>Q500 (500ml)</td>
<td>200µl</td>
<td>0.362</td>
<td>0.196</td>
<td>1.85</td>
<td>1.81µg/ml</td>
<td>362</td>
<td>5.3x10^11</td>
<td>6.83x10^-10</td>
</tr>
<tr>
<td>pZ523 (500ml)</td>
<td>100µl</td>
<td>0.135</td>
<td>0.079</td>
<td>1.71</td>
<td>0.67µg/ml</td>
<td>67.5</td>
<td>5.3x10^11</td>
<td>1.27x10^-10</td>
</tr>
<tr>
<td>NACS (10ml)</td>
<td>100µl</td>
<td>0.011</td>
<td>0.011</td>
<td>1.00</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

**Table 3.1** Evaluation of commercially available columns for the production of plasmid DNA. The starting bacterial culture volume is given under each column. (Q = Qiagen column, ND = not detected, the base line was automatically zeroed with an appropriate blank).
No DNA was recovered from the NACS column and this was confirmed by the UV scan performed in the region of 200-300nm, Figure 3.5 (a). The DNA prepared from each pZ523 column and the Qiagen column appeared to be of good quality as assessed by the 260/280 ratio (section 2.2.3) and by the symmetrical peak around 260nm as seen in Figure 3.5 (a and c respectively). To assess the quality and purity of the DNA further, 1μg of DNA from each column was digested with the restriction enzyme Eco RI and electrophoresed on a 1% agarose gel. The DNA prepared from all the Qiagen columns could be digested to produce the 2.1Kb insert band and the 2.7Kb pUC9 band. However the DNA prepared from the pZ523 column did not digest, indicating that the DNA needed further purification.

The pZ523 method was very long and laborious and although it seemed to produce DNA of a high concentration (1.81μg/ml) and a good 260/280 ratio (1.71), this did not digest with the restriction enzyme Eco RI indicating that something was interfering with the enzyme. The preparation could have been purified further with the use of ethanol precipitation but this was not done since the Qiagen columns produced DNA of suitable quantity and quality. Also Qiagen DNA had the advantage that it was simple to use and required only one day as compared with the three days required to carry out the ethidium bromide/cesium chloride method and it used safe materials. Subsequent to this study, Qiagen columns have been the method of choice for preparing plasmid DNA for use in sequencing and transfection experiments, confirming that the DNA produced is of high quality.

3.4 ISOLATION OF RECOMBINANT CLONES HYBRIDISING TO THE RAT FULL LENGTH 2.1Kb cDNA ENCODING CYP4A1

A rat genomic library in λCh4A was screened by Dr. C. McGeoch, using the 2.1Kb cDNA for CYP4A1 labelled with α-32PdCTP as a probe. From this screen eight positive clones were isolated, which were purified by a further three rounds of
Figure 3.5 A graph of absorbance against wavelength between 200-300nm of plasmid DNA prepared from a) NACS column, b) pZ523 column, and c) Qiagen columns. (The baseline was automatically zeroed using an appropriate blank).
screening. A stock of these eight recombinant phage clones was prepared (section 2.5.1) and stored at 4°C.

3.4.1 Determination of Sizes of the Genomic DNA Inserts in the Recombinant Phage

Initially, DNA was prepared from the eight recombinant lambda phage using a scaled down version of the method in section 2.3.6. From the map of \( \lambda \)Ch4A (Figure 2.1) it can be seen that the recombinant DNA inserts can be excised using the restriction enzyme \( \text{Eco RI} \). This enzyme was used to digest approximately 4\( \mu \)g of DNA from the eight phage clones and the resulting fragments were electrophoresed through a 1% agarose gel. After staining with ethidium bromide the results showed that the DNA had been digested by the restriction enzyme, since the 19.5Kb and 11Kb arms of the phage could be seen, releasing insert fragments of various sizes (Figure 3.6 (A)). To determine the molecular weight of the individual insert fragments, a graph was plotted of the migration of the \( \lambda \text{Hind III} \) digested DNA fragments from the origin, against their known molecular weight (Figure 3.7). The sizes of the inserts were then extrapolated from this standard graph and the results are tabulated in Table 3.2. It can be seen that clones AL4, AR5 and AR6 give the same insert digestion pattern of 8.5, 5.5 and 2.3Kb.

3.4.2 Characterisation of Recombinant Phage Clones

The DNA from both gels in Figure 3.6 (A) were transferred to nylon filters by the method of Southern (section 2.6.1). These filters were hybridised with the rat \( \text{Eco RI/Bgl I} \) \( CYP4A1 \) cDNA 600bp probe which had been labelled with 50\( \mu \)Ci \( \alpha^{32}\text{PdCTP} \) using Multiprime™ (section 2.6.4.1). The unincorporated radiolabelled nucleotides were removed using a NICK™ column and the probe was then denatured and added to 20ml of hybridisation buffer (section 2.6.2). The filters were probed overnight at 42°C and then washed under fairly stringent conditions: two 20min washes of 2 x SSC / 0.1% (w/v) SDS at room temperature, two 20min washes of 0.1 x SSC / 0.1% (w/v)
Figure 3.6 A) Agarose gels of the 8 recombinant phage clones isolated from the 2.1Kb screen. B) Autoradiographs of the above gels hybridised to the 600bp probe

**Gel 1**
Lane 1&14: λ Hind III markers
2&13: pUC9 Eco RI digested
3: -
4: AR4 Eco RI digested
5: AR4 uncut
6: AL7 Eco RI digested
7: AL7 uncut
8: AL4 Eco RI digested
9: AL4 uncut
10: AL2 Eco RI digested
11: AL2 uncut
12: -

**Gel 2**
Lane 1&14: λ Hind III markers
2&13: pUC9 Eco RI digested
3: -
4: AR9 Eco RI digested
5: AR9 uncut
6: AR7 Eco RI digested
7: AR7 uncut
8: AR6 Eco RI digested
9: AR6 uncut
10: AR5 Eco RI digested
11: AR5 uncut
12: -
Figure 3.7 Semi-log plot of the distance travelled by the \(\lambda\) Hind III DNA fragments, from the origin (seen in Figure 3.6), against their known molecular weight.

<table>
<thead>
<tr>
<th>CLONE</th>
<th>FRAGMENT SIZES (Kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL2</td>
<td>8.5, 7.0*</td>
</tr>
<tr>
<td>AL4</td>
<td>8.5*, 5.5, 2.3</td>
</tr>
<tr>
<td>AL7</td>
<td>6.7*, 3.4*, 2.3</td>
</tr>
<tr>
<td>AR4</td>
<td>4.8, 3.4*, 2.5, 1.7, 1.0*</td>
</tr>
<tr>
<td>AR5</td>
<td>8.5*, 5.5, 2.3</td>
</tr>
<tr>
<td>AR6</td>
<td>8.5*, 5.5, 2.3</td>
</tr>
<tr>
<td>AR7</td>
<td>15.5, 8.5</td>
</tr>
<tr>
<td>AR9</td>
<td>8.5, 5.5, 3.5</td>
</tr>
</tbody>
</table>

Table 3.2 Sizes of fragments produced by Eco RI digestion of recombinant phage clone DNA, isolated from the 2.1Kb screen.

* indicates those fragments sharing sequence similarity with the Eco RI/Bgl I 600bp CYP4A1 probe (Figure 3.6, B).
SDS at 55°C and a final rinse in 5xSSC for 20min at room temperature. The filters were then wrapped in Saranwrap™ and exposed to Kodak X-OMAT™ AR5 film at -70°C for 24 hr. Figure 3.6 (B) show the autoradiogram of the two gels. The dark bands indicate those fragments that have a degree of sequence identity with the 600bp probe and which might lie towards the 5' end of their gene. Each clone contained fragments that gave positive hybridisation signals to the 600bp probe (Table 3.2), except for clones AR7 and AR9. These two clones were investigated further and continued to produce no hybridisation signal when probed with either the 2.1Kb or the 600bp probe and therefore were dropped from any further investigation.

3.5 ISOLATION OF RECOMBINANT CLONES HYBRIDISING TO THE Eco RI / Bgl I 600bp FRAGMENT OF RAT CYP4A1

The same rat genomic library in λCh4A was screened again using the Eco RI/Bgl I 600bp fragment of CYP4A1, to try to obtain more recombinant clones with a stronger bias to the 5' end. One million plaques were plated and transferred to duplicate Hybond N™ nylon filters (section 2.3.4). The filters were hybridised with the 600bp probe which had been end labelled with α32P dCTP using Multiprime™ and exposed to X-ray film at -70°C for four days. Thirteen positive clones were identified in the first round of screening but this was reduced to ten positives in the second round as three colonies proved to be false positives. The remaining ten were purified by two further rounds of screening. The filters at each round, after probing with the 600bp fragments were washed at fairly high stringency: two 20min washes of 2 x SSC / 0.1% (w/v) SDS at room temperature, two 20min washes of 0.1 x SSC / 0.1% (w/v) SDS at 55°C, and a final rinse in 5 x SSC at room temperature. Typical results from the further rounds of screening are shown in Figure 3.8. A stock of recombinant phage clones which hybridised to the rat 600bp probe was prepared (section 2.5.1) and stored at 4°C.
Figure 3.8 Autoradiograph of filters from the second (2 plaques positive) and fourth round (all plaques positive) of screening of the λ Ch 4A rat genomic library, using the 600bp probe, showing positive hybridisation signals produced by recombinant phage clone RG4.
3.5.1 Determination of Sizes of the Genomic DNA Inserts in the Recombinant Phage

DNA from each of the ten phage clones was prepared (section 2.3.6), digested with the restriction enzyme *Eco RI* and the resulting fragments were separated by electrophoresis through a 1% agarose gel (Figure 3.9). Table 3.3 shows the fragment sizes for each clone as determined by comparison to the λ *Hind III* markers. It can be seen that clones RG1, 2 and 10 have the same insert *Eco RI* digestion pattern of 6.7, 3.4 and 2.3Kb. It was suspected that RG7, RG6 and RG11 would give the same results, so the *Eco RI* digestion was repeated and the DNA's electrophoresed alongside one another (data not shown). This confirmed that RG7 and RG6 produced the same *Eco RI* restriction pattern as RG1, 2 and 10, but that RG11 produced a different one, namely, 6.7, 3.8 and 2.1Kb. Clones RG3, 4, 8 and 9 also give the same pattern of fragment sizes 7.8 and 6.7Kb. The clone AL7, isolated from the 2.1Kb library screen, also had the same fragment pattern as that of RG1, 2, 6, 7 and 10.

3.6 CHARACTERISATION OF PHAGE CLONES BY SOUTHERN BLOT

Figure 3.10 is a schematic diagram summarising the two library screens and the subsequent grouping of the clones according to their *Eco RI* restriction pattern. Figure 3.11 (A) shows a gel of representative recombinant phage clone DNA from each group digested with *Eco RI* and electrophoresed alongside one another. This shows quite clearly that each group is different. Because so much DNA had been used (10-15μg) per digestion, the DNA had become slightly retarded in the gel as compared to the λ *Hind III* markers, and hence the sizes appear different to those seen before, but the patterns remain the same. This gel was Southern blotted and hybridised to radiolabelled 2.1Kb *CYP4A1* cDNA. The filter was washed under stringent conditions: two 20min washes in 2 x SSC / 0.1% (w/v) SDS at 42°C, two 20min washes in 0.1 x SSC / 0.1% (w/v) SDS at 65°C and then a final rinse in 5 x SSC at room temperature.
Figure 3.9 Agarose gels of the 10 recombinant phage clones isolated from the 600bp screen.

**Gel 1**
- Lane 1&14: λ Hind III markers
- 2&13: pUC9 Eco RI digested
- 3: -
- 4: RG7 Eco RI digested
- 5: RG7 uncut
- 6: RG3 Eco RI digested
- 7: RG3 uncut
- 8: RG2 Eco RI digested
- 9: RG2 uncut
- 10: RG1 Eco RI digested
- 11: RG1 uncut
- 12: 600bp DNA

**Gel 2**
- Lane 1&14: λ Hind III markers
- 2: RG11 Eco RI digested
- 3: RG11 uncut
- 4: RG10 Eco RI digested
- 5: RG10 uncut
- 6: RG9 Eco RI digested
- 7: RG9 uncut
- 8: RG8 Eco RI digested
- 9: RG8 uncut
- 10: RG6 Eco RI digested
- 11: RG6 uncut
- 12: RG4 Eco RI digested
- 13: RG4 uncut
Table 3.3 Sizes of insert fragments produced by *Eco* RI digestion of recombinant phage DNA, isolated from the 600bp screen.

<table>
<thead>
<tr>
<th>CLONE</th>
<th>FRAGMENT SIZES (Kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RG7</td>
<td>6.7</td>
</tr>
<tr>
<td>RG3</td>
<td>7.8, 6.7</td>
</tr>
<tr>
<td>RG2</td>
<td>6.7, 3.4, 2.3</td>
</tr>
<tr>
<td>RG1</td>
<td>6.7, 3.4, 2.3</td>
</tr>
<tr>
<td>RG11</td>
<td>6.7</td>
</tr>
<tr>
<td>RG10</td>
<td>6.7, 3.4, 2.3</td>
</tr>
<tr>
<td>RG9</td>
<td>7.8, 6.7</td>
</tr>
<tr>
<td>RG8</td>
<td>7.8, 6.7</td>
</tr>
<tr>
<td>RG6</td>
<td>6.7</td>
</tr>
<tr>
<td>RG4</td>
<td>7.8, 6.7</td>
</tr>
</tbody>
</table>
Figure 3.10 Schematic diagram, summarising the two library screens and the subsequent grouping of recombinant phage clones, based on their Eco RI digestion pattern.
Figure 3.11 A) Agarose gel of a representative recombinant phage clone digested with Eco RI, B) Southern blot of above gel hybridised to the 600bp probe, C) Southern blot of above gel hybridised to the 2.1Kb probe.

Lane 1: λ Hind III markers
2: Group F (RG11)
3: Group E (RG4)
4: Group D (AR4)
5: Group C (AL7)
6: Group B (AL4)
7: Group A (AL2)
After autoradiography, the filters were stripped of their probe (section 2.6.5), re-probed with the 600bp probe under the same conditions and washed under the same stringent conditions. From the results seen in Figure 3.11 (B) and (C) it was possible to draw up a preliminary restriction map of the orientation of Eco RI fragments within each group (Figure 3.12), since those fragments hybridising to both the 600bp and the 2.1Kb probe would be 5' to those fragments hybridising to the 2.1Kb probe only.

3.7 DISCUSSION

At the beginning of this work, only two members of the CYP4 family had been isolated as cDNA's (CYP4A1 and CYP4B1). CYP4A1 was isolated from a λgt11 expression library constructed from liver mRNA isolated from rats given the peroxisome proliferator clofibrate (Hardwick et al., 1987). CYP4B1 was isolated from a rabbit lung cDNA library (Matsubara et al., 1987). From a Southern blot analysis of rat genomic DNA, Hardwick et al., (1987) deduced that two or three genes existed in the rat CYP4 family. Figure 3.11 (A) is in agreement with this data since six different genomic clones were isolated, however some of these groups may represent overlapping clones. The total size of each recombinant phage clone was found to range from 12.4Kb, group C (Table 3.2, AL7) to 16.3Kb, group B (Table 3.2, AL4). Of those genomic sequences published for other members of the cytochrome P450 superfamily (at the time of this work), CYP21A1 was found to possess the greatest number of exons (10) and was 3.4kb in size (Higashi et al., 1986) and CYP1A1 was the largest in size (6.7kb) and had 7 exons (Gonzalez et al., 1985). It therefore seemed likely that all of the gene for CYP4A1 and the other members of the CYP4 family would be present within the clones isolated.

Figure 3.12 shows the preliminary maps for each clone based on their Eco RI fragments hybridisation to the 600bp and 2.1Kb probes. It can be seen that there is more than one possible orientation for each group since it is impossible to tell whether
Figure 3.12 Preliminary Eco RI (E) restriction maps of the recombinant phage clones, groups A-F.

- represents 600bp probe positive,  represents 2.1kb probe positive, (not to scale).
those fragments that are negative with both probes lie to the 3' or 5' end of the other fragments (groups A, B, D, E and F) and in which orientation (group D). It is also interesting to note that when the clone AR4 (group D) was hybridised to the 600bp probe for the first time (Figure 3.6 (B), gel 1, lane 4) two Eco RI fragments 3.4Kb and 1.0Kb gave a positive signal. However when this experiment was repeated, Figure 3.11 (B), lane 4, no signal was seen. The preliminary maps for both results can be seen in Figure 3.12.
CHAPTER 4

CHARACTERISATION OF RAT CYP4A GENE SEQUENCES
CHAPTER 4
CHARACTERISATION OF RAT CYP4A GENE SEQUENCES

4.1 INTRODUCTION
During this work, the full sequence for the rat CYP4A1 gene was published along with the DNA sequence for a closely related gene, CYP4A2 (Kimura et al., 1989a). The restriction map of these genes can be seen in Figure 4.19 (Kimura et al., 1989a). The cDNA for a third member of this family, designated CYP4A3, was also published at this time (Kimura et al., 1989b). A number of other sequences of members of this expanding family, isolated from different species, were published during this work, including three rabbit cDNAs, CYP4A5, CYP4A6 and CYP4A7 (Johnson et al., 1990) and three orthologous cDNAs of the CYP4B subfamily, designated CYP4BI (human CYP4B1, Nhamburo et al., 1989; rabbit and rat CYP4B1, Gasser and Philpot, 1989). The CYP4B sequences represent a different subfamily since they are ≤ 59% identical to the CYP4A genes at the nucleotide level (Nebert et al., 1989). The full list of CYP4 genes published up until December 1993 can be seen in Table 1.2, Chapter 1. It was decided to further characterise the genes that I had isolated, to establish their identity within the CYP4 family. The Eco RI fragment inserts from representatives of each group were subcloned into the Eco RI site of the plasmid pGEM7Z (for restriction map see Figure 2.2). This plasmid is easily prepared and purified facilitating the isolation of large quantities of insert DNA for analysis.

4.2 PREPARATION OF THE PLASMID pGEM7Z FOR USE IN SUBCLONING
A large scale preparation of pGEM7Z plasmid DNA was carried out (section 2.4.2.2). 5 μg of pGEM7Z DNA was digested with the restriction enzyme Eco RI and 100 ng of this electrophoresed on a 1% agarose gel to check that it had been linearised. The enzyme was removed from the remainder of the digest by phenol/chloroform
extraction (section 2.2.1) and the linear plasmid recovered by ethanol precipitation (section 2.2.2, method A). This was resuspended in distilled water to give a final concentration of 100ng/μl Eco RI cut pGEM7Z.

4.3 PREPARATION OF INSERT DNA FROM RECOMBINANT PHAGE FOR SUBCLONING

Initially a shotgun approach to cloning of insert DNA into pGEM7Z was tried. Shotgun cloning is where the whole preparation of Eco RI digested phage DNA was included in the ligations. In this approach only the insert fragments should be incorporated into the circular plasmid, as the two arms of the phage have only one "sticky end" each. Recombinant DNA from each group (A-F) was prepared on a large scale (section 2.3.6). Approximately 20μg of DNA from each group was digested with Eco RI and 1/10th of the digest electrophoresed on a 0.8% agarose gel in order to check that the DNA had been digested to completion. The enzyme was removed from the remaining digest with phenol/chloroform (section 2.2.1) and the DNA ethanol precipitated (section 2.2.2, method A). The digested DNA was resuspended in distilled water to give a final concentration of 100ng/μl.

The use of the shotgun approach did not always work in the subcloning of all of the Eco RI insert fragments from each group. In such cases a second approach was taken in which the whole digest was electrophoresed on a 0.7% low melting point agarose gel and the DNA fragment of interest excised. The DNA was then extracted from the agarose by either cold phenol (section 2.2.6.2) or Geneclean™ (section 2.2.6.1). This approach was used to maximise the chances of obtaining subclones, since the number of pGEM7Z molecules available for the formation of circular recombinants should be greater if phage arms are not present in the ligations.
4.4 SUBCLONING RESULTS : GROUP A

Ligations were carried out as detailed in section 2.4.3.3 and transformation of competent JM109 cells performed as in section 2.4.4. Table 4.1 shows the results obtained from the first successful experiment using the shotgun approach. No colonies were seen on plate 1, indicating that competent JM109 do not contain any antibiotic resistant contaminants. On plate 2 (undigested pGEM7Z) a number of white colonies, $\frac{1}{10}$th of the total colonies, were present. These represent false positives, since no insert DNA was present in the plasmid. The number of blue colonies seen for the unligated, Eco RI digested pGEM7Z (plate 3, 27 blues) represents $\frac{1}{15}$th of the number seen on the plate with undigested pGEM7Z (plate 2, 400 blues). This implies that most of the pGEM7Z DNA was digested by the restriction enzyme. The blue colonies on plate 3 represent spontaneous religation of the digested plasmid. The number of blue colonies seen on plate 4 (ligated Eco RI digested pGEM7Z) is the same as plate 2. This indicates that the T4 DNA ligase was working efficiently. The number of white colonies seen on this plate, again represent false positives, since no insert DNA is present, and they are of the same order of magnitude seen for the undigested plasmid.

The white colonies seen on plates 5-7 represent recombinant $\beta$-gal$^+$ clones i.e. those plasmids with insert DNA ligated into the multiple cloning site. The molar ratio of 1:1, vector to insert (plates 5a and b), seems to have given the most white colonies and a similar number of blue colonies as compared to the control plate (plate 2). The molar ratio of 1:3 (plates 6a and b) gave only two whites and a dramatic reduction in the number of blue colonies ($\frac{1}{20}$th as compared to plate 2) indicating that the amount of insert DNA present is affecting the religation and hence very few pGEM7Z molecules are religating and hence cell death on ampicilin plates. The molar ratio of 3:1 (plates 7a and b) gave a higher number of white recombinants than the molar ratio 1:3 (plates 6a and b) but less than the 1:1 (plates 5a and b) and also less blue colonies.
<table>
<thead>
<tr>
<th>PLATE</th>
<th>DNA</th>
<th>ENZYME</th>
<th>MOLAR RATIO</th>
<th>WHITE COLONIES</th>
<th>BLUE COLONIES</th>
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<td>5b</td>
<td>pGEM7Z (20ng) + grp A</td>
<td>Eco R1</td>
<td>1:1 (V:I)</td>
<td>37</td>
<td>300</td>
</tr>
<tr>
<td>6a</td>
<td>pGEM7Z (20ng) + grp A</td>
<td>Eco R1</td>
<td>1:3 (V:I)</td>
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<td>20</td>
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<tr>
<td>6b</td>
<td>pGEM7Z (20ng) + grp A</td>
<td>Eco R1</td>
<td>1:3 (V:I)</td>
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<td>30</td>
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<td>3:1 (V:I)</td>
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<td>150</td>
</tr>
</tbody>
</table>

**Table 4.1** Cloning strategy and results of shotgun subcloning of the recombinant phage group A (AL2) inserts into pGEM7Z.

Plates 1-4 were plated at one dilution (100μl)
Plates 5-7 were plated at two dilutions (a = 100μl, b = 200μl)

* = no ligase added

Eco RI = indicates that the plasmid had been linearised before ligation
than the 1:1. However the number of whites on each test plate (5-7) are only in the same order of magnitude as the false positives on the control plates 2 and 4 and therefore could represent false positives.

It was decided to screen the white colonies from plate 5a and 7b (38 in total) for inserted DNA using the cracking method (section 2.4.4.3). Figure 4.1 shows an example of an ethidium bromide stained cracking gel. Lane a1 shows the supercoiled pGEM7Z which was used as a marker. DNA in lanes a2, 5 to 13 and b11 and b12 can be seen to migrate for the same distance as the marker and therefore represent plasmid DNA without insert (false positives). Lanes a3 and a4 show a supercoiling pattern different to that of the marker and represent recombinant plasmid DNA migrating more slowly through the gel. The ten colonies containing recombinant DNA identified in this manner were picked into 10ml of LB media containing ampicillin (150µg/ml) and incubated overnight at 37°C. DNA was prepared by the STET method (section 2.4.1) using 1.4ml of each of the ten overnight cultures. The final DNA preparations were dissolved in 30µl of distilled water and 10µl of each were digested with the enzyme Eco RI and electrophoresed on a 1% agarose gel. As can be seen from Figure 4.2 all ten colonies contain an insert of a larger size than the pGEM7Z plasmid band. The recombinant clones in lanes 2, 4, 5, 12 and 13 contain a 7Kb insert, those in lanes 3, 6, 7 and 11 a 4.7Kb insert and the one in lane 10 contains a 5.7Kb insert. From the Eco RI digestion pattern of the original group A phage DNA, only two different size inserts of 8.5Kb and 7.0Kb were expected (Figure 3.11A), however, it is necessary to run the digested recombinant phage alongside the digested recombinant plasmids to make a true comparison of size. The origin of the 5.7Kb insert is unknown.

4.4.1 Characterisation of Group A Recombinant Subclones

A Qiagen plasmid DNA preparation was performed on the subclones containing the three different sized inserts (section 2.4.2.2). 1µg of DNA of each subclone was digested with Eco RI along with 5µg of phage DNA and electrophoresed on a 1%
**Figure 4.1** Screening of recombinant group A plasmid DNA by supercoiling pattern

Lane a: supercoiled pGEM7Z

Lane b: -

1: supercoiled pGEM7Z
2: A: 7/2/15
3: A: 7/2/14
4: A: 7/2/13
5: A: 7/2/11
6: A: 7/2/10
7: A: 7/2/8
8: A: 7/2/7
9: A: 7/2/6
10: A: 7/2/4
11: A: 7/2/3
12: A: 7/2/2
13: A: 7/2/1
14: λ Hind III marker (sizes shown in Fig.4.2)

**Figure 4.2** Digestion of recombinant group A plasmid DNA with Eco RI. Plasmid DNA prepared by the STET method was digested overnight with the restriction enzyme indicated and then separated on a 1% agarose gel.

Lane 1, 8, 9 and 14: λ Hind III marker

Lane b: -

1: supercoiled pGEM7Z
2: A: 5/1/11
3: A: 5/1/8
4: A: 5/1/6
5: A: 5/1/5
6: A: 5/1/4
7: A: 5/1/2
8: A: 7/2/14
9: A: 7/2/13
10: A: 5/1/23
11: A: 7/2/18
12: A: 7/2/17
13: A: 5/1/19
14: -
agarose gel (Figure 4.3 (A)). From the Eco RI digestion pattern of the original group A phage DNA (lane 5) only two different sized inserts of 4.7Kb and 7.0Kb were expected in the recombinant pGEM7Z subclones, and these were obtained (lanes 2 and 4 respectively).

The DNA from the gel shown in Figure 4.3 (A) was transferred to Hybond N™ nylon filter by the method of Southern (section 2.6.1), and subsequently hybridised to the rat 600bp cDNA CYP4A1 probe. The filter was washed under moderately stringent conditions: two 20min washes in 2 x SSC / 0.1% (w/v) SDS at room temperature, two 30min washes in 0.1 x SSC / 0.1% (w/v) SDS at 55°C and a final rinse in 5 x SSC at room temperature. After autoradiography the filter was stripped to remove the radiolabelled probe (section 2.6.5), re-probed with the rat full length 2.1Kb cDNA CYP4A1 and washed using the same conditions as before. Figure 4.3 (B) shows the two autoradiographs obtained. No hybridisation signal was seen in the group A recombinant phage, lane 5, for either the 2.1Kb or 600bp probe. A positive signal had been obtained previously (figure 3.11 b and c, lane 7). The hybridisation experiment indicated that there was a problem with the probe. Both probes however, did hybridise to the pGEM7Z 3.0Kb band indicating that they were slightly contaminated with some of the vector DNA. Whilst this was annoying, it did provide a positive control for the subsequent hybridisation experiments in which these batches of probes were used.

4.4.2 Sequencing of Group A Subclones
Since the Eco RI restriction pattern of the group A recombinant phage bore no resemblance to the published maps of CYP4A1 or CYP4A2, it was decided to obtain some sequence data for each subclone to see if similarity existed to any of the other published sequences.
Figure 4.3  (A) Digestion of recombinant group A phage and plasmid DNA with the restriction enzyme Eco RI. (B) Southern blot of group A DNA hybridised to rat CYP4Al 600bp and full length 2.1Kb cDNA probes.

Lane 1 and 6 : λ Hind III molecular weight markes
2: A: 5/1/2 : pGEMA4.7
3: A: 7/2/14: pGEMA5.7
4: A: 5/1/5 : pGEMA7.0
5: Recombinant phage group A (AL2)

NB The hybridisation signal seen at 3.0kb, represents probe homology to the plasmid pGEM7Z (refer to section 4.4.1).
6μg of the recombinant plasmids pGEMA7.0 and pGEMA4.7 were denatured by alkali (section 2.7.2) and sequenced using Sequenase™ version 2.0 (section 2.7). The T7 and SP6 polymerase promoters flanking the pGEM7Z plasmid multiple cloning site (Figure 2.2) were used as convenient specific priming sites to sequence each recombinant plasmid in the 5'-3' and 3'-5' direction respectively. After 24hr exposure to X-ray film, an autoradiograph was obtained (Figure 4.4). As shown, approximately 200 nucleotides could be read.

The sequence data obtained was analysed using the Microgenie™ software. Comparison of these sequences with those previously published, namely the genomic sequences CYP4A1 and CYP4A2, revealed no sequence identity. The sequences were then compared to the other published sequences for the CYP4A cDNA's. Again no homology was identified, but since the four recombinant sequences might contain intronic sequences they would not necessarily align to any published cDNA.

The sequences were compared with the database held in the Microgenie software. A 91-96% match was found for pGEMA4.7 and pGEMA7.0 sequences, respectively, with that of bacteriophage lambda (Figure 4.5). The results from the sequencing were confirmed when the filter obtained from the gel in Figure 4.3 (A) was probed with α\(^{32}\)PdCTP labelled lambda DNA. Figure 4.6 shows the autoradiograph from this experiment. Lane 5 quite clearly shows that the inserts in the recombinant phage are homologous to the \(\lambda\) probe as are the insert bands in the recombinant plasmids pGEMA7.0 (lane 4) and pGEMA4.7 (lane 2).

The rat genomic library used in this study was made in the late 1970's and had been obtained by this laboratory in 1986. The first libraries made, such as those made in the late 1970's, seem to suffer from the problem of lambda DNA re-packaging into the phage arms. It was not realised that this was going to prove such a problem until I discovered that group A recombinant phage contained lambda DNA. Subsequently, as
Figure 4.4 An autoradiograph of the sequencing gel from the sequencing of recombinant plasmids pGEMA7.0 and pGEMA4.7.
Figure 4.5 Sequence alignment of recombinant plasmids (I) pGEMA7.0 (sequenced with the T7 primer) and (II) pGEMA4.7 (sequenced with the SP6 primer) to that of bacteriophage lambda.
Figure 4.6 Autoradiograph of Southern Blot obtained from the gel in figure 4.3 (A), hybridised to radiolabelled bacteriophage lambda DNA.

Lane 1 and 6: λ Hind III molecular weight marks
2: A: 5/1/2: pGEMA4.7
3: A: 7/2/14: pGEMA5.7
4: A: 5/1/5: pGEMA7.0
5: Recombinant phage group A (AL2)

NB The hybridisation signal seen at 3.0kb, represents probe homology to the plasmid pGEM7Z (refer to section 4.4.1).
a matter of routine, Southern blots of the remaining groups were probed with a λ probe.

4.5 SUBCLONING RESULTS: GROUP B

Ligations were carried out as detailed in section 2.4.3.3 and transformation of competent JM109 cells performed as described in section 2.4.4. Table 4.2 shows the results obtained from the first attempt at subcloning the insert fragments using the shotgun approach. The control ligation plates (plates 1-4) were very similar to those obtained previously for the subcloning of group A (Table 4.1), indicating that the stored cells were still competent, that the pGEM7Z had been linearized and that the T4 DNA ligase was working efficiently. Of the 97 white colonies (from plates 5, 6 and 7), 48 still remained white after re-streaking on fresh indicator plates, and these were further screened using the cracking procedure (section 2.4.4.3). From this screen, 32 colonies were identified as possibly containing insert DNA and to confirm this, DNA was prepared by the STET method (section 2.4.1), digested with the Eco RI and electrophoresed on a 1% agarose gel. Out of the 32 DNA preparations 18 were shown to contain inserts: three contained a 5.5Kb insert, one a 4.0Kb insert, four a 2.5Kb insert, three a 2.3Kb insert, three a 1.8Kb insert and four contained a 1.0Kb insert (data not shown).

The 8.5Kb fragment was not subcloned using the shotgun approach and therefore an attempt to subclone this fragment by electrophoresing Eco RI digested group B phage DNA (AL4) on a 1% agarose gel and cutting out the 8.5Kb band was made. The DNA was extracted from the agarose with phenol/chloroform (section 2.2.1). Ligations and transformations were carried out as before and the results can be seen in Table 4.3. Once again the control plates, 1-4, indicate that the JM109 cells were still competent, that the pGEM7Z had been linearized and that the T4 DNA was working. The total number of white colonies after re-streaking onto fresh indicator plates was
Table 4.2 Cloning strategy and results of shotgun subcloning of the recombinant phage group B (AL4) inserts into pGEM7Z.

<table>
<thead>
<tr>
<th>PLATE</th>
<th>DNA</th>
<th>ENZYME</th>
<th>MOLAR RATIO</th>
<th>WHITE COLONIES</th>
<th>BLUE COLONIES</th>
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<td>-</td>
<td>-</td>
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<td>Eco R1</td>
<td>1:1 (V:I)</td>
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<td>200</td>
</tr>
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<td>1:1 (V:I)</td>
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Plates 1-4 were plated at one dilution (100μl)
Plates 5-7 were plated at two dilutions (a = 100μl, b = 200μl)
* = no ligase added
Eco RI = indicates that the plasmid had been linearised before ligation.
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<tr>
<th>PLATE</th>
<th>DNA</th>
<th>ENZYME</th>
<th>MOLAR RATIO</th>
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<th>BLUE colonies</th>
</tr>
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<td>4</td>
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<td>3:1 (V:I)</td>
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<td>500</td>
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<td>7b</td>
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<td>Eco R1</td>
<td>3:1 (V:I)</td>
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</table>

Table 4.3 Cloning strategy and results of subcloning of the 8.5Kb Eco RI insert fragment from recombinant phage group B (AL4) into pGEM7Z.

Plates 1-4 were plated at one dilution (100μl)
Plates 5-7 were plated at two dilutions (a = 100μl, b = 200μl)
* = no ligase added
Eco RI = indicates that the plasmid had been linearised before ligation
reduced from 65 to 25. The cracking procedure was performed on these 25 colonies and the results indicated that a possible 7 contained inserts. DNA was prepared from the 7 colonies using the STET method (section 2.4.1) and digested with Eco RI. Four out of the seven colonies were found to contain inserts: two contained a 5.5Kb insert, one a 2.3Kb insert and one contained a 1.8Kb insert. It was obvious that the 8.5Kb fragment had not been subcloned. One further attempt was made to subclone this fragment. Again the group B phage DNA was digested with Eco RI, which was subsequently electrophoresed on a 1% agarose gel. The 8.5Kb band was excised from the gel and the DNA purified using GenecleanTM (section 2.2.6.1). Ligations and transformations were performed as before and once again no recombinant pGEM7Z clone containing an 8.5Kb insert was isolated. Sometimes, for no apparent reason, DNA fragments can be difficult to subclone. In this case the 8.5kb insert should have been digested with another enzyme and subcloned in two fragments, but this was not attempted.

4.5.1 Characterisation of Group B Subclones
A Qiagen plasmid DNA preparation (section 2.4.2.2) was performed on the subclones containing the six different sized inserts. 1μg of DNA of each subclone was digested with Eco RI along with 5μg of phage DNA and electrophoresed on a 1% agarose gel (Figure 4.7A). The 5.5Kb and 2.3Kb fragments of the original phage have been ligated successfully into pGEM7Z (lanes 8 and 4 respectively). The origin of the other four inserts is unknown. The gel in Figure 4.7A was Southern blotted and probed with radiolabelled lambda phage DNA. The only bands to show homology to the probe were the λ Hind III markers and the right and left phage arms of the group B Eco RI digested phage DNA as expected (data not shown).

The filter was then stripped of its probe (section 2.6.5) and re-probed with the rat 600bp cDNA CYP4A1 probe radiolabelled with α32PdCTP (section 2.6.4.1). The filter was washed under stringent conditions: two 20min washes in 2 x SSC / 0.1%
Figure 4.7  (A) Digestion of recombinant group B phage and plasmid DNA with the restriction enzyme *Eco* RI. (B) Southern blot of group B DNA hybridised to rat CYP4A1 600bp and full length 2.1Kb cDNA probes.

Lane 1: λ *Hind* III molecular weight markers
2: pGEMB1.0
3: pGEMB1.8
4: pGEMB2.3
5: pGEMB2.5
6: -
7: pGEMB1.8/4.0
8: pGEMB5.5
9: pGEMB2.9
10: Recombinant phage group B (AL4)

NB The hybridisation signal seen at 3.0kb, represents probe homology to the plasmid pGEM7Z (refer to section 4.4.1).
(w/v) SDS at room temperature, two 30min washes in 0.1 x SSC / 0.1% (w/v) SDS at 55°C and a final rinse in 5 x SSC at room temperature. After autoradiography the filter was washed once more to remove the radiolabelled probe, re-probed with the rat 2.1Kb full length cDNA CYP4A1 and washed under the same stringent conditions. Figure 4.7B shows the two autoradiographs obtained. It can be seen that the 8.5Kb and 2.3Kb band from the recombinant phage (lane 10) gave a positive hybridisation signal, as expected, to the 2.1Kb cDNA probe (although very weak), and that the recombinant plasmid pGEMB2.3 (lane 4) also gave a positive signal confirming that it contains the 2.3Kb phage insert.

4.5.2 Sequencing of Group B Subclones

6 µg of the recombinant plasmids pGEMB5.5 and pGEMB2.3 were denatured by alkali (section 2.7.2) and sequenced (section 2.7) using the T7 and SP6 primers. After 24hr exposure to X-ray film an autoradiograph was obtained and the sequence read. The sequences were analysed using the Microgenie™ software and a comparison was made to the published sequences for the CYP4A family.

The sequence of pGEMB2.3 was analysed by performing a nucleotide matrix comparison to the published sequences of the CYP4A family.

Where significant similarity exists between one sequence and another, a series of dots is generated, totally similar sequences will show a continuous line from the upper left corner to the lower right corner. This type of analysis (data not shown) revealed that the sequence of pGEMB2.3 aligned to CYP4A2 at positions 11,046bp to 13,222bp (Figure 4.8). This result was somewhat surprising, since the preliminary restriction map of group B (Figure 3.12) suggested that it was not the genomic sequence of CYP4A2. At the nucleotide level, CYP4A2 and CYP4A3 share 97% sequence similarity (Kimura et al., 1989a). These results indicate that the group B recombinant phage may represent the genomic sequence of CYP4A3. The sequence of pGEMB2.3 as compared to the CYP4A3 cDNA can be seen in Figure 4.9. The 5' end of this clone, which was sequenced with the T7 primer is obviously intronic sequence,
Figure 4.8 Sequence alignment of pGEMB2.3 to the genomic sequence of CYP4A2. i) pGEMB2.3 sequenced with the T7 primer, ii) pGEMB2.3 sequenced with the SP6 primer.
Figure 4.9  Sequence alignment of pGEMB2.3 to the cDNA sequence of CYP4A3.  i) pGEMB2.3 sequenced with the T7 primer, ii) pGEMB2.3 sequenced with the SP6 primer.
as little homology exists between the clone and the \textit{CYP4A3} cDNA. The 3' end of pGEMB2.3 aligns to \textit{CYP4A3} at positions 2078bp to 2140bp (Figure 4.9, ii.) and exhibits 98.4\% sequence similarity.

The recombinant plasmid pGEM5.5 was also sequenced with both the T7 and SP6 primers, however no sequence identity was observed with either \textit{CYP4A2} or \textit{CYP4A3}. This is perhaps not surprising since the results presented in Figure 3.11B and C and Figure 4.7B and C demonstrate that no homology to either the 600bp or 2.1kb probe exists. The pGEM5.5 sequence was then used to scan the GenEMBL data base using the program FASTA to look for sequences sharing identity with it. This scan revealed nothing of significance (data not shown).

4.6 SUBCLONING RESULTS: GROUP C

Ligations were carried out as detailed in section 2.4.3.3 and transformation of competent JM109 cells performed as described in section 2.4.4. Table 4.4 shows the results obtained from the first attempt at subcloning the insert fragment, using the shotgun approach. Out of the 66 white colonies obtained on the test ligation plates (plates 5-7), 57 remained white after re-streaking on fresh indicator plates and these were further screened using the cracking procedure (section 2.4.4.3). From this screen, 28 colonies were identified as possibly containing insert DNA and to confirm this, DNA was prepared by the STET method (section 2.4.1). Of the 28 DNA preparations, eleven were shown to contain a 2.0Kb insert, six a 6.7Kb insert and eleven of the DNA preparations gave one single band at 3.0Kb. Since pGEM7Z is 3.0Kb in size and an insert of approximately the same size was being looked for, these 3.0Kb clones isolated had to be investigated further. If group C represented the genomic sequence of \textit{CYP4A2}, then according to the restriction map of the published sequence, the 5' end 3.0Kb \textit{Eco RI} fragment would not have a \textit{Bgl I} site. \textit{Bgl I} cuts twice within the pGEM7Z sequence, therefore if a double digest using \textit{Eco RI} and \textit{Bgl I}
Table 4.4 Cloning strategy and results of shotgun subcloning of the recombinant phage group C (AL7) inserts into pGEM7Z.

Plates 1-4 were plated at one dilution (100μl)
Plates 5-7 were plated at two dilutions (a = 100μl, b = 200μl)
* = no ligase added
Eco RI = indicates that the plasmid had been linearised before ligation

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<th>PLATE</th>
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<th>MOLAR RATIO</th>
<th>WHITE colonies</th>
<th>BLUE colonies</th>
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<td>-</td>
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<td>-</td>
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<td>-</td>
<td>18</td>
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<td>4</td>
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<td>Eco RI</td>
<td>-</td>
<td>7</td>
<td>300</td>
</tr>
<tr>
<td>5a</td>
<td>pGEM7Z (20ng)+grp C</td>
<td>Eco RI</td>
<td>1:1 (V:I)</td>
<td>31</td>
<td>200</td>
</tr>
<tr>
<td>5b</td>
<td>pGEM7Z (20ng)+grp C</td>
<td>Eco RI</td>
<td>1:1 (V:I)</td>
<td>6</td>
<td>100</td>
</tr>
<tr>
<td>6a</td>
<td>pGEM7Z (20ng)+grp C</td>
<td>Eco RI</td>
<td>1:3 (V:I)</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>6b</td>
<td>pGEM7Z (20ng)+grp C</td>
<td>Eco RI</td>
<td>1:3 (V:I)</td>
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<td>100</td>
</tr>
<tr>
<td>7a</td>
<td>pGEM7Z (20ng)+grp C</td>
<td>Eco RI</td>
<td>3:1 (V:I)</td>
<td>8</td>
<td>150</td>
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<td>pGEM7Z (20ng)+8.5Kb</td>
<td>Eco RI</td>
<td>3:1 (V:I)</td>
<td>6</td>
<td>100</td>
</tr>
</tbody>
</table>
I was performed on one of these clones then the expected banding pattern (if it does contain a 3.0Kb insert) would be: 3.0Kb, 1.5Kb, 1.3Kb and 0.1Kb. Figure 4.10 shows the ethidium bromide stained gel of one of these clones digested with Eco RI and Bgl I (lane 3). It shows the banding pattern that was expected, indicating that this clone does indeed contain a 3.0Kb insert.

4.6.1 Characterisation of Group C Subclones

A Qiagen plasmid DNA preparation (section 2.4.2.2) was performed on the subclones containing the six different sized inserts. 1µg of DNA of each subclone was digested with Eco RI along with 5µg of phage DNA and electrophoresed on a 1% agarose gel (Figure 4.11 A). As can be seen, the three Eco RI fragments from the original phage (lane 5) have been successfully ligated into pGEM7Z (lanes 2, 3 and 4). This gel was then southern blotted (section 2.6.1) and hybridised to the rat 600bp cDNA CYP4A1 probe (section 2.6.2). The nylon filter was washed under stringent conditions: two 20min washes in 2 x SSC / 0.1% (w/v) SDS at room temperature, two 30min washes in 0.1 x SSC / 0.1% (w/v) SDS at 55°C and a final rinse in 5 x SSC at room temperature. After autoradiography, the filter was washed to remove the radiolabelled probe (section 2.6.5), re-probed with the rat full length 2.1Kb cDNA CYP4A1 and washed under similar stringent conditions. Figure 4.11 B shows the two autoradiographs obtained. The fragments sharing homology to the 600bp probe in the recombinant phage (lane 5) are the 6.7Kb and the 3.0Kb bands. The recombinant plasmids pGEMC6.7 and pGEMC3.0 (lanes 2 and 4 respectively) also show homology to the 600bp probe as expected. When probed with the 2.1Kb probe the 6.7Kb and 2.0Kb fragments lit up in the recombinant phage (lane 5) gave a positive hybridisation signal along with pGEMC6.7 and pGEMC2.0 (lanes 4 and 2 respectively). These results confirm the earlier findings and the preliminary restriction map (Figure 3.12).
Figure 4.10  Digestion of recombinant plasmid pGEMC3.0 with the restriction enzymes Eco RI and Bgl I.

Lane 1: λ Hind III molecular weight markers
2: pGEMC3.0 Eco RI
3: pGEMC3.0 Eco RI/Bgl I digested
Figure 4.11 (A) Digestion of recombinant group C phage and plasmid DNA with the restriction enzyme Eco RI. (B) Southern blot of group C DNA hybridised to the rat *CYP4A1* 600bp and full length 2.1Kb cDNA probes.

Lane 1 and 6: λ*Hind* III molecular weight markers
2: pGEMC2.0
3: pGEMC3.0
4: pGEMC6.7
5: recombinant phage group C (AL7)

NB The hybridisation signal seen at 3.0kb, represents probe homology to the plasmid pGEM7Z (refer to section 4.4.1).
4.6.2 Sequencing of Group C Subclones

In order to confirm that group C is in fact the genomic sequence of CYP4A2 as suspected, it was necessary to obtain some sequence data from the three recombinant plasmids pGEMC6.7, pGEMC3.0 and pGEMC2.0. 6μg of each recombinant plasmid was denatured by alkali (section 2.7.2) and sequenced (section 2.7) using the T7 and SP6 primers. After 24hr exposure to X-ray film an autoradiograph was obtained and the sequence read. The sequences were analysed using the Microgenie™ software and a matrix comparison to the published sequence of CYP4A2 was made (data not shown). This revealed sequence identity between the subclones and CYP4A2, and indicated the relative position of each subclone as compared to CYP4A2 (Figure 4.12).

The subclones were found to be almost identical to the published sequence of CYP4A2 (Kimura et.al., 1989), pGEMC3.0 being similar to bases -1829/+1234, pGEMC7.0 being similar to bases +1877/+8593 and pGEMC2.0 similar to bases +8593/+10,770. It was concluded that group C is in fact CYP4A2 and contains -1829bp of 5' flanking sequence which could be investigated further.

4.7 SUBCLONING RESULTS: GROUP D

Ligations were carried out as detailed in section 2.4.3.3 and transformation of competent JM109 cells performed as described in section 2.4.4. Table 4.5 shows the results obtained from the first attempt at subcloning the insert fragments using the shotgun approach. Although the competency of the cells was reduced (plates 1-4) a number of white colonies was obtained and these were screened further using the cracking procedure (section 2.4.4.3). Out of 34 colonies screened 18 appeared to contain inserts, five containing a 0.92Kb insert, two a 1.0Kb insert, three a 2.5Kb insert, two a 7.2Kb insert and two containing an 11.5Kb insert. The 4.8Kb, 3.4Kb and 1.75Kb fragments present in the original phage, were not subcloned using the shotgun approach therefore an attempt to subclone these fragments by excising the individual
i) pGEMC3.0 sequenced with the T7 primer (top), compared to CYP4A2 (bottom sequence), 95.6% similarity.

1 ..........................GAATTCTTTCAGGGATTTTTGTCATTC 27
600 AACTGTGGATGTGTTCTCTCAGGATTTTTGTCATTC 649
28 CTCTCTGTAGGCTTTCTACTATTATTGTTTTTGCCTTTTCTACTN 77
650 CTCTCTGTAGGCTTTCTACTATTATTGTTTTTGCCTTTTCTACT...CT 697
78 CGGGGGTTCTCATGCTTTCTGGAATTCTTTCAGGGATTTTTGTGATTC 649
698 ACAGGGGTTCCTCATGCTTTCTGGAATTCTTTCAGGGATTTTTGTGATTC 747
128 ATGATTGGTTAAACTAGATCTTGCTTTTCTGGTGTGTTTGGATATTCCGTG 177
748 ATGATTGTTAAACTAGATCTTGCTTTTCTGGTGTGTTTGGATATTCCGTG 797
178 TTTGCTT ............................ 184
798 TTTGCTTTTGGGAGAATGGGGCTCCAGATGCTATGCTTTCTGTT 847

ii) pGEMC3.0 sequenced with the SP6 primer (top) compared to CYP4A2 (bottom sequence), 99.3% similarity.

304 ..........................TTACACACTTAGCCACATTTAAGAATATTAA 274
3350 AGAGGCACTCAGTGGGATTACACACTTAGCCACATTTAAGAATATTAA 3399
273 ACTCAGAGCTCTTTCCCCAGTAGCTTCTCTAAGAAGCCTTAGCTTCATCT 225
3400 ACTCAGAGCTCTTTCCCCAGTAGCTTCTCTAAGAAGCCTTAGCTTCATCT 3449
224 AACAGGNNCCAGGATCTGAGCTTAGGCAAATCCGTGGAGCAGATGCAATGGG 175
3450 AACAGGNNCCAGGATCTGAGCTTAGGCAAATCCGTGGAGCAGATGCAATGGG 3499
174 CTAGATGGAGGNAC.AAAAAGACAGATCAGTCAAGGCAAATTCAGAATTTCT 126
3500 CTAGATGGAGGNAC.AAAAAGACAGATCAGTCAAGGCAAATTCAGAATTTCT 3549
125 ACCTTTTCTGAAACATTTCTGAGCAATGCTCTGTGTTTGGTCTGACATCTC 76
3550 ACCTTTTCTGAAACATTTCTGAGCAATGCTCTGTGTTTGGTCTGACATCTC 3599
75 CCAT.CCACCAAAAGCAGCTAGTAGTCAAAGAATCTTTATGGAATTTATC 27
3600 CCAT.CCACCAAAAGCAGCTAGTAGTCAAAGAATCTTTATGGAATTTATC 3649
26 TTTTCTTTGATGTAATTTCCAAAAGG ............................ 1
3650 TTTTCTTTGATGTAATTTCCAAAAGG 3699
iii) pGEMC6.7 sequenced with the T7 primer (top) compared to CYP4A2 (bottom sequence), 99% similarity.

1 ......................................GAATTCAGTGGGAGGAATGAA 21
4300 TGTTGAGCTCTGAAACCCACATTGAAATTCTAGTGGAGGAATGAA 4349

22 C7RACTCCACAGAGTGTCTCTGAAACCTCAACAGACACTGTAGCAGGT 71
4350 C7RACTCCACAGAGTGTCTCTGAAACCTCAACAGACACTGTAGCAGGT 4399

72 GCACCACAAATAATATTTTTAAAATCTGAAAGAAAAAGCT 121
4400 GCACCACAAATAATATTTTTAAAATCTGAAAGAAAAAGCT 4448

22 CTAACTCCACAGAGTTGTCTTCTGAACTCTACACAGACACTGTAGCAGGT 71
4449 TGIAATTTTGAACACCATCCTCCTATCTGGTCCTCAAGATCTGTCTTACT 4498

172 TTCAGATCCAAAGCCTTATCAATCCCTTGCTCCCTGGATTGGTAAGTAT 221
4499 TGGAGTTTTGAACACCATCCTCCTATCTGGTCCTCAAGATCTGTCTTACT 4498

172 TTTCAGATCCAAAGCCTTATCAATCCCTTGCTCCCTGGATTGGTAAGTAT 221
4549 TTTCAGATCCAAAGCCTTATCAATCCCTTGCTCCCTGGATTGGTAAGTAT 4548

222 ATTAAAATAAGGAGTGTGTTTTCTTCACTACTCTCTGGCTAATGGAGAA. 270
4549 ATTAAAATAAGGAGTGTGTTTTCTTCACTACTCTCTGGCTAATGGAGAAG 4598

iv) pGEM6.7 sequenced with the SP6 primer (top) compared to CYP4A2 (bottom sequence), 95.7% similarity.

1 ................ GTATTCCAATTCTGTGAACTTAAAGCTGTGGAT 33
10800 GTAAGCATTCTTCTGAACTTAAAGCTGTGGAT 10849

34 TAGCTNCCCACAGACAGGCTTCAGATGTGGCATTTTACTGGAAGACATAT 83
10850 TAGCTCCACAGACAGGCTTCAGATGTGGCATTTTACTGGAAGACATAT 10899

84 GACACAGTTTATCCCATAGTGAAGAATGTCATCTTAACCAGCTAAAGCA 133
10900 GACACAGTTTATCCCATAGTGAAGAATGTCATCTTAACCAGCTAAAGCA 10949

134 TACTGCAGCCATTGGAGGATAGGAGAGTCGCAGGTTGTCAAGTTCCTAGC 183
10950 TACTGCAGCCATTGGAGGATAGGAGAGTCGCAGGTTGTCAAGTTCCTAGC 10999

184 TTAAAT .................................... . . . . . . . . 189

11000 TTAAATATCCTTATCCTCTATGCATATTATTGTGTTTTAGAATGGAAT 11049
v) pGEMC2.0 sequenced with the SP6 primer (top) compared to CYP4A2 (bottom sequence), 99.8% similarity. Unfortunately, no sequence was obtained with the T7 primer.

vi) Sequencing strategy of Group C recombinant plasmid clones, and comparison to the genomic Eco RI restriction map of CYP4A2. The arrows represent the direction of each sequencing reaction.

---

Figure 4.12 i-v) Sequence alignment of Group C recombinant plasmid clones to the genomic sequence of CYP4A2. vi) diagram of the sequencing strategy and relative position of each recombinant plasmid clone as compared to CYP4A2.
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<th>BLUE colonies</th>
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<td>-</td>
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<td>1:1 (V:I)</td>
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<td>3:1 (V:I)</td>
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**Table 4.5** Cloning strategy and results of shotgun subcloning of the recombinant phage group D (AR4) inserts into pGEM7Z.

Plates 1-4 were plated at one dilution (100μl)
Plates 5-7 were plated at two dilutions (a = 100μl, b = 200μl)
* = no ligase added
Eco RI = indicates that the plasmid had been linearised before ligation
bands from a 1% agarose gel and extracting the DNA using Geneclean™ was made. Several attempts were made with no success (data not shown).

4.7.1 Characterisation of Group D Subclones

A Qiagen plasmid DNA preparation (section 2.4.2.2) was performed on the subclones containing the five different sized inserts. 1µg of DNA of each subclone was digested with Eco RI along with 5µg of phage DNA and electrophoresed on a 1% agarose gel (Figure 4.13 A). Lanes 3 and 4 indicate that the 1.0Kb and the 2.5Kb bands respectively, from the phage, have been successfully ligated into pGEM7Z. The origin of the 0.92Kb, 7.2Kb and the 11.5Kb inserts are unknown. It was thought that maybe lambda DNA had been subcloned again, therefore the gel was southern blotted and probed with radiolabelled lambda DNA. The only bands to show homology to the probe were the λHind III markers and the right and left phage arms of the group D Eco RI digested phage DNA as expected (data not shown).

The filter was then stripped of its probe (section 2.6.5) and re-probed with the rat 600bp cDNA CYP4A1 probe radiolabelled with α32PdCTP (section 2.6.4.1). The filter was washed under stringent conditions: two 20min washes in 2 x SSC / 0.1% (w/v) SDS at room temperature, two 30min washes in 0.1 x SSC / 0.1% (w/v) SDS at 55°C and a final rinse in 5 x SSC at room temperature. After autoradiography the filter was washed once more to remove the radiolabelled probe, re-probed with the rat 2.1Kb full length cDNA and washed under similar stringent conditions. Figure 4.13 B shows the two autoradiographs obtained. It can be seen that the 4.8Kb and 3.0Kb bands from the original phage (lane 7) gave a positive signal when hybridised to the 2.1Kb cDNA probe, confirming the previous result seen in Figure 3.11 C, lane 4. However, when the same southern was hybridised to the 600bp probe, no bands shared any homology. This is in agreement with Figure 3.11 B, lane 4, but still in disagreement with the result seen in Figure 3.6 B, gel 1, lane 4. It was decided to
Figure 4.13  (A) Digestion of recombinant group D phage and plasmid DNA with the restriction enzyme Eco RI.  (B) Southern blot of group D DNA hybridised to the rat CYP4A1 600bp and full length 2.1Kb cDNA probes.

Lanes 1 and 8: λ Hind III molecular weight markers
2: pGEMD0.92  5: pGEMD7.2
3: pGEMD1.0  6: pGEMD11.5
4: pGEMD2.5  7: recombinant phage group D (AR4)

NB The hybridisation signal seen at 3.0kb, represents probe homology to the plasmid pGEM7Z (refer to section 4.4.1)
sequence the 1.0Kb insert to establish if it shared any sequence similarity to the CYP4A family and also to sequence the 2.5Kb insert.

### 4.7.2 Sequencing of Group D Subclones

6μg of the recombinant plasmids pGEMD2.5 and pGEMD1.0 were denatured by alkali (section 2.7.2) and sequenced (section 2.7) using the T7 and SP6 primers. The sequences were analysed using Microgenie™ software and a comparison to the published sequences for the CYP4A family made.

The sequence obtained from recombinant plasmid pGEMD2.5 revealed no sequence similarity with the published members of the CYP4A family. This was not surprising since no homology to either the 2.1kb or 600bp probe was obtained (Figure 4.13, lane 4). This sequence was then used to scan the GenEMBL data base using the program FASTA, however this revealed nothing of significance (data not shown).

The sequence obtained from pGEMD1.0 (Figure 4.14) was found to have no similarity to the published members of the CYP4A family indicating that the last two 600bp hybridisations were the true result, ie negative. The sequence obtained using the T7 primer (Figure 4.14 i ) contained 15bp repetitive DNA sequence stretching for at least 112bp (Figure 4.14, ii ). This repetitive 15bp sequence along with the sequence for pGEMD1.0 was used to scan the GenEMBL data base using the program FASTA. This revealed no significant homology to the sequences in the data base. The significance of the repetitive element is unknown, but probably indicates the position of pGEMD1.0 to be non-coding.
i) pGEMD1.0 sequenced using the T7 primer. The sequence underlined represents the beginning of a 15bp repetitive element.

```
1   GAATTCTTCA CTTTGCTCAA GGTGTACTTA ACAGGGATGT GGTTCATATAC
51  GGTACRAGAA GCAGTCCCCC TTGCCTGCCCT CCCAGAGCCA AATCTACAGA
101 CTCCCCAGAA GATACTCTGT TCAACCCTTCC AACRATGTAC ACCTGCGGCT
151 CTTCGTGCAC GCTCTAGTGC TGGGCTCTTG CTTCTGTCTC TGTCTCTCAT
201 CACCCCATAG AGGAACCAGC ACTGGGACAG CCAGCAGACTG GACAGCCAGC
251 ACTGGGACAG CCAGCAGACTG GACAGCCAGC ACTGGGACAG CCAGCAGACTG
301 GACAGCCCAAC ACTGAGATGG CCAGCAGACTG
```

ii) Consensus sequence of 15bp repeat element.

```
5' CCAGCAGACTG GACG 3'
```

iii) pGEMD1.0 sequenced with the SP6 primer

```
1   TAGGGCCTAGC CAAACCCTCG CTCAAGTCTG AGAGAGCAGA CGGAAGGTCT
51  GTGGACAGGC CCAGTCTAA AAGAGGCTTG AGGAGCTAAA AAACAAAGGC
101 AGATTTCAAC AAAGAGAAAG TGGAGCTAAA CAAGGGAATA AAACAAAGGC
151 ATTTGTCTAG ATGGGTGCAA AGAAAAATCT CAGGTCCAAC AGTCAGCTGG
201 AAGAAGATCA GTGACGATGA GGGACTTTAT GCTAGCGGGT GTGCAACCCT
251 AGAAAACTCA AATGGAGACG TTCTGGACAA AGAAGCTGTG ACTGCTCTGA
301 CAGTCRAAXC AGTATACGAA XXTGAAAT
```

Figure 4.14  Sequence obtained from recombinant plasmid pGEMD1.0
4.8 SUBCLONING RESULTS: GROUP E

Ligations were carried out as detailed in section 2.4.3.3 and transformation of competent JM109 cells performed as described in section 2.4.4. Table 4.6 shows the results obtained from the first attempt at subcloning the insert fragments using the shotgun approach. Out of the 49 white colonies (plates 5-7), 22 still remained white after re-streaking on fresh indicator plates and these were further screened using the cracking procedure (section 2.4.4.3). From this screen, 9 colonies were identified as possibly containing insert DNA and to confirm this, DNA was prepared by the STET method (section 2.4.1), digested with the restriction enzyme Eco RI and electrophoresed on a 1% agarose gel. Out of the 9 colonies, eight were found to contain a 6.7Kb insert and one to contain a 6.2Kb insert. The expected 7.8Kb fragment from the original phage was not subcloned and further attempts to do so by extracting the band from a gel and purifying the DNA failed (data not shown).

4.8.1 Characterisation of Group E Subclones

A Qiagen plasmid DNA preparation (section 2.4.2.2) was performed on the subclones containing the two different sized inserts. 1μg of DNA of each subclone was digested with Eco RI along with 5μg of phage DNA and electrophoresed on a 1% agarose gel (Figure 4.15 A). As can be seen only the 6.7Kb fragment of the original phage (lane 4) has been successfully ligated into pGEM7Z (lane 3). The origin of the 6.2Kb insert is unknown. The DNA from the gel shown in Figure 4.15 A was transferred to Hybond N™ nylon filter by the method of Southern (section 2.6.1), hybridised to the rat 600bp cDNA CYP4A1 probe and subsequently to the rat 2.1Kb full length cDNA probe after the filter had been stripped of the first probe (section 2.6.5). The filter was washed under the same stringent conditions after each probe: two 20min washes in 2 x SSC / 0.1% (w/v) SDS at room temperature, two 30min washes in 0.1 x SSC / 0.1% (w/v) SDS at 55°C and a final rinse in 5 x SSC at room temperature. Figure 4.15 B shows the two autoradiographs obtained. The fragment sharing homology with the
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<td>280</td>
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<td>5b</td>
<td>pGEM7Z (20ng)+ grp E</td>
<td>Eco R1</td>
<td>1:1 (V:I)</td>
<td>12</td>
<td>520</td>
</tr>
<tr>
<td>6a</td>
<td>pGEM7Z (20ng)+ grp E</td>
<td>Eco R1</td>
<td>1:3 (V:I)</td>
<td>9</td>
<td>200</td>
</tr>
<tr>
<td>6b</td>
<td>pGEM7Z (20ng)+ grp E</td>
<td>Eco R1</td>
<td>1:3 (V:I)</td>
<td>14</td>
<td>600</td>
</tr>
<tr>
<td>7a</td>
<td>pGEM7Z (20ng)+ grp E</td>
<td>Eco R1</td>
<td>3:1 (V:I)</td>
<td>2</td>
<td>160</td>
</tr>
<tr>
<td>7b</td>
<td>pGEM7Z (20ng)+ grp E</td>
<td>Eco R1</td>
<td>3:1 (V:I)</td>
<td>5</td>
<td>400</td>
</tr>
</tbody>
</table>

Table 4.6 Cloning strategy and results of shotgun subcloning of the recombinant phage group E (RG4) inserts into pGEM7Z.

Plates 1-4 were plated at one dilution (100μl)
Plates 5-7 were plated at two dilutions (a = 100μl, b = 200μl)
* = no ligase added
Eco R1 = indicates that the plasmid had been linearised before ligation
Figure 4.15  (A) Digestion of recombinant group E phage and plasmid DNA with the restriction enzyme Eco RI.  (B) Southern blot of group E DNA hybridised to the rat CYP4A1 600bp and full length 2.1kb cDNA probes.

Lanes 1 and 5: λ Hind III molecular weight markers
2: pGEME6.2
3: pGEME6.7
4: Recombinant phage group E (RG4)

NB The hybridisation signal seen at 3.0kb, represents probe homology to the plasmid pGEM7Z (refer to section 4.4.1).
600bp probe in the recombinant phage (lane 4) is the 6.7Kb insert. This 6.7Kb sized insert in recombinant plasmid pGEME6.7 (lane 3) also shares homology to the 600bp probe as expected. These inserts both share homology with the 2.1Kb probe as well. The 7.8Kb insert in the recombinant phage (lane 4) does not share homology with either the 600bp or the 2.1Kb probe.

4.8.2 Sequencing of Group E Subclones

6µg of the recombinant plasmids pGEME6.7 and pGEME6.2 were sequenced as described in section 2.7. The four sequences were analysed using the Microgenie™ software. Matrix homology comparisons of these sequences with those previously published identified pGEME6.7 as being identical to CYP4A2 at position +1877/+8593 (Figure 4.16 (i)), however pGEME6.2 showed no homology to any of the CYP4A sequences or any sequences contained in the Microgenie data bank or the GenENBL data base. Figure 4.16 (i) shows the alignment of the pGEME6.7 sequence with that of CYP4A2. However, if group E was the genomic sequence of CYP4A2, then its Eco RI restriction pattern would be one of 3.0, 0.6, 6.7 and 2.2kb rather than the 7.6 and 6.7Kb seen (Figure 4.15 A, lane 4). It was thought that perhaps group E represented the genomic sequence of CYP4A3, since this cDNA shares 97% nucleotide sequence homology with CYP4A2 (Kimura et al., 1989b). The pGEME6.7 sequence was looked at more closely and it was recognised that it had been sequenced through exon 3, which is a short exon made up of 36bp. Figure 4.16 (ii) is a sequence comparison between exon 3 of CYP4A2 and CYP4A3. It can be seen there are a number of sequence differences resulting in 5 amino acid changes. Figure 4.16 (ii) shows that pGEME6.7 is 100% identical to CYP4A2 in the region of exon 3 (underlined sequence) indicating that it is CYP4A2.

In order to try to determine the origin of the 7.8Kb fragment the filter obtained from a Southern blot of the gel in Figure 4.15 A was re-probed with α32PdCTP labelled lambda DNA. The autoradiograph from this experiment indicated that the 7.8Kb
i) pGEME6.7 sequenced using the T7 primer (top), compared to CYP4A2 (bottom sequence), 100% similarity. The sequence underlined represents exon 3.

```
1 ...........GAATTCAGTGGGAGGAATGAACTAACTCCACAGAGTTGTCT 41
4320 ATTGAATTCTGAGATCTGGGAGGAATGAACTAACTCCACAGAGTTGTCT 4369
42 TCTGAACCTCTACACAGACACTGTAGCAGGTGCACCACAAATAATAAATT 91
4370 TCTGAACCTCTACACAGACACTGTAGCAGGTGCACCACAAATAATAAATT 4419
92 TAAAATCTGGAAGARAGAAAGCTGTTAGTTTTGAACACACATCCT 141
4420 TAAAATCTGGAAGARAGAAAGCTGTTAGTTTTGAACACACATCCT 4469
142 CCTATCTGGTCCTCAAGATCTGTCTTACTTTTCAGATCCAAGCTTATC 191
4470 CCTATCTGGTCCTCAAGATCTGTCTTACTTTTCAGATCCAAGCTTATC 4519
192 AATCCCTTGCTCCCTGGATTGGTAAGTATATTAAAATAAGGAGTGTGTTT 241
4520 AATCCCTTGCTCCCTGGATTGGTAAGTATATTAAAATAAGGAGTGTGTTT 4569
242 TCTTCCTACTCTCTGGCTAATGGAGAAGAACGGGTGACTGGTCACAGTA 291
4570 TCTTCCTACTCTCTGGCTAATGGAGAAGAACGGGTGACTGGTCACAGTA 4619
292 TCATGATCCATTCCTAAATCAAGCATTA ..... 319
4620 TCATGATCCATTCCTAAATCAAGCATTA 4669
```

ii) Sequence comparison of exon 3 (underlined) from CYP4A2 (top) compared to CYP4A3 (bottom sequence).

```
4501 TCAGATCCAAAGCC........TTATCAATCCCTGGCTCCCTGGATTGG 4541
337 TCAGATCCAAAGGGCTCGAAATTATCAATCCCTGGCTCCCTGGATTGG 386
```

Figure 4.16  (i) Sequence alignment of recombinant plasmid pGEME6.7 to the genomic sequence of CYP4A2.  (ii) Sequence comparison of exon 3 from CYP4A2 to CYP4A3.
insert in the recombinant phage was homologous to the probe as were the right and left phage arms and the \(\lambda\)Hind III markers. The recombinant plasmid pGEME6.2 did not show any homology to the lambda probe and its origin remains unknown (data not shown).

4.9 SUBCLONING RESULTS: GROUP F

Ligations were carried out as described in section 2.4.3.3 and transformation of competent JM109 cells performed as described in section 2.4.4. Table 4.7 shows the results obtained from the first attempt at subcloning the insert fragments using the shotgun approach. Out of the 113 white colonies (plates 5-7), 47 were further screened using the cracking procedure (section 2.4.4.3). This identified 28 colonies as possibly containing inserts and to confirm this DNA was prepared by the STET method (section 2.4.1), digested with the restriction enzyme Eco RI and electrophoresed on a 1% agarose gel. Out of the 28 DNA preparations, 25 were found to contain inserts: eleven contained a 2.1Kb insert, nine a 3.8Kb insert and five contained a 6.7Kb insert (data not shown).

4.9.1 Characterisation of Group F Subclones

A Qiagen plasmid DNA preparation (section 2.4.2.2) was performed on the subclones containing the three different sized inserts. 1µg of DNA of each subclone was digested with Eco RI along with 5µg of phage DNA and electrophoresed on a 1% agarose gel (Figure 4.17 A). As can be seen the three Eco RI fragments from the original phage DNA (lane 5) have been successfully ligated into pGEM7Z (lanes 2, 3 and 4). The DNA from this gel was transferred to a nylon filter by the method of Southern (section 2.6.1), which was hybridised to the rat 600bp cDNA CYP4A1
### Table 4.7 Cloning strategy and results of shotgun subcloning of the recombinant phage group F (RG11) inserts into pGEM7Z.

Plates 1-4 were plated at one dilution (100µl)
Plates 5-7 were plated at two dilutions (a = 100µl, b = 200µl)
* = no ligase added

_Eco RI_ = indicates that the plasmid had been linearised before ligation
Figure 4.17  (A) Digestion of recombinant group F phage and plasmid DNA with the restriction enzyme Eco RI. (B) Southern blot of group F DNA hybridised to the rat CYP4A1 600bp and full length 2.1kb cDNA probes.

Lanes 1 and 6: λ Hind III molecular weight markers
2: pGEMF2.1
3: pGEMF3.8
4: pGEMF6.7
5: Recombinant phage group F (RG11)

NB The hybridisation signal seen at 3.0kb, represents probe homology to the plasmid pGEM7Z (refer to section 4.4.1).
probe and subsequently to the rat 2.1Kb full length cDNA probe after the filter had been stripped of the first probe (section 2.6.5). The filter was washed under the same stringent conditions after each probe: two 20min washes in 2 x SSC / 0.1% (w/v) SDS at room temperature, two 30min washes in 0.1 x SSC / 0.1% (w/v) SDS at 55°C and a final rinse in 5 x SSC at room temperature. Figure 4.17 B shows the two autoradiographs obtained. The fragment in the recombinant phage (lane 5) sharing homology to the 600bp probe was the 6.7Kb insert. The recombinant plasmid pGEMF6.7 also shared homology to the 600bp probe as expected. The fragments sharing homology to the 2.1Kb probe were the 6.7Kb and the 2.1Kb inserts in the recombinant phage (lane 5) and the recombinant plasmids pGEMF6.7 (lane 4) and pGEMF2.1 (lane 2). The 3.8Kb insert in the recombinant phage and the recombinant plasmid pGEMF3.8 (lane 3) did not share any sequence identity to either probe and therefore must lie outside of the coding region either to the 5' end or the 3'end of the gene as can be seen in the preliminary restriction map (Figure 3.12).

4.9.2 Sequencing of Group F Subclones

6μg of the recombinant plasmids pGEMF6.7, pGEMF3.8 and pGEMF2.1 were sequenced as described in section 2.7. The six sequences obtained were analysed using the Microgenie™ software and matrix comparisons were performed against sequences previously published for members of the CYP4A family. The sequences for pGEMF6.7 and pGEMF2.1 were found to be homologous to CYP4A2 (Figure 4.18 i-iv) in positions +1877/+8593 and +8593/+10770 respectively and Figure 4.18 vii shows the alignment of the sequences to CYP4A2. However pGEMF3.8 showed no sequence homology to CYP4A2 or any of the other published sequences, indicating that it almost certainly lies to the 3' end of pGEMF2.1 and not as first thought to the 5' end of pGEMF6.7.
i) pGEMF6.7 sequenced using the T7 primer (top), compared to CYP4A2 (bottom sequence), 99% similarity.

1 GAATTCAAGTGGGAGGAATGACTAACTCCACAGAGTGTCTCTGAACCTC 50
4329 GAATTCAAGTGGGAGGAATGACTAACTCCACAGAGTGTCTCTGAACCTC 4378
51 TACACAGAAGCTGTCGACGGTCACCCAAATATATAATTTTTAAAATTTCT 100
4379 TACACAGAAGCTGTCGACGGTCACCCAAATATATAATTTTTAAAATTTCT 4428
101 GAAAAATGAAAGAAAAGCTTGTAGTTTTTGAACACCATCCTCCTATCTG 150
4429 GAAAAATGAAAGAAAAGCTTGTAGTTTTTGAACACCATCCTCCTATCTG 4477
151 GTCTCAAGATCTGTTTAACACAGGGAATGTGTTGTTTTAAATTTACTCT 198
4478 GTCTCAAGATCTGTTTAACACAGGGAATGTGTTGTTTTAAATTTACTCT 4527
199 GCTCCCTGGATTGGTATGATATTAAATAAGGAGTGTGTGTGTTTT 237
4528 GCTCCCTGGATTGGTATGATATTAAATAAGGAGTGTGTGTGTTTT 4570

ii) pGEMF6.7 sequenced using the SP6 primer (top), compared to CYP4A2 (bottom sequence), 100% similarity.

154 AGACATATGACACAGTTTATCCCATAGTGAAGAATGTCATCTTAACCAGC 105
10892 AGACATATGACACAGTTTATCCCATAGTGAAGAATGTCATCTTAACCAGC 10941
104 TAAAAGCATACTGCAGCCATTGGAGGATAGGAGAGTCGCAGGTTGTCAAG 55
10942 TAAAAGCATACTGCAGCCATTGGAGGATAGGAGAGTCGCAGGTTGTCAAG 10991
54 TTCTAGCTTAATATCCCTATCCTCTATGCTATATTAGTGTTTTTAGA 5
10992 TTCTAGCTTAATATCCCTATCCTCTATGCTATATTAGTGTTTTTAGA 11041
4 ATTC 1
11042 ATTC 11045

iii) pGEMF2.1 sequenced using the T7 primer (top), compared to CYP4A2 (bottom sequence, 98% similarity.

1 GAATTCAACTCTATTAATTCAATTCAGTAATGAAAAGAATTTTTAATTATGTACTTT 50
11046 GAATTCAACTCTATTAATTCAATTCAGTAATGAAAAGAATTTTTAATTATGTACTTT 11095
51 TTTTCTGCCACGTTCCTTGCTGAGACAGGAGAATGTAGCCAATCACTTAT 100
11096 TTTTCTGCCACGTTCCTTGCTGAGACAGGAGAATGTAGCCAATCACTTAT 11145
101 ACCTGATGTACGAGCGATGAGTGTATTCCTCTCTTCGAGGAGTGTACTTTT 149
11146 ACCTGATGTACGAGCGATGAGTGTATTCCTCTCTTCGAGGAGTGTACTTTT 11195
150 CACTGACA. .CTATGAAA. .GTACACACTCTCT 178
11196 CACTGACA. .CTATGAAA. .GTACACACTCTCT 11228
iv) pGEMF2.1 sequenced using the SP6 primer (top), compared to CYP4A2 (bottom sequence), 99.8% similarity.

```
iv) pGEMF2.1 sequenced using the SP6 primer (top), compared to CYP4A2 (bottom sequence), 99.8% similarity.

141 ATTAATTTCTGTTCTATATTTTTATTTATAGATACAGAAGCTTTACTCTCGCG
13081 ATTAATTTCTGTTCTATATTTTTATTTATAGATACAGAAGCTTTACTCTCGCG
91 TGAACCTGCTATGCTCCCTGACAGCCATCAGCAGAAGCAGG
13131 TGAACCTGCTATGCTCCCTGACAGCCATCAGCAGAAGCAGG
42 CTGTGGCCATCTCCTCTCCCATGTTAGAGATGGAGATGGAGAGAG
13181 CTGTGGCCATCTCCTCTCCCATGTTAGAGATGGAGATGGAGAGAG
```

ev) pGEMF3.8 sequenced using the T7 primer.

```
ev) pGEMF3.8 sequenced using the T7 primer.

1 GAATTCTGTGTAAGATATGACACCATTATTTTTCTGTTAGAGATGGAGATGGAGAGAG
51 TTAATATAGTTAGATATGTCACCATTATTCTTCTGTTAGAGATGGAGATGGAGAGAG
101 TGTATGATACTTGTATGACTTGTATGACTTGTATGACTTGTATGACTTGTATGACT
151 CTGTGGGGGGTTGTATGACTTGTATGACTTGTATGACTTGTATGACTTGTATGACT
201 TGTATGACTTGTATGACTTGTATGACTTGTATGACTTGTATGACTTGTATGACT
```

vi) pGEMF3.8 sequenced using the SP6 primer.

```
vii) Sequencing strategy of Group F recombinant plasmid clones, and comparison to the genomic Eco RI restriction map of CYP4A2. The arrows represent the direction of each sequencing reaction.

vii) Sequencing strategy of Group F recombinant plasmid clones, and comparison to the genomic Eco RI restriction map of CYP4A2. The arrows represent the direction of each sequencing reaction.
```

Figure 4.18 i-iv) Sequence alignment of group F recombinant plasmid clones to the genomic sequence of CYP4A2. v-vi) Sequence obtained from pGEMF3.8 using the T7 and SP6 primers. vii) Diagram of the sequence strategy and relative position of each recombinant plasmid clone as compared to CYP4A2 and proposed position of pGEMF3.8.
4.10 DISCUSSION

The publishing of the genomic sequences for *CYP4A1* and *CYP4A2* (Kimura et al., 1989a), enabled me to ascertain the identity of some of the clones that I had isolated. Figure 4.19 indicates the *Eco RI* restriction map of the rat *CYP4A1* and *CYP4A2* genes. Comparing these maps to the preliminary restriction maps in Figure 3.12, it can be seen that group C is the only one that appears to have a similar *Eco RI* digestion pattern. Although if group C represented *CYP4A2* then a 0.6Kb fragment was missing from the recombinant phage. This 0.6Kb fragment may have been missed on the gel (ie electrophoresed off the bottom), but repeated *Eco RI* digests of group C recombinant phage DNA showed it not to be present. The *Eco RI* restriction pattern of group F recombinant phage DNA, identified it as possibly being *CYP4A2*, an overlapping clone of group C with 3.8Kb of previously unpublished 3' flanking sequence. Sequence analysis (Figures 18) gave strong support to this suggestion.

The recombinant phage in group B produced an interesting *Eco RI* restriction pattern made more so by the fact that when pGEMB2.3 was sequenced it appeared to be part of the *CYP4A2* gene (Figure 4.8). However, the *Eco RI* restriction pattern was not that of *CYP4A2* (Figure 4.19). Since the cDNA of *CYP4A3* and the coding sequence of *CYP4A2* are 97% homologous at the nucleotide level (Kimura et al., 1989b), it was thought that group B recombinant phage may represent the genomic sequence of *CYP4A3*. However, it was impossible to tell just by comparing the sequence of subclone pGEMB2.3 with that of *CYP4A3* (Figure 4.9).

The identity of the recombinant phage in group D remains unknown. The *Eco RI* restriction pattern is completely dissimilar to that of *CYP4A1* and *CYP4A2* and the sequences obtained from pGEMD2.5 and pGEMD1.0 (Figure 4.14) share no homology with the other published *CYP4A* cDNA sequences. This is perhaps not
Figure 4.19 The restriction map of the rat CYP4A1 and CYP4A2 genes. The boxes represent exons; solid boxes are coding sequence and open boxes are noncoding sequence. E and B denote Eco RI and Bam HI sites respectively. (Taken from Kimura et al., 1989a).
surprising since pGEM2.5 and pGEM1.0 do not hybridise to either the rat 600bp or 2.1Kb probe (Figure 4.13 B) and therefore represent non-coding sequences. The recombinant phage in group D may represent a new member of the rat CYP4A family.

The recombinant phage in groups E and A both proved to contain bacteriophage lambda DNA fragments. Figure 4.16 indicated that the recombinant plasmid pGEME6.7 was homologous to CYP4A2, however, as discussed previously, it did not have the correct Eco RI restriction pattern to be CYP4A2, and therefore it was thought that it might represent the genomic sequence of CYP4A3. The sequence around exon 3 in CYP4A2 and CYP4A3 was known to contain several basepair differences. Figure 4.16 showed that the sequence for pGEME6.7 spanned exon 3 and was 100% identical to CYP4A2 in this region. The origin of the 7.8Kb fragment in the group E recombinant phage DNA was confirmed when this fragment hybridised to α¹³²PdCTP labelled bacteriophage lambda DNA.

The two Eco RI fragments in the group A recombinant phage DNA were also positive when hybridised to radiolabelled bacteriophage lambda DNA (Figure 4.6) confirming the sequencing of the subclones, recombinant plasmids pGEMA7.0 and pGEMA4.7 (Figure 4.5). How then was this clone originally isolated? In the original phage there was probably a CYP4A2 Eco RI fragment, size 6.7Kb, that when digested with Eco RI was electrophoresed to the same position as the bacteriophage lambda piece of DNA isolated in recombinant plasmid pGEMA7.0. Hence when the library was originally screened with the 2.1Kb CYP4A1 cDNA probe this clone would have been positive and therefore isolated. However when the Eco RI fragments were subcloned into pGEM only the bacteriophage lambda sequences were subcloned and not the CYP4A2 6.7Kb sequence. Figure 3.11, lane 7, would confirm this hypothesis since a positive signal is seen for both the 600bp and 2.1Kb probes and this fragment is the same size as those positive signals seen for group C (lane 5), group E (lane 3) and group F (lane 2), all containing a CYP4A2 6.7Kb sequence.
The re-packaging of bacteriophage lambda back into the λCh4A arms seems to be a common event in the fact that 5 out of 16 of the total number of recombinant phage clones isolated, (≈33%), contain lambda DNA as an insert. This is known to be a common problem of libraries made in the late 1970's, early 1980's. The method of Benton and Davis (1977) for screening phage genomic libraries is efficient and has been extensively used in many laboratories. However, a large number of clones need to be screened in order to isolate single copy genes. Clarke and Carbon, (1976), derived a formula which relates the probability of including any one DNA sequence in a random library of genomic recombinants with the number of recombinant phage as follows:

\[ N = \frac{\ln (1-P)}{\ln (1-f)} \]

Where \( N \) = number of recombinants,
\( P \) = probability of isolating the gene,
\( f \) = the fractional proportion of the genome in a single recombinant, ie fragment size (bp) / genome size (bp)

From this formula, with an average size of recombinant insert of 20Kb (such as that produced by random fragment insertion into λCh4A), 6.5 x 10^5 phage need to be screened in order to have a 99% chance of isolating a single copy gene. Thus it was gratifying that as described in Chapter 3 when approximately 2 x 10^6 phage were screened (only a factor of 3 greater than the predicted number needed) a number of recombinants which hybridised to the rat CYP4A1 cDNA were isolated, eleven of which appear to contain some CYP4A2 sequences. However, it was surprising that no CYP4A1 sequences had been isolated. Kimura et al., (1989a), isolated an 18Kb BamHI fragment containing the coding sequences for CYP4A1 by screening an unamplified partial genomic library, hybridised to the rat CYP4A1 cDNA, previous attempts at screening amplified libraries having failed. This may then be a reason why the genomic sequence for CYP4A1 was not isolated from the screening of the amplified genomic library in λCH4A.
There were a number of inserts subcloned into pGEM7Z that were not present in the original phage. The origin of these inserts remains unknown, although it is possible that due to the method used to subclone the recombinant phage inserts that bacterial DNA was subcloned instead. In order to establish which group might represent the genomic sequence for \textit{CYP4A3} as opposed to \textit{CYP4A2}, it was decided to characterise groups B, C and F further.

The sequences presented in this chapter cannot be taken as 100\% accurate since the reaction was only performed in one direction. To be absolutely certain of the sequence, both strands of the DNA must be sequenced at least twice. This was not pursued as only an indication as to the clones identity was required.
CHAPTER 5

GENE-SPECIFIC ANALYSIS OF CYP4A SEQUENCES USING OLIGONUCLEOTIDES
CHAPTER 5
GENE-SPECIFIC ANALYSIS OF CYP4A SEQUENCES USING OLGONUCLEOTIDES

5.1 INTRODUCTION

Since the cDNA of CYP4A3 shares 97% nucleotide sequence similarity to CYP4A2 (Kimura et al., 1989b) it was necessary to develop a method which would distinguish between the two groups thought to represent the genomic sequence of CYP4A2 (groups C and F) and CYP4A3 (group B). Two gene-specific oligonucleotide probes were used, made to the 3' end of the genes. The P450 4A2 and 4A3 oligonucleotides are homologous to the corresponding regions of the CYP4A2 gene (nucleotides +10,127/+10,146) and CYP4A3 cDNA (nucleotides 1691-1710), respectively, and are based on Kimura et al. (1989b).

In 1990 Issemann and Green reported cloning and characterisation of a member of the nuclear hormone receptor family that can be activated by peroxisome proliferators (PPAR). These studies showed that a chimeric receptor constructed using the DNA binding domain of the oestrogen receptor and the putative ligand binding domain of PPAR was able to activate an oestrogen-responsive gene in the presence of peroxisome proliferators. A comparison of primary amino acid sequences within the steroid hormone receptor family revealed considerable identity between the DNA recognition helix of the DNA binding domain of PPAR and the other nuclear hormone receptors that bind to the sequence TGACCT (Schwabe et al., 1990; Umensono and Evans, 1989; Smith et al., 1991). Issemann and Green (1990) therefore predicted that PPAR would recognize a similar motif and on the basis of this the oligonucleotide GTOX285 was synthesised (at ICI) and used as a probe (by myself) to identify possible sites of transcriptional regulation within the genes that I had isolated.
5.2 USE OF CYTOCHROME P450 4A2 AND 4A3 Oligonucleotides in Southern Blot Analysis

The two specific oligonucleotides are shown below. It can be seen that they differ by only four nucleotides (indicated in bold), but this is sufficient to discriminate between CYP4A2 and CYP4A3 sequences by using the appropriate washing conditions.

5'-ACTCCAGACACCTTCCCAGC-3'  4A2
5'-CCTCCAGACTCCATCCCAGT-3'  4A3

15 pmol of each oligonucleotide were labelled with 50 μCi of γ-32p ATP using 10 units of T4 polynucleotide kinase (section 2.6.4.2). The nylon filters obtained from the gels seen in Figure 4.7 A (group B), Figure 4.11 A (group C) and Figure 4.17 A (group F) were prehybridised in 40 ml of hybridisation solution (section 2.6.3) for five hours at 50°C and hybridised in 10 ml of fresh hybridisation solution overnight at the same temperature. The washing conditions used were those described by Kimura et al., namely an initial 5 min wash in 3 x SSC / 0.5% (w/v) SDS at 50°C followed by two 20 min washes in 3 x SSC / 0.5% (w/v) SDS at 55°C. The filters were autoradiographed for 2 and 18 hours at -70°C (section 2.2.5) and then stripped of the first probe as detailed in section 2.6.5.

Figure 5.1 shows the autoradiographs obtained at 2 and 18 hours after hybridisation to the 4A2 specific oligonucleotide. It can clearly be seen that the 2.1 Kb fragment in the recombinant plasmid pGEMC2.0 (lane 2) and pGEMF2.1 (lane 2) and the corresponding fragment in their respective phage DNA (lane 5) hybridised to the 4A2 oligonucleotide as expected. The recombinant plasmid pGEMB2.3 in lane 4 showed no hybridisation signal, even after an 18 hour exposure.
Figure 5.1  Autoradiograph of Southern blots obtained from recombinant DNA in group B, group C and group F (gels seen in Figure 4.7 A, 4.11 A and 4.17 A respectively. Details for each gel can be found in the appropriate Figure legend), hybridised to radiolabelled CYP4A2 oligonucleotide.
Figure 5.2 shows the autoradiographs obtained after hybridising to the 4A3 specific oligonucleotide. After a 2hr exposure the group B film shows a band at 2.1Kb in lane 4 representing the recombinant plasmid pGEMB2.3. The corresponding band in the group B phage DNA can be seen in lane 10, although it is very faint. Both bands are more clearly visualised after an 18hr exposure. The group C and group F recombinant plasmids pGEMC2.0 and pGEMF2.1, as for the 4A2 oligonucleotide, hybridised to the 4A3 oligonucleotide (lane 2 respectively). This result was unexpected since I was certain group C was in fact CYP4A2 and therefore implied the hybridisation and washing conditions were not correct to achieve total specificity for each oligonucleotide probe. However, the fact that pGEMB2.3 only showed homology to the 4A3 oligonucleotide and not the 4A2 oligonucleotide under the same conditions, was a good indication that this was in fact CYP4A3. It was decided to use the 4A2 and 4A3 oligonucleotides as specific primers in a sequencing reaction using pGEMB2.3, pGEMC2.0 and pGEMF2.1 as templates to confirm these findings.

### 5.3 USE OF CYTOCHROME P450 4A2 AND 4A3 OLIGONUCLEOTIDES AS SPECIFIC PRIMERS FOR SEQUENCING

6µg of the recombinant plasmids pGEMB2.3, pGEMC2.0 and pGEMF2.1 were denatured by alkali (section 2.7.2) and sequenced, using the P450 4A2 and 4A3 oligonucleotides, according to section 2.7, except that the amount of primer used for each reaction was 1pmol. pGEMB2.3 only sequenced with the P450 4A3 oligonucleotide and this sequence aligned to the CYP4A3 cDNA in positions 1761/1889 (Figure 5.3 (i)). This sequence was also compared to CYP4A2 (Figure 5.3 (ii)) which demonstrated 5bp differences that had not been seen in Figure 5.3 (i). The plasmid pGEMB2.3 had been sequenced twice with the P450 4A3 oligonucleotide, indicating that these 5bp differences were not due to sequencing error.
Figure 5.2 Autoradiograph of Southern blots obtained from recombinant DNA in group B, group C and group F (gels seen in Figure 4.7 A, 4.11 A and 4.17 A respectively. Details for each gel can be found in the appropriate Figure legend), hybridised to radiolabelled CYP4A3 oligonucleotide.
i) pGEMB2.3 sequenced (nucleotides 1 to 140) using the P450 4A3 specific oligo (underlined sequence) aligned to the CYP4A3 sequence

1791  CCTCCAGACTCCATCCAGTTTGGTGCTGGGACCCATCCATCTGTTT 1739
12  ...ACTTGCCGCCAGGCTGTCTCCAGCC 40
1740  TCACCTCTGAGCCGTCAGCTGTCCGCCAGCC 1789
41  TGCTTACCCTCTATTATCTTTGACTAAAGAAGATGTAATTTTAGAAG 90
1790  TGCTTACCCTCTATTATCTTTGACTAAAGAAGATGTAATTTTAGAAG 1839
91  GGAAACTCTTGCACTTTATCTCTCTTCAGTTCTCAGAGTCTTGGGACAAT 140
1840  GGAAACTCTTGCACTTTATCTCTCTTCAGTTCTCAGAGTCTTGGGACAAT 1889

ii) pGEMB2.3 sequenced (nucleotides 1 to 140) using the P450 4A3 specific oligonucleotide (the position of the P450 4A2 specific oligonucleotide is underlined), aligned to the CYP4A2 sequence. The differences between the two sequences are highlighted in bold.

ACTCCAGACACTCCCATCCAGTTTGGTGCTGGGACCCATCCATCTGTTT 12650
12651  TTGCTGCGTGGGACCCATTTGCATCTGGTTTTCAGCTGACCCCTAGCC 12700
12  ACTTGCCGCCAGGCTGTCTCCAGCC 61
12701  ACCCTGCGCAGCCAGCAGGTCCCTCTGACCCCTATCATCATCATT 12750
62  GGACTAAACGAGAAGATGTAATTTTAGAAGGGAACCTCTTGACTTTATCT 111
12751  GGACTAAACGAGAAGATGTAATTTTAGAAGGGAACCTCTTGACTTTATCT 12800
112  CTCTTCAGTTCTGCAGTCTTTGGGACAAT 140
12801  CTCTTCAGTTCTGCAGTCTTTGGGACAAT 12829

Figure 5.3 Sequence obtained from group B recombinant plasmid pGEMB2.3 using a P450 4A3 specific oligonucleotide, compared to i) CYP4A3 and ii) CYP4A2.
The sequences obtained for pGEMC2.0 and pGEMF2.1 with the 4A2 oligonucleotide were 100% identical to CYP4A2 (data not shown). These plasmids only sequenced with the 4A2 oligonucleotide and taken together with the data presented in Chapter 4, indicate that these are overlapping genomic clones of CYP4A2.

### 5.4 USE OF A PPRE-SPECIFIC OLIGONUCLEOTIDE IN SOUTHERN BLOT ANALYSIS

In an attempt to elucidate whether the genomic DNA inserts from any of the recombinant groups contained a binding sequence for the recently discovered PPAR, an oligonucleotide (GTOX285) was synthesised (section 2.7.5) which can be seen below. The letters in bold represent the direct repeat of a hexanucleotide sequence thought to represent the PPAR DNA binding site (known as the PPRE, the peroxisome proliferator response element).

5'-AGCTTGACCTcTGACCTG-3' GTOX285

The first approach used was one of spotting 10μl of phage stock (section 2.5.1) from each group onto a freshly prepared growing lawn of LE392 cells on an LB agarose plate. The plate was left to harden for 1hr at room temperature and then incubated at 37°C overnight to allow the phage to lyse the bacterial cells. Filter lifts were performed as described in section 2.3.4 in duplicate and then prehybridised at 42°C for 8hrs. 15pmol of GTOX285 was labelled with 50μCi of γ-32P ATP using 10 units of T4 polynucleotide kinase (section 2.6.4.2) and added to 10ml of hybridisation buffer (section 2.6.3). Hybridisation was performed at 67°C for the first 40min and then at 28°C overnight. The filters were washed at low stringency for 2 x 20min in 6 X SSC / 0.1% (w/v) SDS at room temperature and then autoradiographed for 2hr at -70°C.
Figure 5.4 shows the autoradiograph obtained. The area of lysis from the bacteriophage of each group are outlined and each group clearly marked. Group A was present as a negative control, since this was known to contain a lambda DNA insert and therefore should not have hybridised to the probe GTOX285. As a positive control, phage containing some 20Kb of 5' CYP4A1 sequence with a putative PPAR response element known to be positive to the GTOX285 probe, was used (T. Aldrich, ICI, unpublished). As can be seen in Figure 5.4 no clear hybridisation signal was obtained from any of the groups and more importantly none could be seen for the positive control. A second attempt was made using the Southern blots prepared previously for each group (Chapter 4), and as a positive control a Southern blot, of the 5' end of CYP4A1 DNA, described above was used. The filters were hybridised to GTOX285 under the same conditions as above and autoradiographed for 24hr at -70°C. The positive control showed a single band, although it was very weak in intensity. No bands could be seen on any of the other filters (data not shown).

5.5 DISCUSSION

Kimura et al., (1989b), investigated the regulation of CYP4A1, CYP4A2 and CYP4A3 by the use of gene specific oligonucleotide probes. Using the same gene specific oligonucleotides for CYP4A2 and CYP4A3, under the same hybridisation conditions, I was able to identify the recombinant phage in group B as probably the genomic clone representing CYP4A3 (Figure 5.2, group B recombinant phage and plasmid DNA, lanes 4 and 10 respectively). However this same CYP4A3 oligonucleotide probe also hybridised to the 2.1Kb fragment in group C and group F (recombinant phage and plasmid, lanes 2 and 5 respectively), which indicated that the conditions of hybridisation used were not totally specific. Taken together with the fact that the CYP4A2 oligonucleotide did not hybridise to the recombinant plasmid pGEMB2.3 and the corresponding size fragment in the group B recombinant phage (Figure 5.1, lanes 4 and 10, respectively), indicated that group B probably
**Figure 5.4** Autoradiograph of filter from recombinant phage spot plate, hybridised to radiolabelled oligonucleotide GTOX285.

- Group A (AL2)
- Group B (AL4)
- Group C (AL7)
- Group D (AR4)
- Group E (RG4)
- Group F (RG11)
represented part of the genomic sequence of CYP4A3. This was confirmed by using the 4A3 specific oligonucleotide as a sequencing primer as only the recombinant plasmid pGEMB2.3 produced any sequence. Figure 5.3 shows the sequence compared to that of CYP4A2 and CYP4A3. It suggests that the group B recombinant phage represents the genomic sequence of CYP4A3.

The use of the putative PPAR binding site as an oligonucleotide probe, proved to be inconclusive. It appeared from the results obtained that no such site was present in the recombinant phage clones that I had isolated, however the probe seemed to bind non-specifically to high molecular weight DNA (data not shown). Further to the work of Issemann and Green (1990), Tugwood et al. (1992), identified a PPAR response element (PPRE) in the 5' flanking region of the rat acyl CoA oxidase gene. This response element, located 570bp upstream of the acyl CoA oxidase gene, contains a direct repeat of the sequence motifs TGACCT and TGTCCT and binds PPAR. More recently, Muerhoff et al. (1992a) identified a PPRE in the 5' flanking region of the rabbit CYP4A6 gene. A comparison of these two response elements to the GTOX285 oligonucleotide can be seen below:

5' - ACGTGACCTTGT CCTGG - 3'  ACO PPRE (-572/-555bp)
5' - CCCC TGACCTTGT CCTAC - 3'  CYP4A6 PPRE (-724/-741bp)
5' - AGGCTTGACCTcTGACCT - 3'  GTOX285

The number of base pair differences from the actual PPRE's to the putative GTOX285 oligonucleotide PPRE used and the actual hybridisation conditions may account for the lack of a hybridisation signal. Alternatively no PPRE site exists in the CYP4A genomic DNA sequences that I have isolated.
CHAPTER 6

CYP4A PROMOTER ASSAYS USING AN IN VITRO DNA TRANSFECTION METHOD
CHAPTER 6
CYP4A PROMOTER ASSAYS USING AN IN VITRO DNA TRANSFECTION METHOD

6.1 INTRODUCTION

Since expression of the rat CYP4A2 gene had not been examined in relation to PPAR regulation, my CYP4A clones were used to investigate this aspect. Despite the fact that the 2Kb of upstream sequence isolated in the group C recombinant plasmid had already been sequenced (Kimura et al., 1989a) and found not to contain a recognisable PPRE sequence, it was decided that this would provide a good starting point in a transfection system.

In order to study the effects of putative promoters or enhancers in vitro, these sequences must first be linked to a reporter gene and transfected into eukaryotic cells. Such reporter gene systems are able to provide quantitation of transcription in whole cells in response to transcriptional activators. The reporter gene system used in this chapter is the CAT (chloramphenicol acetyltransferase) gene. Since CAT is a bacterial gene, levels of CAT enzyme can be easily assayed with little or no background from endogenous cellular gene activity.

The transfection level measured for a reporter gene is highly dependent on the efficiency of transfection obtained with the target cell and therefore it is necessary to include an internal control in each transfection reaction so that results can be compared from experiment to experiment. The plasmid pCH110 (Stratagene) is designed for this purpose. It contains a functional lacZ gene which is expressed from the SV40 early promoter producing β-galactosidase which can then be easily assayed.

A number of methods have been devised to introduce foreign DNA into eukaryotic
cells, the most commonly used being DNA/calcium phosphate co-precipitation or methods based on the use of high molecular weight polycations such as DEAE-dextran. Although extensively used for cell-line transfection, these methods suffer from high variability of efficiency or of cell survival and need to be adapted to each cell line. The transfection method used in this work is based on compacted cationic lipid-coated plasmid DNA (Felgner et al., 1987). The reagent used, \( N-[1-(2,3-diyoleyloxy)propyl]-N,N,N-trimethylammonium chloride \) (DOTMA), forms positively charged liposomes which complex with the nucleic acids. The lipid-nucleic acid complex, when applied to cultured cells, fuses with plasma membranes and transfers the nucleic acid into the cells. This technique is highly efficient and also lacks any detectable toxic side effects at the concentration used.

### 6.2 EVALUATION OF THE TRANSFECTION SYSTEM USING STRUCTUALLY DIFFERENT PEROXISOME PROLIFERATORS

In order to investigate the promoter/enhancer activity of the 5' flanking sequences isolated it was necessary to set up the cell culture facilities in the laboratory and to recreate the transfection system used by Tugwood et al. (1992). To this end the mouse Hepal cells, carrier plasmid pBluescript, expression vector pSG5, the PPAR expression vector pSG5.PPAR, the \( \beta \)-galactosidase expression plasmid pCH110 and the reporter vectors pG.CAT and pACO(-1273/-471)G.CAT were all kindly donated by S. Green, ICI.

Qiagen plasmid DNA preparations (section 2.4.2.2) were performed on the above plasmids and diluted to a concentration of \( 1 \mu\text{g}/\mu\text{l} \). The reporter plasmid pACO(-1273/-471)G.CAT was transfected into the mouse hepatoma cell line Hepal (section 2.10) in the presence or absence of the PPAR expression vector (pSG5.PPAR) that contained the complete coding sequence of the mouse receptor, and in the presence
or absence of the potent peroxisome proliferator Wy 14643 and the structurally dissimilar peroxisome proliferator perfluorodecanoic acid (PFDA), as indicated in Table 6.1. The cells were harvested from each plate and cytosol extracted by three rounds of freeze thaw cycles. Extracts were normalized for β-galactosidase activity as described in section 2.10.1 and a CAT assay performed as described in section 2.10.2.

The autoradiograph of the TLC plate can be seen in Figure 6.1 and the results from the TLC scanner in Table 6.1. Figure 6.1 shows the parent 14C chloramphenicol at the bottom of the autoradiograph and the two acetylated products, that have migrated at slightly different rates, further up the autoradiograph. It can be seen that there is an increase in intensity of the top metabolite as there is an increase in Wy 14643 (Figure 6.1 A) concentration and this is reflected in the graph of Figure 6.2. The graph indicates that the maximal induction of the CAT enzyme is seen at a Wy 14643 concentration of 10⁻⁵M and that this response plateaus at 10⁻⁴M. In fact concentrations of Wy 14643 greater than 10⁻⁴M were found to be toxic, as indicated by cell detachment. Figure 6.2 also indicates that PFDA does not activate PPAR to increase the amount of CAT enzyme produced. When PPAR was not co-transfected with pACO(-1273/-471)G.CAT in the absence of inducer (Wy 14643), the percent of acetylated chloramphenicol was 4.6. This is probably due to the rabbit β-globin promoter driving some CAT production. When PPAR was co-transfected with the reporter plasmid still in the absence of inducer, the percent of acetylated chloramphenicol was 21.5. This value was thought to be extremely high, since the percent of acetylated chloramphenicol in the presence of PPAR and Wy 14643 at 10⁻⁵M was 45 ± 1.2, representing only a 2 fold induction. A lower value for the absence of inducer along with a 4 fold induction was expected (Tugwood et al., 1992).

It was thought that perhaps because the Wy 14643 solutions were made in plastic
Table 6.1 Transfection strategy and results of CAT assay. Wy indicates peroxisome proliferator Wy 14643, PF indicates peroxisome proliferator PFDA, AcCh indicates acetylated $^{14}$C chloramphenicol and SEM indicates standard error of the mean.
Figure 6.1 Stimulation of pACO(-1273/-471)G.CAT reporter gene by increasing concentrations of: A) the peroxisome proliferator Wy 14643 (Wy) and B) by the structurally dissimilar peroxisome proliferator, PFDA (PF), in the absence (-R) or presence (+R) of the PPAR receptor. The position of 14C-chloramphenicol (Chl) and acetylated 14C-chloramphenicol (1/3 Ac) after thin layer chromatography is indicated. The concentration of Wy 14643 (I) is indicated at the bottom of each figure.
Figure 6.2 Dose response of pACO(-1273/-471)G.CAT stimulation using the peroxisome proliferators Wy 14643 (Wy) and PFDA (PF).
eppendorfs that the DMSO used to dissolve the inducer may have caused the leaching of some phthalate esters from the tubes giving rise to an increase in activation of the PPAR. To test this theory, an experiment was set up using just DMSO, in plastic or glass, to activate the PPAR (Table 6.2). The results indicate that there is no significant difference in CAT activity when using DMSO (ACS or Analar grade) stored in either glass or plastic. In fact it can be said that the DMSO is not affecting CAT activity since CAT activity was within the same range when sterile Milli Q water was used instead. The value of CAT activity in the absence of PPAR receptor (3.6% ± 0.3) was similar to that observed before (Table 6.1, 4.6% ± 0.7). The values obtained in the presence of PPAR (6.2 to 8.3% acetylated chloramphenicol) were at least half the value of that observed before (Table 6.1, 21.5%), however, this result was obtained from two plates only. Taken together these results indicate that there is slight variation between experiments, despite the fact that each experiment has an internal control (the pCH110 vector).

The transfection of pACO(-1273/-471) with or without PPAR in the presence of increasing concentrations of Wy 14643 was repeated again, with controls, all performed in triplicate, as described above. The mean values of % acetylated chloramphenicol are given in Table 6.3. The co-transfection of pACO(-1273/-471)G.CAT with PPAR in the absence of Wy 14643 gave a percent of acetylated chloramphenicol of 12.12 ± 1.67 which is very similar to the values seen in Table 6.2 for the same experiment. Once again, an increase in CAT activity is seen with an increase in Wy 14643 concentration.
Table 6.2 Transfection strategy for looking at the effect of two different DMSO's and results of the CAT assay performed. ACS indicates ACS grade from Sigma, Analar indicates analar grade from Fisons, stored in either glass or plastic. AcCh = acetylated $^{14}$C chloramphenicol and SEM indicates standard error of the mean.

<table>
<thead>
<tr>
<th>PLATE</th>
<th>DMSO</th>
<th>MEAN % AcCh ±SEM (‰ total $^{14}$C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pBluescript</td>
<td>pSGS</td>
</tr>
<tr>
<td>1</td>
<td>ACS, Glass</td>
<td>10</td>
</tr>
<tr>
<td>2/3/4</td>
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<td>ACS, Glass</td>
<td>5</td>
</tr>
<tr>
<td>8/9/10</td>
<td>Analar, Glass</td>
<td>5</td>
</tr>
<tr>
<td>11/12/13</td>
<td>Analar, Plastic</td>
<td>5</td>
</tr>
<tr>
<td>14/15/16</td>
<td>H2O</td>
<td>5</td>
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</table>
### Table 6.3 Transfection strategy and results of CAT assay

<table>
<thead>
<tr>
<th>PLATE</th>
<th>LIGAND</th>
<th>PLASMIDS (µg)</th>
<th>MEAN % AcCh ±SEM ( % total 14C)</th>
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<tr>
<td></td>
<td></td>
<td>pBlueScript</td>
<td>pSGS</td>
</tr>
<tr>
<td>1/2</td>
<td>DMSO</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>3/4/5</td>
<td>DMSO</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>6/7/8</td>
<td>DMSO</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>9/10/11</td>
<td>WY 10⁻⁸M</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>12/13/14</td>
<td>WY 10⁻⁷M</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>15/16/17</td>
<td>WY 10⁻⁶M</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>18/19/20</td>
<td>WY 10⁻⁵M</td>
<td>5</td>
<td>-</td>
</tr>
</tbody>
</table>

Wy indicates peroxisome proliferator Wy 14643, AcCh indicates acetylated 14C chloraphenicol and SEM indicates standard error of the mean.
6.3 SUBCLONING OF RAT CYP4A2 5' FLANKING SEQUENCE

6.3.1 PCR Amplification of 5' CYP4A2 Sequence

Oligonucleotides were synthesised (section 2.7.5) based on the published sequence of the rat CYP4A2 gene promoter (Kimura et al., 1989a) and used to amplify a region corresponding to positions -1871 to +20 (containing the gene start site), and positions -1871 to -100 (gene start site absent), from the recombinant plasmid pGEMC3.0 using the polymerase chain reaction (PCR). The three oligonucleotides used and their position within the 5' flanking sequence of CYP4A2 can be seen in Figure 6.3. To aid the subcloning of the PCR fragments into the expression vector pG.CAT, a Sph I enzyme restriction site was incorporated into the middle of each oligonucleotide (indicated in bold letters in Figure 6.3). The annealing temperature in the thermal cycle and the number of cycles were modified in order to reduce non-specific amplification and to produce the best yield of the specific DNA product required (data not shown). PCR was performed for both primer sets as described in section 2.8 and electrophoresed on a 1% agarose gel. Figure 6.4 shows the ethidium bromide stained gel. Lanes 1 and 2 represent the negative control for each primer set and the lack of bands in these two lanes indicate that no contaminating DNA was present since none had been amplified. It can be seen that there is more than one band present in each lane indicating that the 5' primer is not specific. The 1.9Kb bands (lanes 3-5) representing DNA amplified from primers S1/S2 and the 1.8Kb bands (lanes 6-9) representing DNA amplified from primers S1/S3 were excised from the gel and purified by the Geneclean method (section 2.2.6.1).
Figure 6.3 PCR oligonucleotides S1, S2 and S3. Their position in relation to the 5' flanking sequence of CYP4A2 is indicated and the Sph I restriction endonuclease site incorporated into the oligonucleotides can be seen in bold.

S1 5' GAATTCTTTCAGGGAGCATGCTTTTTGTGATTCCTC 3'
S2 5' ACCCATGGTTAATGGGCATGCCTTCTGGATCACTAG 3'
S3 5' AGTGACCTCAGACTCGCATGCCAAAGTTTATTACTC 3'

Figure 6.3 PCR oligonucleotides S1, S2 and S3. Their position in relation to the 5' flanking sequence of CYP4A2 is indicated and the Sph I restriction endonuclease site incorporated into the oligonucleotides can be seen in bold.
Figure 6.4 Amplification of 5' flanking sequence of the rat CYP4A2.

Lane 1: no template DNA + primer set S1/S2
2: no template DNA + primer set S1/S3
3: pGEMC3.0 (template) + primer set S1/S2
4: pGEMC3.0 (template) + primer set S1/S2
5: pGEMC3.0 (template) + primer set S1/S2
6: pGEMC3.0 (template) + primer set S1/S3
7: pGEMC3.0 (template) + primer set S1/S3
8: pGEMC3.0 (template) + primer set S1/S3
9: pGEMC3.0 (template) + primer set S1/S3
10: λ Hind III molecular weight markers
### 6.3.2 Ligation of Amplified 5' CYP4A2 Sequences into the Expression Vector pG.CAT

5μg of Qiagen prepared pG.CAT DNA was digested with the restriction enzyme *Sph* I (12 units) at 37°C overnight and then dephosphorylated with calf intestinal alkaline phosphatase as described in section 2.2.7.2, and then purified by phenol/chloroform extraction (section 2.2.1). The DNA was recovered by ethanol precipitation (section 2.2.2, method B) and dissolved in water to a final concentration of approximately 100ng/μl.

The purified PCR products were digested with the restriction enzyme *Sph* I (12 units) at 37°C overnight and then purified by phenol/chloroform extraction (section 2.2.1). The DNA was recovered by ethanol precipitation (section 2.2.2, method A) and dissolved in water to a final concentration of approximately 50ng/μl.

Ligations were carried out as detailed in section 2.4.3.3 and transformation of competent TB1 cells performed as described in section 2.4.4. The expression vector pG.CAT does not contain the lac α-peptide and hence recombinant DNA-plasmid colonies cannot be selected by blue/white colour screening. Instead they are selected for by their ability to resist the antibiotic ampicillin, the colonies appearing white on the selective plate. However, since antibiotic resistance is conferred to the bacterial host cell by the plasmid and not just by the recombinant plasmid, a number of false positives are undoubtedly formed. Table 6.4 shows the results obtained from the subcloning of the PCR products into the expression vector pG.CAT. It can be seen that the TB1 cells are extremely confluent (plate 2) in that the whole plate was covered with white colonies. The number of white colonies was reduced when the plasmid had been digested with the restriction endonuclease *Sph* I (plate 3), however due to the presence of the plasmids compatible ends this number of colonies was still quite large (the white colonies representing self religations as no T4 DNA ligase was
<table>
<thead>
<tr>
<th>PLATE</th>
<th>DNA</th>
<th>ENZYME</th>
<th>CIAP</th>
<th>MOLAR RATIO</th>
<th>WHITE colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>*No DNA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>*pG.CAT (10ng)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>*pG.CAT (20ng)</td>
<td>Sph I</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>*pG.CAT (20ng)</td>
<td>Sph I</td>
<td>CIAP</td>
<td>-</td>
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<tr>
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<td>pG.CAT (20ng)</td>
<td>Sph I</td>
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<td>1:1 (V:I)</td>
<td>3200</td>
</tr>
<tr>
<td>6</td>
<td>pG.CAT (20ng)</td>
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<td>CIAP</td>
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<tr>
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<td>Sph I</td>
<td>CIAP</td>
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<td>CIAP</td>
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<td>CIAP</td>
<td>1:3 (V:I)</td>
<td>52</td>
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<tr>
<td>12</td>
<td>pG.CAT (20ng) + S1/S3</td>
<td>Sph I</td>
<td>CIAP</td>
<td>1:6 (V:I)</td>
<td>49</td>
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</tbody>
</table>

Table 6.4 Cloning strategy and results of subcloning of the PCR products from the two primer sets, S1/S2 and S1/S3 into the expression vector pG.CAT.

* indicates no ligase included
Sph I indicates the plasmid had been linearised before ligation
CIAP indicates the plasmid had been dephosphorylated with calf intestinal alkaline phosphatase before ligation
ND indicates that there were too many colonies to count
These self religations were further reduced by dephosphorylating the plasmid (plate 4) preventing the plasmid from religating. The T4 DNA ligase was seen to be working since the number of whites doubled on plates 5 and 6 as compared to plates 3 and 4 respectively. The greatest number of colonies for both primer sets appeared to be at the 1:1 vector:insert molar ratio (plates 7 and 10). 64 colonies were picked at random from plates 7, 8 and 9 and another 64 from plates 10, 11 and 12 and screened further using the cracking procedure (section 2.4.4.3). This screen identified 11 colonies as possibly containing insert DNA and these were further investigated by preparing DNA by the STET method (section 2.4.1). The DNA from these 11 colonies were digested with the restriction enzyme Sph I and electrophoresed on a 1% agarose gel. Only a linearised plasmid of 5.5Kb size was produced, characteristic of pG.CAT without an insert (data not shown). Further attempts to subclone the two PCR fragments using differing molar ratios of 0.5:1.0 and 1:2 (vector:insert) also failed (data not shown).

6.3.3 Ligation of pGEMC3.0 Insert DNA into Expression Vector pG.CAT

A second approach taken was to try to subclone the 5' flanking sequence of CYP4A2 by taking the entire 3.0Kb of insert DNA from recombinant plasmid pGEMC3.0 and subcloning it into the expression vector pG.CAT. The vector was treated in the following way. 5μg of Qiagen prepared pG.CAT (section 2.4.2.2) was digested with the restriction endonuclease Xba I (10 units) at 37°C for four hours after which time the 3' recessed ends created, were filled using the Klenow fragment of DNA polymerase as described in section 2.2.7.3. The DNA was then purified by phenol/chloroform extraction (section 2.2.1) followed by ethanol precipitation (section 2.2.2, method A) and resuspended in water to a final concentration of 40ng/μl.
5µg of the recombinant plasmid pGEMC3.0 was digested with the restriction enzymes Eco RI and Bgl I as described in section 4.6 and electrophoresed on a 1% agarose gel. The 3.0Kb band was excised from the gel and the DNA purified using Geneclean (section 2.2.6.1). The 3' recessed ends created from the Eco RI digestion were filled using the Klenow fragment of DNA polymerase (section 2.2.7.3) and then treated with T4 polynucleotide kinase (section 2.2.7.4) to phosphorylate the DNA. The DNA was then purified by phenol/chloroform extraction (section 2.2.1) followed by ethanol precipitation (section 2.2.2, method A) and resuspended in water to a final concentration of 40ng/µl.

Blunt end ligations were carried out as detailed in section 2.4.3.3 and transformation of competent TB1 cells performed as described in section 2.4.4. Table 6.5 shows the results obtained. Digesting the plasmid DNA with Xba I and treating with Klenow (plate 3) produced only a few white colonies, 14, representing self ligation. When compared to plate 3 in table 6.3 (1360 whites) it could be seen that the filling of the 3' recess had been successful. Plate 4 (Table 6.5) indicated that blunt end religation of the plasmid had taken place and hence that the T4 DNA ligase was active. The greatest number of colonies was produced on plate 5 by a vector to insert molar ratio of 1:0.5. One hundred of these colonies were picked at random and screened further using the cracking procedure (section 2.4.4.3), however none were found to contain insert DNA (data not shown). Unfortunately, time restraints prevented furtherance of this work.

6.4 PROMOTER ACTIVITY OF HUMAN CYP4A 5' FLANKING SEQUENCES

In parallel to my work using the rat genomic library other members of the laboratory, (S. Hood and C. McGeogh), screened a human genomic DNA library with the same CYP4A1 probe and isolated positive hybridising recombinant phage clones. One
Table 6.5 Cloning strategy and results of subcloning of the 3.0Kb insert from the vector pGEMC3.0 into the expression vector pG.CAT.

* indicates no ligase included

Xba I indicates the plasmid has been linearised before ligation

Klenow indicates the 3' overhangs of the plasmid has been filled before ligation

ND indicates that there were too many colonies to count

<table>
<thead>
<tr>
<th>PLATE</th>
<th>DNA</th>
<th>ENZYME</th>
<th>KLENOW</th>
<th>MOLAR RATIO</th>
<th>WHITE colonies</th>
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<td>*No DNA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>*pG.CAT (10ng)</td>
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<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>*pG.CAT (20ng)</td>
<td>Xba I</td>
<td>K</td>
<td>-</td>
<td>14</td>
</tr>
<tr>
<td>4</td>
<td>pG.CAT (20ng)</td>
<td>Xba I</td>
<td>K</td>
<td>-</td>
<td>1000</td>
</tr>
<tr>
<td>5</td>
<td>pG.CAT (20ng) + 3.0kb</td>
<td>Xba I</td>
<td>K</td>
<td>1:0.5 (V:I)</td>
<td>1600</td>
</tr>
<tr>
<td>6</td>
<td>pG.CAT (20ng) + 3.0kb</td>
<td>Xba I</td>
<td>K</td>
<td>1:1 (V:I)</td>
<td>1000</td>
</tr>
<tr>
<td>7</td>
<td>pG.CAT (20ng) + 3.0kb</td>
<td>Xba I</td>
<td>K</td>
<td>1:3 (V:I)</td>
<td>1000</td>
</tr>
</tbody>
</table>
clone, when characterised, was found to contain the 3' end of a human CYP4A gene. This DNA fragment was used to screen a second human genomic DNA library, from which two positive hybridising recombinant phage clones were isolated. The two clones were overlapping and the larger of the two (designated λB1) was found to contain a complete human CYP4A gene and 9.0Kb of 5' flanking sequence. The restriction map of λB1 can be seen in Figure 6.5. The DNA sequence of λB1 shows extensive homology with the recently described CYP4AII cDNA (Palmer et al., 1993). The Xba I and Xba I/Xho I fragment, indicated in Figure 6.5, were subcloned into the plasmid pGEM7Z and characterised by DNA sequencing. In order to investigate promoter activity in response to PPAR I subcloned the 5' flanking sequence (present in recombinant plasmids HG22 and HG23) of this human CYP4A gene into the expression vector pG.CAT.

6.4.1 Subcloning of Human CYP4AII Gene 5' Flanking Sequence

5μg of Qiagen prepared pG.CAT DNA was digested with the restriction enzyme Xba I (10 units) at 37°C overnight, dephosphorylated with calf intestinal alkaline phosphatase as described in section 2.2.7.2, and purified by phenol/chloroform extraction (section 2.2.1). The DNA was recovered by ethanol precipitation (section 2.2.2, method B) and dissolved in water to a final concentration of approximately 100ng/μl.

5μg of Qiagen prepared recombinant plasmids HG22 and HG23 were digested with the restriction enzyme Xba I (10 units) at 37°C overnight and then electrophoresed on a 1% agarose gel. The 4Kb and 2.4Kb inserts were excised from the agarose gel and the DNA extracted by the Geneclean™ method (section 2.2.6.1).

Ligations were carried out as detailed in section 2.4.3.3 and transformation of competent XL1-blue cells performed as described in section 2.4.4. Table 6.6 shows the results from this subcloning experiment. It can be seen that plates 7 and 8
Figure 6.5 Restriction map of human *CYP4A* recombinant phage (λB1), showing the regions sequenced (recombinants subcloned into the vector pGEM7zf(+) shown below sequence, the numbers represent exons).
<table>
<thead>
<tr>
<th>PLATE</th>
<th>DNA</th>
<th>ENZYME</th>
<th>CIAP</th>
<th>MOLAR RATIO</th>
<th>WHITE colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>*No DNA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>*pG.CAT (20ng)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>800</td>
</tr>
<tr>
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<td>*pG.CAT (20ng)</td>
<td>Xba I</td>
<td>-</td>
<td>-</td>
<td>90</td>
</tr>
<tr>
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<td>CIAP</td>
<td>-</td>
<td>15</td>
</tr>
<tr>
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<td>Xba I</td>
<td>-</td>
<td>-</td>
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</tr>
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<td>Xba I</td>
<td>CIAP</td>
<td>-</td>
<td>50</td>
</tr>
<tr>
<td>7</td>
<td>pG.CAT (20ng) + HG22</td>
<td>Xba I</td>
<td>CIAP</td>
<td>1:2 (V:I)</td>
<td>200</td>
</tr>
<tr>
<td>8</td>
<td>pG.CAT (20ng) + HG23</td>
<td>Xba I</td>
<td>CIAP</td>
<td>1:2 (V:I)</td>
<td>150</td>
</tr>
</tbody>
</table>

Table 6.6 Cloning strategy and results of subcloning of the human CYP4A 5' flanking sequences into the expression vector pG.CAT.

* indicates no ligase included

Xba I indicates the plasmid had been linearised before ligation

CIAP indicates the plasmid had been dephosphorylated with calf intestinal alkaline phosphatase before ligation
produced, respectively, four and three times as many colonies as the control plate (plate 6). 5 colonies from each test plate were screened further. The five colonies from plates 7 and 8 were picked at random and DNA prepared by the STET method (section 2.4.1). The DNA was digested with the restriction enzyme Xba I along with the recombinant plasmids HG22 and HG23, which were used as appropriate size markers, and electrophoresed on a 0.7% agarose gel (Figure 6.6). Two bands can be seen in lane 1, the 5.5Kb band representing the plasmid pG.CAT and the 4Kb band representing the HG23 insert. Lane 2 contained the recombinant plasmid HG23 digested with Xba I, which gave rise to a band the same size as that seen in lane 1, namely the 4Kb HG23 insert, and also produced a 3Kb band representing the plasmid pGEM7Z. Lane 3 shows an insert of size 2.4Kb equivalent to that seen when the recombinant plasmid HG22 is digested with Xba I (lane 4). Sequencing, using the -40 universal primer, confirmed that the inserts in the recombinant plasmids pHG22G.CAT and pHG23G.CAT were the human genomic fragments from the recombinant phage λB1 (data not shown).

6.4.2 Activity of Reporter Plasmids Containing Human CYP4A11 5' Flanking Sequences in Response to the Peroxisome Proliferator Wy 14643

DNA from the recombinant plasmids pHG22G.CAT and pHG23G.CAT was prepared as described in section 2.4.2.2 and diluted to a concentration of 1μg/μl. These two reporter plasmids were transfected into the mouse hepatoma cell line Hepa1, using the procedure described in section 2.10, in the presence or absence of PPAR and in the presence or absence of the peroxisome proliferator Wy 14643. A concentration of 10^{-5}M, which was previously determined to give maximal induction of the CAT enzyme, was used (Table 6.7). The reporter plasmid pACO(-1273/-471)G.CAT was also co-transfected with PPAR, since this was known to contain a PPRE, and therefore served as a positive control. The cells were harvested from each plate and cytosol extracted by three rounds of freeze/thaw cycles. Extracts were normalized for
Figure 6.6  Digestion of recombinant plasmids pHG22.GEM and pHG23.GEM along with recombinant human CYP4A pG.CAT clones, with the restriction enzyme Xba I.

Lane 1: clone 16a: pHG23G.CAT  
2: pHG23.GEM  
3: clone 14d: pHG22G.CAT  
4: pHG22.GEM  
5: λ Hind III molecular weight markers
Table 6.7 Transfection strategy for human CYP4A 5' flanking sequence reporter constructs and results of CAT assay. Wy indicates the peroxisome proliferator Wy 14643 at a concentration of 10^-5 M and AcCh indicates acetylated 14C chloramphenicol.
β-galactosidase as described in section 2.10.1 and a CAT assay performed as described in section 2.10.2.

The autoradiograph of the TLC plate can be seen in Figure 6.7 and the results from the TLC scanner in Table 6.7. As expected, the reporter plasmid pACO(-1273/-471)G.CAT was stimulated when co-transfected with the pSG5.PPAR expression plasmid in the presence of Wy 14643 (Figure 6.7, lanes 3 and 4). However, the potent peroxisome proliferator Wy 14643 was unable to stimulate CAT activity in the human CYP4A11 5' flanking sequence CAT constructs (Figure 6.7, lanes 8-10 and 14-16).

6.5 DISCUSSION

Unfortunately, subcloning of the rat CYP4A2 5' flanking sequence was unsuccessful. Numerous attempts were made to subclone the 5' flanking PCR fragments shown in Figure 6.4, using various molar ratios of vector to insert DNA (Table 6.4) none of which yielded a recombinant clone. However only 55% and 38% of the total number of colonies of the ligation of the PCR products from the primer sets S1/S2 and S1/S3 respectively, into the expression vector pG.CAT, were screened. It is possible that a recombinant clone existed in the colonies that were not screened. An approach to consider in the future for subcloning the PCR fragments would be to use a specially designed PCR cloning vector such as that produced by Invitrogen Corporation or Promega. Both vectors are provided as a linearised plasmid modified to contain dT overhangs and cloning of PCR products takes advantage of the single dA added to the 3' end of double-stranded DNA molecules by thermostable polymerases. Since I had already managed to subclone the 3.0Kb Eco RI fragment of CYP4A2 into the vector pGEM7Z, an attempt was made to subclone this fragment into the expression vector pG.CAT. The vector pG.CAT, however, did not contain an Eco RI site in its multiple cloning site, therefore it was necessary to perform a blunt end ligation. The
Figure 6.7  Stimulation of reporter gene constructs in the presence (+) or absence (-) of the peroxisome proliferator Wy 14643 (Wy), at a concentration of $10^{-5}$M, and PPAR (R). The position of $^{14}$C-chloramphenicol (Chl) and acetylated $^{14}$C-chloramphenicol (1/3 Ac) after thin layer chromatography is indicated.
results seen in Table 6.5 indicated that the ligation had been successful (plate 5 as compared to plate 4). However, a vast number of colonies were obtained, approximately 18,000 (plates 5, 6, and 7) of which only 100 were screened, none of which contained a recombinant clone. If time had allowed then the transformation of competent TB1 cells would have been performed with less DNA and plated at a lower density to give a more manageable number of colonies to screen.

The transient co-transfection assay was established in the laboratory with some success. Table 6.1 shows the results from the first experiment. The average percent acetylated chloramphenicol for the co-transfection of PPAR with the reporter plasmid in the absence of an inducer (plates 5 and 6) was 21.5%. As stated previously, this was felt to be quite high, however this experiment was only performed in duplicate. In the subsequent experiments this value was reduced (Table 6.3) to 12.12% \pm 1.67, a value comparable to that reported by Tugwood et al., (1992). The results in Table 6.1 demonstrate that some CAT activity is produced by the reporter vector alone (4.5% acetylated chloramphenicol). This could be due to the rabbit \( \beta \)-globin promoter driving CAT production, or it may represent the presence of PPAR in the cell. The fact that the cotransfection of pSG5.PPAR stimulates the reporter plasmid (21.5% acetylated chloramphenicol) in the absence of a peroxisome proliferator may indicate either that PPAR has some partial constitutive activity or that there are small levels of a natural PPAR ligand present either in the culture medium or endogenous to the cells.

The results in Table 6.1 and 6.3 indicate that PPAR is activated by the peroxisome proliferator Wy 14643 (45 \pm 2.9 % and 33.6 \pm 2.3 % acetylated chloramphenicol, respectively, at 10^{-5}M concentration) and not at all by the peroxisome proliferator PFDA (21.9 \pm 1.8 % acetylated chloramphenicol at 10^{-4}M concentration). PFDA is a perfluorocarbon that is used in the chemical industry. Generally, perfluorocarbons are considered metabolically inert and relatively non-toxic compounds (Sargent and Seff,
1970). Recently, however, perfluorinated compounds such as perfluorodecanoic acid (PFDA) have been shown to be peroxisome proliferators (Ikeda et al., 1985, Borges et al., 1992). The result obtained in the cotransfection assay may indicate that PFDA does not exert its peroxisome proliferator action via the PPAR, however this experiment needs to be repeated. It has been shown that PFDA administration to rats increases the plasma triglyceride levels while causing a decrease in plasma cholesterol levels. This finding was in contrast to that observed for other classical peroxisome proliferators, such as the hypolipidaemic agent, clofibrate, and has led to the classification of PFDA as a "non-hypotriglyceridaemic" peroxisome proliferator (Borges et al., 1990).

Figure 6.7 demonstrates that the potent peroxisome proliferator Wy 14643, at a concentration of 10^-5M, was unable to stimulate the human 5' CAT constructs in the absence or presence of co-transfected pSG5.PPAR (lanes 5-16). This is consistent with the lack of a PPRE in the sequence so far obtained (S. Hood, personal communication). However there are a number of possibilities as to why no response was seen:

i) There is no PPRE present.

ii) A PPRE may be present further upstream than that tested.

iii) There may exist a negative cis regulatory region exerting an effect (as seen in the rat acyl CoA oxidase gene, Osumi et al., 1991).

iv) An auxiliary factor maybe required such as RXR.

The significance of these possibilities will be discussed in more detail in Chapter 7.
CHAPTER 7

DISCUSSION
CHAPTER 7
DISCUSSION

The phenomenon of peroxisome proliferation has invariably been associated with induction of member(s) of the CYP4A subfamily enzymes. Regulation of the members of the rat CYP4A family has not been fully studied at the molecular level and despite publication of the rat CYP4A1 and CYP4A2 genomic sequences (Kimura et al., 1989a), no functional assays have been performed to determine the mechanism of their transcriptional activation by peroxisome proliferators.

In this thesis I report on the isolation and sequencing of three different members of the rat CYP4A family. With the recent discovery that mPPAR mediates the transcriptional activation of the rat acyl-CoA oxidase gene (Tugwood et al., 1992) and also the rabbit CYP4A6 gene (Muerhoff et al., 1992b) by peroxisome proliferators, the presence of a PPAR response element (PPRE) in the 5' regulatory DNA sequences of the three different rat CYP4A genes isolated here was investigated. I also examined the effect of mPPAR on the transcriptional activation of the human CYP4A11 gene, the first such report.

7.1 THE ISOLATION AND CHARACTERISATION OF THE RAT CYP4A GENES

A rat genomic recombinant phage library in λCh4A was screened using the rat CYP4A1 cDNA (Earnshaw et al., 1988) as a probe and a total of 16 clones were isolated. These were segregated into 6 different groups according to their Eco RI restriction pattern (Figure 3.1). The library had been screened with medium stringency so as not to exclude the isolation of other isoforms of CYP4A1 as it had been demonstrated by Southern blot, that at least two or three genes existed in the rat CYP4 family (Hardwick et al., 1987).
The six different recombinant phage clones were characterised by Southern blot using a more 5' located 600bp of \textit{CYP4A1} cDNA as a probe (representing the first 5 exons). The fragments were subcloned into the plasmid pGEM7 and partly sequenced. Figure 3.11B demonstrated that all groups, except group D, exhibited hybridisation to the $^{32}$P labelled 600bp probe. \textit{Eco} RI fragments hybridising only to the 600bp probe appeared to lie upstream to those \textit{Eco} RI fragments showing homology to both the 600bp and full length \textit{CYP4A1} cDNA and a preliminary restriction map was made based upon these results (Figure 3.12).

From the sequence analysis presented in chapter 4, I concluded that group A and E disappointingly, represented λ DNA sequences (Figure 4.5 and Figure 4.19) as well as containing a 6.7kb \textit{Eco} RI fragment from \textit{CYP4A2} which seemed to be very well represented in this particular genomic library. I concluded that group B probably represented the genomic clone of \textit{CYP4A3}.

It is now known that a great deal of multiplicity exists within the \textit{CYP4A} family (Table 1.2) with four members in the rat family, four in the rabbit, two in the mouse, one in the guinea pig and two (?) in the human family identified to date. The relationship at the amino acid level of the individual members of the \textit{CYP4A} family can be seen in Table 7.1. In view of the fact that the members of the \textit{CYP4A} family are so closely related (ranging from 70-97% amino acid similarity), it was surprising that none of the six different recombinant phage isolated from the library screen, represented the rat \textit{CYP4A1} genomic sequence (section 4.10). This must be a reflection of the quality of the recombinant library and not the conditions used to screen it. The quality of the library was also reflected by the number of recombinant phage clones isolated that contained lambda DNA as an insert (5 out of the original 16, Figure 4.6).

The cytochrome P450s are thought to have evolved from a common ancestral gene some 3.5 billion years ago. The diversity seen within the cytochrome P450 family is
<table>
<thead>
<tr>
<th></th>
<th>CYP4A1</th>
<th>CYP4A2</th>
<th>CYP4A3</th>
<th>CYP4A4</th>
<th>CYP4A5</th>
<th>CYP4A6</th>
<th>CYP4A7</th>
<th>CYP4A8</th>
<th>Cyp4a-10</th>
<th>CYF4A11</th>
<th>Cyp4a-12</th>
<th>CYP4A13</th>
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<tbody>
<tr>
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<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
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<tr>
<td>CYP4A2</td>
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</tr>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>93%</td>
<td>ND</td>
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<tr>
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<td>80%</td>
<td>ND</td>
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</tr>
</tbody>
</table>

Table 7.1 Amino acid similarities between members of the CYP4A family. The percentages were obtained from the references quoted in Table 1.2 and are based on gap alignment data. ND indicates not determined.
thought to have arisen as the result of species specific gene duplication and conversion events which have conferred an evolutionary advantage on the animals concerned. On the basis of this, Nebert (1990) has proposed that the oldest cytochrome P450 enzymes may play an important role in maintaining the steady-state levels of endogenous ligands involved in ligand-modulated transcriptional regulation of genes effecting homeostasis, growth, differentiation and neuroendocrine function. So far the CYP4 genes have the highest number of introns among the eukaryotic P450 genes, from 10 in CYP4B1 (Yokotani et al., 1990) to 12 in CYP4A1 (Kimura et al., 1989a). A hypothesis has been proposed that as genes evolved, intron loss occurred (Gilbert et al., 1986). Therefore the CYP4 genes may preserve the most "ancient" structure among known eukaryotic cytochrome P450 genes (Degtyarenko and Archakov, 1993).

The multiplicity of genes seen in the CYP4A subfamily can be related to the multiplicity of their substrates (ω-hydroxylation of fatty acids and prostaglandins), their tissue specificity (expressed in the liver, kidney, lung, small intestine and prostate), and their species specificity. These all relate to endogenous functions (reviewed in section 1.2.2) which for the most part remain unknown.

7.2 THE ROLE OF PPAR IN THE TRANSCRIPTIONAL REGULATION OF CYP4A GENES AND IN PEROXISOME PROLIFERATION

The balance of evidence produced over the last four years indicates that the steroid hormone receptor PPAR (Isseman and Green, 1990), mediates some of the biological responses to peroxisome proliferators. Since the demonstration that PPAR recognises a response element (PPRE) present in the 5' upstream region of the rat peroxisomal acyl CoA oxidase gene causing an increase in its transcription (Tugwood et al., 1992), a number of other PPRE's have been identified. They have also been found in the genes for the rat peroxisomal bifunctional enzyme (Zhang et al., 1992a, Bardot et al.,
1993), the rat peroxisomal 3-ketothiolase gene (Kliewer *et al.*, 1992b), *CYP4A6*, the major rabbit lauric acid 12-hydroxylase (Muerhoff *et al.*, 1992b), and rat liver fatty acid binding protein (Issemann *et al.*, 1992, Simon *et al.*, 1993). More recently it has also been demonstrated that PPAR activates these genes through heterodimer formation with the retinoid receptor RXR (Kliewer *et al.*, 1992b, Issemann *et al.*, 1993, Gearing *et al.*, 1993, Keller *et al.*, 1993, Bardot *et al.*, 1993). A scheme for the involvement of PPAR and RXR in peroxisome proliferator dependent enzyme induction is shown in Figure 7.1.

As can be seen in Figure 7.1 a number of questions remain about the control of enzyme induction by PPAR, for instance;

1) does a PPRE exist in the 5' upstream region of genes such as *CYP4A1/4A2/4A3/4A11* and cell cycle regulatory genes such as *c-myc/c-fos*?

2) what is the natural ligand for PPAR?

3) what is the function of the heterodimerisation with RXR?

4) which other members of the steroid hormone receptor family can interact with PPAR?

5) how do peroxisome proliferators activate PPAR?

6) are all the pleiotropic effects of peroxisome proliferators mediated through PPAR?

7) what is the role of the peroxisome proliferator dependent induction of PPAR (Gebel *et al.* 1992, McNae personal communication)?

The identification that clofibrate-activated PPAR could bind to the regulatory sequence at position -727bp upstream of the *CYP4A6* gene and stimulate transcriptional activity (Muerhoff *et al.*, 1992b), strongly suggests that the induction by clofibrate of the closely related rat fatty acid ω-hydroxylases *CYP4A1/4A2/4A3* (Table 7.1) also involves PPAR. These peroxisome proliferator inducible genes, including acyl CoA oxidase, do not contain a consensus TATA box in their promoter.
Figure 7.1 Schematic diagram indicating the role of PPAR and RXR in peroxisome proliferator dependent enzyme induction. Retinoid X receptor (RXR), peroxisome proliferator activated receptor (PPAR), heat shock protein (HSP).
sequence (Kimura et al., 1989a, Osumi et al., 1991, Muerhoff et al., 1992a), however several putative regulatory sequences have been identified in the 5' flanking regions of these genes. Two highly conserved sequences have been identified in the upstream regions of CYP4A1 and CYP4A2. These are a 19bp sequence located 42 and 48bp upstream and a 6bp (GATTTA) sequence located 20 and 25bp upstream of the transcriptional start sites of CYP4A1 and CYP4A2 respectively (Kimura et al., 1989a). The function of these sequences are not yet known. A consensus GC rich sequence, the core binding site for the transcription factor Sp1, has been identified in the 5' flanking regions of both CYP4A1 and CYP4A6 (Kimura et al., 1989a, Muerhoff et al., 1992a). In addition an 11bp sequence (the function of which is unknown) is strongly conserved between CYP4A1 and CYP4A6 (Muerhoff et al., 1992a).

The CYP4A6 PPRE is present in a reverse orientation to that in the rat peroxisomal acyl CoA oxidase gene at position -558bp (Figure 7.1), indicating that the PPRE is an inducible enhancer element. The fact that enhancers can act at a considerable distance from the transcriptional start site might explain why a PPRE has not been identified in the -1.0kb and -2.5kb of upstream sequence of CYP4A1 and CYP4A2 respectively (Kimura et al., 1989a). It has been suggested that a PPRE is present at -5.0kb upstream of the CYP4A1 (Green, personal communication), however, others have suggested that no PPRE exists up to -20kb of CYP4A1 (Gonzalez, personal communication).

It should not be assumed however that the transcriptional activation of rat CYP4A fatty acid ω-hydroxylases, and acyl CoA oxidase is equally sensitive to PPAR otherwise results such as those of Milton et al. (1990) cannot be explained. These workers observed a biphasic induction of the apoprotein content, catalytic activity and mRNA levels of CYP4A1 after a single dose of clofibrate. The first and second peaks of induction were observed at about 30 minutes and 18 hours respectively. However, the mRNA levels of acyl CoA oxidase and palmitoyl CoA oxidase were induced in a
monophasic manner after clofibrate treatment with peaks at about 18 hours and 18 to 24 hours post dose respectively (Milton et al., 1990). In addition the clofibrate-induced level of CYP4A1 mRNA 24 hours after clofibrate treatment was not affected by co-treatment with cycloheximide, but the induction of acyl CoA oxidase mRNA was abolished by cyclohexamide treatment. The results of this study suggest that the basal level of the PPAR is sufficient for the induction of CYP4A1 while the transcriptional induction of acyl CoA oxidase might require the de novo synthesis of additional regulatory factor(s). This second phase may be due to recycling of the PPAR and RXR.

In the search for the endogenous ligand of PPAR, several workers have reported that long chain fatty acids can activate PPAR (Gottlicher et al., 1992, Issemann et al., 1993) but the relative potency compared to activation by the peroxisome proliferators themselves was low. However, in contrast Keller et al. (1993) demonstrated that polyunsaturated fatty acids activated the *Xenopus laevis* PPAR receptor as efficiently as the most potent peroxisome proliferator, Wy 14643. Ultimately, binding studies are required to determine whether fatty acids are the endogenous ligand of PPAR or whether a fatty acid metabolite, such as the α-hydroxy metabolite of arachidonic acid, could be the true ligand.

It is possible that the increase in fatty acid α-hydroxylation, due to the early induction of CYP4A (Milton et al., 1990), prior to the induction of peroxisomal β-oxidation results in the generation of the true ligand of PPAR. However, an inhibitor of CYP4A1, 1-aminobenzotriazole, did not inhibit PPAR activation by fatty acids in transfected hepatocytes (Gottlicher et al., 1992). The substrate overload theory suggests that an accumulation of fatty acids occurs after administration of the peroxisome proliferator due to an inhibition of the mitochondrial β-oxidation which occurs at an early time point (Lock et al., 1989). Therefore, CYP4A has access to excess fatty acid substrate which could then lead to production of the endogenous
ligand for PPAR, thereby leading to induction of peroxisomal β-oxidation. In support of this theory is the observation that induction of peroxisomal acyl CoA oxidase is dependent on an earlier phase of protein synthesis (Milton et al., 1990). This early phase of protein synthesis might represent the synthesis of CYP4A1 since it has been shown that mechanism based inhibitors of CYP4A1 can substantially inhibit peroxisome proliferation in vivo (Ortiz de Montellano, 1992).

Another question raised above is how is RXR heterodimerisation involved in the regulation of PPAR responsive genes? It has been demonstrated that the DNA-binding capacity of receptors such as the Drosophila ecdysone (EcR), the thyroid hormone receptor (TR), retinoic acid receptor (RAR) and the vitamin D3 receptor (VDR) with their target response elements is potentiated by the heterodimerisation of the receptor with the retinoid X receptor (RXR) (Yao et al., 1992, Leid et al., 1992, Zhang et al., 1992b, Kliewer et al., 1992a, Carlberg et al., 1993). More recently it has been shown that RXR can heterodimerise with PPAR, in the presence of 9-cis retinoic acid (the ligand for RXR) and peroxisome proliferators, to act synergistically in the induction of acyl CoA oxidase, 3-ketothiolase and bifunctional protein genes (Kliewer et al., 1992b, Bardot et al., 1993). Presumably, the ability of RXR to contribute to transcriptional activation of the heterodimer will depend upon the context of the response element within the promoter, and also the cell type, thus providing fine tuning of the response. As has been stated above, there are now a number of PPREs that have been identified, all with slight variations in the TGACCT direct repeat (Figure 7.1). These slight differences may lead to differences in the affinity of PPAR/RXR binding, thus leading to differential induction of the relevant genes by the PPAR/RXR heterodimer.

The interaction of PPAR with other steroid hormone receptors such as COUP-TF (Chicken Ovalbumin Upstream Promoter Transcription Factor) has not been investigated. It is known that COUP-TF can recognise a direct repeat separated by
either 1bp (the same specificity exhibited by PPAR) or 2bp (Green, 1993). COUP-TF appears capable of exerting either a negative or positive effect on target gene transcription (O'Malley and Conneely, 1992) and may therefore represent another type of control over the peroxisome proliferator dependent induction of certain genes.

The multiplicity of PPAR was discussed in Chapter 1 (section 1.6). It is known that as well as the mouse PPAR (Issemann and Green, 1990) there are 3 PPARs (α, β, γ) present in *Xenopus laevis* (Dreyer *et al.*, 1992), one in rat (Gottlicher *et al.*, 1992), one in human (Sher *et al.*, 1993) and a human PPAR-like receptor, Nuc-1 (Schmidt *et al.*, 1992). It is likely that there are more PPAR isoforms present in mouse, rat and human, especially as it is known 3 isoforms of RXR exist (α, β, γ) (Mangelsdorf *et al.*, 1990). The presence of these isoforms could also produce fine tuning of the response by each having different affinities for ligands and for DNA binding sites.

It has been demonstrated that the human PPAR, when co-transfected into mouse Hepa1 cells in the presence of peroxisome proliferators Wy 14643, nafenopin and clofibrate, has the ability to trans-activate the rat acyl CoA oxidase promoter and the rabbit *CYP4A6* promoter, comparable to that of the mouse receptor (Sher *et al.*, 1993). The existence of an active human PPAR would indicate the possible presence of PPREs upstream of the human genes orthologous to those shown in Figure 7.1. Although it has been demonstrated that the peroxisomal enzymes and β-oxidation system do exist in human cells (Watkins *et al.*, 1991), unfortunately none of the human peroxisomal β-oxidation genes have been isolated to determine the existence of a PPRE. The work presented in Chapter 6 is the first report of an investigation of the possible role of PPAR in the induction of a human gene, namely *CYP4A11*. Two constructs were made, pHG23G.CAT (-3.2kb/-20bp of *CYP4A11*) and pHG22G.CAT (-5.8kb/-3.2kb), both of which were co-transfected with the PPAR expression plasmid into the mouse hepatoma cell line, Hepa1. In the presence of the peroxisome proliferator Wy 14643 (10^-5M), no CAT activity was observed (Figure 6.7). This lack
of response, although consistent with the sequencing data so far obtained for this region (however, this is far from complete) could be due to a number of factors (some of which have already been discussed), for example;

1) negative cis regulatory regions may exist which exert a dominant negative effect, such as that observed in the rat acyl CoA oxidase gene (Osumi et al., 1991) and the rat bifunctional enzyme (Bardot et al., 1993). The only way to test this hypothesis would be to sequentially delete the sequence from both the 5' and 3' end and place these truncated sequences upstream of the CAT reporter vector to see if a response to Wy 14643 could be elicited.

2) an auxiliary factor such as RXR, may be required to elicit a full response.

3) a PPRE exists, but further upstream than the -5.8kb tested.

4) no PPRE exists.

The lack of a response of the CYP4A11 to PPAR is not totally unexpected when considering the species differences that exist in response to peroxisome proliferators. Peroxisome proliferation has not been clearly demonstrated in humans (Hanefeld et al., 1983). It may well be that the mechanism of regulation of the response element controlling the human CYP4A11 and perhaps other human genes (such as acyl CoA oxidase) induced by PPAR and its activators/ligands, fundamentally differs from that of rats or mice, resulting in low responsiveness to peroxisome proliferators. One possibility is that the human genes have lost the cis-acting regulatory elements found in rodent genes. It would be interesting to determine whether there is an inducible human acyl CoA oxidase response element which can be activated by human PPAR in the presence of a suitable activator, or whether some inhibition of the induction process can account for the low responsiveness of humans to peroxisome proliferators.

The ω-hydroxylated product of CYP4A metabolism requires further oxidation to the carboxylic acid before it can enter the β-oxidation pathway, one of these oxidations being performed primarily by alcohol dehydrogenase. Oxidation of primary alcohols is
catalysed by a family of enzymes, the alcohol dehydrogenases, each with a different substrate specificity (Vallee and Bazzone, 1983). Alcohol dehydrogenase is known to catalyse the rate limiting step of the oxidation of retinol to retinoic acid. Examination of the 5' flanking regions of one of these alcohol dehydrogenases revealed the presence of a retinoic acid response element (RARE), suggesting that a positive feedback loop operates resulting in increased formation of retinoic acid (Harding and Duester, 1992). This RARE consists of a direct repeat, separated by 5bp and therefore represents a target for RAR/RXR heterodimers. Since the ligand for RXR, 9-cis retinoic acid, is a stereoisomer of retinoic acid, it is possible that this feedback loop also operates to increase 9-cis retinoic acid. It seems that alcohol dehydrogenase may be intimately involved in the mechanism of peroxisome proliferation, not only in oxidising ω-hydroxy fatty acids, but also by increasing the formation of retinoic acid from retinol (Figure 7.2).

Retinoic acid is involved in cell differentiation and proliferation processes and therefore an increase in cellular concentration of this mediator and its involvement in peroxisome proliferation may indicate that a receptor-mediated mechanism of peroxisome proliferator-induced cell proliferation and hepatocarcinogenesis may exist. Genes containing PPRE or RARE sequences involved in cell proliferation undoubtably exist and await further investigation. For example c-myc and c-fos are known to be induced by peroxisome proliferators (Cherkaoui-Malki et al., 1990, Bentley et al., 1988).

Another question raised above is how do peroxisome proliferators activate PPAR? This could be achieved by a number of mechanisms. The peroxisome proliferator might bind directly to the PPAR inducing a conformational change in the PPAR molecule, although, to date, no evidence for this has been produced. However, recent investigations with computer aided molecular modelling (D. Lewis, personal communication) have suggested that, although peroxisome proliferators have diverse
Figure 7.2 The role of positive feedback regulation in peroxisome proliferation. Retinoic acid receptor (RAR), retinoid X receptor (RXR), peroxisome proliferator activated receptor (PPAR), alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH) and stimulation of activity (+).
chemical structures, many share some common structural features and functional
groups and a putative ligand-binding site which can accommodate numerous peroxisome
proliferators has been proposed (Lewis and Luke, 1993). Alternatively, conformational change in the PPAR molecule might be caused by an intermediate, peroxisome proliferator activated factor.

It is known that the 70kD and 90kD heat shock proteins (HSP70 and HSP90 respectively) are associated with the steroid hormone receptors and are vital in controlling receptor function (Landers and Spelsberg, 1992), for example, dissociation of HSP90 from the glucocorticoid receptor results in activation of the receptor without ligand binding (Daniel et al., 1991). It has been demonstrated that peroxisome proliferators can bind to heat shock proteins in vitro (Alvares et al., 1990) and this may provide a mechanism of activation of PPAR, by peroxisome proliferators sequestering certain heat shock proteins.

Phosphorylation provides another possible mechanism for the activation of PPAR by peroxisome proliferators. Many steroid hormone receptors have been found to be phosphorylated (Daniel et al., 1991) and this affects the transcriptional efficiency of these receptors. It is interesting to note that peroxisome proliferators can activate protein kinase C (PKC) via formation of their CoA-thioesters, which are rapidly formed by hepatocytes (Bronfman et al., 1986, 1989). The PKC gene family codes for at least nine serine/threonine protein kinases (Nishizuka, 1992) that are present, in quiescent cells, as cytosolic inactive enzymes. Each isoform has specific requirements for diacylglycerol, phosphatidylserine and calcium. Some PKC isoforms are known to be the intracellular receptors for phorbol ester tumour promoters such as 12-o-tetradecanoylphorbol-13-acetate (Nishizuka, 1984), demonstrating a crucial role of these enzymes in cell growth and differentiation. Recently a functional link between the PKC and retinoid pathways has been established (Tahayato et al., 1993). The
authors demonstrated that selective inhibition of PKC lead to the loss of ligand dependent transcription of a retinoic acid inducible promoter.

7.3 SUGGESTIONS FOR FUTURE WORK

In order to progress this work further, the following lines of investigation may be fruitful:

1. To fully characterise the group B recombinant phage clone, establishing its identity as the genomic sequence of CYP4A3 by sequencing the exons, using exon specific primers, and to use PCR to establish intron length. Characterisation of the 5' flanking region could then be carried out and investigations into this genes inducibility by peroxisome proliferators (Kimura et al., 1989b) and the differences in this inducibility observed between male and female (Sundseth and Waxman, 1992) performed. Control elements may also be present downstream of the promoter.

2. Further attempts to investigate the role of PPAR in the induction of CYP4A2 should be made. Screening of a second library using the recombinant plasmid pGEMC3.0 as a probe should be performed to pull out further 5' sequence to the -2.5kb already published (Kimura et al., 1989a).

3. Investigations into the control of the basal level of transcription of CYP4A2 and CYP4A3 should be carried out by incubating the 5' ends of these genes with nuclear and cytosol extracts from liver and kidney from a control male and female rat. Using the techniques of gel retardation and footprinting any DNA/protein interactions could be established and characterised in regard to the site of DNA binding. Isolating these proteins would be more difficult.

4. The results presented in Chapter 6 indicate that the peroxisome proliferator PFDA does not activate the PPAR, suggesting that not all peroxisome proliferators mediate
their response via the PPAR. This experiment should be repeated, using a variety of
different peroxisome proliferators, (ranging in potency as regarding their ability to
induce hepatocarcinogenesis in rodents), as well as PFDA, at different concentrations
so that a full characterisation of the transfection system in our hands could be
established.

5. Further analysis of the human 5' flanking region of CYP4A11 should be carried out
as suggested in the previous discussion. In total, -9.0kb of upstream region has been
isolated, but only -5.8kb has been tested.

6. Analysis of the regulatory regions of other human genes such as acyl CoA oxidase
would give a better understanding of the observed species differences in response to
peroxisome proliferators.

7. An attempt to establish whether the induction of CYP4A1, by peroxisome
prolifers, is causal or casual in the ensuing peroxisome proliferation could be made
by stably transfecting a cell line (whose response to peroxisome proliferators has been
characterised) with an expression vector, such as the one shown below:-

The integration of the construct into the cells could be selected for using a neomycin
resistance cassette. However, no CYP4A1 would be produced from this vector as
there is a lengthy stop sequence placed inbetween the SV40 promoter and the CYP4A1 cDNA. This stop sequence is flanked by FLP recombinase recognition sites. Transient transfection of a FLP recombinase expression vector into these cells would flip out the stop sequence, thereby bringing the CYP4A1 cDNA under control of the SV40 promoter switching on its expression. The effect of induction of CYP4A1 on the rest of the cell could then be monitored.
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