Molecular characterisation of xenobiotic-metabolising enzymes in Australian marsupials

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Contents

List of Tables viii
List of Figures ix
Abbreviations xii
Summary xiv
Declaration xvi
Acknowledgements xvii
Publications xviii
(1) Papers
(2) Manuscripts in Preparation
(3) Abstracts
(4) Additional Presentations

Chapter 1: General Introduction

1.1. Geological history of Australia 2
1.2. Origins of Australia's marsupial fauna 4
1.3. Classification of Australian mammalian fauna 5
1.3.1. Monotremes (subclass Prototheria) 5
1.3.2. Marsupials (subclass Marsupialia) 6
1.3.3. Placental mammals (subclass Eutheria) 7
1.4. Australian marsupials as unique scientific research models 7
1.5. Metabolism of xenobiotics 8
1.6. Cytochromes P450 10
1.6.1. History 10
1.6.2. Structure 11
1.6.3. Regulation 13
1.6.4. Diversity and nomenclature 13
1.6.5. *CYP2* family 17
1.6.5.1. CYP2C subfamily 17
1.7. UDP glucuronosyltransferases 21
1.7.1. Structure 21
1.7.2. Diversity and nomenclature 21
1.7.3. The *UGT1* family 24
1.7.4. The *UGT2* family 24

1.8. Xenobiotic metabolism in Australian marsupials 25
1.8.1. Phase I metabolism- mixed function oxidase system 25
1.8.2. Phase II metabolism 28
1.8.3. Metabolism of dietary terpenes by Australian marsupials 30
1.9. Aims of the project 32

Chapter 2: Materials and Methods

2.1. Materials 35
2.1.1. Marsupial tissues 35
2.1.2. cDNA libraries 35
2.1.3. Chemicals, Kits and Enzymes 35
2.1.4. Antibodies 38
2.1.5. Equipment 38
2.1.6. Bacterial culture media 40
2.1.6.1. Solid media 40
2.1.6.2. Liquid media 40
2.1.6.3. Antibiotics 40
2.2. Methods 40

Protein methodologies
2.2.1. Microsome preparation 40
2.2.2. Lowry assay 41
2.2.3. Cytochrome *c* NADPH reductase assay 41
2.2.4. Total cytochrome P450 assay 42
2.2.5. SDS-PAGE 42
2.2.5.1. Preparation of samples 42
2.2.5.2. SDS-PAGE of samples 42
2.2.6. Immunoblotting 42

General molecular biology techniques
2.2.7. Total RNA isolation 43
2.2.7.1. mRNA isolation from total RNA 44
2.2.7.2. Quantification of RNA 45
2.2.8. Ligation of PCR products into pGEM®-T and pGEM®-T Easy vectors 45
2.2.8.1. Preparation of competent E.coli DH5α cells 45
2.2.8.2. Transformation of plasmids into E.coli DH5α competent cells 45
2.2.8.3. Small scale purification of plasmids (alkaline lysis) 46
2.2.8.4. Screening for inserts in pGEM®-T and pGEM®-T Easy clones 46
2.2.9. DNA analysis 47
2.2.9.1. Quantification of DNA 47

2.2.9.2. Restriction digestion of DNA 47
2.2.9.3. Gel electrophoresis and visualisation of DNA 47
2.2.10. DNA sequencing 48
2.2.10.1. Sequencing reaction 48
2.2.10.2. Sequencing reaction primers 49
2.2.10.3. DNA sequence analysis 49
2.2.11. Isolation, purification and radiolabelling of cDNA probes 50
2.2.11.1. Low-melting point gel electrophoresis of amplified cDNA clones 50
2.2.11.2. Gel extraction and purification of cDNA fragments 50
2.2.11.3. Radioactive labelling of cDNA probes with α-^{32}P-dCTP 50
2.2.11.4. Hybridisation of filters with α-^{32}P labelled cDNA probes 51
2.2.12. Northern blot analysis 52
2.2.12.1. RNA sample preparation 52
2.2.12.2. Gel preparation and electrophoresis 52
2.2.12.3. Capillary blotting 52
2.2.12.4. Hybridisation of membranes with α-^{32}P labelled koala cDNA probes 53

Polymerase chain reaction techniques
2.2.13. Reverse transcription of RNA 53
2.2.14. Polymerase chain reaction (PCR) 53
2.2.14.1. PCR amplification of marsupial CYP2C and UGT sequences 54
2.2.15. 5' Rapid Amplification of cDNA ends (RACE) of a koala *CYP2C* cDNA sequence 54
2.2.15.1. First strand cDNA synthesis 55
2.2.15.2. GLASSMAX spin cartridge purification of cDNA 55
2.2.15.3. TdT-tailing of purified koala 5' RACE cDNA 56
2.2.15.4. PCR amplification of the 5' end of a koala CYP2C cDNA sequence 56
2.2.16. 3' RACE of a koala *CYP2C* cDNA sequence 56
2.2.16.1. First strand cDNA synthesis 57
2.2.16.2. PCR amplification of the 3' end of a koala CYP2C cDNA sequence 57

cDNA library screening techniques
2.2.17. cDNA library screening 57
2.2.17.1. Liquid culture preparation of E.coli XL1-Blue MRF' cells 58
2.2.17.2. Library plating 58
2.2.17.3. Plaque transferral to nylon membranes 58
2.2.17.4. Hybridisation of filters with α-^{32}P labelled marsupial cDNA probes 59
2.2.17.5. In vivo excision of positive clones 59
2.2.17.6. Sequencing of excised library clones 60

Chapter 3: Marsupial Tissue Bank

3.1. Introduction 62
3.2. Research strategy 62
3.3. Methods 63
3.3.1. Collection and storage of marsupial tissue samples 63
3.4. Results 64
3.5. Discussion 66

Chapter 4: Cytochrome P450 expression in representative Australian marsupial species

4.1. Introduction 68
4.2. Research strategy 69
4.3. Results 70
4.3.1. Enzyme assays 70
4.3.2. SDS-PAGE analysis of koala, tammar wallaby, wombat and rat liver microsomes 71
4.3.3. Immunoblot analysis of hepatic microsomes prepared from the koala, tammar wallaby, wombat and rat 73
4.3.3.1. CYP2C 73
4.3.3.1.1. Hepatic Expression 73
4.3.3.1.2. Extrahepatic Expression 76
4.3.3.2. CYP2D 78
4.3.3.3. CYP2E1 80
4.3.3.4. CYP3A 82
4.3.3.5. NADPH-cytochrome P450 reductase 84
4.4. Discussion 85
4.4.1. Enzyme Assays 85
4.4.2. Immunoblot analyses of marsupial hepatic microsomes 86
4.4.2.1. CYP2C 86
4.4.2.2. CYP2D 89
4.4.2.3. CYP2E1 90
4.4.2.4. CYP3A 91
4.4.2.5. NADPH-cytochrome P450 reductase 92

Chapter 5: Identification and cloning of cytochrome P450 2C (*CYP2C*) cDNA sequences from representative Australian marsupial species

5.1. Introduction 95
5.2. Research strategy 96
5.3. Methods 98
5.3.1. Primer design 98
5.4. Results 100
5.4.1. Amplification of marsupial *CYP2C* cDNA sequences by polymerase chain reaction 100
5.4.2. Sequence analysis of amplified marsupial polymerase chain reaction products 104
5.4.2.1. Western quoll CYP2C cDNA sequences 104
5.4.2.2. Tammar wallaby CYP2C cDNA sequences 108
5.4.2.3. Koala CYP2C cDNA sequences 108
5.4.3. Rapid Amplification of cDNA ends (RACE) of a koala *CYP2C* cDNA sequence 117
5.4.4. Sequence analysis of 3' RACE koala amplified products 123
5.4.5. Screening of a koala liver cDNA library with a 700 bp partial koala *CYP2C* sequence 124
5.4.6. Screening of a koala liver cDNA library with a 400 bp partial koala *CYP2C* sequence 124
5.4.7. Sequence analysis 125
5.4.8. RNA blot analysis 132
5.5. Discussion 134
5.5.1. Isolation of *CYP2C* cDNAs from Australian native marsupial species 134
5.5.1.1. Analysis of western quoll CYP2C cDNA sequences 134
5.5.1.2. Analysis of tammar wallaby CYP2C cDNA sequences 135
5.5.1.3. Analysis of koala CYP2C cDNA sequences 136

Chapter 6: Identification and cloning of UDP glucuronosyltransferases from representative Australian marsupial species

6.1. Introduction 143
6.2. Research strategy 144
6.3. Methods 146
6.3.1. Primer design 146
6.4. Results 148
6.4.1. Immunoblot analysis of marsupial liver microsomes with anti-mouse UGT2 IgG 148
6.4.2. Amplification of UDP glucuronosyltransferase sequences from marsupial cDNA by polymerase chain reaction 150
6.4.3. Sequence analysis of amplified marsupial polymerase chain reaction products 156
6.4.4. Screening of a koala liver cDNA library with a 600 bp partial koala *UGT2* sequence 161
6.4.5. Sequence analysis 162
6.4.6. Screening of a koala liver cDNA library with a partial koala 800 bp *UGT1* sequence 162
6.4.7. Screening of a tammar wallaby liver cDNA library with a partial koala 800 bp *UGT1* sequence 162
6.4.8. RNA blot analysis 163
6.5 Discussion 165
6.5.1. Immunoblot analysis of adult and pouch young marsupial liver microsomes 165
6.5.2. Isolation of *UGT1* sequences from Australian native marsupial species 166
6.5.2.1. Analysis of UGT1 sequences from Australian marsupial species 166
6.5.2.2. Analysis of UGT2 sequences from Australian marsupial species 167

Chapter 7: General Discussion

References 176

Appendix A 194
Appendix B 196
Appendix C 197

List of Tables

1.1.	Geological time scale of evolutionary events	3
1.2.	Classification of Marsupials	6
1.3.	Vertebrate cytochrome P450 families	15
1.4.	A list of mammalian *CYP2C* genes	18
1.5.	A list of mammalian *UGT* families and subfamilies	23
2.1.	Reagents and Kits used in experimental procedures	36
2.2.	Equipment, manufacturer and the reason used during each research protocol	39
3.1.	Marsupial tissue bank	65
4.1.	CYP content and NADPH-cytochrome P450 reductase activity in koala, tammar wallaby and rat hepatic microsomes	70
5.1.	CYP2C primer sequences	99
5.2.	Amino acid sequence comparison of the KLC#38 and KLC#26 clones with selected mammalian CYP2C proteins	138
6.1.	UGT primer sequences	147

List of Figures

1.1. Relative components and interactions of the mixed function oxidase system 11
1.2 Three dimensional structure of the bacterial CYP, CYP101 12
4.1. Coomassie brilliant blue stain of hepatic microsomes purified from the koala, tammar wallaby, wombat and rat 72
4.2. Immunoblot analysis of marsupial liver microsomes with anti-rat CYP2C6 IgG 73
4.3. Immunoblot analysis of marsupial liver microsomes with anti-rat CYP2C6 IgG 74
4.4. Immunoblot analysis of hepatic pouch young and adult koala liver microsomes with anti-rat CYP2C6 IgG 75
4.5. Immunoblot analysis of marsupial liver microsomes with anti-rat CYP2C11 IgG 76
4.6. Immunoblot analysis of marsupial extrahepatic microsomes with anti-rat CYP2C6 IgG 77
4.7. Immunoblot analysis of marsupial liver microsomes with anti-human CYP2D6 IgG 78
4.8. Immunoblot analysis of koala pouch young liver microsomes with anti-human CYP2D6 IgG 79
4.9. Immunoblot analysis of marsupial liver microsomes with anti-rat CYP2E1 IgG 80
4.10. Immunoblot analysis of koala pouch young liver microsomes with anti-rat CYP2E1 IgG 81
4.11. Immunoblot analysis of marsupial liver microsomes with anti-rabbit CYP3A6 IgG 82
4.12. Immunoblot analysis of koala pouch young liver microsomes with anti-rabbit CYP3A6 IgG 83
4.13. Immunoblot analysis of marsupial liver microsomes with anti-human NADPH-cytochrome P450 reductase IgG 84
5.1. Research strategy to isolate a marsupial *CYP2C* cDNA sequence 97
5.2. Analysis of marsupial PCR products by agarose gel electrophoresis 101
5.3. Analysis of koala PCR products by agarose gel electrophoresis 103
5.4. Sequence analysis of three partial quoll *CYP2C* cDNA clones 105
5.5. Alignment of the deduced amino acid sequences of quoll clones *CYP2C#1*, *#2* and *#4* with mouse, rat and human CYP2C proteins 107
5.6. Sequence analysis of three partial tammar wallaby *CYP2C* cDNA clones 109

5.7. Alignment of the deduced amino acid sequences of tammar wallaby clones *CYP2C#2*, *#3* and *#4* with mouse, rat and human CYP2C proteins 111
5.8. Sequence analysis of three partial koala *CYP2C* cDNA clones 112
5.9. Alignment of the deduced amino acid sequences of koala clones *CYP2C#4*, *#8* and *#9* with mouse, rat and human CYP2C proteins 114
5.10. Sequence analysis of two partial 400 bp koala *CYP2C* cDNA clones 115
5.11. Alignment of the deduced amino acid sequences of the 400 bp koala *CYP2C* clones with mouse, rat and human CYP2C proteins 116
5.12. Analysis of 5' RACE koala PCR products by agarose gel electrophoresis 118
5.13. Analysis of TdT-tailed 5' RACE koala PCR products by agarose gel electrophoresis 120
5.14. Analysis of 3' RACE koala PCR products by agarose gel electrophoresis 121
5.15. Alignment of the deduced amino acid sequence of the 3'RACE koala *CYP2C* clone with mouse, rat and human CYP2C proteins 123
5.16. Alignment of the deduced amino acid sequence of the KLC#38 *CYP2C* cDNA clone with multiple CYP2C proteins 126
5.17. RNA blot analysis of koala, tammar wallaby and rat liver mRNA and total RNA probed with a 700 bp partial koala *CYP2C* sequence 133
5.18. Alignment of the deduced amino acid sequences of the two koala library clones with rat and human CYP2C proteins 140
6.1. Research strategy to isolate a marsupial *UGT* cDNA sequence 145
6.2. Immunoblot analysis of marsupial liver microsomes with anti-mouse UGT2 IgG 148
6.3. Immunoblot analysis of koala pouch young liver microsomes with anti-mouse UGT2 IgG 149
6.4. Analysis of western quoll and tammar wallaby PCR products by agarose gel electrophoresis 151
6.5. Analysis of koala PCR products by agarose gel electrophoresis 152
6.6. Analysis of marsupial *UGT2* PCR products by agarose gel electrophoresis 154
6.7 Sequence analysis of the western quoll and tammar wallaby *UGT1* clones with selected mammalian *UGT1* sequences 157
6.8. Alignment of the deduced amino acid sequences of the western quoll and tammar wallaby *UGT1* clones with mouse, rat and human UGT1 proteins 158
6.9. Alignment of the deduced amino acid sequence of the koala *UGT1* clone with mouse, rat and human UGT1 proteins 159
6.10. Sequence analysis of the tammar wallaby and koala *UGT2* clones 160

6.11. Alignment of the deduced amino acid sequences of the tammar wallaby and koala *UGT2* clones with human, rat and rabbit UGT2 proteins 161
6.12. RNA blot analysis of koala, tammar wallaby and rat liver mRNA and total RNA probed with a 800 bp partial koala *UGT1* sequence 164

Abbreviations

A	Adenine
aa	amino acid
ß-gal	beta galactosidase
bp	base pair
BSA	bovine serum albumin
4CN	4-chloro-1-naphthol
C	Cytosine
cDNA	complementary deoxyribonucleic acid
CYPs	Cytochrome P450 enzymes
DAB	3,3'-diaminobenzidine
dAMP	Deoxyadenosine mono-phosphate
DEPC	Diethyl pyrocarbonate
DNA	deoxyribonucleic acid
dNTPs	pooled deoxyribonucleotide triphosphates
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetra acetic acid disodium salt
ER	endoplasmic reticulum
EtBr	ethidium bromide
g	grams
G	Guanosine
HRP	horse radish peroxidase
hr	hours
IMVS	Institute of Medical and Veterinary Science
IPTG	isopropyl-ß-D-1-thiogalactopyranoside
kbp	kilobase pair
kDa	kilodaltons
LB	Luria-Bertoni
M	molar
MCS	multiple cloning site
mg	milligrams
min	minutes
ml	millilitres
mM	millimolar

μM	micromolar
μl	microlitre
μg	microgram
MOPS	3-N-Morpholinopropane-sulphonic acid
mRNA	messenger ribonucleic acid
ng	nanograms
nm	nanometres
nmol	nanomoles
nt	nucleotide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG_{8000}	polyethylene $glycol_{8000}$
pfu	plaque forming units
pmole	picomoles
RNA	ribonucleic acid
rpm	revolutions per minute
RT-PCR	reverse transcription-polymerase chain reaction
SDS	sodium dodecyl sulphate
SSC	0.3M trisodium citrate, 3M sodium chloride (pH 7.0)
T	Thymidine
TAE	40mM Tris-acetate, 1mM EDTA (pH 8.0)
TBS	16mM Tris-acetate, 500mM sodium chloride
TE	10mM Tris-HCl, 1mM EDTA (pH 8.0)
TEMED	N, N, N', N'-tetramethylethylenediamine
Tris	Tris (hydroxymethyl) aminomethane
TRNA	transfer ribonucleic acid
TTBS	0.05% v/v Tween-20 in TBS
U	units
UAP	universal amplification primer
UV	ultraviolet light
V	volts
v/v	volume per volume
w/v	weight per volume
X-gal	5-Bromo-4-chloro-3-indoly-ß-D-galactopyranoside
x g	number of times unit gravitational force

Summary

Australia's native marsupials are descendants of animals isolated on the continent following Australia's separation from Antarctica approximately 26 million years ago. During their isolation, Australia's marsupial species have evolved into an array of herbivorous and carnivorous species exhibiting unique physiological, reproductive and dietary characteristics. A number of marsupial species possess the ability to ingest and metabolise a range of dietary compounds that would be toxic to other mammalian species. The enzymatic processes governing the ability of marsupials to metabolise dietary- and environmentally-derived xenobiotic compounds are not well defined.

Mammalian species have evolved several families of xenobiotic-metabolising enzymes including the cytochromes P450 (CYPs) and UDP glucuronosyltransferases (UGTs). CYPs comprise a large superfamily of enzymes which catalyse the oxidative metabolism of many xenobiotic and endogenous substrates. The specific induction of CYPs by xenobiotic compounds has provided strong evidence that the principle role of some CYPs, primarily from families CYP1, CYP2 and CYP3, is the metabolism and detoxication of those and related compounds from the body. The UGTs comprise a smaller family of enzymes which catalyse the conjugation of a glucuronic acid moiety to small hydrophobic molecules (aglycone), thus facilitating their excretion as water-soluble products. Both CYPs and UGTs are regulated in a tissue-specific and temporal manner.

Prior to the commencement of this project, no studies had been conducted into CYP activity or expression in many marsupial species including the koala, tammar wallaby and wombat. In addition, no CYP or UGT forms had been identified or cloned from any of Australia's marsupial species. To provide insights into the xenobiotic-metabolising capacity of marsupials, this project employed molecular biology and biochemical techniques to identify and study CYPs and UGTs from representative Australian marsupial species. Species studied included the koala (*Phascolarctos cinereus*), tammar wallaby (*Macropus eugenii*), western quoll (*Dasyurus geoffroii*) and southern-hairy nosed wombat (*Lasiorhinus latifrons*).

To facilitate this work, it was necessary to acquire tissue samples from a variety of Australian marsupial species. The formal establishment of a Marsupial Tissue Bank at the School of Pharmacy and Medical Sciences therefore represented a major focus of this project. Multiple tissues were collected from herbivorous species including the koala, eastern grey, western grey and red kangaroos, brushtail and ringtail possums, tammar wallaby, wombat and brushtailed bettong. The tissue bank also contains carnivorous species such as the western and eastern quoll. The availability of the Marsupial Tissue Bank has greatly facilitated marsupial research in the School over the last 3 years.

Determination of total hepatic cytochrome P450 complement in marsupial species revealed high levels in koala relative to other marsupial species. Immunoblot studies were then performed on tissues prepared from multiple adult marsupial species and pouch-young koalas. Antibodies utilised included anti-CYP2C, anti-CYP2D, anti-CYP2E1, anti-CYP3A, anti-UGT2 and anti-NADPH-cytochrome P450 reductase. Findings included high CYP2C immunoreactivity and a relative lack of CYP2D immunoreactivity in all species. Collectively, these immunoblot studies highlight the unique CYP complement of marsupial species relative to mammalian species. Significant interspecies differences between marsupials were also observed.

To isolate hepatic *CYP2C* and *UGT* cDNA sequences from representative Australian marsupial species, degenerate oligonucleotide primers, designed from the conserved regions of known mammalian *CYP2C* and *UGT* sequences, were used in polymerase chain reaction (PCR) experiments. Three partial *CYP2C* cDNAs were isolated from each of the western quoll, tammar wallaby and koala. In addition, partial clones encoding *UGT1* and *UGT2* sequences were isolated from each species. A koala liver cDNA library screened with a partial koala *CYP2C* sequence facilitated the isolation of two full-length clones,*CYP2C47* and *CYP2C48*, the first full length CYPs isolated from any marsupial. Five *CYP2C* cDNAs were isolated from the koala in total.

The work in this thesis has provided valuable information into the CYP450 content and expression in a number of Australian marsupial species. The isolation and comparison of a koala CYP2C sequence with other mammalian forms provides important evolutionary and structural information regarding the CYP450 enzyme system.

Declaration

"I declare that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge it does not contain any materials previously published or written by another person except where due reference is made in the text."



Brett Ryan Jones

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Publications

The following papers and abstracts support the work presented in this thesis.

1. Papers

Stupans, I., Kong, S., Kirlich, A., Murray, M., Bailey, E., **Jones, B.R.,** and McKinnon, R.A. (1999). Hepatic microsomal enzyme activity in the koala and tammar wallaby: high 17β-hydroxysteroid oxidoreductase activity in koala liver microsomes. *Comp. Biochem. Physiol. Part C.* 123: 67-73.

Stupans, I., **Jones, B.R.,** and McKinnon, R.A. Xenobiotic metabolism in marsupials. *Comp. Biochem. Physiol. Part C.* (2000 - in press)

2. Manuscripts in Preparation

Jones, B.R., Stupans, I., and McKinnon, R.A. Immunoexpression analysis of CYP enzymes in selected Australian marsupial species. (to be submitted to *Comp. Biochem. Physiol. Part C.*)

Jones, B.R., Stupans, I., and McKinnon, R.A. Identification of five *CYP2C* genes in the koala (*Phascolarctos cinereus*). (to be submitted to *Animal Genetics*)

Woolley, M.W., Stupans, I., **Jones, B.R.,** and McKinnon, R.A. CYP1A1 in Australian Marsupials, the Tammar Wallaby (*Macropus eugenii*) and Koala (*Phascolarctos cinereus*): Cloning and Characterisation. (to be submitted to *Bulletin of Environmental Contamination and Toxicology*).

3. Abstracts

Jones BR, Stupans I and McKinnon RA. Identification of partial cytochrome P450 cDNAs from the Western Quoll (*Dasyurus geoffroii*) and Koala (*Phascolarctos cinereus*). *Proceedings of the Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists (ASCEPT).* 4:115 (1997)

Stupans I, Kirlich A, Murray M, Jones BR, Bailey E and McKinnon RA. Hepatic microsomal xenobiotic metabolism in representative Australian native animals. *Archives of Pharmacol.* 358 Suppl 2:R416 (1998)

Jones BR, Stupans I and McKinnon RA. Characterisation of cytochromes P450, and cloning of partial cytochrome P450 cDNAs in Tammar Wallaby (*Macropus eugenii*) and Koala (*Phascolarctos cinereus*). *Proceedings of the International Society for the Study of Xenobiotics* (*ISSX*). 13:129 (1998)

Jones BR, Stupans I and McKinnon RA. Cytochrome P450 expression and partial cloning of P450 cDNAs in Tammar Wallaby (*Macropus eugenii*). *Proceedings of the Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists (ASCEPT).* 5:29 (1998)

Jones BR, Stupans I and McKinnon, RA. Isolation of two cytochrome P450 *CYP2C* cDNA sequences from an Australian native marsupial, the Koala (*Phascolarctos cinereus*). *Proceedings of the Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists (ASCEPT).* (2000)

4. Additional Presentations

Adelaide Pharmacology Group (October, 1997)

Adelaide Marsupial Research Group (September, 1998)

Chapter 1

General Introduction

Australia's native marsupial fauna comprises a vast array of carnivorous and herbivorous species exhibiting unique physiological, reproductive and dietary characteristics. A number of marsupial species possess the ability to ingest and metabolise a range of dietary compounds that would be toxic to other mammalian species. The enzymatic mechanisms governing the ability of marsupials to metabolise dietary xenobiotic compounds is discussed in the following sections.

Cytochromes P450 (CYPs) comprise a superfamily of membrane-bound haemoproteins that catalyse the oxidative metabolism of a diverse array of xenobiotic compounds and endogenous substrates. CYPs are regulated in a tissue-specific and temporal manner and are critically important in detoxifying and eliminating drugs, chemicals and environmental pollutants that may be encountered during a lifetime. In mammals, various CYPs also participate in the biosynthesis and metabolism of sterols and steroid hormones. The history, structure and function of the cytochromes P450 is reviewed in the following sections. The isolation and characterisation of cytochromes P450 in marsupials will aid in the understanding of their response to environmental chemical exposure and in addition, provide important evolutionary information on the cytochrome P450 superfamily and marsupials in general.

The UDP glucuronosyltransferases (UGTs) comprise a smaller family of xenobiotic-metabolising enzymes which, like CYPs, catalyse the metabolism of a variety of diverse compounds. The nomenclature, diversity and function of UGTs is discussed in the following sections.

1.1. Geological history of Australia

In order to appreciate the significance of Australia's unique indigenous fauna it is necessary to have some understanding of the geological and biological history of the continent. During the early Jurassic period (190 million years ago) the Australian continent comprised part of the great southern super-continent known as Gondwana (Strahan, 1983). At this time, Gondwana also included the landmasses now recognised as Africa, South America, India, Antarctica, New Zealand and Madagascar (Strahan, 1983). Due to the warm global temperatures existing during this period, the supercontinent was covered with dense tropical rainforests, remnants of which can be seen today in Tasmania and in isolated locations of Victoria and Queensland (Nedin, 1994). Approximately 160 million years ago, Africa began to separate from east Gondwana through continental drift and move northwards, eventually colliding into southern Europe (Strahan, 1983). Then, by the late Jurassic (130 million years ago), India split from Western Australia, drifted northwards and collided with Asia resulting in the formation of the Himalayas (Strahan, 1983). New Zealand separated from the remaining continents and started moving eastwards into the Pacific Ocean about 80 million years ago (Strahan, 1983). The rift between Australia and New Zealand which occurred during this time, resulted in the eastern edge of the continent rising to form the Great Divide (Nedin, 1994). A geological time scale highlighting important time points for animals is provided in **Table 1.1.**

Australia, Antarctica and South America remained attached until about 55 million years ago (Strahan, 1983). Then, during the Miocene epoch, Australia and Antarctica finally split, leaving Australia as a single isolated landmass (Strahan, 1983). The geographical isolation created by the separation of the Australian continent from Antarctica and all other landmasses, enabled the independent evolution of animals and plants within Australia at the time to develop into a unique flora and fauna (Strahan, 1983).

The Australian continent (including New Guinea) continued to move further northwards, a movement which still continues to this today, and enabled some of the tropical rainforest areas to survive the increased aridity that accompanied global cooling. Interestingly, it has been

hypothesised that as a result of this increased aridity, eucalypts and acacias also began to evolve from the rainforest species (Nedin, 1994).

Table 1.1. Geological time scale of evolutionary events (Keeton and Gould, 1986)

Era	Period	Epoch	millions of years ago	Animal life
Cenozoic	Quaternary	Recent	0.01	Rise of civilisations
		Pleistocene	2.5	First *Homo sapiens* Nth/ Sth America redock
		Pliocene	7	
	Tertiary	Miocene	35	Antarctica/ Australia split
		Oligocene	38	Marsupials extinct in Asia/ Nth Africa/ Europe Marsupials in Australia/ Asia
		Eocene	54	Marsupials extinct in Nth America Marsupials reach Antarctica Radiation of placentals in Nth America Sth/ Nth America split
		Palaeocene	65	
	Cretaceous	Late	136	Sth American marsupials First marsupials, Nth America
Mesozoic	Jurassic		190	

The Australian continent, including New Guinea, drifted across the Indian Ocean towards Asia and the northern edge of its continental shelf collided with that of southeastern Asia about 15 million years ago (Strahan, 1983). The resulting geological stress created the New Guinea highlands and enabled the invasion of Asian plants to the northeastern regions of Australia.

The Pliocene era, 2-10 million years ago, was characterised by a period of global warming. Australia was warm and wet during this time, and large lakes occurred in what is now the Nullarbor Plain and lower Murray basin. The Pliocene era also heralded the first appearance of hominoids on the planet. The more modern *Homo sapiens* evolved later during the Pleistocene era,

about 100,000 years ago, and the first aboriginals are believed to have entered Australia from Asia during periods of low sea level between 50,000 and 60,000 years ago. At this time, Australia and New Guinea still formed a single landmass and were only separated from the rest of Asia by narrow channels of sea no wider than seventy kilometres (Strahan, 1983).

1.2. Origins of Australia's marsupial fauna

The native mammals that now comprise the Australian fauna are derived from two sources (Strahan, 1983). The oldest inhabitants, such as marsupials and monotremes, are descendants of animals that were already on the continent when it separated from Antarctica, and continued as passengers while the landmass drifted through the Indian Ocean (Strahan, 1983; Cox, 2000). Bats and rodents, the only naturally occurring placental mammals in Australia, began to move onto the continent, probably through New Guinea first, as the continent drifted towards the Indonesian islands (Strahan, 1983; Cox, 2000).

The evolution of marsupials prior to their isolation on the Australian continent is well understood. Fossilised marsupial remains dating around 100 million years old have been uncovered in North America (Strahan, 1983). It is hypothesised that marsupials then migrated into Europe, North Africa and also Asia by the Oligocene era, however none of these groups were able to survive (Nedin, 1994; Cox, 2000). During the Late Cretaceous period, marsupials had also inhabited South America, where they subsequently evolved into a variety of species while becoming extinct in the northern continent (Nedin, 1994; Cox, 2000). South America still possesses almost one-third of the living species of marsupials, most of which belong to the opossum family, Didelphidae (Strahan, 1983). Although the relationship between them is unclear, it is generally accepted that the Didelphidae family is ancestral to the Australian marsupials (Strahan, 1983; Cox, 2000).

South America separated from North America during the Eocene era, effectively blocking the radiation of placental mammals existing in North America at the time from spreading to South America (Nedin, 1994; Cox, 2000). It was also during the Eocene era that marsupials reached Antarctica, which was still attached to South America and Australia (Nedin, 1994; Cox, 2000).

The discovery in 1982 of a fossil marsupial in Antarctica supports the theory that marsupials migrated to Australia through Antarctica (Strahan, 1983). Although a marsupial fossil has been discovered in Asia, it closely resembles the European didelphid *Peratherium*, and possesses few characteristics of Australian forms (Nedin, 1994). Therefore, colonisation of Australia from the north is not considered a viable theory. Australian marsupials, both living and extinct, share many characteristics with South American marsupials and extinct Antarctic forms, therefore indicating a southern migration route for marsupials and also explaining the lack of placental mammals in Australia (Cox, 2000).

After Australia separated from Antarctica in the Miocene, the marsupial fauna was effectively isolated on the continent (Nedin, 1994; Cox, 2000). During the next 30-40 million years when Australia was drifting across the Indian Ocean, the resident marsupials underwent a significant adaptative radiation (Strahan, 1983). The carnivorous species, such as the quolls, dunnarts and antechinuses, are considered to be the most closely related to the original inhabitants, while the kangaroos, possums and wallabies are examples of the most extensively specialised herbivorous species (Strahan, 1983).

1.3. Classification of Australian mammalian fauna

The Australian mammal fauna comprises a wide variety of animal species each possessing distinguishable characteristics. Each species has been classified into categories according to traditional biological naming systems while taking into account their evolutionary relationships (Strahan, 1983). Three separate groups comprise the Australian mammalian fauna, the monotremes, the marsupials and the placental mammals (Strahan 1983).

1.3.1. Monotremes (subclass Prototheria)

Members of this group belong to only one order, the Monotremata, and include the egg-laying mammals such as the platypus and echidna (Strahan, 1983).

1.3.2. Marsupials (subclass Marsupialia)

Marsupials possess characteristics, both physiological and reproductive, not seen in any other class of animals. For example, marsupials are born as a tiny neonate with few functional organs, and then undergo all of their growth in an air-breathing environment (Tyndale-Biscoe, 1973).

Marsupials have essentially evolved into four main groups: the South American opossums; the carnivorous Australian marsupials; the omnivorous bandicoots; and the herbivorous kangaroos, wallabies and possums (Strahan, 1983). Classified according to the traditional method based on dentition, marsupials are grouped into two orders, the carnivorous Polyprotodonta and the herbivorous Diprotodonta (Strahan 1983). A list of the biological names used to classify Australian marsupials is provided in **Table 1.2.**

Table 1.2. Classification of Marsupials (Strahan, 1983).

CLASS MAMMALIA

SUBCLASS Marsupialia

ORDER Polyprotodonta
- **SUBORDER Didelphimorphia (South American marsupials)**
- **SUBORDER Dasyuromorphia**
  - **SUPERFAMILY Dasyuroidae**
    - **FAMILY Dasyuridae (quolls, dunnarts, antechinuses, planigales, ningauis, Tasmanian Devil)**
    - **FAMILY Thylacinidae (Thylacine)**
    - **FAMILY Myrmecobiidae (Numbat)**
- **SUBORDER Notoryctemorphia**
  - **SUPERFAMILY Notoryctoidae**
    - **FAMILY Notoryctidae (Marsupial Mole)**
- **SUBORDER Peramelomorphia**
  - **SUPERFAMILY Perameloidea**
    - **FAMILY Peramelidae (bandicoots)**
    - **FAMILY Thylacomyidae (bilbies)**

ORDER Diprotodonta
- **SUPERFAMILY Vombatidae**
  - **FAMILY Phascolarctidae (Koala)**
  - **FAMILY Vombatidae (wombats)**
- **SUPERFAMILY Phalangeroidea**
  - **FAMILY Petauridae (ringtail possums, larger gliders)**
  - **FAMILY Phalangeridae (brushtail possum)**
  - **FAMILY Burramyidae (pygmy-possums, feathertail gliders)**
- **SUPERFAMILY Tarsipedoidea**
  - **FAMILY Tarsipedidae (Honey-possum)**
- **SUPERFAMILY Macropodoidea**
  - **FAMILY Potoroidae (potoroos, bettongs, rat-kangaroos)**
  - **FAMILY Macropodidae (kangaroos, wallabies, rock-wallabies, nailtail-wallabies, forest wallabies, hare-wallabies, Swamp Wallaby, pademelons, Quokka, tree-kangaroo)**

1.3.3. Placental mammals (subclass Eutheria)

Clearly the most successful of the three subclasses, placental mammals are simply defined as those mammals not exhibiting characteristics of either monotreme or marsupial species. Of the 17 orders that comprise this subclass, only the bats (order Chiroptera), rats and mice (order Rodentia) and seals (order Pinnipedia) form part of Australia's native fauna (Strahan, 1983).

1.4. Australian marsupials as unique scientific research models

Marsupials, usually only regarded as curiosities by the early settlers, were recognised very early as having the potential to make a significant contribution to the understanding of mammalian reproductive processes (Renfree, 1995). Consequently, work on various aspects of marsupial reproduction was undertaken at the end of the nineteenth century (Griffiths, 1978; Renfree, 1995). During this period several important findings were published. For example, the early development and basic reproductive biology of both monotremes and marsupials was established (Semon, 1894; Bresslau, 1912, 1920; Hill, 1900, 1910; *cited in* Renfree, 1995).

The first description of the phenomenon of embryonic diapause in a marsupial by Sharman (1955) stimulated renewed interest into marsupial scientific research after a long period of dormancy. The principles governing embryonic diapause, the process by which 100 cell embryos are stored by a number of marsupial species for several months during which no growth, cell division or differentiation occurs, have been the focus of intensive studies (Renfree and Tyndale-Biscoe, 1973; Tyndale-Biscoe *et al.*, 1974; Renfree, 1980; Renfree, 1981; Ward, 1988; Ward and Renfree, 1988; Renfree, 1993). During this period, marsupial chromosomes were also found to be relatively few in number (Sharman and Barber, 1952; Sharman, 1961; Hayman and Martin, 1965) and lactation was reported to play a significant role in the control of reproduction (Sharman, 1962, 1965; Tyndale-Biscoe, 1963).

During the last two decades a significant amount of scientific research has been conducted into marsupial reproduction (ie. gametes, fertilisation, sex determination and differentiation, early development), ecology and reproductive physiology (Rodger, 1991; Selwood, 1992; Curlewis, 1994; Selwood, 1994; Hinds, 1994; Taggart, 1994; Temple-Smith, 1994; Renfree, 1995).

Consequently, these areas are now well understood (Hinds, 1994; Renfree, 1995). The long lactation and dependence of the young on the mother, combined with the accessibility of the developing organ systems have made the marsupial a valuable tool in the study of mammalian reproduction and development (Renfree, 1992).

Finally, various aspects of marsupial research are slowly eroding the misconception that marsupials only represent a simple subclass of mammals and the only value in studying them is to provide an insight into the evolutionary pathways of eutherian mammals. The establishment and sustenance of a number of marsupial species as captively bred colonies, for example the tammar wallaby (*Macropus eugenii*), South American opossum (*Monodelphis domestica*) and Fat-tailed Dunnart (*Sminthopsis crassicaudata*), at a number of Australian universities can only further promote their research potential (Saunders and Hinds, 1997).

1.5. Metabolism of xenobiotics

On any given day, an average person may be exposed to literally thousands of different molecules. Some of these substances, such as carbohydrates and proteins, are necessary sources of nutrition and are processed in refined biochemical systems. However, humans and other biological organisms are also exposed throughout their lifetime to a vast array of xenobiotics including pharmacological drugs, environmental pollutants and natural toxins. Although the immune system plays an important role in the elimination of undesirable molecules, organisms have evolved unique detoxication mechanisms involving a variety of enzyme systems to facilitate the detoxication and removal of xenobiotic compounds encountered throughout their lifetime. The enzymatic transformations of a foreign compound that result in its detoxication, or loss of pharmacological activity, are traditionally classified into functionalisation (phase I) and conjugation (phase II) reactions. Functionalisation reactions include oxidation, hydroxylation, reduction and hydrolysis and in the process of metabolism sometimes create a more toxic metabolite, a process termed bioactivation. Functionalisation reactions are predominantly catalysed by the cytochrome P450 system, although other enzymes including xanthine oxidases, alcohol and aldehyde dehydrogenases, amine oxidases and flavin-containing monooxygenases are sometimes involved (Armstrong, 1987; Kalant and Roschlau, 1989). In these enzymatic reactions,

a new functional group (usually an oxygen atom) is introduced into the substrate molecule or an existing functional group is modified, thus making the xenobiotic more polar and readily excreted.

Conjugation (phase II) reactions are enzymatic syntheses whereby a functional group is masked by the addition of a new hydrophilic endogenous group, which can include acetate, sulphate or glucuronic acid, to facilitate excretion. Phase II reactions can be catalysed by a number of different enzyme families including UDP glucuronosyltransferases, N-acetyltransferases, glutathione *S*-transferases, sulphotransferases, methyltransferases and hydrolyases (Armstrong, 1987; Kato *et al.*, 1989). The majority of xenobiotic molecules undergo both functionalisation and conjugation reactions sequentially, with the products usually being less active biologically.

The oxidation of xenobiotic substrates in Phase I metabolism is almost exclusively catalysed by a family of membrane-bound haemoproteins located in the smooth endoplasmic reticulum of the liver and other extrahepatic tissues, collectively termed cytochromes P450 (CYPs). The cytochrome P450 family also catalyses the oxidative metabolism of a variety of endogenous substrates, including steroids, fatty acids, prostaglandins and bile acids. The cytochrome P450 enzyme system exhibits broad substrate specificity with multiple CYP isoforms possessing diverse and sometimes overlapping substrate specificities. CYP proteins are located primarily in the endoplasmic reticulum where they utilise electrons from reduced nicotinamide adenine dinucleotide (NADPH) to insert an oxygen atom into a substrate. CYPs have also been isolated in the mitochondria of eukaryotic organisms where they are involved in the metabolism of cholesterol and its derivatives. A current review of the cytochrome P450 enzyme family, including historical aspects, structure and diversity, is provided in the following sections.

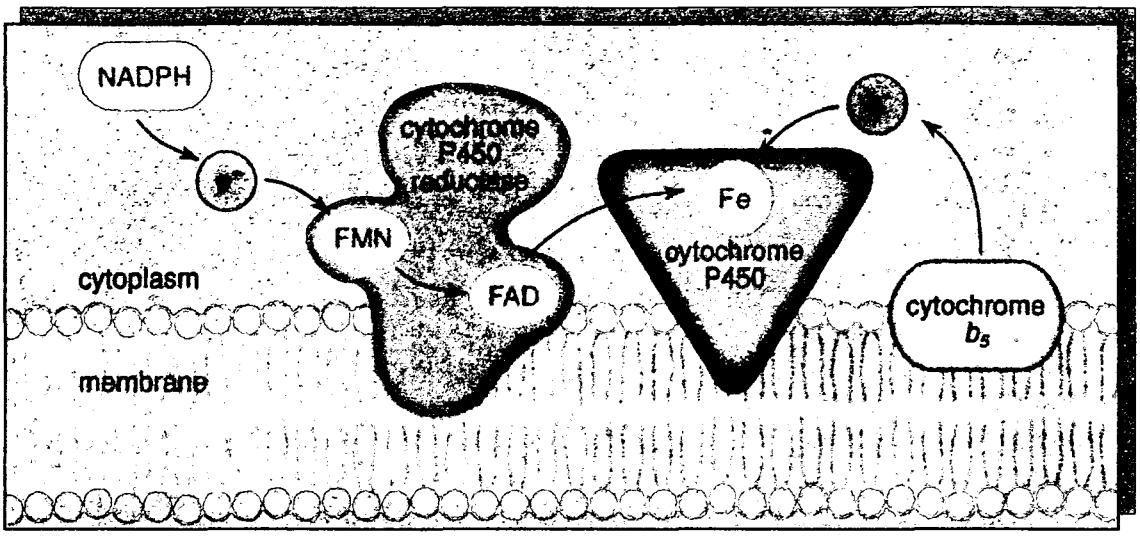
1.6. Cytochromes P450

1.6.1. History

The exploration in the 1940s of a unique class of enzymatic reactions that involved molecular oxygen, now known as mixed-function oxidation reactions, marked the discovery of an enzyme system which is still being elucidated to this day. The enzyme system, observed in microsomal preparations, utilised the insertion of an oxygen atom into a substrate through the collaboration of O_2 and a reducing equivalent from NADPH (Sweat and Lipscomb, 1955; Ryan and Engel, 1956; Conney *et al.*, 1956; Conney *et al.*, 1957; Brodie *et al.*, 1958).

The term "cytochrome P450" was first used in the naming and characterisation of a microsomal membrane-bound haemoprotein possessing a unique 450nm optical absorption peak of its carbon monoxide-bound form, which had no known physiological functions (Omura and Sato, 1961; Omura and Sato, 1964a; Omura and Sato, 1964b). However, the existence of an unknown pigment with a unique CO binding region and spectra which was highly unstable to solubilisation treatments with detergents, was previously observed by both Klingenberg (1958) and Garfinkle (1958) in rat liver hepatic microsomes. The key enzymatic components of the cytochrome P450-mediated oxidation system were first reported by Lu and Coon, 1968. The oxidase system was resolved into three major components, a cytochrome P450 haemoprotein, a flavoprotein termed NADPH-cytochrome P450 reductase, and phospholipids from the cell membrane.

Figure 1.1. Relative components and interactions of the mixed function oxidase system



The molecular constituents of the cytochrome P450 system reside within the membrane of the endoplasmic reticulum. Before the cytochrome P450 enzyme *(blue)* can oxidise a substrate, the iron atom in the enzyme's haem group must accept two electrons from either the nicotinamide-adenine dinucleotide phosphate (NADPH) molecule *(orange)* located in the cytoplasm, or the cytochrome b_5 molecule *(aqua)* embedded in the membrane. The NADPH-cytochrome P450 reductase protein *(purple)* which comprises two subcomponents, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), acts as an intermediary in the transfer of electrons from NADPH (reproduced from Guengerich, 1993).

1.6.2. Structure

Cytochromes P450 of eukaryotic organisms are tightly embedded in the membranes of the endoplasmic reticulum or mitochondria, whereas the bacterial CYPs are cytoplasmic. The hydrophobic character of eukaryotic CYPs has made it extremely difficult to crystallise the protein, due to its tendency to aggregate rather than form crystals. Consequently, the first CYP to be purified and crystallized was a water-soluble bacterial CYP, CYP101 (or $P450_{cam}$) (Dus *et al.*, 1970; Yu and Gunsalus, 1974). The tertiary molecular structure of CYP101 has since been elucidated by X-ray crystallography (see Figure 1.2) (Poulos *et al.*, 1985; Poulos *et al.*, 1987). The general structure of CYP101 represented an asymmetrical triangular prism consisting of 12 α-helices and 5 anti-parallel β-sheets (Poulos *et al.*, 1985). The haem was found to be situated

between two long α-helices, at the bottom of a large internal pocket which is surrounded by hydrophobic amino acid residues to accommodate hydrophobic substrates (Poulos *et al.*, 1985). The recent purification and X-ray crystallographic analysis of several other bacterial CYPs have revealed similar overall structures (Graham and Pedersen, 1999). Williams *et al* (2000) recently reported the first successful determination of the tertiary structure of an animal microsomal CYP, CYP2C5. It confirmed that the overall conformation of eukaryotic membrane-bound CYPs is similar to that of the previously described bacterial CYPs. Finally, all microsomal CYP enzymes possess a highly hydrophobic "signal anchor sequence" composed of 20-25 amino acid residues at their amino-terminus, which targets and anchors the molecule to the membrane bilayer (Sakaguchi *et al.*, 1987).

Figure 1.2. Three dimensional structure of the bacterial CYP, CYP101



The enzyme contains two binding sites, one for a molecule of oxygen at its haem group *(pink and blue honeycomb structure)* and the other for the substrate it modifies. Structural twists of the constituent amino acids result in alpha helices *(yellow cylinders)* and beta sheets *(green ribbons with arrows)* (reproduced from Guengerich, 1993).

1.6.3. Regulation

The induction of some particular forms of cytochrome P450 by foreign chemical compounds has been the focus of intensive studies for more than 30 years. The first indication of the inducibility of CYPs was the finding that rats treated with the polycyclic aromatic hydrocarbon, 3-methylcholanthrene, exhibited an increased capacity to metabolise certain aminoazo dyes (Conney *et al.*, 1956). The enzyme activities induced by 3-methylcholanthrene include the aryl hydrocarbon hydroxylase (AHH), now termed CYP1A1 (Conney *et al.*, 1957; Nebert *et al.*, 1972). The specific induction of CYPs by xenobiotic compounds has provided strong evidence that the principal role of some CYPs, primarily from families CYP1, CYP2 and CYP3, is the metabolism and detoxication of those and related compounds from the body (Conney, 1967; Omura, 1999).

Each known inducing compound exhibits a characteristic pattern of CYP enzyme induction. Although a single form of CYP often accounts for most of the total CYP content in microsomes after induction by a foreign compound, the expression of other CYP forms can also be affected. The induction of these secondary targets, although expressed in smaller amounts than the major form following induction, can significantly affect the reaction rates they catalyse in microsomes.

1.6.4. Diversity and nomenclature

Cytochrome P450 is now the collective name of a large superfamily of haemoproteins which is thought to have diversified from a single ancestral protein during the course of evolution (Nebert *et al.*, 1987; Nebert and McKinnon, 1994). Modern advances in molecular biology techniques initiated an explosion of research into the field of cytochrome P450 enzymes (Nebert *et al.*, 1987; Nebert and Gonzalez, 1987; Gonzalez, 1990). The plethora of trivial names and increasing confusion with the identity of new *CYP* genes, particularly to those outside of the field, led to the development of a standard nomenclature system for the *CYP* gene superfamily based on divergent evolution (Nebert *et al.*, 1987). CYP enzymes are now classified into different families in accordance with the degree of homology of amino acid sequence in their protein structures (Nebert *et al.*, 1987). CYP enzymes exhibiting <40% homology in their amino acid sequence are classified in different families which are designated by Arabic numbers, for example, CYP1. Each family is further divided into subfamilies of enzymes. The enzymes within a mammalian

subfamily possess >55% sequence homology and are designated by capital letters, for example, CYP1A (Nebert *et al.*, 1987). Arabic numbers are used for designating individual enzymes within a subfamily, eg. CYP1A1 and CYP1A2, and enzymes are generally numbered in the order in which they are identified. The CYP nomenclature system is updated regularly, and subsequently has been extended and reviewed in four published listings (Nebert *et al.*, 1989b; Nebert *et al.*, 1991; Nelson *et al.*, 1993; Nelson *et al.*, 1996). It is now apparent from the examination of numerous mammalian and prokaryotic CYP genes, where several regions exhibit similarity, that the CYP superfamily is ancient and has evolved from a single ancestral form (Nebert and Gonzalez, 1987; Nelson and Strobel, 1987; Gonzalez, 1988; Nebert *et al.*, 1989a; Gonzalez and Nebert, 1990). Although difficult to accurately estimate, it has been proposed that the ancestral *CYP* gene existed more than 3 billion years ago (Nelson and Strobel, 1987; Gonzalez, 1990; Gonzalez and Nebert, 1990; Rendic and Di Carlo, 1997). All CYPs identified to date exhibit a conserved 10 amino acid "signature" motif which includes the invariant cysteine residue that ligates the haem iron (Nelson *et al.*, 1993; Nelson *et al.*, 1996).

According to the latest published CYP update, 481 genes and 22 pseudogenes have been identified from 85 eukaryote (including vertebrates, invertebrates, fungi and plants) and 20 prokaryote species (Nelson *et al.*, 1996). Due to the pace of CYP research, a database of newly identified CYPs is maintained at http://drnelson.utmem.edu/CytochromeP450.html. According to this database, 17 mammalian CYP families comprising 37 subfamilies have now been identified. An overview of the vertebrate CYP families with their relative enzyme functions identified to date is provided in **Table 1.3**.

Table 1.3. Vertebrate cytochrome P450 families (Nelson *et al.*, 1996).

FAMILY	PHYLUM IDENTIFIED	FUNCTION
CYP1	**Vertebrates**	**xenobiotic metabolism**
CYP2	**Vertebrates**	**drug/xenobiotic metabolism**
CYP3	**Vertebrates**	**drug/xenobiotic metabolism**
CYP4	**Vertebrates**	**fatty-acid hydroxylases**
CYP5	**Vertebrates**	**thromboxane synthase**
CYP7A	**Vertebrates**	**cholesterol 7α-hydroxylase**
CYP7B	**Vertebrates**	**unknown function(s)**
CYP8	**Vertebrates**	**prostacyclin synthase**
CYP11	**Vertebrates**	**cholesterol side-chain cleavage, steroid 11β-hydroxylase, and aldosterone synthase (mitochondrial enzyme)**
CYP17	**Vertebrates**	**steroid 17α-hydroxylase**
CYP19	**Vertebrates**	**aromatisation of androgens**
CYP21	**Vertebrates**	**steroid 21-hydroxylase**
CYP24	**Vertebrates**	**steroid 24-hydroxylase**
CYP26	**Vertebrates**	**retinoic acid metabolism**
CYP27	**Vertebrates**	**steroid 27-hydroxylase**
CYP39	**Vertebrates**	**unknown**
CYP41	**Vertebrates**	**cholesterol 24-hydroxylase**
CYP51	**Vertebrates, Invertebrates**	**sterol biosynthesis**

In mammals, CYPs can be functionally subdivided into two major classes; those involved in the synthesis of steroids and other endogenous substrates and those that primarily metabolise xenobiotics (Gonzalez, 1990; Coon *et al.*, 1992; Omura, 1999). It is now clear that CYPs also play important roles in the maintenance of the steady-state levels of endogenous ligands involved in the transcription of genes effecting growth, differentiation and other critical cellular processes (Nebert, 1994). It is now generally accepted that the steroidogenic and cholesterol-metabolising CYPs were amongst the earliest to emerge, and probably evolved in early organisms to synthesise lipids and steroids needed for the maintainenance of the plasma membrane (Nebert and Gonzalez, 1985; Gonzalez, 1988; Gonzalez, 1990; Omura, 1999). All of these enzymes are well conserved in mammals with regards to their catalytic activities and substrate specificities, as any variation through mutational events would have been highly deleterious or fatal (Gonzalez, 1990).

The xenobiotic-metabolising CYPs probably diverged from the steroidogenic CYPs about 900 million years ago (Nelson and Strobel, 1987; Gonzalez, 1988; Gonzalez, 1990). The apparent explosion of many new *CYP* genes during the past 800 million years, particularly in the *CYP2* gene family, has been related to the emergence of mammals onto land, and subsequent 'animal-plant warfare' (Nelson and Strobel, 1987; Gonzalez, 1988; Nebert *et al.*, 1989; Gonzalez, 1990; Oguri *et al.*, 1994). As animals diverged from plants then began to utilise them as a food source, the plants evolved toxic and undigestable compounds to ensure their survival (Nelson and Strobel, 1987; Gonzalez, 1990). In return, animals responded by developing new enzymes to metabolise the new toxic plant compounds (Nelson and Strobel, 1987; Gonzalez, 1990; Nebert and McKinnon, 1994). As many pesticides and modern drugs are derived from plant metabolites or exhibit similar chemical structures, it is hardly surprising that animals possess a sufficiently diverse array of CYP enzymes to handle almost any newly synthesised chemical (Nebert *et al.*, 1989; Gonzalez, 1990; Gonzalez and Gelboin, 1994).

Cytochrome P450 genes within the *CYP1*, *CYP2*, *CYP3* and *CYP4* families predominantly encode for microsomal enzymes involved in the metabolism and detoxication of xenobiotic compounds. These CYPs are primarily expressed in hepatic tissue but can also be found to a lesser extent in tissues which are in contact with the outside of the body (eg. lung, small intestine, skin) (Omura, 1999). The specific induction of several CYP forms, particularly in the *CYP1* and *CYP2* gene families, by certain xenobiotics has enhanced the view that their principle role is the detoxication of foreign compounds (Nebert and McKinnon, 1994; Omura, 1999). The following section will be limited to a discussion on xenobiotic-metabolising mammalian CYPs within the *CYP2C* gene subfamily.

1.6.5. *CYP2* Family

The *CYP2* gene family is the most complex and diverse CYP family, and has undergone a number of gene duplication events during the course of evolution (Nebert *et al.*, 1989). The *CYP2* gene family currently comprises 20 subfamilies (*CYP2A*, *CYP2B*, *CYP2C*, *CYP2D*, *CYP2E*, *CYP2F*, *CYP2G*, *CYP2H*, *CYP2J*, *CYP2K*, *CYP2L*, *CYP2M*, *CYP2N*, *CYP2P*, *CYP2Q*, *CYP2R*, *CYP2S*, *CYP2T*, *CYP2U*, *CYP2V*) (Nelson, 2000). It is hypothesised that the radiation of the CYP2 family occurred around 370 million years ago, in response to animal-plant warfare (Gonzalez and Nebert, 1990; Lewis *et al.*, 1998). Further expansion of the CYP2 family is estimated to have taken place 230 million years ago during the emergence of mammalian species (Lewis *et al.*, 1998). It is postulated that the CYP2A, CYP2B, CYP2C and CYP2E subfamilies diverged during this period (Lewis *et al.*, 1998). The various subfamilies of the CYP2 family exhibit differing, but sometimes overlapping substrate specificities (Lewis *et al.*, 1998).

1.6.5.1. *CYP2C* subfamily

The *CYP2C* gene subfamily represents the largest *CYP* subfamily thus far identified in experimental animals. Members of this subfamily are generally constitutively expressed in hepatic and extrahepatic tissues (Gonzalez, 1990; Zaphiropoulos and Wood, 1993; Nebert and McKinnon, 1994; Zaphiropoulos *et al.*, 1995). Currently, the *CYP2C* subfamily consists of 42 genes (9 rabbit, 8 rat, 6 pig, 6 mouse, 4 hamster, 4 human, 2 monkey, 1 dog, 1 goat, 1 chicken) and 2 pseudogenes (1 rat, 1 pig) (Nelson *et al.*, 1996; Nelson, 2000). A list of mammalian *CYP2C* genes characterised to date with the corresponding gene numbers and references is provided in **Table 1.4.** Investigations in the rat and rabbit have further demonstrated that several *CYP2C* genes are regulated in a developmental and sex-specific manner (Dannan *et al.*, 1986; Gonzalez *et al.*, 1986; Yoshioka *et al.*, 1987; Zaphiropoulos *et al.*, 1988; McClellan-Green *et al.*, 1989).

Table 1.4. A list of mammalian *CYP2C* genes (Nelson, 2000)

GENE SYMBOL	SPECIES	REFERENCE
CYP2C1	rabbit	Zhao *et al.*, 1990; Genbank D26152
CYP2C2	rabbit	GenBank K01521
CYP2C3	rabbit	Chan and Kemper, 1990
CYP2C4	rabbit	Zhao *et al.*, 1990
CYP2C5	rabbit	Zhao *et al.*, 1987; GenBank M55664
CYP2C6	rat	Imaoka *et al.*, 1987; PIR A41425
CYP2C7	rat	Westin *et al.*, 1990; GenBank X12595
CYP2C8	human	Ged and Beaune, 1991; Genbank Y00498
CYP2C9	human	Ohgiya *et al.*, 1992; Genbank S46963
CYP2C11	rat	Yoshioka *et al.*, 1987; GenBank U33173
CYP2C12	rat	Matsumoto *et al.*, 1986
CYP2C13	rat	McClellan-Green *et al.*, 1989; GenBank X79810
CYP2C14	rabbit	GenBank D00190
CYP2C15	rabbit	GenBank M19234
CYP2C16	rabbit	GenBank M18376
CYP2C18	human	Romkes *et al.*, 1991; GenBank L16876
CYP2C19	human	Romkes *et al.*, 1991; Swiss P33261
CYP2C20	monkey	Komori *et al.*, 1992; GenBank S53046
CYP2C21	dog	PIR A37222
CYP2C22	rat	Nagata *et al.*, 1987
CYP2C23	rat	Karara *et al.*, 1993; GenBank U04733
CYP2C24	rat	Zaphiropoulos, 1991
CYP2C25	hamster	Sakuma *et al.*, 1994; GenBank X63022
CYP2C26	hamster	Sakuma *et al.*, 1994; GenBank D11435
CYP2C27	hamster	Sakuma *et al.*, 1994; GenBank D11436
CYP2C28	hamster	Sakuma *et al.*, 1994; GenBank D11437
Cyp2c29	mouse	Matsunaga *et al.*, 1994; Genbank D17674
CYP2C30	rabbit	Noshiro, unpublished; Genbank D26153
CYP2C31	goat	Zeilmaker *et al.*, 1994
CYP2C32	pig	Zaphiropoulos *et al.*, 1995
CYP2C33	pig	Zaphiropoulos *et al.*, 1995
CYP2C34	pig	Zaphiropoulos *et al.*, 1995
CYP2C35	pig	Zaphiropoulos *et al.*, 1995
CYP2C36	pig	Zaphiropoulos *et al.*, 1995
Cyp2c37	mouse	Luo *et al.*, 1998
Cyp2c38	mouse	Luo *et al.*, 1998
Cyp2c39	mouse	Luo *et al.*, 1998
Cyp2c40	mouse	Luo *et al.*, 1998
CYP2C41	dog	Bai, unpublished; no acc. no.
CYP2C42	pig	Nissen *et al.*, 1998; Genbank Z93098
CYP2C42P1	pig	Nissen *et al.*, 1998; GenBank Z93100
CYP2C43	monkey	Ohmori, unpublished; no acc. no.
Cyp2c44	mouse	Helvig, unpublished; no acc. no.
CYP2C45	chicken	Baader, unpublished; no acc. no.

Three forms corresponding to the *CYP2C* subfamily were initially isolated in the rabbit (Leighton *et al.*, 1984). To date, a total of eight forms have been identified in this species from a variety of

Three forms corresponding to the *CYP2C* subfamily were initially isolated in the rabbit (Leighton *et al.*, 1984). To date, a total of eight forms have been identified in this species from a variety of tissues (Leighton and Kemper, 1984; Tukey *et al.*, 1985; Johnson *et al.*, 1987; Imai *et al.*, 1988; Hassett and Omiecinski, 1990; Pendurthi *et al.*, 1990).

A number of constitutively expressed rat *CYP2C* cDNAs have also been isolated and characterised (Stroem *et al.*, 1988; Legraverend *et al.*, 1994). Waxman and Walsh (1984) and Ryan *et al.* (1984) both reported the isolation of developmentally regulated rat CYP2C enzymes, now designated CYP2C6 and CYP2C7 respectively, which exhibited broad substrate specificities. An adult male specific rat CYP, CYP2C11, has also been characterised by several laboratories (Cheng and Schenkman, 1982; Guengerich *et al.*, 1982; Kamataki *et al.*, 1983; Waxman, 1984; Matsumoto *et al.*, 1986). Members of the rat CYP2C subfamily exhibit stereoselectivity in the hydroxylation of steroids, notably testosterone. Imaoka *et al.* (1993) have also reported the identification of a constitutively expressed *CYP2C* cDNA in rat kidney, termed *CYP2C23*, which was found to catalyse the epoxygenation of arachidonic acid when expressed using baculovirus.

The human *CYP2C* subfamily has been studied extensively due to the existence of at least two genetic polymorphisms, one associated with the metabolism of mephenytoin (Wilkinson *et al.*, 1989), and a much rarer polymorphism in the metabolism of tolbutamide and phenytoin (Kutt *et al.*, 1964; Scott and Poffenbarger, 1979; Vasko *et al.*, 1980; Vermeij *et al.*, 1988; Page *et al.*, 1991). Human enzymes belonging to the CYP2C subfamily have been purified by a number of different laboratories (Wang *et al.*; 1983: Gut *et al.*, 1986: Shimada *et al.*, 1986; Wrighton *et al.*, 1987; Ged *et al.*, 1988; Wrighton *et al.*, 1993). Four members of the human *CYP2C* subfamily, *CYP2C8*, *CYP2C9*, *CYP2C18*, *CYP2C19*, have been identified to date (Okino *et al.*, 1987; Umbenhauer *et al.*, 1987; Ged *et al.*, 1988; Romkes *et al.*, 1991). *CYP2C9* was initially thought to encode the major *S*-mephenytoin hydroxylase enzyme, however subsequent cDNA expression studies have indicated that *CYP2C9* and its allelic variants exhibit minimal catalytic activity toward *S*-mephenytoin (Kimura *et al.*, 1987; Ged *et al.*, 1988). More recent investigations have reported that CYP2C9 has a high catalytic activity towards tolbutamide, and is the major tolbutamide hydroxylase enzyme (Brian *et al.*, 1989; Relling *et al.*, 1990; Srivastava *et al.*, 1991; Goldstein *et al.*,

1994). Finally, the metabolism of mephenytoin has been correlated to the more recently purified CYP2C19 enzyme, even though it is present in much lower concentrations in human liver than either CYP2C8 or CYP2C9 (Wrighton *et al.*, 1993; Goldstein *et al.*, 1994).

A recent investigation into the extrahepatic tissue distribution of mRNAs for the four human CYP2Cs detected expression in a variety of tissues, including brain, uterus, kidney, ovary and duodenum (Klose *et al.*, 1999). However, CYP2C19 mRNA expression was found only in the duodenum (Klose *et al.*, 1999). The developmental regulation of the human CY2C subfamily has also been investigated in foetal, newborn, infant and adult liver samples (Ratanasavanh *et al.*, 1991; Treluyer *et al.*, 1997). CYP2C proteins were not detected in foetal human livers by immunoblotting, but dramatically increased in the first few weeks after birth (Ratanasavanh *et al.*, 1991; Treluyer *et al.*, 1997). Similarly, the hydroxylation of tolbutamide, a marker for CYP2C9 was not detected in foetal microsomes, but rose during the first month after birth (Treluyer *et al.*, 1997).

Four new members of the mouse CYP2C subfamily have been cloned and characterised (Luo *et al.*, 1998). CYP2C37, CYP2C38, CYP2C39 and CYP2C40 all metabolised arachidonic acid with differing metabolic profiles and catalytic rates (Luo *et al.*, 1998). CYP2C38 and CYP2C40 mRNA was detected in a number of extrahepatic tissues, while CYP2C37 and CYP2C39 mRNAs were only detected in the liver (Luo *et al.*, 1998).

Four distinct *CYP2C* cDNAs, *CYP2C25*, *CYP2C26*, *CYP2C27* and *CYP2C28*, have also been cloned from the hamster (Sakuma *et al.*, 1994; Sakuma *et al.*, 1995). CYP2C28 protein expressed in yeast catalysed the methylation of aminopyrine and benzphetamine, but did not catalyse the hydroxylation of tolbutamide, testosterone or progesterone (Sakuma *et al.*, 1995). Each of the hamster CYP2C forms was induced by phenobarbital, while 3-methylcholanthrene only induced the expression of CYP2C25 and CYP2C26 (Sakuma *et al.*, 1995).

1.7. UDP glucuronosyltransferases (UGTs)

The mammalian UDP glucuronosyltransferases are endoplasmic reticulum-bound enzymes which catalyse the conjugation of a glycosyl group from a nucleotide sugar to a small hydrophobic molecule (aglycone) (Mackenzie *et al.*, 1997).

1.7.1. Structure

UGT enzymes are localised in the membrane of the endoplasmic reticulum and evidence to date supports the view that the UGT catalytic site faces the lumenal side of the ER. It is hypothesised that the ER membrane acts as a permeability barrier between the substrate and the active site of the enzyme located in the lumen of the ER. This model agrees with the finding that all UGT activities are highly latent in the native environment (Vanstapel and Blanckaert, 1988; Shepherd *et al.*, 1989; Mackenzie, 1991).

1.7.2. Diversity and nomenclature

Whilst initial evidence for the existence of UGTs came via enzyme activity and protein purification studies, the isolation of cDNAs encoding UGTs has enabled researchers to recognise the full extent of their expression and regulation. The initial cDNA cloning of three *UGT* cDNAs, *UGT2B1*, *UGT2B3* and *UGT2B4*, from rats in 1984 has since been followed by the identification of a number of *UGTs* in a variety of species using cDNA cloning techniques (Mackenzie *et al.*, 1984; Mackenzie *et al.*, 1997).

The accompanying confusion in the naming of newly characterised UGTs led to the development of a unified nomenclature system based on divergent evolution (Burchell *et al.*, 1991). UGT genes are now named in a similar fashion to that previously explained for the cytochrome P450 gene superfamily (Nebert *et al.*, 1987; Burchell *et al.*, 1991). The first review of the new nomenclature system for the UDP glucuronosyltransferase gene superfamily appeared in 1991, and has been updated once (Burchell *et al.*, 1991; Mackenzie *et al.*, 1997). From the latest update, 110 *UGT* cDNAs comprising 33 families have been identified in a number of bacterial, plant and animal species (Mackenzie *et al.*, 1997). In mammals, 47 cDNAs/genes comprising 3 families, *UGT1*, *UGT2* and *UGT8*, have been described (Mackenzie *et al.*, 1997. A list of the

mammalian *UGT* families and subfamilies characterised to date with the corresponding gene numbers and references is shown in **Table 1.5.**

All UDP glucuronosyltransferases so far identified contain a conserved 44 amino acid "signature sequence" in their carboxy-terminal halves (Mackenzie *et al.*, 1997). It has been hypothesised that this region is involved in the binding of the UDP moiety of the nucleotide sugar, as it exhibits some similarity to regions of other nucleotide-binding sugars (Mackenzie *et al.*, 1997). It also appears from the comparison of plant, animal and bacterial sequences that UGTs have evolved from a single ancestral gene before the divergence of eukaryotes and prokaryotes (Mackenzie, 1995; Mackenzie *et al.*, 1997). Due to the ancestry of the *UGT* gene superfamily, it has been postulated that they may play an important role in maintaining the steady-state levels of endogenous ligands involved in ligand-modulated transcription of genes that cause growth, differentiation and cellular homeostasis (Mackenzie *et al.*, 1997). Similar hypotheses have been previously been put forward for other families of xenobiotic-metabolising enzymes, including the cytochromes P450.

Table 1.5. A list of mammalian *UGT* families and subfamilies (Mackenzie *et al.*, 1997)

GENE SYMBOL	SPECIES	REFERENCE
UGT1 families		
UGT1A1	Rat	Iyanagi, 1991
	Human	Ritter *et al.*, 1991
Ugt1a1	Mouse	Kong *et al.*, 1993; Genbank L02333
UGT1A2	Rat	Sato *et al.*, 1990; Genbank M34007
	Rat	Emi *et al.*, 1995; GenBank D38066
UGT1A2p	Human	Ritter *et al.*, 1992b
UGT1A3	Rat	Emi *et al.*, 1995
	Human	Ritter *et al.*, 1992b
UGT1A4p	Rat	Emi *et al.*, 1995
UGT1A04	Rabbit	Phillip *et al.*, 1994
UGT1A4	Human	Ritter *et al.*, 1991
UGT1A5	Rat	Emi *et al.*, 1995
	Human	Ritter *et al.*, 1992b
UGT1A6	Rat	Iyanagi *et al.*, 1986 and Jackson *et al.*, 1988
	Human	Harding *et al.*, 1988
	Rabbit	Lamb *et al.*, 1994
Ugt1a6	Mouse	Lamb *et al.*, 1994
UGT1A7	Rat	Emi *et al.*, 1995
	Human	GenBank U39570
UGT1A8	Rat	Emi *et al.*, 1995
	Human	GenBank U42604
UGT1A9p	Rat	Emi *et al.*, 1995
UGT1A9	Human	Wooster *et al.*, 1991; Genbank AF056188
Ugt1a9	Mouse	Lamb *et al.*, 1994
UGT1A10	Human	GenBank U39550
UGT1A11p	Human	GenBank U39551
UGT1A12p	Human	GenBank U39552
UGT2 families		
UGT2A1	Rat	Lazard *et al.*, 1991; Genbank X57565
	Human	GenBank AJ006054
UGT2B1	Rat	Mackenzie *et al.*, 1986
UGT2B2	Rat	Mackenzie *et al.*, 1986
UGT2B3	Rat	Mackenzie, 1986
UGT2B4	Human	Jackson *et al.*, 1987
Ugt2b5	Mouse	Kimura and Owens, 1987
UGT2B6	Rat	Mackenzie, 1990b
UGT2B7	Human	Ritter *et al.*, 1990b
UGT2B8	Rat	GenBank U27518
UGT2B9	Monkey	Belanger *et al.*, 1997
UGT2B10	Human	Jin *et al.*, 1993
UGT2B12	Rat	Green *et al.*, 1995
UGT2B13	Rabbit	Tukey *et al.*, 1993; GenBank L01081
UGT2B14	Rabbit	Tukey *et al.*, 1993
UGT2B15	Human	Chen *et al.*, 1993
UGT2B16	Rabbit	Li *et al.*, 1997
UGT2B17	Human	Beaulieu *et al.*, 1996
UGT2C1	Rabbit	GenBank L01083

1.7.3. The *UGT1* family

The *UGT1* gene locus is unique in that it comprises at least 12 first exons (exons 1) that have their own promoter and appear to be capable of splicing with a common set of 4 downstream exons (exons 2-5). This results in each *UGT1* gene product exhibiting different *N*-terminal halves but identical C-terminal halves (Owens and Ritter, 1992; Jansen *et al.*, 1992). Use of the promoter and first exon closest to the four common exons (exons 2-5) results in the *UGT1A1* gene, and accordingly use of the twelfth promoter and first exon combined with exons 2 through 5 results in the *UGT1A12* gene (Mackenzie *et al.*, 1997). Three of the 12 promoter/exons, *UGT1A2p*, *UGT1A11p* and *UGT1A12p* were found to encode pseudogenes (Mackenzie *et al.*, 1997). In the latest UGT superfamily update, each first exon has been designated as a distinct gene (e.g. *UGT1A1*, *UGT1A2*,.....*UGT1A12*), even though the carboxy-terminal half of each of the genes is identical (Mackenzie *et al.*, 1997).

The *UGT1* gene family comprises two subfamilies, *UGT1A* and *UGT1B*, with only *UGT1A* being isolated in mammalian species examined to date (Mackenzie *et al.*, 1997). To date, *UGT1* forms have only been identified in humans (12 forms), rat (10 forms), mouse (3 forms) and rabbit (2 forms) (Mackenzie *et al.*, 1997). The UGT1 family of enzymes exhibit distinct, but overlapping substrate specificities and include forms that are capable of glucuronidating bilirubin, quinones and phenols (Jansen *et al.*, 1992). Investigations into human UGT1 forms have revealed that the glucuronidation of steroids is not a significant feature.

1.7.4. The *UGT2* family

From the latest nomenclature review, the *UGT2* gene family comprises three subfamilies, *UGT2A*, *UGT2B* and *UGT2C* (Mackenzie *et al.*, 1997). To date, the *UGT2* family consists of 20 genes (8 rat, 5 human, 4 rabbit, 1 mouse, 1 monkey and 1 cow) (Mackenzie *et al.*, 1997). Members of the UGT2 family, unlike UGT1 forms, appear to be encoded by separate genes and are involved in the glucuronidation of endogenous steroids and biogenic amines (Mackenzie *et al.*, 1997).

1.8. Xenobiotic metabolism in Australian marsupials

1.8.1. Phase I metabolism- mixed function oxidase system

Investigations into the enzymatic mechanisms governing the ability of any marsupial species to metabolise xenobiotics have been scarce. McManus and Ilett (1976) investigated various components of the hepatic oxidative metabolism pathway of an Australian marsupial, the quokka (*Setonix brachyurus*). Focussing primarily on the mixed function oxidase (MFO) system, they reported that the quokka generally had lower rates of oxidative drug metabolism when compared to the rat (McManus and Ilett, 1976). The authors postulated that this was due to the lower metabolic rate exhibited by marsupials in comparison to other eutherian mammals (Brown and Main, 1967; Kinnear and Brown, 1967; McManus and Ilett, 1976). However, measurements of some components of the cytochrome P450 pathway in hepatic microsomes revealed that both male and female quokkas exhibited higher cytochrome P450, b_5 and NADPH- cytochrome *c* reductase activities than the rat (McManus and Ilett, 1976). The quokka, like eutherian mammals, also exhibited sex differences in oxidative drug metabolism (Gillette, 1971; Davies *et al.*, 1969; Schenkman *et al.*, 1967; McManus and Ilett, 1976). In addition, the quokka MFO system was induced by phenobarbital pre-treatment with male quokkas exhibiting a 100% increase in the levels of cytochrome P450 and NADPH-cytochrome *c* reductase activity (McManus and Ilett, 1976). The study by McManus and Ilett (1976) therefore indicated that a marsupial, the quokka, had the ability to respond to environmental contaminants by increasing its MFO enzyme concentration and rate of metabolism, despite possessing a generally lower capacity of xenobiotic metabolism than the rat.

McManus and Ilett (1977) further assessed microsomal xenobiotic metabolism in a range of Australian marsupial species. They studied the quokka (*Setonix brachyurus*), grey kangaroo (*Macropus fuliginosus*), bettong (*Bettongia penicillata*), short-nosed bandicoot (*Isoodon obesulus*) and the brushtail possum (*Trichosurus vulpecula*). They reported as with their previous study, that for each xenobiotic substrate tested (ie. aniline, 3,4-benzpyrene and ethylmorphine) marsupials generally possessed a lower rate of hepatic oxidative metabolism when compared to the rat. McManus and Ilett (1977) also reported similar levels of cytochrome P450, cytochrome b_5, and NADPH-

cytochrome *c* reductase activity in liver microsomes between marsupials and the rat (McManus and Ilett, 1976; McManus and Ilett, 1977). However no sex differences were observed for any of the marsupial species examined (McManus and Ilett, 1977). The extrahepatic xenobiotic metabolism of one marsupial species, the quokka (*Setonix brachyurus*), was also investigated (McManus and Ilett, 1977). The concentrations of components of the cytochrome P450 pathway in extrahepatic tissues, ie. lung, kidney and intestine, of the male quokka were comparable to other eutherian species (Tredger *et al.*, 1976; Litterst *et al.*, 1975; Chabra *et al.*, 1974; McManus and Ilett, 1977). Finally, McManus and Ilett (1977) postulated that the overall hepatic xenobiotic-metabolising capacities of the marsupials studied were disadvantaged by their lower liver to body weight ratios, when compared to the rat.

The development of the hepatic mixed function oxidase system has also been investigated in pouch young and joey quokkas (*Setonix brachyurus*) (McManus *et al.*, 1978). Pouch life in the quokka joey lasts about 180 days, and the young continue to suck from the mother for a further two months once out of the pouch (Shield, 1965; Cronin, 1971 *cited in* McManus *et al.*, 1978). The study examined the development of components of the cytochrome P450 pathway in pouched and joey quokkas ranging in age from 20 to 255 days (McManus *et al.*, 1978). Cytochrome P450 levels were first detected in pouch young quokkas at 35 days, and reached adult levels between 155 and 255 days after birth (McManus *et al.*, 1978). A similar pattern of development was also observed for cytochrome b_5 levels. NADPH-cytochrome *c* reductase levels were reported to be approximately one-fifth of adult levels at 45 days and reached adult levels by 80-105 days of life (McManus *et al.*, 1978). McManus *et al.* (1978) summarised that the pattern of cytochrome P450, cytochrome b_5, NADPH-cytochrome *c* reductase and benzpyrene hydroxylase activity observed in the development of the quokka more closely resembled the developmental pattern reported for the human foetus, rather than that seen for other eutherian species.

The mixed function oxidase system has more recently been investigated in the brushtail possum (*Trichosurus vulpecula*) (Bolton and Ahokas, 1997a). The authors reported that hepatic cytochrome P450, cytochrome b_5 and NADPH-cytochrome *c* reductase levels of the possum were comparable to reported eutherian values (Bolton and Ahokas, 1997a). Similarly, 7-

ethoxyresorufin O-deethylase (EROD) and 7-ethoxycoumarin O-deethylase (ECOD) activities were also in the range of values for eutherian mammals (Bolton and Ahokas, 1997a). The authors also noted that measured hepatic cytochrome P450 content, cytochrome b_5 and ECOD activity was significantly greater in non-urban brushtail possums in comparison to individuals from an urban population (Bolton and Ahokas, 1997a). Sex differences in cytochrome P450 isoenzyme expression were also reported (Bolton and Ahokas, 1997a).

In summary, the study by Bolton and Ahokas (1997a) indicated that the brushtail possum possessed comparable levels of hepatic MFO activity to other eutherian mammals. Their findings contrast with previous studies that reported lower values for components of the cytochrome P450 pathway in selected marsupial species compared to other eutherian mammals (McManus and Ilett, 1976; McManus and Ilett, 1977; McManus *et al.*, 1978; Bolton and Ahokas, 1997a).

Bolton and Ahokas (1997d) also investigated the ontogenic expression of various enzymes involved in phase I and II metabolism in the brushtail possum, *Trichosurus vulpecula*. They reported that the youngest age group studied, 60 days old, exhibited enzyme expression 5-10% of adult levels (Bolton and Ahokas, 1997d). A gradual increase in CYP, glutathione *S*-transferase and cytochrome b_5 enzyme expression was then observed, with adult levels being obtained during the weaning period (150-180 days) (Bolton and Ahokas, 1997d). In conclusion, the authors hypothesised that dietary xenobiotics may influence the developmental expression of detoxication enzymes in this species (Bolton and Ahokas, 1997d).

Pass *et al.* (1999) recently investigated the induction of xenobiotic-metabolising enzymes, specifically cytochromes P450, in the brushtail possum (*Trichosurus vulpecula*) by *Eucalyptus* terpenes. The authors reported that possums fed a diet containing 1,8-cineole, *p*-cymene, α-pinene and limonene for 10 days exhibited a 53% higher cytochrome P450 content and 45% increase in aminopyrine demethylase activity when compared to control possums (Pass *et al.*, 1999). Immunoblot analyses of hepatic microsomes from test animals using antibodies raised against CYP2C6, CYP2C11 and CYP2E1 also showed stronger immunoreactive bands in comparison to the control groups (Pass *et al.*, 1999). The study was the first to show

experimentally that dietary terpenes induce xenobiotic-metabolising enzymes in folivorous marsupials.

1.8.2. Phase II metabolism

The hepatic glutathione transferase family of enzymes has been investigated in a selected number of Australian marsupial species (Bolton and Ahokas, 1992; Bolton and Ahokas, 1997b; Bolton and Ahokas, 1997c). Glutathione *S*-transferases (GSTs) are a multifunctional family of enzymes that catalyse the conjugation of electrophilic and hydrophobic compounds with cellular glutathione, to facilitate their excretion from the organism (Daniel, 1993). GSTs aid in the detoxication of many endogenous and xenobiotic substrates (Motoyama and Dauterman, 1980; Ketterer and Meyer, 1989).

Hepatic GSTs have been investigated in two marsupial species to date, the brown antechinus, *Antechinus stuartii* and the brushtail possum, *Trichosurus vulpecula* (Bolton and Ahokas, 1992; Bolton and Ahokas, 1997b; Bolton and Ahokas, 1997c). Five GST isoenzymes belonging to families alpha, pi and mu were detected in the hepatic cytosol of the adult brown antechinus (Bolton and Ahokas, 1997b). In contrast, the brushtail possum exhibited a slightly different GST isoenzyme profile (Bolton and Ahokas, 1997c). The possum also predominantly expressed a single alpha GST isoenzyme, however GST isoenzymes belonging to other classes were not detected (Bolton and Ahokas, 1997c). The predominantly single hepatic alpha GST isoenzyme profile exhibited by both marsupial species contrasts with previously studied eutherian mammals (Jernstrom *et al.*, 1988; Bolton and Ahokas, 1997b). The functional importance of the enzyme pattern exhibited by the two marsupial species studied to date is presently unclear. It has been hypothesised that the major alpha GST isoenzyme plays a critical role in the detoxication of plant allelochemicals present in the marsupials' diet (Bolton and Ahokas, 1997b; Bolton and Ahokas, 1997c).

Baudinette *et al.* (1980) investigated the metabolic fate of injected phenol in nine marsupial and twelve eutherian species. The marsupial species included herbivorous, carnivorous, omnivorous and insectivorous examples (Baudinette *et al.*, 1980). Significant differences in phenol metabolism were observed between marsupial species with differing diets (Baudinette *et al.*, 1980). The

folivorous marsupials, specifically the brushtail possum and koala, exhibited a severely limited capacity to conjugate with sulphate groups, and relied heavily on glucuronide for conjugation (Baudinette *et al.*, 1980). The brushtail possum has previously been reported to possess a limited capacity for sulphate conjugation (Roy, 1963 *cited in* Baudinette *et al.*, 1980). It has been hypothesised that the reported inability of the koala and brushtail possum to form sulphate conjugates may be due to a low dietary supply of sulphate, or an enzymatic deficiency in sulphatase activity (Eberhard *et al.*, 1975 *cited in* Baudinette *et al.*, 1980). In contrast, the carnivorous and omnivorous species relied predominantly on sulphate for conjugation (Baudinette *et al.*, 1980).

The acetylation of sulphanilamide has been investigated in four marsupial species and a monotreme (McLean *et al.*, 1983). The urinary metabolites of sulphanilamide, dosed intraperitoneally, were examined in the Tasmanian devil, brushtail possum, pademelon, eastern-barred bandicoot (marsupials), echidna (monotreme) and rat (eutherian). The major urinary metabolite in the Tasmanian devil, brushtail possum and pademelon was N^4-acetylsulphanilamide (6-17%), significantly less than the rat which excreted 40% of the dose as this metabolite. Significantly, the bandicoot and echidna were both virtually unable to acetylate sulphanilamide. The only detectable acetylated metabolite in the urine of both species was N^1-acetylsulphanilamide, 0.3-1.5% for the bandicoot and 0.1-0.2% in the echidna. The bandicoot and echidna do not possess similar diets, and the reason for their unique metabolic defect is unknown (McLean *et al.*, 1983).

Finally, the fate of benzoic acid has been investigated in seven marsupial species and the rat (Awaluddin and McLean, 1985). The authors reported that the overall pattern of benzoic metabolism in the marsupial species examined was comparable to other mammals (Awaluddin and McLean, 1985).

1.8.3. Metabolism of dietary terpenes by Australian marsupials

The *Eucalyptus* forests of Australia are an important food source for a number of Australian marsupial species. Spencer (1968, *cited in* Eberhard *et al.*, 1975) reported the approximate composition of dried *Eucalyptus microtheca* leaf included: protein 6.4%, fat 2.4%, fibre 13.6%, ash 8.2% and nitrogen-free extract 69.4%. In addition, *Eucalyptus* leaves are chemically defended by high levels of phenolics and essential oils (Morrow and Fox, 1980). The essential oil in *Eucalyptus* species is comprised of a complex and variable mixture of monoterpenes, whose composition changes depending on the species of *Eucalyptus*, age of the leaf, season and locality (Eberhard *et al.*, 1975). The major constituents of distilled Eucalyptus oil include cineole (70% w/w), and significant amounts of α-pinene, *d*-limonene and *p*-cymene (Corrigan, 1992 *cited in* McLean and Foley, 1997). Specifically, 1,8-cineole is the most abundant terpene present in *Eucalyptis* species, and can sometimes account for more than 90% of the terpene content (Boland and Brophy, 1991; Li *et al.*, 1994).

Arboreal marsupial species that ingest *Eucalyptus* leaves on a daily basis include the koala (*Phascolarctos cinereus*), greater glider (*Petauroides volans*), common ringtail possum (*Pseudocheirus peregrinus*) and common brushtail possum (*Trichosurus vulpecula*) (Eberhard *et al.*, 1975; Strahan 1983). These species therefore ingest significant quantities of eucalyptus oil, and its toxic constituents, as part of their select diet (Eberhard *et al.*, 1975; McLean and Foley, 1997). Similar quantities would be potentially fatal to many other mammalian species (Webb and Pitt, 1993; Barnes, 1996 *cited in* McLean and Foley, 1997). It is hypothesised that the *Eucalyptus* leaf-eating marsupial species have therefore evolved novel mechanisms and pathways for the detoxification of the ingested terpenes (McLean and Foley, 1997).

Monoterpenes are highly lipophilic compounds which require metabolism to facilitate excretion (Gershenzon and Croteau, 1991 *cited in* McLean and Foley, 1997). Relatively little ingested dietary terpene in the koala and brushtail possum was reported to be excreted unchanged in the faeces or urine (Eberhard *et al.*, 1975; Southwell *et al.*, 1980). A number of studies have researched the metabolic fate of dietary terpenes and other toxic plant secondary products in *Eucalyptus* foliage consumed by marsupial species. Cleland (1946) (*cited in* Eberhard *et al.*, 1975) fed a cineole-rich

diet to the brushtail possum, *Trichosurus vulpecula*, and reported an increase in glucuronic acid excretion in the urine. Similarly, Hinks and Bollinger (1957a) observed that brushtail possums switched to a diet of *Eucalyptus* foliage from a diet of fruit and vegetables, exhibited a two- to threefold increase in the excretion of glucuronic acid conjugates in the urine. The authors hypothesised that the formation of large amounts of glucuronides was connected with the ingestion and necessary detoxication of the toxic components in the essential oils present in *Eucalyptus* foliage (Hinks and Bollinger, 1957a). Preliminary examinations of the koala (*Phascolarctos cinereus*), wombat (*Vombatus ursinus*), tree kangaroo (*Dendrolagus lumholtzi*), Tasmanian devil (*Sarcophilus harrisii*), and the tiger cat (*Dasyurus maculatus*), also revealed the presence of glucuronides in the urine (Hinks and Bollinger, 1957b). Significantly, the amount of urinary glucuronic acid reported for the koala was twice that observed previously for the brushtail possum (Hinks, 1956; Hinks and Bollinger, 1957a; Hinks and Bollinger, 1957b; Marsh, 1969).

Southwell (1975) reported that koalas fed *E.punctata* leaf excreted novel monoterpenoid lactones. In contrast, brushtail possums dosed with α- and β-pinene only exhibited less-oxidised metabolites in the urine (Southwell *et al.*, 1980). Significantly, the authors noted that koalas and brushtail possums exhibited different oxidative pathways and also greater oxidative activity was observed with increased dietary terpene consumption (Southwell *et al.*, 1980). The koala therefore appears to have evolved a greater capacity to oxidise dietary terpenes than the brushtail possum, based on the metabolic products of α- and β-pinene (Southwell, 1975; Southwell *et al.*, 1980; McLean and Foley, 1997).

The metabolic fate of dietary terpenes has also been investigated in the ringtail possum, *Pseudocheirus peregrinus* (McLean *et al.*, 1993). The majority of metabolites were oxidised, with no other conjugates of terpenes detected (McLean *et al.*, 1993). In contrast, Boyle *et al.* (1999) recently reported that significant amounts of metabolites in the brushtail possum and rat, fed diets of *p*-cymene, were glucuronidated. However, there was no evidence of glucuronidation of *p*-cymene metabolites in specialist marsupials (ie. ringtail possum, greater glider) (Boyle *et al.*, 1999). It was therefore hypothesised that the preferred method of elimination of *p*-cymene in specialist marsupial species is extensive oxidation, rather than glucuronidation (Boyle *et al.*, 1999).

A recent investigation by Boyle *et al.* (2000) into the fate of the dietary terpene, *p*-cymene, in the koala produced similar findings. *p*-Cymene metabolism in the koala resulted in the formation of extensively oxidised metabolites which have increased polarity and renal excretion (Boyle *et al.*, 2000). Flynn and Southwell (1979) investigated the fate of 1,8-cineole in the brushtail possum and also reported the presence of oxidised metabolites. A more recent study by Boyle *et al.* (2000-in press) reported similar findings, however glucuronic acid conjugation was also detected.

In summary, the specialist *Eucalyptus*-eating marsupials, such as the koala and ringtail possum, seem to detoxify dietary terpenes through extensive oxidation culminating in the production of polar, acidic metabolites which exhibit enhanced renal excretion (Southwell, 1975; Southwell *et al.*, 1980; McLean and Foley, 1993; Boyle *et al.*, 1999). Marsupials with a more varied diet (ie. the brushtail possum), do not appear to oxidise terpenes as extensively (Southwell *et al.*, 1980; Boyle *et al.*, 2000). Finally, only minimal conjugation of metabolites has been observed in specialist marsupials (McLean *et al.*, 1993). It has been hypothesised that this may be due to the poor nutritional content and toxic component of *Eucalyptus* leaves, and the need to conserve amino acids and carbohydrates (McLean and Foley, 1997).

1.9. Aims of the project

From the preceding discussion, it is clear that there is a significant lack of information regarding the mechanisms governing the metabolism of both xenobiotic compounds and endogenous substrates by Australian marsupial species at the molecular level. The availability of such information in Australian marsupials would be important, not only for its intrinsic scientific value, but also to gain an understanding of the relative components of the mixed function oxidase pathway responsible for the metabolism and subsequent excretion of man-made chemicals. These mechanisms, which have been well characterised in a host of other mammalian species, are largely unknown in most Australian marsupial species. From an evolutionary perspective, the lack of data is also relevant as Australian marsupials comprise a unique phylogenetic class exhibiting dietary, reproductive and physiological characteristics not seen in any other mammalian species. Investigations into the xenobiotic-metabolising enzymes of

Australian marsupial species may therefore result in the elucidation of novel enzymes possessing unique catalytic properties.

To summarise, the isolation and characterisation of xenobiotic-metabolising enzymes, notably cytochromes P450, in Australian marsupials will aid in the understanding of the response of these unique mammals to environmental chemical exposure. The studies will also provide important evolutionary information on both the cytochrome P450 superfamily and marsupial species in general.

It was therefore a primary aim of this study to assemble tissue samples from representative Australian marsupial species to initiate a comprehensive and continuing analysis into the xenobiotic-metabolising enzymes of Australian marsupial fauna. This would involve establishing links with wildlife parks, educational institutions and the public to acquire tissues from as diverse array of species as possible, suitable for molecular biology use.

The availability of such tissues would enable investigations into the CYP content and complement of representative marsupial species, by conducting traditional CYP assays and immunoblots using a selection of polyclonal antibodies raised against the major xenobiotic-metabolising CYPs. This information, while providing an initial insight into the expressed CYP families in marsupials would also enable the identification of potential targets for subsequent cloning studies.

The isolation of xenobiotic-metabolising enzyme cDNA sequences, particularly *CYPs* and *UGTs*, by RT-PCR would provide direct evidence for the expression and multiplicity of such enzymes in Australian marsupials. In conclusion, the isolation of an entire coding region for any particular cDNA sequence encoding a xenobiotic-metabolising enzyme would allow subsequent investigations into the catalytic activity of the expressed enzyme against chemicals of toxicological significance.

Chapter 2

Materials and Methods

2.1. Materials

2.1.1. Marsupial tissues

Road-injured marsupial species from the Adelaide Hills, South Australia, were euthanased by pentobarbitone injection at the Department of Anatomical Sciences, University of Adelaide. Following death, various tissues were collected (ie. liver, lung, kidney) and stored by fixation in formalin or snap-frozen in liquid nitrogen. Collection of scavenged marsupial tissues was approved by the Animal Ethics Committee, IMVS, Adelaide. Other marsupial species tissues were procured in a similar fashion, and a more detailed explanation of their origins is outlined in section 3.3.1. of Chapter 3.

2.1.2. cDNA libraries

A male Adelaide Hills koala (*Phascolarctos cinereus cinereus*) liver cDNA library was produced specifically for this project. The library was prepared in collaboration with Professor Mick McManus and Dr Robyn Bolton (Department of Physiology and Pharmacology, University of Queensland). Koala liver tissue was shipped directly to the Department of Genetics, Southwest Foundation for Biomedical Research, San Antonio, Texas, USA in an ultra-cold container for library construction. The cDNA library was constructed using the Lambda ZAP Express CMV-XR vector, with the cDNA fragments cloned unidirectionally with sticky *EcoR I* and *Xho I* ends for the 5' and 3' ends respectively.

2.1.3. Chemicals, Kits and Enzymes

Reagents and kits used for the various analytical, molecular and assay procedures, including product manufacturers, are listed alphabetically in **Table 2.1.** General laboratory supplies have not been listed. They were of analytical reagent grade.

Table 2.1. Reagents and Kits used in experimental procedures.

SUPPLIER	REAGENT or KIT
Amersham - Sydney, Australia	Nitrocellulose Membrane
	Aqueous Counting Scintillant (ACS)
AGP Technologies - Queensland,	DNA grade agarose
Australia	Hi-Strength Low Melting Agarose
Bio-Rad Laboratories - California, USA	Tween-20 (poloxyethylene sorbitan monolaurate)
	HRP Colour Development Reagent, 4CN
	Coomassie Brilliant Blue
	HRP Colour Development Reagent, DAB
	Ammonium Persulphate (APS)
	TEMED
Geneworks Ltd. - Adelaide, Australia	Stabilised α-^{32}P Deoxycytosine 5'- triphosphate
	PCR primers
	M13 universal forward primer
	M13 reverse primer
	RNAzol^{TM} B
	100bp DNA ladder (molecular weight marker)
	Gigaprime DNA Labelling Kit
Life Technologies (Gibco BRL),	Luria Broth Base
Scotland	Restriction enzymes
	Restriction enzyme buffers
	Taq DNA polymerase
Pharmacia Biotechnology	mRNA Purification Kit
Uppsala, Sweden	First Strand cDNA Synthesis Kit
	100mM Deoxynucleotide triphosphate (dNTP) set
Progen Industries Ltd. - Queensland,	X-gal
Australia	IPTG

	T_4 DNA Ligase
	Ampicillin
	Kanamycin
	Tetracycline
Promega Corporation - Sydney,	pGEM®-T, pGEM®-T Easy Vector Systems
Australia	RNAse I Ribonuclease
Qiagen Pty. Ltd. - Victoria, Australia	QIA Nucleotide Removal Kit
	QIAEX II Gel Extraction Kit
	QIAprep Spin Miniprep Kit
Sigma - NSW, Australia	MOPS
	Polyethylene glycol 8000
	β-NADPH
	Horse heart cytochrome C reductase
	Ethidium bromide
	Trizma Base
Silenus - Victoria	anti-goat, anti-human secondary IgG
Stratagene Pty Ltd. - NSW	XL1-Blue MRF' host cells
	ExAssist® Interference Helper Phage with
	XLOLR strain
Perkin-Elmer Corporation - NSW	ABI Prism™ Terminator Cycle Australia
	Sequencing Ready Reaction Kit (with
	Ampli-*Taq*® DNA Polymerase, FS)

2.1.4. Antibodies

CYP and associated polyclonal antibodies were utilised in immunoblot analyses with hepatic microsomes prepared from koala, koala pouch young, tammar wallaby, wombat and rat tissue samples. The goat anti-rabbit CYP3A6 IgG and goat anti-human NADPH-cytochrome P450 reductase IgG were previously obtained by repeated injection of adult goats with the respective purified proteins. These antibodies were kind gifts from Professor M McManus (University of Queensland), and both have been previously used to detect the relevant protein in rat microsomes (McClure and Stupans, 1995). The anti-human CYP2D6 IgG, was previously obtained from human sera and also recognises CYP2D1 in the rat (Yamamoto *et al.*, 1993). The goat anti-rat CYP2E1 IgG was a gift from Dr ME Veronese, and has previously detected a single form in rat microsomes (Ieva Stupans, personal communication). The rabbit anti-rat CYP2C11 IgG was a gift from A/Prof M Murray (University of New South Wales) and also recognises a single form in rat microsomes (Ieva Stupans, personal communication). The goat anti-mouse UGT2 IgG was a kind gift from Professor P Mackenzie (Flinders University) and recognises multiple UGT2 enzymes in rat and mouse (Mackenzie *et al.*, 1984). The goat anti-rat CYP2C6 IgG and sheep anti-rat CYP2E1 IgG were both purchased from Daiichi Pure Chemical Co. Pty., Tokyo, Japan. According to the manufacturers, the goat anti-rat CYP2C6 polyclonal antiserum detects rat CYP2C6 with a minor cross-reactivity with rat CYP2C13 in microsomes prepared from phenobarbital-treated rats. In addition, the polyclonal anti-rat CYP2E1 antisera immunoblots rat CYP2E1 with a minor cross-reactivity with rat CYP2C11 and CYP2C13 in liver microsomes from acetone-treated rats.

2.1.5. Equipment

The equipment used for each analytical, assay and molecular biology protocol is summarised in Table 2.2.

Table 2.2. Equipment, manufacturer and the reason used during each research protocol.

EQUIPMENT	MANUFACTURER	REASON USED
Centrifuges		
Sorvall RC-2B centrifuge	Dupont Ltd	Microsome preparation RNA isolation
Optima XL-80 ultracentrifuge	Beckman, CA, USA	Microsome preparation
Hettich Zentrifugen Rotina 48	Beckman, CA, USA	total RNA isolation mRNA isolation
Spectrophotometers		
U-3200 Recording Spectrophotometer	Hitachi Ltd, Tokyo, Japan	Total CYP450 and NADPH assay
120-02 spectrophotometer	Shimadzu Corp, Kyoto, Japan	DNA, RNA absorbance
Radioactivity measurements		
2200CA Tri-Carb Liquid Scintillation Analyzer	Packard, Meriden, CT, USA	P^{32} labelling incorporation of cDNA probe
Incubators		
Model 2000 Hybridisation Incubator	Robbin Scientific, CA, USA	library screening northern blotting
Orbital Mixer incubator Model OM10	Ratek Instruments, Australia	bacterial cultures plate incubations
Autoradiography		
Hypercassette™	Amersham Life Science, Eng	membrane exposures
UVC 500UV Cross-Linker	Hoefer Instruments Pty Ltd	DNA, RNA fixation
Electrophoresis		
DNA Sub Cell™ Electrophoresis System	Bio-Rad Laboratories CA, USA	DNA, RNA separation electrophoresis
EPS 600	Pharmacia Biotech, Sweden	Electrophoresis Power Supply

2.1.6. Bacterial culture media

2.1.6.1. Solid media

The following solid media was used for all bacterial cultivation. All "Bacto" media was obtained from Difco Laboratories, MI, USA. Luria-Bertoni (LB) agar plates were prepared by adding 1.5% (w/v) bactoagar to prepared LB. The LB plates used in the cDNA library plating process were prepared the same way, however $MgSO_4$ was added to a final concentration of 10mM.

2.1.6.2. Liquid media

Luria-Bertoni (LB) (1% peptone, 0.5% yeast extract and 20mM NaCl) was the sole growth medium for all bacterial and phage cultivation. Incubations were at 37°C unless otherwise specified. Generally, liquid cultures were grown in 10ml sterile tubes with aeration.

2.1.6.3. Antibiotics

Ampicillin (Progen Industries, Queensland) was used at a final concentration of 100μg/ml in broth and solid media for the cultivation of transformed *E.coli* bacteria. Tetracycline (Progen Industries, Queensland) and Kanamycin (Progen Industries, Queensland) were also used at concentrations of 12.5μg/ml in broth and solid media for the selection of *E.coli* host cells and cloning vectors during the library screening process.

2.2. Methods

Protein methodologies

2.2.1. Microsome preparation

Microsomes were prepared from rat, koala, koala pouch young, tammar wallaby and wombat liver tissues using the method previously described by McManus *et al.*, (1988). Briefly, tissues were placed in 0.1M potassium phosphate buffer (pH 7.4) containing 0.15% (w/v) potassium chloride prior to microsome preparation. Samples were then homogenised in four volumes of the above buffer using a Potter-Elvenheim glass teflon homogeniser attached to a power drill. The

resulting homogenate was centrifuged at 10,000 x g (4°C) for 20 min to remove nuclei, mitochondria and cell debris. The supernatant fraction was collected and further centrifuged at 105,000 x g (4°C) for 1 hr to obtain the microsomal pellet. Microsomal protein was then manually resuspended in 0.1M potassium phosphate buffer (pH 7.4) using a glass homogeniser, and further centrifuged at 105,000 x g (4°C) for 1 hr to recover the microsomal protein. The pellet was finally resuspended in 0.1M potassium phosphate buffer (pH 7.3) containing 20% (v/v) glycerol. Microsome preparations were stored at -80°C until use.

2.2.2. Lowry assay

Protein determinations were performed as outlined by Lowry *et al.* (1951). Briefly, it involved preparing a solution combining solution A (3% (w/v) Na_2CO_3/ 0.4% (w/v) NaOH), solution B (4% (w/v) NaK tartrate) and solution C (2% (w/v) $CuSO_4.5H_2O$) in a 100:1:1 ratio. Protein samples were diluted with distilled water to a final volume of 300µl, and 5ml of solution ABC was added to it. The samples were allowed to stand at room temperature for 15 min after which 0.5ml of diluted Folin-Ciocalteau reagent (1:2) was added. Samples were then vortexed and left at room temperature for a further 15 min. The absorbance of each protein sample was then measured at 660nm. Final protein concentrations were determined using a bovine serum albumin standard curve.

2.2.3. Cytochrome *c* NADPH reductase assay

Briefly, 600µl of 1mM KCN (100mM Potassium cyanide diluted in 0.3M phosphate buffer, pH 7.7) and 150µl of 150µM horse heart cytochrome c (in 0.3M phosphate buffer, pH 7.7) was added to two cuvettes, inverted to mix, then autozeroed, and a baseline reading taken for 1 min at 550nm using a Hitachi scanning spectrophotometer. Finally, 10µl of marsupial microsomes (1:10 dilution in 1mM phosphate buffer, pH 7.7) was then added to one cuvette, inverted to mix, followed by 25µl of 1M NADPH (β-Nicotinamide adenine dinucleotide phosphate, Sigma). The cuvette was quickly inverted three times, autozeroed, and the absorbance measured for 1 min at 550nm.

2.2.4. Total cytochrome P450 assay

Microsomes were diluted to 1mg/ml using 0.1M phosphate buffer (pH 7.4) and kept on ice prior to the assaying procedure. Following the addition of a small spatula of sodium dithionite (May and Baker, Dagenham, England), 700µl of microsomes was added to two cuvettes, the samples autozeroed and a baseline reading taken between 500nm to 390nm using a Hitachi scanning spectrophotometer. 20-25 bubbles of carbon monoxide were then bubbled through one cuvette, and the absorbance values again measured from 500nm to 390nm.

2.2.5. SDS-PAGE

2.2.5.1. Preparation of samples

Microsomes were diluted in 1M phosphate buffer (pH 7.4) to the required concentration. An equal volume of PAGE solubilisation buffer containing 0.5M Tris, 36% (v/v) glycerol, 11% (w/v) SDS, 18% (v/v) 2- mercaptoethanol and 0.05% (w/v) bromophenol blue was added to the sample. The solution was heated to 100°C for 5 min to denature the proteins before loading onto a SDS-polyacrylamide gel.

2.2.5.2. SDS-PAGE of samples

Samples were analysed using an SDS-polyacrylamide gel consisting of a 4.5% (w/v) stacking gel and 7.5% (w/v) separating gel prepared according to the protocol of Laemmli (1970). After the addition of samples, the proteins were stacked at 100V for 1 hr then separated at 200V for 2 hrs. Gels were stained with Coomassie brilliant blue stain consisting of 0.3% (w/v) Coomassie brilliant blue, 45% (v/v) methanol and 9% (v/v) glacial acetic acid for 1 hr with agitation. Gels were then destained overnight in a solution of 20% (v/v) methanol and 10% (v/v) glacial acetic acid.

2.2.6. Immunoblotting

Following the SDS-PAGE of sample proteins, as described previously, the apparatus was dissembled and the gel placed in distilled water for 5 min. The gel was soaked in transblotting buffer consisting of 21mM Tris, 192mM Glycine and 20% (v/v) methanol for 10 min. A

nitrocellulose membrane, several pieces of blotting paper and some sponge pads were also soaked in transblotting buffer. The gel sandwich was then assembled and the proteins were transferred to the membrane at 50mA for 16 hr. The membrane was washed with TBS containing 16mM Tris and 500mM NaCl before being incubated in a blocking solution composed of 5% (w/v) skim milk in TBS for 1 hr with agitation. The membrane was washed twice in TTBS containing 0.05% tween-20 in TBS for 5 min per wash. Each cytochrome P450 antibody was diluted 1:1000 with antibody buffer consisting of 0.02% (w/v) skim milk in TTBS, the membrane was incubated with the diluted primary antibody for at least 16 hr with agitation. The nitrocellulose membrane was washed twice in TTBS for 5 min then incubated with the secondary antibody, 30µl of anti-sera in 15ml of antibody buffer, for 1 hr. After two more washes with TTBS for 5 min each, the membrane was washed in TBS twice for 5 min. Detection of the presence of bound proteins to the nitrocellulose membrane was achieved by utilising the horseradish peroxidase (HRP) conjugated to the secondary antibody. HRP Colour Development Reagent (Bio-Rad, California, USA) containing 4-chloro-1-naphthol or 3,3'-diaminobenzidine, forms a coloured precipitate at the site of secondary antibody conjugation. Immunoblot colour development was achieved by dissolving 0.06% (w/v) HRP colour development reagent in 20ml of ice-cold methanol, 81ml of TBS and 90µl of Hydrogen peroxide (100vol.). The membrane was incubated in this solution with agitation until bands indicating bound proteins became clearly visible. The membrane was washed in water for 10 min in order to terminate the colour development reaction, dried, then stored between two sheets of Whatman paper.

General molecular biology techniques

2.2.7. Total RNA isolation

Prior to the RNA extraction process, all homogenising equipment, centrifuge tubes and glassware were treated with 1% Hydrogen peroxide (H_2O_2). Equipment was then soaked in 0.1% diethyl pyrocarbonate (DEPC) treated water in order to inhibit the action of RNAses, and thus guard against the degradation of the RNA.

Total RNA was isolated from koala, tammar wallaby and rat liver tissue using a modification of the guanidinium method of Chomczynski and Saachi (1987), as per the manufacturers protocol (RNAzol™B RNA isolation protocol, Geneworks, Adelaide). Portions of each marsupial liver tissue used to extract RNA were first weighed in a plastic weighing tray, then finely cut into small fragments using a sterile scalpel blade. The sample was transferred to a glass homogenising tube with the specified volume of RNAzol™B (2ml per 100mg of tissue), and homogenised using a Teflon rod attached to a drill. An appropriate amount of chloroform (0.2ml/2ml of homogenate) was added to the homogenate, and mixed into the sample by vigorously shaking for at least 15 sec. Following a brief incubation at 4°C for 5 min, the sample was centrifuged at 9,000 x g for 15 min (4°C) in a Sorvall RC-2B ultra-centrifuge.

The upper aqueous phase was removed and transferred to a new centrifuge tube and an equal volume of isopropanol was added. The sample was stored at 4°C for at least 15 min, then centrifuged again at 9,000 x g for 15 min (4°C). The RNA pellet was washed in 8ml of 75%(v/v) ethanol by vortexing then re-centrifuged at 7,500 rpm for 8 min (4°C). The pellet was transferred to a 1.5ml eppendorf tube and dried briefly under vacuum for 5 min using a SpeedVac RC10.10. (Medos Company, Melbourne). The RNA was resuspended in an appropriate volume of DEPC-treated water, and stored at -70°C until use.

2.2.7.1. mRNA isolation from total RNA

Purification of mRNA from rat, koala and tammar wallaby liver total RNA samples was achieved using an mRNA purification kit (Amersham Pharmacia Biotech, Sydney, Australia) as per manufacturers instructions. Briefly, 1.5mg of denatured total RNA, dissolved in 1ml of elution buffer and 0.2ml of sample buffer (supplied) was applied to a previously equilibrated oligo(dt)-cellulose column. The RNA sample was allowed time to enter the column under gravity, before centrifugation at 350xg for 2 min. To remove rRNA and other impurities the column was twice washed with 0.25ml aliquots of high salt buffer (supplied) and washed three times with 0.25ml aliquots of low salt buffer (supplied) by centrifuging at 350xg for 2 min between each wash. The bound poly(A)+ RNA was then eluted into a 1.5ml microcentrifuge tube by washing the column with four successive 0.25ml aliquots of elution buffer (supplied), prewarmed to 65°C, and

centrifuging at 350xg for 2 min after each application. Samples were immediately precipitated by the addition of 50μl potassium acetate solution (supplied), 10μl of glycogen solution (supplied) and 1ml of 100% ethanol and incubating at -20°C for at least 2hr. Poly(A)+ RNA was recovered by centrifuging at 13000 rpm for 15 min at 4°C. After briefly vacuum-drying the pellet, the RNA was redissolved in elution buffer and stored at -70°C until use.

2.2.7.2. Quantification of RNA

RNA was quantified using a UV-120-02 Spectrophotometer by measuring the absorbance at wavelengths 260nm and 280nm. DEPC-treated water was used as a blank. The concentration of the corresponding RNA solution was calculated by assuming that 1 absorbance unit corresponded to 40μg/ml of RNA (Sambrook *et al.*, 1989).

2.2.8. Ligation of PCR products into pGEM®-T and pGEM®-T Easy vectors

Amplified PCR products were ligated into pGEM®-T Vector using the manufacturers' protocols and reagents (Promega, Madison, WI, USA). Briefly, 4-5μl of PCR product was added to 1μl of T4 DNA ligase 10X buffer, 1μl of pGEM®-T vector (50ng), 1μl of T4 DNA ligase (1 Weiss unit/μl) and water to a final volume of 10μl. The mixture was incubated overnight at 4°C, then transformed into DH5α competent cells, described in section 2.2.8.2.

2.2.8.1. Preparation of competent *E.coli* DH5α cells

1ml of an overnight *E.coli* strain DH5α culture was innoculated into 100ml of LB broth and incubated until log growth was established. Cells were harvested by centrifugation and resuspended in cold $CaCl_2$ (50mM). The cells were pelleted by further centrifugation and again resuspended in cold $CaCl_2$, before being placed on ice for 45 min. Glycerol was added to a final concentration of 15%(v/v) and cells were finally stored in aliquots at -70°C.

2.2.8.2. Transformation of plasmids into *E.coli* DH5α competent cells

2μl of plasmid DNA was transformed into 100μl of DH5α competent cells by initially incubating on ice for 30 min. Following a heat-shock at 42°C for 2 min, 1ml of Luria-Bertoni (LB) medium (1% tryptone, 0.5% yeast extract, 20mM NaCl) was added to the mixture and incubated at 37°C

for 1 hr. The sample was spun at 13,000 rpm for 30 sec and all but 100μl of the supernatant was discarded. 8μl of 50mg/ml X-gal (5-bromo-4-chloro-3-indolyl-B-D-galactoside; Life Technologies, Sydney) and 5μl of 0.8M IPTG (isopropyl-B-D-1-thiogalactopyranoside; Life Technologies, Sydney) was added and the pellet was resuspended by vortexing, all of the suspension was then plated onto Luria Broth (LB) agar plates (1.5% agar, 1% tryptone, 0.5% yeast extract, 20mM NaCl) containing ampicillin (100μg/ml). Plates were incubated overnight at 37°C and recombinant clones were selected based on blue/white colour selection.

2.2.8.3. Small scale purification of plasmids (alkaline lysis)

Small scale plasmid DNA preparations were performed using an alkaline lysis method as described by Sambrook *et al.* (1989). Isolated white colonies were innoculated into 5ml of Luria Broth (1% tryptone, 0.5% yeast extract, 20mM NaCl) containing 100μg/ml ampicillin using a sterile toothpick. Cultures were incubated overnight at 37°C in a shaking incubator. 1.5ml of each culture was then transferred into a sterile 1.5ml eppendorf tube and spun at 13,000rpm for 1 min. After removal of the supernatant, 100μl of GET buffer (50mM glucose, 10mM EDTA, 25mM Tris, pH 8) was added, the solution was vortexed and left to incubate for 5 min. 200μl of Solution B (0.2M NaOH, 1% SDS) was added, the tube inverted then incubated for 5 min on ice. Following the addition of 150μl of 3M NaAc (pH 4.8) the tube was inverted several times then spun at 13,000 rpm for 5 min. The supernatant was transferred to a clean eppendorf tube and an equivalent amount of phenol-chloroform extract was added and mixed by vortexing. The sample was spun for 2 min at 13,000 rpm. The upper aqueous layer was transferred to another sterile eppendorf tube and 1ml of 100% ethanol was added, mixed, and incubated on ice for at least 5 min. Following a 5 min spin at 13,000 rpm, 1ml of 70% ethanol was added to the precipitate and the sample lightly vortexed. After another 5 min spin at 13,000 rpm the supernatant was drained and the precipitate dried for 5 min using a SpeedVac RC10.10. The dried precipitate was finally resuspended in 20μl of sterilised, deionised water.

2.2.8.4. Screening for inserts in pGEM®-T and pGEM®-T Easy clones

The plasmid DNA extracted from bacterial cells via small scale plasmid purification (section 2.2.8.3), was restricted with *Pst I* and *Apa I* for pGEM®-T vector or *EcoR I* for pGEM®-T Easy

vector systems to excise the insert, as described in section 2.2.9.2. Restriction digests were separated by gel electrophoresis and visualised under UV radiation (section 2.2.9.3) to determine fragment sizes and ascertain which plasmid preparations contained the desired inserts. Suitable plasmids were stored at -20°C, until purification for sequencing (section 2.2.10), or fragment extraction and purification for cDNA probe radiolabelling (section 2.2.11).

2.2.9. DNA analysis

2.2.9.1. Quantification of DNA

DNA was quantified using a UV-120-02 Hitachi scanning spectrophotometer by measuring the absorbance at wavelength 260nm using sterile distilled as a blank. GeneQuant was also used to measure more dilute DNA samples. The concentration of the corresponding DNA solution was calculated by assuming that 1 absorbance unit corresponded to 50mg/ml of double-stranded DNA.

2.2.9.2. Restriction digestion of DNA

Following plasmid isolation and purification, restriction digests were performed to excise the desired insert. Restriction digests were performed using 1-5μg of DNA, 1μl of each restriction enzyme (10U/μl), 2μl of the appropriate restriction enzyme buffer (10X), 1μl of RNAse I Ribonuclease (1mg/ml) and distilled water to a final volume of 20μl. The reaction mixture was incubated for at least 1 hr in a 37°C water bath.

2.2.9.3. Gel electrophoresis and visualisation of DNA

Restriction digest DNA fragments and PCR products were separated by agarose gel electrophoresis. Between 1 and 2% (w/v) DNA grade agarose gels were prepared in 40mM Tris-acetate, 1mM EDTA (TAE) gel running buffer with an aliquot (10μl) of either the digest or PCR sample loaded onto the gel. Prior to loading samples, 2μl of gel loading buffer (30% (w/v) glycerol, 0.25% (v/v) xylene cyanol, 0.25% (v/v) bromophenol blue) was added to each sample. Agarose gels were stained with 2μl of ethidium bromide (10mg/ml) per 40ml gel solution. DNA fragments were visualised using a long wave ultraviolet transilluminator and photographed using

a Sony video camera module linked to a Sony UP-D860E digital graphic printer. Double-stranded DNA molecular weight markers were used to estimate DNA sample sizes. Listed below are the molecular weight markers used.

Lambda DNA/Hind III (Geneworks, Adelaide, Australia)

100bp DNA Ladder (Life Technologies, Sydney, Australia)

100bp DNA Ladder (Geneworks, Adelaide, Australia)

2.2.10. DNA sequencing

DNA intended for use in sequencing reactions had to be of high quality for adequate results to be obtained. Therefore, a QIAprep Spin Miniprep Kit (Qiagen Pty Ltd, Australia) utilising a silica-gel membrane chromatography technique was employed as per manufacturers' protocols. Briefly, bacterial cells from a 3ml overnight culture were pelleted by centrifugation at 13,000 rpm for 1 min and resuspended in buffer P1 (supplied). Cells were lysed and the remaining cellular debris precipitated after the addition of buffer P1 and N3 (supplied) respectively. Following centrifugation at 13,000 rpm for 10 min, the supernatant was transferred to a QIAprep spin column where the plasmid DNA was immobilised after a brief centrifugation. The spin column was washed with buffer PE (supplied) and the purified plasmid DNA eluted with 50μl of sterile, distilled water by centrifugation at 13,000 rpm for 1 min. The purified plasmid DNA was quantified (section 2.2.9.1) and stored at -20°C ready for use in sequencing reactions (section 2.2.10.1).

2.2.10.1. Sequencing reaction

Each sequencing reaction contained 500-1000ng of plasmid DNA, 8μl of ABI Prism™ dye terminator reaction mix (A/C/G/T- dye terminators and Amplitaq DNA polymerase (Perkin Elmer)), 3.2*p*mole primer and sterile, distilled water to a final reaction volume of 20μl. The reaction mix was overlayed with mineral oil before undergoing a sequencing reaction in a Perkin Elmer DNA Thermal Cycler (Model 480).

Temperature (°C)	Step	Time (min:sec)	Cycles
96	**denaturation**	**0:30**	**25**
50	**annealing**	**0:15**	**25**
60	**extension**	**4:00**	**25**
4	**storage**		

Following the sequencing reaction, DNA was ethanol precipitated by adding 2µl of 3M sodium acetate (pH 5.2) and 50µl of ice-cold 100% ethanol to the entire 20µl of sequencing reaction mix. To facilitate DNA precipitation, samples were incubated at -20°C for 1 hr before being spun at 13,000 rpm for 20 min. DNA pellets were washed with 200µl of 70% ethanol and finally dried in a heating block at 65°C for approximately 15 min.

Samples were forwarded to the Molecular Pathology Sequencing Centre, IMVS, Adelaide for gel electrophoresis performed using a Perkin Elmer ABI Prism 377 Sequencer.

2.2.10.2. Sequencing reaction primers

Primers used in the sequencing reactions were purchased desalted from Geneworks, Adelaide and diluted in sterile, distilled water to a working concentration of 0.01µg/µl.

M13 Universal Forward primer: 5' GTAAAACGACGGCCAGT 3'

M13 Universal Reverse primer: 5' CACACAGGAAACAGCTATGACCAT 3'

T3 primer 5' AATTAACCCTCACTAAAGGG 3'

T7 primer 5' GTAATACGACTCACTATAGGGC 3'

2.2.10.3. DNA sequence analysis

Sequencing information was analysed using the Macvector™ (version 6.5.3) computer programme (Oxford Molecular Ltd, 2000).

2.2.11. Isolation, purification and radiolabelling of cDNA probes

2.2.11.1. Low-melting point gel electrophoresis of amplified cDNA clones

Selected cDNA clones were used as templates in PCR amplification experiments in conjunction with the degenerate CYP2C primers. Following verification of positive insert amplification, the remaining PCR product was isolated and purified using agarose gel electrophoresis. Briefly, a 1.5% (w/v) low-melting point agarose gel was prepared in 1X TAE gel-running buffer. The PCR product was loaded onto the gel with the appropriate amount of gel-loading buffer and the samples electrophoresed until sufficient separation of the insert had been achieved. The DNA fragments were viewed briefly under UV light and the insert excised using a sterile scalpel blade. Finally, the excised gel piece containing the desired insert was placed into an 1.5ml eppendorf tube ready for purification.

2.2.11.2. Gel extraction and purification of cDNA fragments

DNA fragments separated on low melting-point agarose gels were extracted using a QIAEX II Gel Extraction Kit (QIAGEN Pty. Ltd, Australia) as per the recommended manufacturers' protocols. Briefly, QIAEX II emulsion and Solubilisation Buffer was added to the gel slice containing the DNA fragment and the mixture heated to 50°C for 10 min. DNA fragments adsorb to the QIAEX II particles and were recovered by centrifugation. The pellet was thoroughly washed to remove residual agarose and salt contaminants, then allowed to air dry. DNA was eluted by resuspending the pellet in 20μl of sterile, distilled water, centrifuging and transferring the supernatant to a fresh eppendorf tube. The elution step was repeated to increase DNA yield.

2.2.11.3. Radioactive labelling of cDNA probes with α-^{32}P-dCTP

Radioactive labelling of the purified cDNA fragments was achieved using the Gigaprime DNA labelling kit (Geneworks, Adelaide, SA) as per the manufacturers' protocols. The DNA fragment sample (25ng) was resuspended to a final volume of 7μl in sterile, distilled water then denatured by heating at 100°C for 5 min. The denatured DNA was immediately chilled on ice, before being quickly centrifuged to re-concentrate the sample. The following components from the Gigaprime

DNA labelling Kit were then added to the DNA sample, 6μl of Decanucleotide solution (Tube 1) and 6μl of Nucleotide/Cocktail Buffer (Tube 2C). 5μl of [α-^{32}P]-dCTP (3000Ci/mmol, 10μCi/μl) was then added to the sample followed by 1μl of Klenow enzyme (Tube 3), the solution mixed, then centrifuged to ensure that the contents were concentrated at the bottom of the tube. The mixture was incubated at 37°C for 25 min and the reaction stopped by the addition of 2μl of 0.5M EDTA. Radiolabelled DNA probes were then purified, as per the manufacturers protocols, using the QIAquick Nucleotide Removal Kit (QIAGEN Pty Ltd, Australia) in order to remove any unincorporated radioactivity. Incorporation efficiency was determined by liquid scintillation spectrophotometry.

2.2.11.4. Hybridisation of filters with α-^{32}P labelled cDNA probes

Filters were wet with 1X SSC and placed in hybridisation tubes with 20ml of prehybridisation solution (50% deionised formamide, 5X SSC, 5X Denhardt's solution, 0.1% w/v SDS, 100μg/ml denatured herring sperm DNA). The filters were hybridised at the chosen temperature for at least 1 hr in a Robbins Scientific Micro Hybridisation Incubator (Model 2000, RS, USA). Equivalent amounts of ^{32}P-labelled probes were added to each tube and the filters re-incubated overnight. Following hybridisation, filters were removed and initially washed with 2X SSC and 0.5% SDS at room temperature for 30 min. The filters were additionally washed with 1X SSC and 0.1% SDS at 50°C for 1 hr to prevent any non-specific binding of the probe.

Washed filters were finally placed in autoradiograph cassettes, plaque side up, with film and incubated at -70°C for at least 2 days. Duplicate filter images were aligned and positive spots appearing on both filters were marked. Positive plaques were picked from the corresponding plate using a sterile transfer pipette tip and innoculated in 1ml of SM buffer. The phages were then re-plated in significantly less densities and transferred to nylon filters and hybridised as previously explained. The process was repeated until single, positive plaques were obtained.

2.2.12. Northern blot analysis

2.2.12.1. RNA sample preparation

The methods outlined in this section for RNA sample preparation and gel electrophoresis have been adapted from those of Lehrach *et al.* (1977), Goldberg (1980) and Seed (1982).

RNA samples were prepared by mixing total RNA (~15μg) or mRNA (~15ng) with 2μl of 10X morpholinopropanesulphonic acid (MOPS), 3.5μl of 37% formaldehyde, 10μl of deionised formamide and 1μl of 1mg/ml ethidium bromide. The mixture was incubated at 65°C for 15 min, then snap cooled on ice for a further 10 min. While on ice, 4μl of RNA loading dye (50% (w/v) glycerol, 1M EDTA, 0.25% (v/v) bromophenol blue, 0.25% (v/v) xylene cyanol) was added to each sample. The RNA samples were then loaded onto the gel.

2.2.12.2. Gel preparation and electrophoresis

Electrophoresis of both total RNA and mRNA samples was performed using a 1% agarose-2.2M formaldehyde gel. Prior to the loading of the RNA samples, the gel was pre-electrophoresed at 35V for 10 min. The gel, containing samples, was electrophoresed at 35V until the indicator dye had migrated sufficiently. The quality of RNA preparations was assessed by UV-irradiation, before being transferred to a nylon membrane via a capillary blotting technique.

2.2.12.3. Capillary blotting

Following electrophoresis of the RNA samples, the gel was rinsed in DEPC-treated water for approximately 30 min, then inverted and placed onto the wick of the RNA transfer apparatus. The wick had been prepared from 2 sheets of Whatman 3M paper soaked in 10X sodium citrate/sodium chloride buffer (SSC). The gel was then overlayed with a piece of Hybond™ -nylon membrane pre-soaked initially in deionised water and then 2X SSC. An additional three sheets of pre-soaked Whatman 3M paper were placed over the membrane, a 5-7cm high stack of absorbent paper was placed on top of this and a 500g weight was rested over the paper to facilitate capillary action. The blot was surrounded with plastic wrap and the transfer was allowed to proceed overnight in 10X SSC. Following transfer, the gel was stained with ethidium bromide and viewed

under UV illumination to ensure the complete transferral of RNA onto the nylon membrane. Once verified, the RNA was cross-linked onto the membrane by UV radiation. RNA membranes were placed between two pieces of Whatman paper, wrapped in aluminium foil and stored at 4°C until needed.

2.2.12.4. Hybridisation of membranes with α-^{32}P labelled koala cDNA probes

Membranes were hybridised with α-^{32}P labelled koala *CYP2C* and *UGT* cDNA probes as described in section 2.2.11.4. of this chapter.

Polymerase chain reaction techniques

2.2.13. Reverse transcription of RNA

First-strand cDNA was synthesised from marsupial total RNA using the First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Sydney, Australia) according to manufacturer protocols. Approximately 5μg of total RNA was added to a 0.5ml microcentrifuge tube and brought to a volume of 8μl with DEPC-treated water. The RNA solution was heated to 65°C for 10 min then quickly chilled on ice. 5μl of Bulk First-Strand cDNA Reaction Mix (Cloned, FPLC*pure*® Murine Reverse Transcriptase, RNAguard (porcine), RNAse/DNAse-Free BSA, dATP, dCTP, dGTP, and dTTP in aqueous buffer), 1μl of DTT solution (200mM aqueous solution) and 1μl of *Not*I-d(T)18 primer (0.2μg/μl) was added to the heat-denatured RNA and mixed by pipetting up and down several times. The solution was incubated at 37°C for 1 hr, and the first-strand cDNA reaction product was stored at -20°C until use. The first-strand cDNA reaction product was directly utilised in PCR amplification experiments.

2.2.14. Polymerase chain reaction (PCR)

PCR was used to amplify short fragments of marsupial *CYP2C* cDNA sequences from first-strand cDNA using degenerate oligonucleotide primers. The amplified fragments were then utilised as probes to screen a marsupial cDNA library. PCR was also used to amplify *UGT* sequences from marsupial first-strand cDNA. PCR reactions were performed using *Taq* polymerase (Life Technologies, Sydney).

2.2.14.1. PCR amplification of marsupial *CYP2C* and *UGT* sequences

Oligonucleotide primers (see section 5.3.1. and section 6.3.1. of Chapters 5 and 6 for primer details) at concentrations of 150ng/μl were utilised in PCR amplification experiments with koala, western quoll and tammar wallaby liver cDNA. Generally, 1μl of first-strand cDNA reaction product was used in conjunction with 2μl of 10X PCR buffer (no $MgCl_2$), 1μl of forward primer (150ng/μl), 1μl of reverse primer (150ng/μl), 1μl of dNTPs (10 mM), 0.5-2μl of 50 mM $MgCl_2$ and 0.3μl of *Taq* DNA polymerase (5 U/μl) and adjusted to a final volume of 20μl with sterile, distilled water. Reaction samples were gently mixed and briefly spun before being overlayed with mineral oil. PCR amplifications were performed using a Perkin Elmer DNA thermal cycler (model 480) under variable conditions optimised to the following: 94°C for 3 min; 35-40 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min; followed by a final extension of 72°C for 10 min.

On completion, PCR products were analysed for expected sizes using 2% (w/v) agarose gels and visualised with ethidium bromide and UV illumination (section 2.2.9.3). PCR products were subcloned directly into pGEM®-T vector and sequenced using M13 Universal forward and reverse primers (section 2.2.10).

2.2.15. 5' Rapid Amplification of cDNA ends (RACE) of a koala *CYP2C* cDNA sequence

As an alternative to library screening, 5' RACE-PCR was used in an attempt to isolate the 5' region of a koala *CYP2C* cDNA sequence. The 5' RACE System for Rapid Amplification of cDNA Ends, Version 2.0 Kit (Life Technologies, USA) was used and the manufacturers reagents and protocols implemented. In summary, the technique involved synthesising the 5' end of first-strand cDNA from koala total RNA using a primer specific for a koala *CYP2C* cDNA sequence (KGSP2C). Following purification, the first-strand cDNA was tailed using TdT enzyme. Once tailed, PCR amplification is performed using two primers: one a gene-specific koala CYP2C primer that anneals slightly 5' of KGSP2C; the other a universal amplification primer (UAP) provided with the system that binds to the TdT-tailed 5' region of the cDNA molecule.

2.2.15.1. First strand cDNA synthesis

Briefly, approximately 100ng of koala total RNA was added to 10ng of a gene-specific anti-sense primer, KGSP2C (see section 5.3.1. of Chapter 5 for primer details), then made up to a final volume of 15.5μl with DEPC-treated water. The mixture was incubated at 70°C for 10 min in order to denature the RNA. After chilling for 1 min on ice, the mixture was briefly centrifuged to reconcentrate the solution at the bottom of the tube. The following solutions were then added in the order given: 2.5μl of 10X PCR Buffer (200mM Tris-HCl (pH 8.4), 500mM KCl), 2.5μl of 25mM $MgCl_2$, 1μl of 10mM dNTP mix (10mM each dATP, dCTP, dGTP, dTTP) and 2.5μl of 0.1M DTT. The solution was gently mixed, briefly centrifuged, then incubated for 1 min at 42°C. 1μl of Superscript™ II Reverse Transcriptase (200 units/μl) was added, mixed gently and the solution was further incubated for 50 min at 42°C. The sample mixture was incubated at 70°C for 15 min in order to terminate the first-strand synthesis reaction. Following centrifugation for 10 sec, the mixture was placed at 37°C. 1μl of RNAse mix was added, mixed thoroughly, then incubated for a further 30 min at 37°C. The final first-strand cDNA solution was stored at -20°C.

2.2.15.2. GLASSMAX spin cartridge purification of cDNA

The first strand cDNA product was purified from excess primer and nucleotides using the manufacturers kit and protocols (Life Technologies, USA). Briefly, 120μl of binding solution (6M NaI) was added to the first strand cDNA reaction product. The cDNA/ NaI solution was transferred to a GLASSMAX spin cartridge and centrifuged at 13,000 rpm for 20 sec. The flowthrough was removed and 400μl of cold 1X wash buffer was added to the spin cartridge. The cartridge was spun at 13,000 rpm for 20 sec. This step was repeated three additional times. The cartridge was then washed twice with 400μl of cold 70%(v/v) ethanol as described in the previous step, and spun for an additional minute at 13,000 rpm. After transferring the spin cartridge into a fresh sample recovery tube, 50μl of sterilised, distilled water (preheated to 65°C) was added to the cartridge. The cartridge was spun at 13,000 rpm for 20 sec to elute the cDNA.

2.2.15.3. TdT-tailing of purified koala 5' RACE cDNA

The following components were added to a 0.5ml eppendorf tube in the order given: 6.5μl of DEPC-treated water, 5μl of 5X tailing buffer (50 mM Tris-HCl (pH 8.4), 125 mM KCL, 7.5 mM $MgCl_2$), 2.5μl of 2 mM dCTP and 10μl of column purified koala cDNA. The mixture was incubated at 94°C for 3 min then chilled on ice. Following a brief centrifugation, 1μl of TdT was added and the mixture incubated at 37°C for 10 min. The TdT was inactivated by incubating the mixture at 65°C for 10 min. The reaction mixture was stored at -20°C until use.

A variation of the TdT-tailing procedure was also performed which involved incubating the mixture at 37°C for 30 min following the addition of TdT.

2.2.15.4. PCR amplification of the 5' end of a koala *CYP2C* cDNA sequence

PCR amplification experiments were conducted as previously described in section 2.2.14.1 of this chapter. Three gene-specific primers designated KGSP2A, KGSP2B and KGSP2C (see section 5.3.1. of Chapter 5 for primer details) were designed based on the sequence analysis of the koala *CYP2C#9* clone (section 5.4.2.3. of Chapter 5). The KGSP2C primer was used for first-strand synthesis while the other two internal primers, KGSP2B and KGSP2A were utilised in PCR experiments.

2.2.16. 3' RACE of a koala *CYP2C* cDNA sequence

The 3' RACE system for the rapid amplification of cDNA ends, Version 2.0 kit (Life Technologies, USA) was used in order to amplify the 3' region of a koala *CYP2C* cDNA sequence. In summary, the process involved synthesising first-strand cDNA at the poly(A) tail of the mRNA, using an adaptor primer provided with the system. Following the digestion of the original mRNA template, PCR amplification was performed using two primers: one was a gene-specific koala *CYP2C* primer (KGSP2H) that anneals to a site located within the cDNA molecule; the other was a universal amplification primer (UAP) provided with the system that binds to the 3' region of the cDNA template.

2.2.16.1. First strand cDNA synthesis

Briefly, approximately 1μg of koala total RNA was made up to a final volume of 11μl with DEPC-treated water. 1μl of 10μM universal amplification primer (UAP) solution was added before the mixture was incubated at 70°C for 10 min. After chilling for 1 min on ice, the mixture was briefly centrifuged to reconcentrate the solution at the bottom of the tube. The following solutions were then added in the order given: 2μl of 10X PCR Buffer (200mM Tris-HCl (pH 8.4), 500mM KCl), 1μl of 10mM dNTP mix (10mM each dATP, dCTP, dGTP, dTTP), 2μl of 0.1M DTT and 2μl of 25mM $MgCl_2$. The solution was gently mixed, briefly centrifuged, then incubated for 2 min at 42°C. 1μl of Superscript^{TM} II Reverse Transcriptase (200 units/μl) was added, mixed gently and the solution further incubated for 50 min at 42°C. Following centrifugation for 10 sec, the mixture was placed on ice and 1μl of RNAse H was added, mixed thoroughly, then incubated for a further 20 min at 37°C. The first-strand cDNA solution was utilised directly in PCR amplification experiments and was stored at –20°C until use.

2.2.16.2. PCR amplification of the 3' end of a koala *CYP2C* cDNA sequence

PCR amplification experiments were conducted as previously described in section 2.2.14.1. of this Chapter. Amplified PCR products were subcloned (section 2.2.8) and sequenced (section 2.2.10) to determine their identity.

cDNA library screening techniques

2.2.17. cDNA library screening

Koala and tammar wallaby liver cDNA libraries were screened in order to isolate full-length marsupial *CYP2C* and *UGT* cDNA sequences. In summary, the technique involved immobilising plaques containing cDNA sequences onto nylon filters and hybridising with a specific radioactively-labelled cDNA probe. The filters were then washed to remove any unbound probe and subjected to autoradiography.

2.2.17.1. Liquid culture preparation of *E.coli* XL1-Blue MRF' cells

A single, isolated colony from a primary working plate was innoculated in 10ml LB broth +10mM $MgSO_4$ and 0.2% maltose and incubated overnight in a 37°C shaking incubator. The culture was centrifuged at 2,700 rpm for 20 min in a swing-out centrifuge to pellet the host bacteria. The cells were finally resuspended in 5ml of 10mM $MgSO_4$.

2.2.17.2. Library plating

A dilution of the koala and tammar wallaby cDNA libraries were prepared such that they would yield approximately 50,000 pfu for each 150mm LB + $MgSO_4$ agar plate. The library dilution was prepared in 100µl of SM buffer, then combined with 600µl of an overnight XL1-BLUE MRF' culture in 10ml sterile tubes. The tubes were then incubated at 37°C for 15 min. Prior to plating, ten 150mm LB + $MgSO_4$ plates were dried for at least 1 hr at 37°C to guard against the soft-top agarose layer peeling off during the lifting procedure, and to also ensure that the plaques did not run together. 8ml of molten LB soft-top agarose + 10mM $MgSO_4$ was added to each of the cell suspensions, mixed quickly, then poured onto the prewarmed plates. Plates were then incubated in an inverted position at 37°C until the plaques reached approximately 1.5mm in diameter.

2.2.17.3. Plaque transferral to nylon membranes

A Nitrobind Nylon Membrane (Geneworks, Adelaide) was numbered using a permanent pen and layed onto the hardened LB soft top agarose. The filter was marked in three asymmetrical locations by stabbing through the filter and into the agar of the plate with a sterile needle immersed in ink. After 2 min, the filter was carefully removed using sterile forceps and layed onto Whatman paper soaked in DNA denaturing solution (1.5M NaCl, 0.5M NaOH). After 5 min, the filter was removed and layed onto Whatman paper soaked in neutralising solution (1.5M NaCl, 0.5M Tris-HCl (pH 8.0)). After another 5 min incubation, the filter was placed onto Whatman paper soaked in 2X SSC. After 5 min, the filter was placed into a UVC- 500UV crosslinker and irradiated for 20 sec. The UV-irradiation process permanently cross-links the DNA to the filter. The filter was finally left to dry between two sheets of paper. A second filter was then placed onto the same plate and marked with ink at the same three locations as for the original filter. The

filter was removed after 3 min, then denatured, neutralised and rinsed as explained for the original filter.

2.2.17.4. Hybridisation of filters with α-^{32}P labelled marsupial cDNA probes

Filters were hybridised with a α-^{32}P labelled cDNA probe in order to detect larger size cDNA fragments (section 2.2.11.4). Unbound probe was removed from the filters by washing with 2X SSC and 0.5% SDS at room temperature for 30 min, followed by washing at higher temperatures (50-60°C) with 1X SSC and 0.1% SDS. The filters were then layed on Whatman paper and autoradiographed for 24-48 hr. Positive plaques were isolated and subjected to three rounds of screening before undergoing *in vivo* excision.

2.2.17.5. *In vivo* excision of positive clones

Single plaques of interest were cored from the agar plate and transferred to a sterile 1.5ml microcentrifuge tube containing 500μl of SM buffer and 20μl of chloroform. After brief vortexing, the tube was incubated overnight at 4°C.

Separate overnight cultures of XL1-Blue MRF' cells, supplemented with 0.2 % (w/v) maltose and 10mM $MgSO_4$, and XLOLR cells in LB broth were prepared at 37°C. The next day, the XL1-Blue MRF' and XLOLR cells were spun down (1250 x g for 20 min) and resuspended at an OD_{600} of 1.0 in 10mM $MgSO_4$. The following components were then combined in a Falcon polypropylene tube: 200μl of XL1-Blue MRF' cells at an OD_{600} of 1.0, 250μl of phage stock (containing >1 x 10^5 phage particles) and 1μl of the ExAssist helper phage (>1 x 10^6 pfu/μl). After an incubation at 37°C for 15 min, 3ml of LB broth was added to the tube and re-incubated overnight at 37°C with shaking. Next day, the tube was heated to 65°C for 50 min, then spun at 1000 x g for 15 min. The supernatant was decanted into a new, sterile polypropylene tube. This stock contains the excised pBK-CMV phagemid packaged as filamentous phage particles. The excised phagemids were plated by adding 50μl of the phage supernatant to 200μl of XLOLR cells (OD_{600} = 1.0) in a 1.5 ml microcentrifuge tube. After an incubation at 37°C for 15 min, 200μl of cell mixture was plated onto LB-kanamycin plates (50μg/ml). The plates were incubated

overnight at 37°C. Colonies appearing on the plate contain the pBK-CMV double-stranded phagemid with the cloned cDNA insert.

2.2.17.6. Sequencing of excised library clones

Selected clones were sequenced as described previously (section 2.2.10). Briefly, T3 and T7 primers were utilised to determine the 5' and 3' ends of the cDNA insert, respectively. In addition, specific internal primers were designed to sequence the remaining segments.

Chapter 3

Marsupial Tissue Bank

3.1. Introduction

A number of Australian marsupial species are now classified as either threatened, endangered or vulnerable. The critical lack of numbers of many marsupials, combined with their tourist and cultural value, has made it difficult to study any marsupial for molecular scientific research. In addition, scientific research into marsupials has in the past been dogged with political, public and funding issues. Consequently, during the past few decades the number of studies into the xenobiotic metabolism of Australia's native species has been severely limited.

At the commencement of this project, only a single sample of Western quoll (*Dasyurus geoffroii*) liver tissue was available for use. Therefore, to facilitate the proposed studies of xenobiotic metabolism in marsupial species, it was necessary to assemble a Marsupial Tissue Bank at the School of Pharmacy and Medical Sciences. The underlying aim of this work was to acquire tissue samples from representative Australian marsupial species, thereby facilitating a comprehensive and continuing analysis of marsupial enzyme expression and function. Through the continuing help and generosity of Cleland Wildlife Park, the Adelaide Zoo, veterinarians, educational institutions and the general public, a diverse array of marsupial tissues are now available for research use. The tissue samples collected from each species are discussed in the following sections.

3.2 Research strategy

The research strategy, to obtain tissue samples from a variety of Australian marsupial species suitable for molecular biology use initially involved establishing links between other educational institutions involved in marsupial research, and wildlife parks. Once aware of our research and needs, each institution was generous in providing their time and materials, where possible, to further promote research into Australia's marsupial fauna.

3.3. Methods

3.3.1. Collection and storage of marsupial tissue samples

The scavenged marsupial tissue samples utilised in each research experiment were obtained with the approval of the Institute of Medical and Veterinary Science Animal Ethics Committee. Western Grey kangaroo, pouch young koala and tammar wallaby (adult and pouch young) tissue samples were obtained from Kangaroo Island, South Australia as part of a government-approved sterilisation and culling programme. Road-injured adult koalas were obtained from the Adelaide Hills and were euthanased using phenobarbital at the Department of Anatomical Sciences, University of Adelaide, if deemed to possess terminal injuries. Eastern grey and red kangaroos were euthanased at the Cleland Wildlife Park, South Australia by park officers acting within the terms of an authorised culling programme. Brushtail and ringtail possums were captured in metropolitan Adelaide and also euthanased using phenobarbital at the University of Adelaide, after which scavenged tissue was taken. Southern-hairy nosed wombats were euthanased in the Riverland region, South Australia as part of an ecological study conducted by Monash University, Victoria. Finally, the Brush-tailed Bettong, Eastern and Western Quoll, Spinifex Hopping mice, Plains rat, Long-nosed Potoroo and Yellow-footed Rock Wallaby tissue samples were kind donations from Chris Leigh, Department of Anatomical Sciences, University of Adelaide.

Selected tissue samples were excised from the animal within 5 min of death and immediately snap-frozen in liquid nitrogen. The samples remained frozen in nitrogen until transferred to a -70°C freezer for long-term storage.

3.4. Results

The individual species from which tissue samples were collected are tabulated in **Table 3.1.** As the major sites of xenobiotic metabolism are the liver, lung and kidneys, these tissues have been exclusively collected. The bank contains tissues from various herbivorous species such as the koala, eastern grey, western grey and red kangaroos, brushtail and ringtail possums, wallabies, wombat and bettong. Of the eight families that comprise the Order Diprotodonta, the tissue bank contains representatives from six of them. The tissue bank also comprises carnivorous members from the Order Polyprotodonta such as the Western and Eastern Quolls. Of the six families which represent the order, the bank contains individuals from one of them. Further attempts to obtain tissue from this order have been hindered by the endangered status of a number of its members.

Finally, tissues from juvenile koalas at varying stages of pouch development have also been obtained. The ages of the pouch young koalas were estimated based on their body weight and head size. The youngest individual was estimated to be 140 days old, while the oldest 400 days. In addition, liver samples from two pouch young tammar wallabies (one male and one female) have also been collected. The ages of the juvenile wallabies are estimated to be less than 200 days.

Table 3.1. Marsupial tissue bank

The Australian marsupial species from which liver, lung and/or kidney samples were collected.

Marsupial	Species Name	No. of Specimens
Koala	*Phascolarctos cinereus*	13
Koala pouch young	*Phascolarctos cinereus*	4
Tammar wallaby	*Macropus eugenii*	9
Tammar wallaby pouch young	*Macropus eugenii*	2
Southern hairy-nosed wombat	*Lasiorhinus latifrons*	1
Long-nosed potoroo	*Potorous tridactylus*	1
Eastern quoll	*Dasyurus viverrinus*	1
Western quoll	*Dasyurus geoffroii*	1
Brush-tailed bettong	*Bettongia penicillata*	1
Yellow-footed rock-wallaby	*Petrogale xanthopus*	1
Eastern grey kangaroo	*Macropus giganteus*	10
Red kangaroo	*Macropus rufus*	3
Western grey kangaroo	*Macropus fuliginosus*	3
Common brushtail possum	*Trichosurus vulpecula*	5
Common brushtail possum pouch young	*Trichosurus vulpecula*	1
Common ringtail possum	*Pseudocheirus peregrinus*	1
Plains rat	*Pseudomys australis*	5
Spinifex hopping-mouse	*Notomys alexis*	5

3.5. Discussion

The marsupial tissue bank now housed at the School of Pharmacy and Medical Sciences, University of South Australia represents the most complete and diverse array of marsupial tissue samples collected for biochemical work anywhere in Australia. All specimens are of sufficient quality to allow RNA extraction and subsequent cloning studies. Investigations into the species-specific, tissue-specific and sex-specific expression of any enzyme family can now be achieved utilising this resource. In addition, the availability of a range of juvenile tissue samples from the koala and tammar wallaby should initiate research into the developmental expression of xenobiotic-metabolising enzymes. Such studies may elucidate any role these enzymes play in unique characteristics of marsupials including their dietary tolerance and reproductive strategy.

Chapter 4

Cytochrome P450 expression in representative Australian marsupial species

4.1. Introduction

The Australian marsupial fauna comprises a variety of species exhibiting unique dietary and physiological characteristics. For example, a number of marsupial species survive on the unlikely food source of *Eucalyptus* foliage. Such arboreal animals, which include the koala, greater glider and ringtail possum ingest significant quantities of eucalyptus oil, and its toxic constituents (eg. terpenes, phenols), on a daily basis (Eberhard *et al.*, 1975; McLean and Foley, 1997). The evolution of novel mechanisms and pathways for the detoxification of ingested dietary toxins by *Eucalyptus*-eating marsupial species therefore seems likely (McLean and Foley, 1997).

Investigations into the metabolism of dietary terpenes by specialist *Eucalyptus*-eating marsupials revealed that they metabolised the compounds through extensive oxidation resulting in the formation of acidic, highly polar metabolites (Southwell, 1975; Southwell *et al.*, 1980; McLean and Foley, 1997; Boyle *et al.*, 1999). Marsupials that exhibit a more varied diet, for example the brush-tailed possum, do not appear to oxidise terpenes as extensively (Southwell *et al.*, 1980; Boyle *et al.*, 2000). Further studies investigating the metabolic fate of dietary chemicals in a variety of marsupial species also revealed that folivorous marsupials, such as the brushtail possum and koala, exhibited a limited capacity to conjugate with sulphate groups, and relied heavily on glucuronide for conjugation (Baudinette *et al.*, 1980).

Relatively few studies have examined the enzymatic mechanisms driving xenobiotic metabolism in marsupials, and prior to this work no studies have reported data obtained from koala, tammar wallaby or wombat liver. An initial investigation by McManus and Ilett (1976) into components of the hepatic mixed function oxidative pathway of the quokka (*Setonix brachyurus*), revealed that quokkas generally exhibited lower rates of oxidative drug metabolism compared to other eutherian animals. However, measurements of some components of the cytochrome P450 pathway in hepatic microsomes revealed that the quokka possessed similar levels of cytochrome P450, b_5 and NADPH- cytochrome *c* reductase activities to the rat (McManus and Ilett, 1976). An additional study by McManus and Ilett (1977) which assessed microsomal xenobiotic metabolism in a range of Australian marsupial species, including the grey kangaroo (*Macropus fuliginosus*), bettong (*Bettongia penicillata*) and brushtail possum (*Trichosurus vulpecula*), also reported

similar MFO enzyme activities between the marsupials and the rat. A more recent study by Bolton and Ahokas (1997a) with the brushtail possum reported similar findings.

The development of the hepatic mixed function oxidase system in Australian marsupials has been investigated in the quokka (*Setonix brachyurus*) and brushtail possum (McManus *et al.*, 1978; Bolton and Ahokas, 1997d).

One aim of this project was to examine selected components of the mixed function oxidation pathway in two marsupial species, the koala and tammar wallaby, by assessing their total CYP content and NADPH-cytochrome *c* reductase enzyme activities. In addition, immunoblot analyses were conducted with three marsupial species, the koala (including pouch young), tammar wallaby and the wombat, in order to gain an understanding of their CYP enzyme profile.

4.2. Research strategy

The research strategy, to investigate CYP content and complement in a selection of marsupial species, involved conducting traditional CYP assays and immunoblot studies utilising a panel of polyclonal antibodies raised against the major xenobiotic-metabolising CYPs. Immunoblot studies focused on liver microsomes from adult koala, tammar wallaby and wombat, in addition to pouch young koala liver. The use of antibodies raised against various CYP proteins in different species has previously been shown to be successful in investigating the CYP complement of species for which no information exists.

Polyclonal antibodies raised against the following xenobiotic-metabolising CYPs were used: human CYP2D6, rat CYP2E1, rabbit CYP3A6, rat CYP2C6 and rat CYP2C11. This panel of antibodies covers all of the major xenobiotic-metabolising CYP families other than the CYP1 family. Studies of the CYP1 family were conducted by Mark Woolley (PhD candidate) in parallel with the studies reported here. Finally, the NADPH-cytochrome P450 reductase protein, a critical component of the mixed function oxidase system, was investigated in marsupial liver microsomes using an antibody raised against the human NADPH-cytochrome P450 reductase protein.

4.3. Results

4.3.1. Enzyme assays

Results of total CYP content determinations and NADPH-cytochrome P450 reductase activity assays in koala (two male and two female) and tammar wallaby (five male and one female) are shown in **Table 4.1.** Rat microsomes, prepared from male Hooded Wistar rats as per section 2.2.1, were included in these analyses for comparative purposes. All animals (koalas, wallabies and rats) were sexually mature.

For each experiment, the data are expressed as the mean of triplicate observations. Variability of the population is indicated by the standard deviation (S.D). Statistical analysis was carried out using one-way analysis of variance (STATVIEW, Berkley, USA) followed by a Scheffe *F*-test to detect differences.

Table 4.1. CYP content and NADPH-cytochrome P450 reductase activity in koala, tammar wallaby and rat hepatic microsomes.

Content or activity	Koala (n=4)	Tammar Wallaby (n=6)	Rat (n=4)
Total CYP content (nmol/mg microsomal protein)	**0.87 ± 0.18**	**0.24 ± 0.04***	**0.65 ± 0.07**
NADPH-cytochrome P450 reductase (nmol/mg protein/min)	**150 ± 34**	**167 ± 34.8**	**158 ± 18.5**

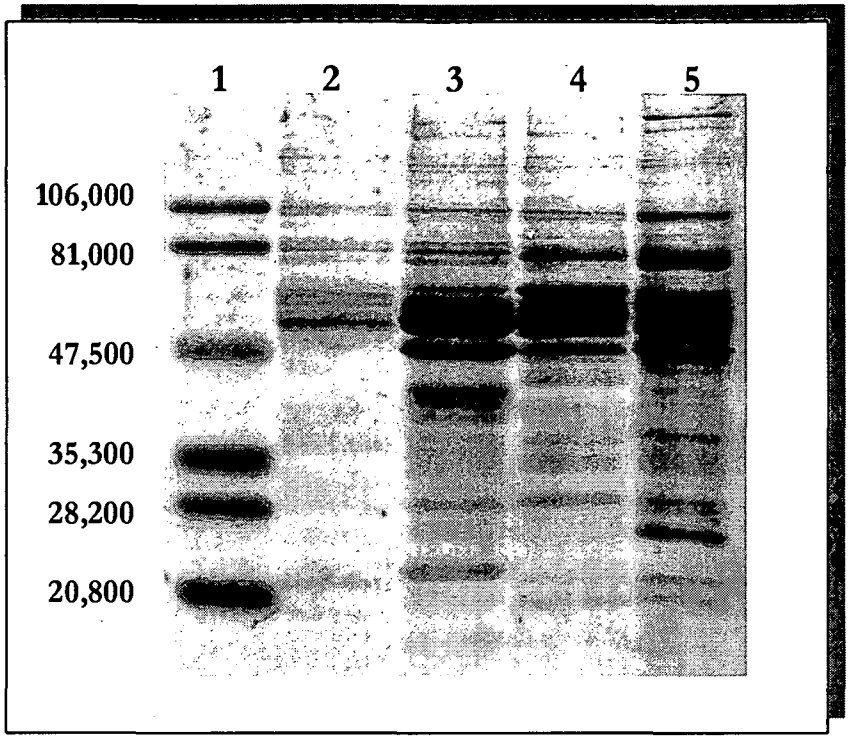
*** Different from koala microsomes at $P<0.05$.**

The results indicate that total CYP content in hepatic microsomes from the koala (0.87 +- 0.18 nmol/mg microsomal protein) is slightly higher to that obtained for the rat (0.65 +- 0.07 nmol/mg microsomal protein). The tammar wallaby hepatic microsomes exhibited lower CYP content than either the koala ($P < 0.05$) or rat samples. The measured NADPH-cytochrome P450 reductase activities were similar in each of the species tested.

4.3.2. SDS-PAGE analysis of koala, tammar wallaby, wombat and rat liver microsomes

Microsomes prepared from male koala, tammar wallaby, wombat and rat were subjected to SDS-PAGE as outlined in Section 2.2.5. The resultant gel was stained with Coomassie brilliant blue to visualise proteins. SDS-PAGE analysis of 30μg of the purified hepatic microsomes revealed that each of the marsupial species exhibited intense protein banding ranging from 47-70 kDa (Figure 4.1, lanes 3-5). Cytochrome P450 proteins characterised in other mammalian species range from 45-60 kDa in size. The purified rat hepatic microsomes exhibited a similar protein banding profile (Figure 4.1, lane 2).

Figure 4.1: Coomassie brilliant blue stain of hepatic microsomes purified from the koala, tammar wallaby, wombat and rat.



30μg of purified hepatic microsomes from the koala, tammar wallaby, wombat and rat were separated by SDS PAGE (10% gel) and stained with Coomassie brilliant blue stain. **Lane 1:** pre-stained marker (Life Technologies), relative sizes of the bands, in daltons, are shown to the left. **Lane 2:** rat hepatic microsomes. **Lane 3:** koala hepatic microsomes. **Lane 4:** tammar wallaby hepatic microsomes. **Lane 5:** wombat hepatic microsomes.

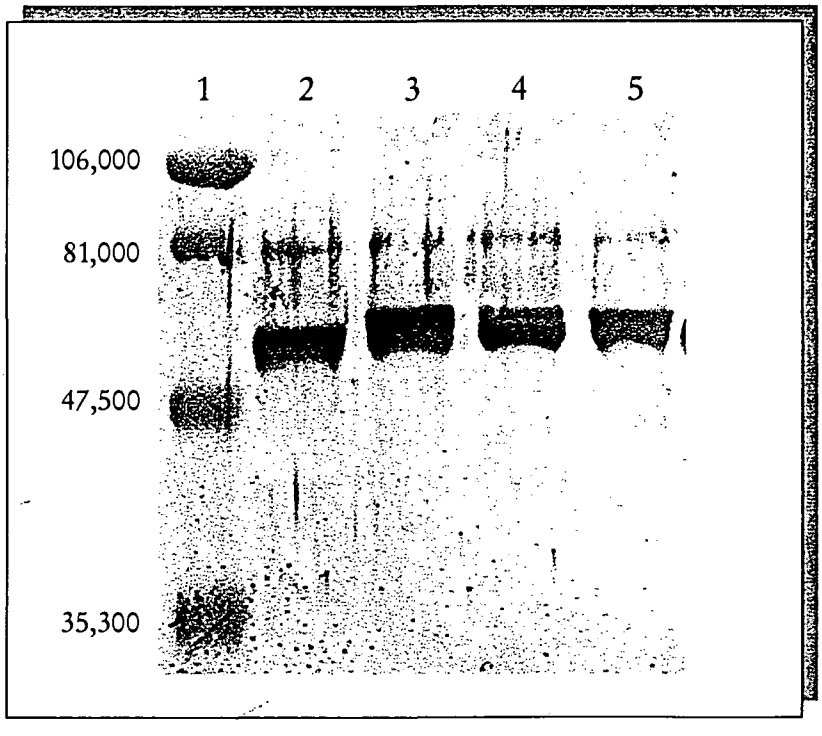
4.3.3. Immunoblot analysis of hepatic microsomes prepared from the koala, tammar wallaby, wombat and rat

4.3.3.1 CYP2C

4.3.3.1.1 Hepatic Expression

Immunoblotting was performed as described in section 2.2.6. To detect CYP2C proteins in marsupial liver microsomes, membranes were probed with a commercially available goat anti-rat CYP2C6 polyclonal IgG (Daiichi Pure Chemical Co., Japan). Immunoblotting experiments were conducted with 10μg of purified hepatic microsomes from each marsupial species. The anti-CYP2C6 IgG identified multiple intense bands of appropriate size (55-60kDa) in the rat and marsupial samples. A representative immunoblot is shown in Figure 4.2. Due to the band intensity, it is unclear precisely how many immunoreactive bands are present.

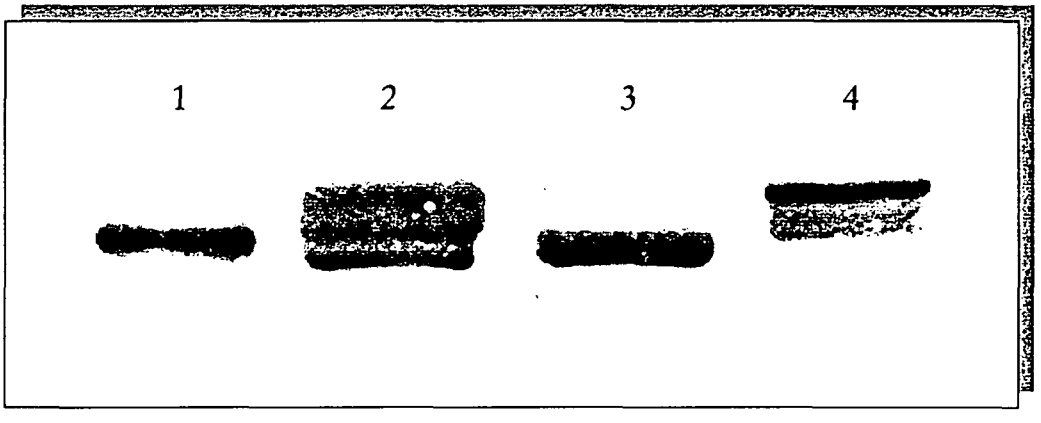
Figure 4.2. **Immunoblot analysis of marsupial liver microsomes with anti-rat CYP2C6 IgG**



Hepatic microsomal fractions (10μg) from koala, tammar wallaby, wombat and rat were separated by SDS PAGE (7.5% gel), transferred to a nitrocellulose membrane, and probed with goat anti-rat CYP2C6 IgG. **Lane 1:** pre-stained marker (Life Technologies). **Lane 2:** rat hepatic microsomes. **Lane 3:** koala hepatic microsomes. **Lane 4:** tammar wallaby hepatic microsomes. **Lane 5:** wombat hepatic microsomes.

In an attempt to more clearly define individual CYP2C6 immunoreactive bands, an additional immunoblot was performed using 5μg of hepatic microsomes from each species (Figure 4.3). Using significantly less protein, a single immunoreactive band corresponding to CYP2C6, was obtained for the rat sample (Figure 4.3, lane 1). The tammar wallaby and wombat microsomal samples both exhibited a single intense immunoreactive band in conjunction with bands of less intensity (Figure 4.3, lanes 3,4). The koala hepatic microsomal sample exhibited at least three immunoreactive bands suggesting that at least three CYP2C enzymes are expressed in the liver of this species (Figure 4.3, lane 2).

Figure 4.3: Immunoblot analysis of marsupial liver microsomes with anti-rat CYP2C6 IgG

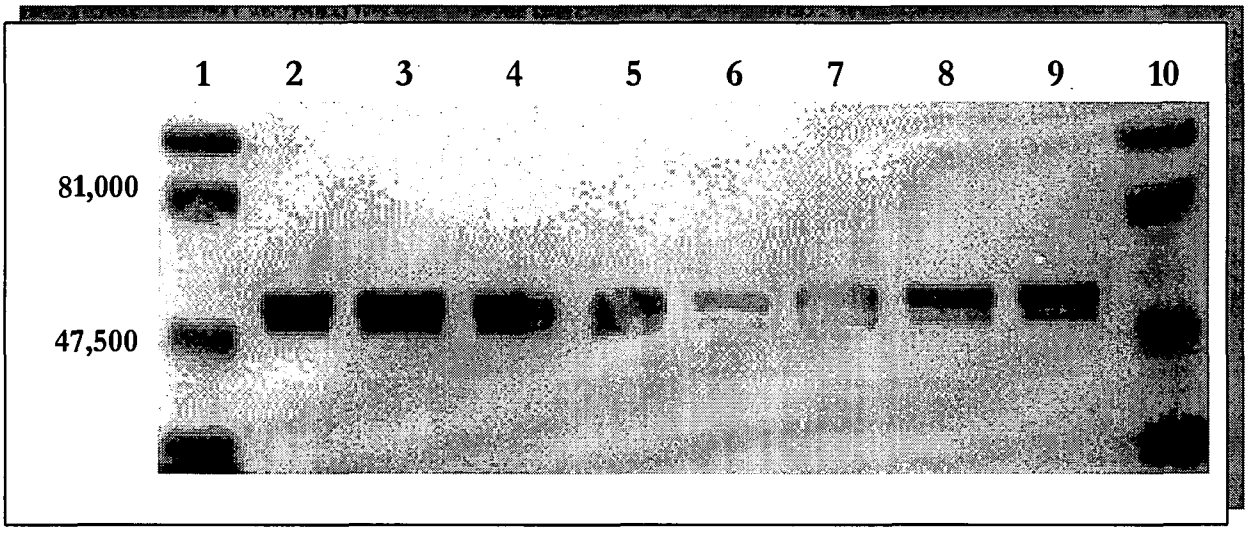


5μg of koala, tammar wallaby, wombat and rat hepatic microsomes were separated by SDS PAGE (7.5% gel), transferred to a nitrocellulose membrane, and probed with goat anti-rat CYP2C6 IgG. **Lane 1:** rat hepatic microsomes. **Lane 2:** koala hepatic microsomes. **Lane 3:** tammar wallaby hepatic microsomes. **Lane 4:** wombat hepatic microsomes.

To identify any age-related differences in CYP2C expression, an immunoblot analysis was performed on pouch young and adult koala hepatic microsomal samples (10μg) using the goat anti-rat CYP2C6 IgG (Figure 4.4). The pouch young koala liver samples included individuals ranging from 180-400 days old. In addition, both male and female samples were analysed to investigate for the presence of any sex differences. Each of the adult liver samples exhibited multiple immunoreactive bands as expected, however no sex differences were observed between the adult samples (Figure 4.4, lanes 2-5, N.B. an air bubble inhibited protein transfer in lane #5

during the immunoblotting process). Furthermore, no significant sex differences were detected for the pouch young koala microsomes (Figure 4.4, lanes 6-9). However, CYP2C immunoreactivity was observed to increase with the advancing development of the pouch young samples (Figure 4.4, lanes 6-9). For example, relatively faint immunoreactive bands were detected in the 240 gm (180 day old) sample, while significant immunoreactivity was detected in the oldest pouch young sample (430 gms).

Figure 4.4. Immunoblot analysis of hepatic pouch young and adult koala liver microsomes with anti-rat CYP2C6 IgG

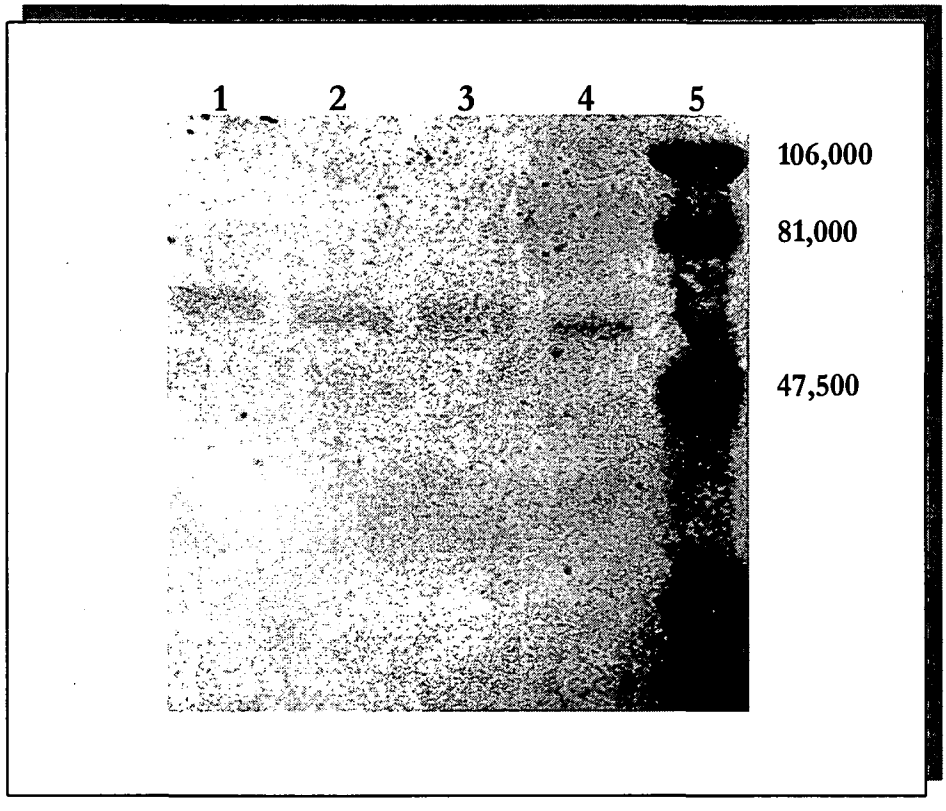


Hepatic microsomal fractions (10μg) purified from male and female pouch young and adult koalas were separated by SDS PAGE (7.5% gel), transferred to a nitrocellulose membrane, and probed with goat anti-rat CYP2C6 IgG. **Lanes 1 and 10:** pre-stained marker. **Lane 2:** adult female koala hepatic microsomes. **Lane 3:** adult female koala hepatic microsomes. **Lane 4:** adult male koala hepatic microsomes. **Lane 5:** adult female koala hepatic microsomes. **Lane 6:** male koala (180-250 days old, 240 gms) hepatic microsomes. **Lane 7:** male koala (180-250 days old, 390 gms) hepatic microsomes. **Lane 8:** female koala (180-250 days, 370 gms) hepatic microsomes. **Lane 9:** male koala (250-400 days, 430 gms) hepatic microsomes.

The availability of a second CYP2C antibody enabled further characterisation of CYP2C expression in marsupials. This antibody was raised against the rat CYP2C11 enzyme.

Immunoblot analysis of 20μg of hepatic microsomes from the koala, tammar wallaby, wombat and rat using the rabbit anti-rat CYP2C11 IgG revealed faint immunoreactive bands for each of the species (Figure 4.5, lanes 1-4). The bands corresponded to the expected size, however were too faint to analyse. Due to the low immunoreactivity of the antibody, it was decided not to perform an immunoblotting experiment with the pouch young koala microsomes.

Figure 4.5. Immunoblot analysis of marsupial liver microsomes with anti-rat CYP2C11 IgG



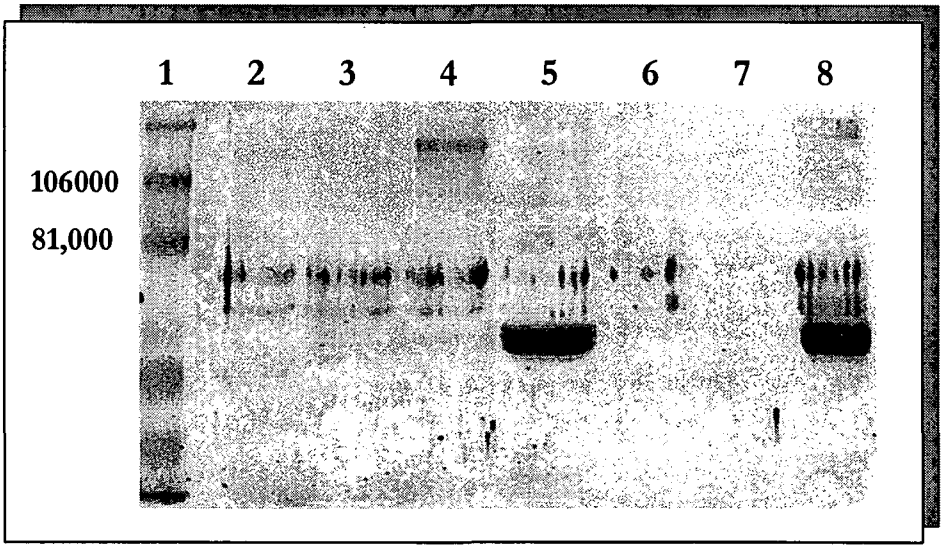
Hepatic microsomal fractions (20μg) from the koala, tammar wallaby, wombat and rat were separated by SDS PAGE (7.5% gel), transferred to a nitrocellulose membrane, and probed with rabbit anti-rat CYP2C11 IgG. **Lane 1:** wombat hepatic microsomes. **Lane 2:** tammar wallaby hepatic microsomes. **Lane 3:** koala hepatic microsomes. **Lane 4:** rat hepatic microsomes. **Lane 5:** pre-stained marker (Life Technologies).

4.3.3.1.2. Extrahepatic Expression

To assess the extrahepatic expression of CYP2C enzymes, immunoblot analysis was performed on a range of extrahepatic marsupial tissues using the goat anti-rat CYP2C6 IgG (Figure 4.6). The

rat lung microsomal sample exhibited a faint immunoreactive band at the size expected (Figure 4.6, lane 4). Multiple rat CYP2C isoforms have previously been detected in the kidney and prostate (Zaphiropoulos and Wood, 1993; Imaoka *et al.*, 1993). The tammar wallaby lung microsomal sample exhibited three faint immunoreactive bands of the expected size (Figure 4.6, lane 3). No CYP2C immunoreactivity was detected in koala lung microsomes (Figure 4.6, lane 2). The koala kidney microsomal sample exhibited three intense, immunoreactive bands which co-migrated with the bands observed in tammar wallaby lung (Figure 4.6, lane 5). The strong intensity of the kidney koala bands most likely reflects unequal loading relative to lung samples (see legend to Figure 4.6).

Figure 4.6. Immunoblot analysis of marsupial extrahepatic microsomes with anti-rat CYP2C6 IgG

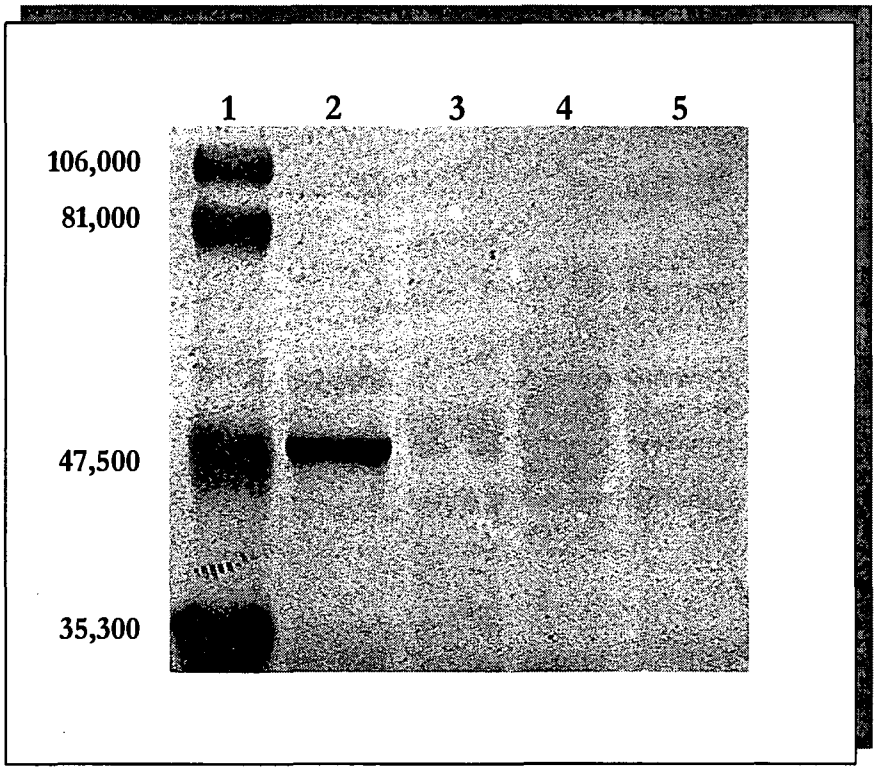


Koala, tammar wallaby, fat-tailed dunnart and rat extrahepatic microsomes were separated by SDS PAGE (7.5% gel), transferred to a nitrocellulose membrane, and probed with goat anti-rat CYP2C6 IgG. **Lane 1:** pre-stained marker (Life Technologies). **Lane 2:** koala lung microsomes (15μg). **Lane 3:** tammar wallaby lung microsomes (15μg). **Lane 4:** rat lung microsomes (20μg). **Lane 5:** koala kidney microsomes (45μg). **Lane 6:** dunnart kidney microsomes (10μg). **Lane 7:** blank. **Lane 8:** rat liver microsomes (20μg) (control).

4.3.3.2. CYP2D

An immunoblotting experiment performed with 20μg of koala, tammar wallaby, wombat and rat hepatic microsomes using anti-human CYP2D6 IgG revealed minimal immunoreactivity in each of the three tested marsupial species (Figure 4.7, lanes 3-5). The rat microsomal sample exhibited a single, strong immunoreactive band (Figure 4.7, lane 2). Given the apparent multiplicity of the rat CYP2D subfamily, this single band most likely reflects several CYP2D isoforms. An additional immunoblotting experiment conducted using 75μg of hepatic microsomes for each species with the CYP2D6 antibody revealed faint immunoreactive bands in each marsupial sample (data not shown).

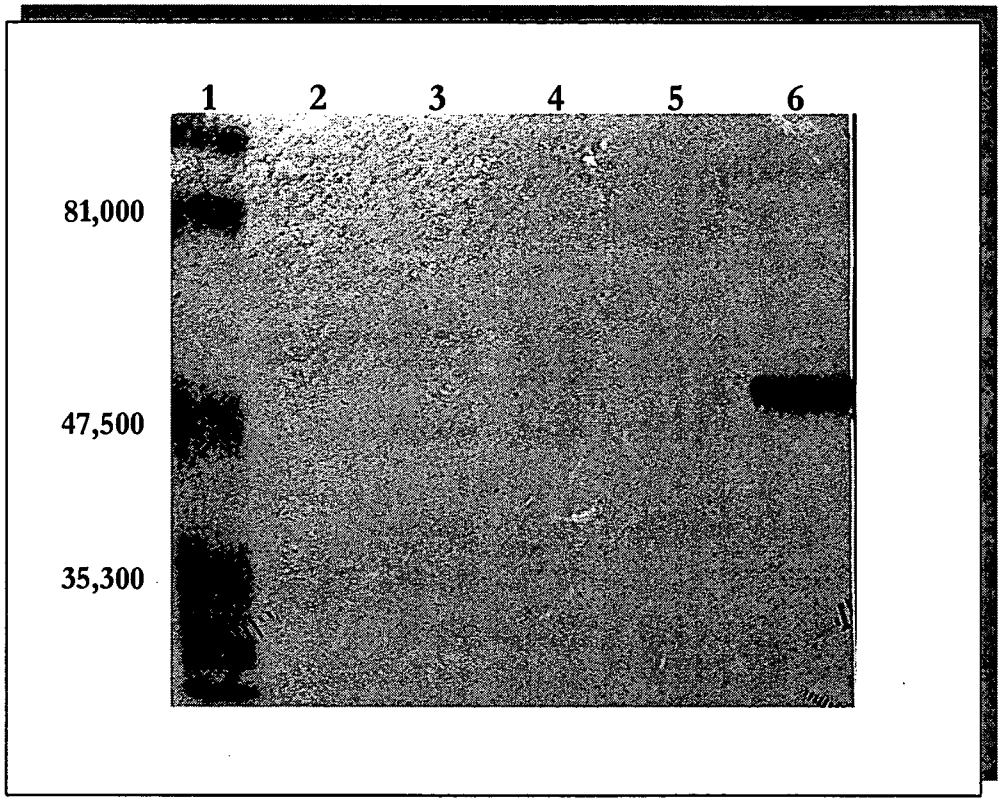
Figure 4.7: Immunoblot analysis of marsupial liver microsomes with anti-human CYP2D6 IgG



20μg of koala, tammar wallaby, wombat and rat liver microsomes were separated by SDS PAGE (7.5% gel), transferred to a nitrocellulose membrane, and probed with anti-human CYP2D6 IgG. **Lane 1:** pre-stained marker (Life Technologies). **Lane 2:** rat hepatic microsomes. **Lane 3:** koala hepatic microsomes. **Lane 4:** tammar wallaby hepatic microsomes. **Lane 5:** wombat hepatic microsomes.

Immunoblot analysis of 20μg of koala pouch young koala hepatic microsomes using the anti-human CYP2D6 IgG also failed to detect immunoreactive bands in any samples (Figure 4.8, lanes 2-5). A strong immunoreactive band was detected in the rat hepatic microsomal sample (Figure 4.8, lane 6).

Figure 4.8. Immunoblot analysis of koala pouch young liver microsomes with anti-human CYP2D6 IgG.

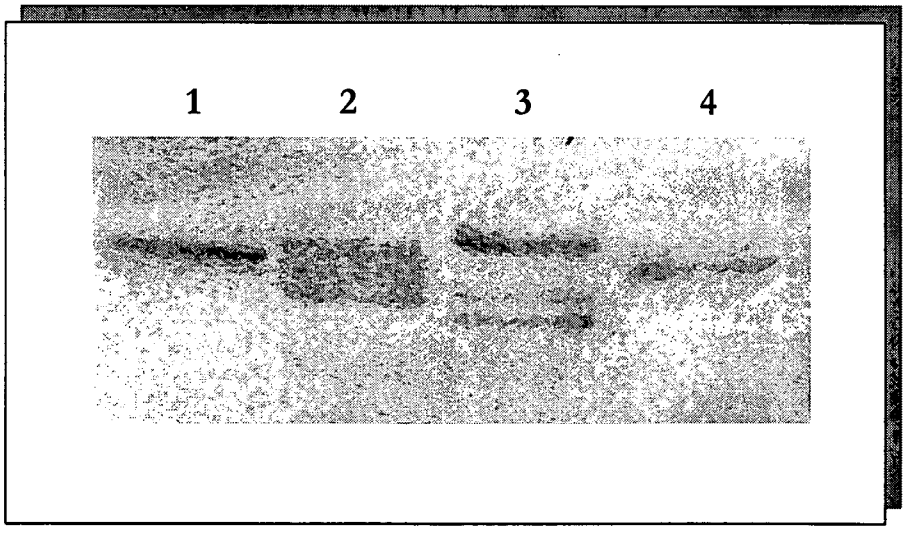


Hepatic microsomal fractions (20μg) purified from male and female pouch young koalas aged between 180-400 days were separated by SDS PAGE (7.5% gel), transferred to a nitrocellulose membrane, and probed with anti-human CYP2D6 IgG. **Lane 1:** pre-stained marker. **Lane 2:** male koala (180-250 days old, 240 gms) hepatic microsomes. **Lane 3:** male koala (180-250 days old, 390 gms) hepatic microsomes. **Lane 4:** female koala (180-250 days, 370 gms) hepatic microsomes. **Lane 5:** male koala (250-400 days, 430 gms) hepatic microsomes. **Lane 6:** rat hepatic microsomes (control).

4.3.3.3. CYP2E1

An immunoblotting experiment performed with 20μg of hepatic marsupial and rat microsomes using a goat anti-rat CYP2E1 antibody revealed immunoreactive bands for each of the selected marsupial species (Figure 4.9, lanes 2-4). This anti-rat CYP2E1 IgG has been extensively characterised and recognises a single isoform in rat, rabbit and humans (Ieva Stupans, personal communication). The tammar wallaby hepatic microsomes exhibited a strong immunoreactive band, most likely representing the marsupial CYP2E1 (Figure 4.9, lane 3). Smaller bands of less intensity were also present in this species. A single predominant band was also observed in wombat microsomes (Figure 4.9, lane 4). The koala microsomes exhibited a multitude of bands (Figure 4.9, lane 2). As expected from previous results, a single immunoreactive band was detected in the rat hepatic microsomal sample (Figure 4.9, lane 1).

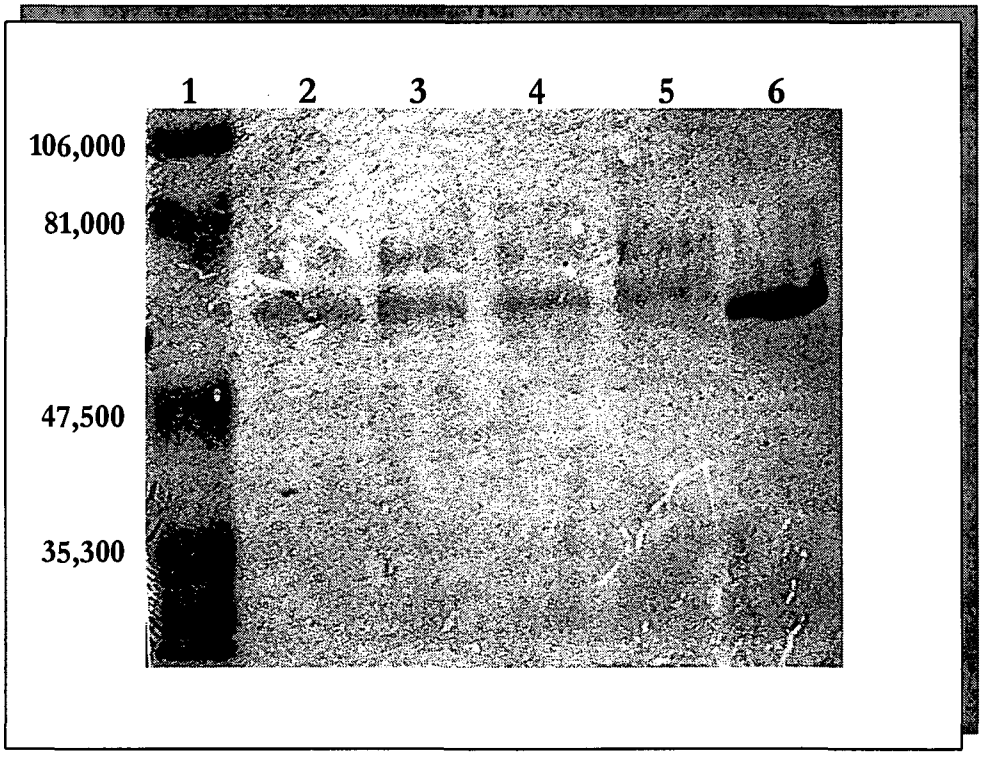
Figure 4.9. Immunoblot analysis of marsupial liver microsomes with anti-rat CYP2E1 IgG.



Hepatic microsomal fractions (20μg) from the koala, tammar wallaby, wombat and rat were separated by SDS PAGE (7.5% gel), transferred to a nitrocellulose membrane, and probed with goat anti-rat CYP2E1 IgG. **Lane 1:** rat hepatic microsomes. **Lane 2:** koala hepatic microsomes. **Lane 3:** tammar wallaby hepatic microsomes. **Lane 4:** wombat hepatic microsomes.

Immunoblot analysis was also performed with a second commercially available sheep anti-rat CYP2E1 antibody (Daiichi Pure Chemical Co., Japan) using 20μg of koala pouch young liver microsomes (Figure 4.10). The pouch young koala microsomes exhibited two immunoreactive bands at each developmental stage. The faster migrating band most likely represents koala CYP2E1 (Figure 4.10, lanes 2-5). The larger immunoreactive band detected in each sample likely reflects non-specific binding of the antibody. The rat hepatic microsomal sample exhibited a strong immunoreactive band at about 60kDa (Figure 4.10, lane 6).

Figure 4.10. Immunoblot analysis of koala pouch young liver microsomes with anti-rat CYP2E1 IgG

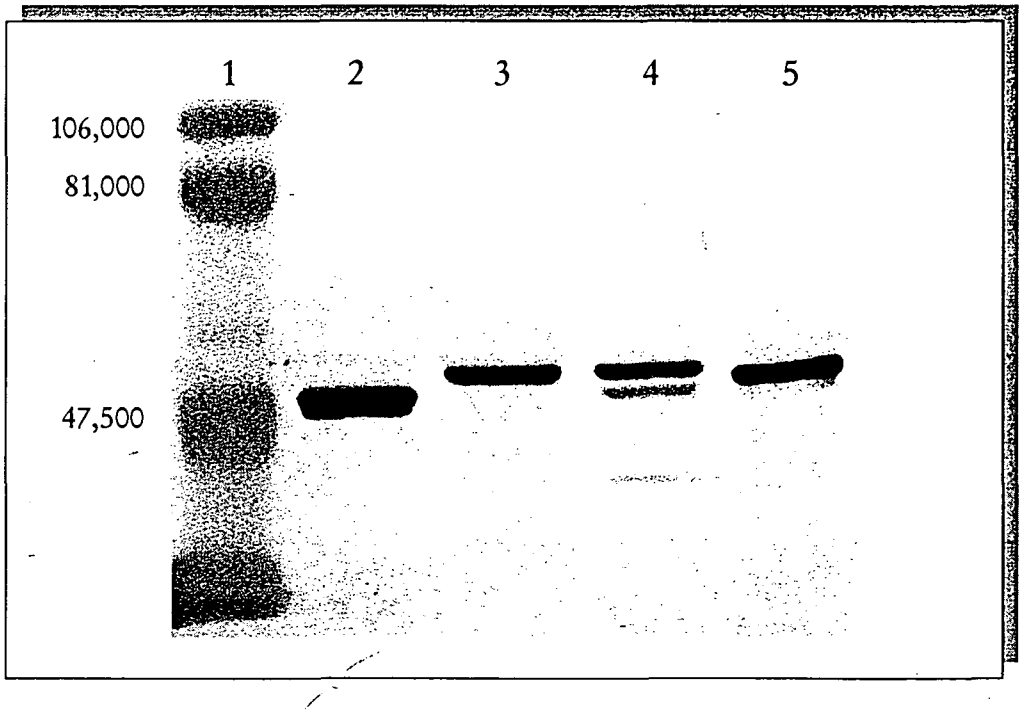


Hepatic microsomal fractions (20μg) purified from male and female pouch young koalas aged between 180-400 days were separated by SDS PAGE (7.5% gel), transferred to a nitrocellulose membrane, and probed with sheep anti-rat CYP2E1 IgG. **Lane 1:** pre-stained marker. **Lane 2:** male koala (180-250 days old, 240 gms) hepatic microsomes. **Lane 3:** male koala (180-250 days old, 390 gms) hepatic microsomes. **Lane 4:** female koala (180-250 days, 370 gms) hepatic microsomes. **Lane 5:** male koala (250-400 days, 430 gms) hepatic microsomes. **Lane 6:** rat hepatic microsomes (control).

4.3.3.4. CYP3A

Immunoblot analysis performed with 20μg of koala, tammar wallaby, wombat and rat liver microsomes with a goat anti-rabbit CYP3A6 IgG detected significant immunoreactivity in each of the tested species (Figure 4.11). The rat microsomal sample exhibited a pair of intense immunoreactive bands of similar size (~48kDa) (Figure 4.11, lane 2). The rat CYP3A subfamily comprises at least four forms, therefore it is likely that the two bands represent multiple CYP3A isoforms. The koala microsomal sample exhibited a single immunoreactive band (Figure 4.11, lane 3). The tammar wallaby and wombat liver microsomes exhibited an intense immunoreactive band in conjunction with a less intense form (Figure 4.11, lanes 4-5). The smaller immunoreactive band detected in the tammar wallaby sample is too small to be a cytochrome P450 protein, and is considered to be a result of non-specific antibody binding (Figure 4.11, lane 4).

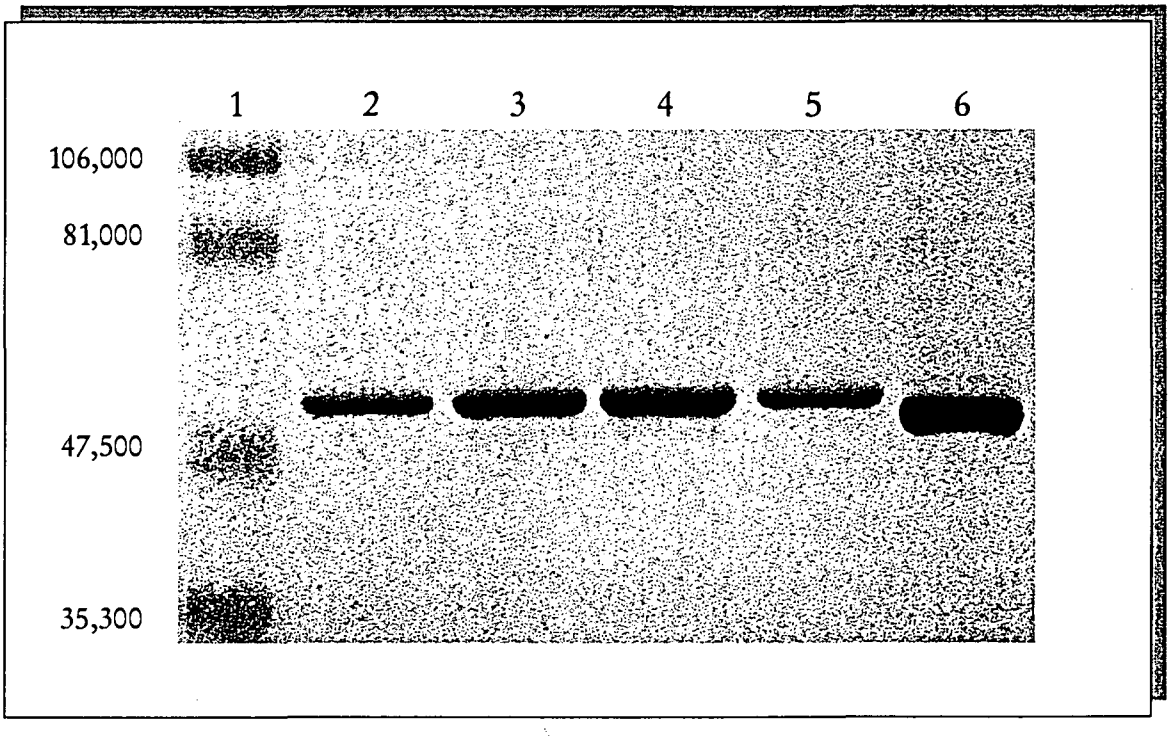
Figure 4.11. Immunoblot analysis of marsupial liver microsomes with anti-rabbit CYP3A6 IgG



Hepatic microsomal fractions (20μg) from the koala, tammar wallaby, wombat and rat were separated by SDS PAGE (7.5% gel), transferred to a nitrocellulose membrane, and probed with goat anti-rabbit CYP3A6 IgG. **Lane 1:** pre-stained marker (Life Technologies). **Lane 2:** rat hepatic microsomes. **Lane 3:** koala hepatic microsomes. **Lane 4:** tammar wallaby hepatic microsomes. **Lane 5:** wombat hepatic microsomes.

Immunoblot analysis of 20μg of koala pouch young liver microsomes using the anti-rabbit CYP3A6 IgG produced a comparable result to that previously seen with the adult koala liver sample (Figure 4.12). The koala liver microsomes exhibited a single, intense immunoreactive band of approximately 49 kDa at each developmental stage although the intensity of this band varied significantly (Figure 4.12, lanes 2-5). A doublet of immunoreactive bands were again detected in the rat hepatic microsomal sample (Figure 4.12, lane 6).

Figure 4.12. Immunoblot analysis of koala pouch young liver microsomes with anti-rabbit CYP3A6 IgG

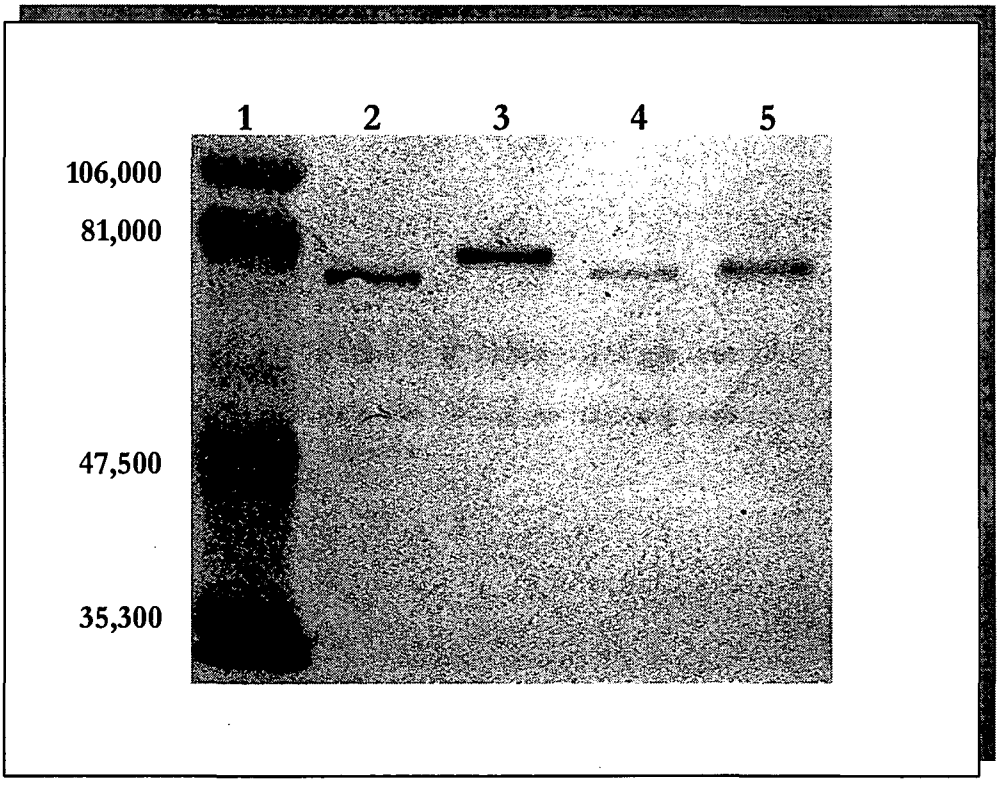


20μg of hepatic microsomes purified from male and female pouch young koalas aged between 180-400 days were separated by SDS PAGE (7.5% gel), transferred to a nitrocellulose membrane, and probed with goat anti-rabbit CYP3A6 IgG. **Lane 1:** pre-stained marker. **Lane 2:** male koala (180-250 days old, 240 gms) hepatic microsomes. **Lane 3:** male koala (180-250 days old, 390 gms) hepatic microsomes. **Lane 4:** female koala (180-250 days, 370 gms) hepatic microsomes. **Lane 5:** male koala (250-400 days, 430 gms) hepatic microsomes. **Lane 6:** rat hepatic microsomes (control).

4.3.3.5. NADPH-cytochrome P450 reductase

Immunoblot analysis of 5μg of marsupial liver microsomes with a goat anti-human NADPH-cytochrome P450 reductase IgG is shown in Figure 4.13. The anti-human NADPH-cytochrome P450 reductase IgG recognises a single band in rat, rabbit and human microsomes (McClure and Stupans, 1995). The koala liver microsomal sample exhibited an immunoreactive band which migrated slower in comparison to the other tested marsupial species and the rat (Figure 4.13, lane 3). The tammar wallaby and wombat samples also exhibited slightly slower migrating immunoreactive bands compared to rat microsomes (Figure 4.13, lanes 4-5). The NADPH-cytochrome P450 reductase molecule is generally 78 kDa in most eukaryotic cells (Porter, 1991).

Figure 4.13. Immunoblot analysis of marsupial liver microsomes with anti-human NADPH-cytochrome P450 reductase IgG.



Hepatic microsomal fractions (5μg) from the koala, tammar wallaby, wombat and rat were separated by SDS PAGE (7.5% gel), transferred to a nitrocellulose membrane, and probed with goat anti-human NADPH-cytochrome P450 reductase IgG. **Lane 1:** pre-stained marker (Life Technologies). **Lane 2:** rat hepatic microsomes. **Lane 3:** koala hepatic microsomes. **Lane 4:** tammar wallaby hepatic microsomes. **Lane 5:** wombat hepatic microsomes.

4.4. Discussion

This chapter outlines the first biochemical and immunochemical data to be obtained for components of the mixed function oxidase system in three representative Australian marsupial species, the koala, tammar wallaby and southern hairy-nosed wombat. The total CYP content and NADPH-cytochrome P450 reductase activity was measured in the koala and tammar wallaby, with significant differences observed. The availability of several polyclonal antibodies raised against major mammalian xenobiotic-metabolising CYPs also enabled studies into the CYP complement and expression in these Australian marsupial species. A number of interspecies differences in CYP expression and multiplicity were observed between the marsupial species as well as in comparison to the rat control. The procurement of pouch young koala samples of differing developmental stages also enabled the first examination of the developmental expression of the major xenobiotic-metabolising CYPs in this previously unstudied species.

4.4.1. Enzyme Assays

The measured total CYP content for koala liver microsomes (n=4) was not significantly higher than that observed for the rat (n=4). In addition, the total CYP content for the koala was 3.5-fold higher than that measured in tammar wallaby liver microsomes (n=6). CYP content was elevated in koala liver microsomes regardless of the timing of tissue collection or geographical origins of the animal. Although the specific reason behind this difference is unclear, the results support the hypothesis that the unique *Eucalyptus* diet of the koala requires significant CYP enzyme activity to facilitate the metabolism and subsequent excretion of digested terpenes. The tammar wallaby, although a herbivorous feeder, does not feed on *Eucalyptus* foliage, and therefore does not require as extensive metabolism of ingested dietary compounds. However, it remains likely that the tammar wallaby ingests man-made chemicals through its feeding habits.

The exact nature and form of the CYP enzymes, which may be elevated in response to dietary terpenes, cannot be determined from total CYP measurements. Significantly, subsequent immunoblot analyses of major xenobiotic-metabolising CYPs did not reveal marked overexpression of any CYP2D, CYP2E or CYP3A enzymes in the koala, relative to other species examined.

The measured NADPH-cytochrome P450 reductase activities were similar in all of the three species studied. The requirement for the reductase protein as an intermediary in the transfer of electrons to the CYP enzyme is common to most CYPs across mammalian species. Given this general housekeeping role, reductase activity was not expected to be significantly different in marsupials. Unlike individual CYPs, reductase levels are not readily modulated by chemical inducers. A typically strong CYP inducer such as phenobarbitone produces only a 2-3 fold increase in NADPH-cytochrome P450 reductase levels, while other compounds such as ethanol have little or no effect. NADPH-cytochrome P450 reductase expression is predominantly regulated by the pituitary-thyroid gland and thyroid hormone (T3) is necessary to maintain its expression (Porter, 1991).

4.4.2. Immunoblot analyses of marsupial hepatic microsomes

Immunoblot analyses were performed with marsupial hepatic and extrahepatic microsomes using a range of polyclonal antibodies raised against the major-xenobiotic metabolising CYPs. These studies represent the first analyses of specific CYP forms for any Australian marsupial species by immunoblotting.

4.4.2.1. CYP2C

Immunoblot analysis of marsupial liver microsomes with anti-rat CYP2C6 IgG detected a multitude of intense immunoreactive bands for each species (Figure 4.2). Due to the intensity, individual bands could not be resolved. An additional immunoblot with the anti CYP2C6 IgG using less microsomal protein enabled a more accurate identification of the CYP2C forms for each marsupial species (Figure 4.3). At least two CYP2C forms were detected in the tammar wallaby and wombat microsomal samples. The koala exhibited a minimum of three intense immunoreactive bands, indicating the expression of at least three CYP2C forms in koala liver. The koala sample exhibited significantly greater immunoreactivity to the anti CYP2C6 IgG in comparison to the other tested marsupial species. The significance of this finding remains unclear. However, it may be hypothesised that the CYP2C subfamily plays an important role in the metabolism and subsequent excretion of compounds contained in the koala's unique *Eucalyptus* diet. In support of this hypothesis, *Eucalyptus* terpenes have recently been shown by

immunoblotting to increase CYP2C expression in brushtail possums (Pass *et al.*, 1999). The isolation of full-length koala *CYP2C* cDNA sequences and subsequent characterisation of their catalytic activities towards compounds of dietary significance, would provide the most accurate determination of the role of the CYP2C subfamily in xenobiotic metabolism in the koala.

In humans, the CYP2C subfamily metabolises a number of important therapeutic drugs including mephenytoin, tolbutamide, warfarin and selected barbiturates (Goldstein and Morais, 1994). The detection of multiple CYP2C forms in the three tested marsupial species is comparable to other studied mammalian species. For example, nine forms have been identified to date in the rabbit, eight in the rat, and four in both the hamster and humans (Nelson, 2000).

A developmental analysis of hepatic pouch young and adult koala microsomes with anti-rat CYP2C6 IgG elucidated a significant finding (Figure 4.4). It should be noted that a limited number of samples were utilised in these studies, and additional pouch young individuals would need to be tested to confirm these results. CYP2C immunoreactivity was observed to increase with the advancing age of the tested pouch young liver samples. Using a standard amount of protein, the youngest pouch young samples (~180 days), as determined by weight, both exhibited less CYP2C immunoreactivity than the ~400 day old sample. In addition, the oldest pouch young sample did not appear to exhibit equal levels of CYP2C immunoreactivity to any of the adult samples. The gradual increase in CYP2C expression exhibited by the pouch young koalas during development may reflect the gradual exposure of the pouch young koalas to xenobiotic compounds contained in their diet.

Pouch young koala's weigh less than 0.5 g at birth and remain in the pouch for 7-8 months (210-240 days), suckling on a diet of milk (Strahan, 1983). Weaning commences in the last few months of pouch life, and the young animals change to a diet of leaves is initiated by its feeding on soft faeces donated from the caecum of the mother (Strahan, 1983). It is hypothesised that this process innoculates the infant with the micro-organisms enabling its own caecum to function (Strahan, 1983). By the age of 12 months the koala is fully weaned and independent of its mother (Strahan, 1983).

The youngest koala pouch young samples (180 days) utilised in this study are therefore representative of individuals that have most likely commenced weaning and are nearing the end of pouch life. In contrast, the 400 day old koala sample is probably fully weaned, nearing independence from its mother and feeding solely on a diet of *Eucalyptus* leaves. The differing diets of the two pouch young samples would therefore support the hypothesis that the increase in CYP2C immunoreactivity detected in the oldest individual koala is reflective of an increase in dietary *Eucalyptus* compounds. A similar hypothesis has previously been postulated for the brushtail possum, where adult levels of detoxication enzymes were achieved during the weaning period (Bolton and Ahokas, 1997d).

Finally, a study performed using pouch young koalas less than 180 days old, may elucidate whether the onset of CYP2C expression in the koala is correlated to the weaning process or some earlier developmental stage.

This work represents the first data available on the developmental expression of any CYP enzyme family in any Australian marsupial species through immunoblotting. McManus *et al.* (1978) reported that total CYP and cytochrome b_5 levels in pouch young and juvenile quokkas (*Setonix brachyurus*) reached adult levels 155-255 days after birth. Bolton and Ahokas (1997d) reported that the ontogenic expression of detoxication enzymes was delayed in the brushtail possum (*Trichosurus vulpecula*), in comparison to other eutherian species.

The developmental regulation of the CYP2C subfamily has also been investigated in humans (Ratanasavanh *et al.*, 1991; Treluyer *et al.*, 1997). CYP2C proteins were not detected in human foetal livers by immunoblotting, but dramatically increased in the first few weeks after birth (Ratanasavanh *et al.*, 1991; Treluyer *et al.*, 1997).

Immunoblot analysis of extrahepatic marsupial microsomes also revealed some unique findings (Figure 4.6). Multiple CYP2C immunoreactive bands were detected in koala kidney and tammar wallaby lung microsomes with the anti-rat CYP2C6 IgG. The multitude of intense bands detected in the koala kidney sample, although indicative of the large amount of protein used, indicates the

expression of a number of CYP2C isoforms in the tissue of this species. The presence of multiple bands in tammar wallaby lung is also significant as the lung can come into contact with chemical carcinogens and xenobiotic chemicals in inhaled air.

CYP2C isoforms have previously been detected in extrahepatic tissues including brain, uterus, kidney, ovary and duodenum from the rat, human, mouse and hamster (Zaphiropoulos *et al.*, 1995; Luo *et al.*, 1998; Klose *et al.*, 1999). In addition, CYP2C isoforms have also been detected in mouse and rabbit lung tissue (Luo *et al.*, 1998; Zhu *et al.*, 2000).

The identification of multiple CYP2C bands in all of the marsupial species tested to date represents an opportunity for the successful cloning of multiple *CYP2C* sequences from an Australian marsupial. The isolation of multiple *CYP2C* cDNA sequences from three Australian marsupial species is reviewed in Chapter 5.

4.4.2.2. CYP2D

Immunoblot analysis of 20μg of marsupial liver microsomes with anti-human CYP2D6 IgG failed to detect any immunoreactive bands (Figure 4.7). In addition, no CYP2D6 immunoreactivity was detected in pouch young koala liver microsomes (Figure 4.8). The absence of immunoreactive bands with the anti-human CYP2D6 IgG for each of the marsupial species, except at large protein concentrations, was unexpected. The reasons behind the absence of one of the major xenobiotic-metabolising CYPs from all of the tested Australian marsupial species are yet to be elucidated.

A number of possibilities exist to explain the absence of CYP2D immunoreactivity in the tested marsupial species. One hypothesis is that marsupial CYP2D enzymes may have evolved differently to previously studied mammalian species and consequently the anti-human CYP2D6 IgG may be unable to recognise the specific isoform in marsupials. Also, CYP2D enzymes may be expressed in marsupials at such low levels that the anti-human CYP2D6 IgG is unable to detect any immunoreactivity. The faint immunoreactive bands detected in each marsupial species with the anti-human CYP2D6 IgG using an excessive amount of protein (75μg) would tend to

support the latter hypothesis. In this immunoblot, multiple immunoreactive bands were detected in the tammar wallaby sample, while single bands were revealed in both the koala and wombat microsomal samples.

CYP2D6 is one of the major human hepatic CYPs important in the metabolism of a number of clinical and therapeutic drugs (Nebert and McKinnon, 1994). Currently, three *CYP2D* genes have been identified in humans, however two of these are pseudogenes (Nelson, 2000). Multiple *CYP2D* genes have also been cloned from the rat, mouse and rabbit (Nelson, 2000).

The absence of a major xenobiotic-metabolising CYP subfamily, such as CYP2D, from Australian marsupials has the potential to make these animals more susceptible to man-made chemical exposure. However, a more critical analysis of this subfamily in Australian marsupials is required in order to support this hypothesis.

4.4.2.3. CYP2E1

Immunoblot analysis of adult marsupial liver microsomes using anti-rat CYP2E1 IgG detected an intense immunoreactive band, corresponding to CYP2E1, in the tammar wallaby sample (Figure 4.9). Multiple bands of less intensity were revealed in both the koala and wombat liver samples (Figure 4.9). The presence of multiple bands in the koala and wombat may represent the detection of additional CYP2 subfamilies in both species.

CYP2E1 is an important xenobiotic-metabolising enzyme as it is involved in the metabolism of numerous low molecular weight compounds, including carcinogens (Nebert and McKinnon, 1994). It is constitutively expressed in the liver and a variety of other tissues. CYP2E1 is also induced by and catalyses the metabolism of ethanol (Nebert and McKinnon, 1994).

Immunoblot analysis of pouch young koala liver samples with a commercially available anti-rat CYP2E1 IgG detected two immunoreactive bands in each sample (Figure 4.10). The smaller band most likely represents the koala CYP2E1. CYP2E1 expression was revealed at each

developmental stage of the pouch young koalas, from 180-400 days old. No differences between samples were detected.

CYP2E1 expression has previously been detected in human foetal (16-24 weeks) liver microsomal samples by immunoblotting (Carpenter *et al.*, 1996; Hakkola *et al.*, 1998). Furthermore, the CYP2E1 expressed in human foetal liver was functionally similar to the adult form, and was inducible in culture by xenobiotics, including ethanol (Carpenter *et al.*, 1996).

4.4.2.4. CYP3A

Immunoblot analysis of adult marsupial liver microsomes with anti-rabbit CYP3A6 IgG revealed some significant findings (Figure 4.11). A single immunoreactive band was detected in the koala, while the tammar wallaby and wombat microsomal samples both exhibited two bands of differing intensity.

The CYP3A subfamily is critically important in the metabolism of numerous chemically diverse compounds administered to humans (Nebert and McKinnon, 1994). It is the most abundant group of CYP enzymes in the liver of mammalian species (de Wildt *et al.*, 1999b). In humans, CYP3A4 is the most highly expressed CYP in the liver and accounts for approximately 30-40% of the total CYP content in human adult liver and small intestine (de Wildt *et al.*, 1999b). CYP3A7 is the major CYP isoform detected in human embryonic, foetal and newborn liver and is involved in steroid metabolism (Oesterheld, 1998). CYP3A7 is also present in adult liver but at a significantly lower level than CYP3A4 (de Wildt *et al.*, 1999b).

It may be hypothesised that the intense and faint immunoreactive bands detected in the tammar wallaby and wombat microsomal samples represent the expression of at least two CYP3A isoforms. The possibility that the faint bands correspond to the foetal CYP3A form in each species cannot be discounted.

Immunoblot analysis of koala pouch young liver microsomes with anti-rabbit CYP3A6 IgG detected a single, intense immunoreactive band at each developmental stage tested (Figure 4.12). A doublet of bands were again detected for the adult rat liver microsomal sample. No significant

sex differences were observed for the koala pouch young samples. However, the intensity of the male and female samples estimated at 180-250 days old was significantly greater than that exhibited by both the slightly younger and older samples. The significance of this finding remains unclear, and requires further analysis.

Finally, it is unable to be determined from the immunoblot whether the immunoreactive band exhibited by the pouch young samples is representative of an embryonic CYP3A isoform, or is the same form that was detected in the adult koala liver sample.

In summary, the detection of an apparent single CYP3A form in the koala microsomal sample was the most surprising result using this antibody. The possibility that the single immunoreactive band may correspond to two CYP3A forms of identical size cannot be discounted, and could only be confirmed through cloning experiments. At least two CYP3A forms were detected in each of the other species tested.

4.4.2.5. NADPH-cytochrome P450 reductase

Immunoblot analysis of marsupial liver microsomes with anti-human NADPH-cytochrome P450 reductase IgG detected a sharp immunoreactive band for each sample, at the expected size (Figure 4.13). A significantly larger immunoreactive band, most likely corresponding to NADPH-cytochrome P450 reductase, was detected in the koala sample in comparison to the other marsupial species and the rat.

As explained previously, the NADPH-cytochrome P450 reductase protein is an essential component of the microsomal cytochrome P450-dependent monooxygenase system as it catalyses the transfer of electrons from NADPH to the cytochromes P450 (Guengerich, 1993). An NADPH-cytochrome P450 reductase molecule is comprised of both a flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) group. It is hypothesised that the NADPH-cytochrome P450 reductase arose through the fusion of the ancestral genes for these two types of flavoproteins (Porter, 1991). Furthermore, the universality of electron transfer across eukaryotic

and prokaryotic species is evidence that these molecules were utilised in the earliest stages of evolution (Porter, 1991).

The large NADPH-cytochrome P450 reductase protein detected for the koala, ~80 kDa, was a surprising finding. The exact size of the koala NADPH-cytochrome P450 reductase protein is unable to be accurately determined from the immunoblot, however rat liver NADPH-cytochrome P450 reductase has previously been reported at 76 kDa (Porter and Kasper, 1985). In addition, both rabbit liver and *Saccharomyces cerevisiae* NADPH-cytochrome P450 reductase proteins exhibit a molecular weight of 76 kDa (Katagiri *et al.*, 1986; Porter, 1991). Further studies are currently being conducted into the cloning and characterisation of the koala NADPH-cytochrome P450 reductase cDNA by Sandra Kong (PhD candidate).

The tammar wallaby and wombat liver microsomal samples also exhibited slightly larger NADPH-cytochrome P450 reductase proteins in comparison to the rat, but not to the same extent as the koala.

In summary, with the exception of the CYP2D subfamily, the Australian marsupial species tested generally exhibited comparable levels of CYP immunoreactivity to the major xenobiotic-metabolising enzymes when compared to the rat. The significant multiplicity of CYP2C immunoreactivity detected in koala liver microsomes, in comparison to the other marsupial species, indicates the presence of multiple CYP2C forms and the importance of this CYP subfamily for xenobiotic metabolism in this species. In addition, the detection of each of the major xenobiotic-metabolising enzymes, except CYP2D, in 180-400 day old pouch young koalas indicates that this species may be exposed to dietary xenobiotic compounds at an early developmental stage.

In conclusion, the studies outlined in this chapter have also provided initial data for a number of CYP families in Australian marsupials, which can be further utilised in subsequent cloning studies.

Chapter 5

Identification and cloning of Cytochrome P450 2C (*CYP2C*) cDNA sequences from representative Australian marsupial species

5.1. Introduction

Cytochrome P450 enzymes play a pivotal role in numerous fundamental cellular biosynthetic pathways in addition to their involvement in metabolising virtually all drugs and foreign chemicals. A critical component of the microsomal mixed function oxidase system, initial protein purification studies into CYPs revealed the existence of multiple CYPs in mammals and other species. Studies conducted with purified CYP preparations, reconstituted with lipid and NADPH-cytochrome P450 reductase, demonstrated that individual CYPs can exhibit either highly specific or broad overlapping substrate specificities.

Initial investigations into the isolation and characterisation of *CYP* cDNAs exploited the inductive properties of a number of specific CYPs from animals treated with certain chemicals (eg. phenobarbitone, 3-methylcholanthrene) (Gonzalez and Kasper, 1980a; Gonzalez and Kasper, 1980b). Using this methodology, rat phenobarbital-induced, rat steroid-induced, and mouse 3-methylcholanthrene-induced *CYP* cDNA clones were isolated for the first time (Adesnik *et al.*, 1981; Fujii-Kuriyama *et al.*, 1981; Negishi *et al.*, 1981; Gonzalez and Kasper, 1982; Hardwick *et al.*, 1983; Gonzalez *et al.*, 1984). In order to isolate *CYP* cDNAs that were not readily inducible, antibodies raised against specific CYP forms were used to screen cDNA expression libraries. A number of constitutively expressed *CYP* cDNA clones were isolated using these screening procedures (Gonzalez *et al.*, 1985; Beaune *et al.*, 1986; Gonzalez *et al.*, 1986; Song *et al.*, 1986; Hardwick *et al.*, 1987; Nagata *et al.*, 1987; Umbenhauer *et al.*, 1987). Recent advances in molecular biology techniques have enabled more detailed analyses of the evolution and phylogenetic comparison of the CYP superfamily and the roles of CYP enzymes in any given organism. Furthermore, recombinant DNA techniques have intensified studies into the multiplicity, catalytic activities, and expression of isolated *CYP* genes. Presently, over 1000 *CYP* genes have been identified from a multitude of bacterial, plant and animal species (http://drnelson.utmem.edu/CytochromeP450.html).

Prior to the commencement of this work, no *CYP* cDNA, partial or full-length, had been isolated from any Australian marsupial species. The identification and characterisation of CYPs in Australia's native marsupials is critically important in assessing their ability to cope with

environmental chemical exposure. It is also important to characterise CYPs in marsupials in order to provide new evolutionary data on the cytochrome P450 superfamily from a unique phylogenetic class.

From the results of immunoblot studies, it is apparent that the CYP2C subfamily is highly expressed in marsupials. In addition, these studies provided evidence that multiple CYP2C forms are expressed in marsupial liver. Based on this data, the CYP2C subfamily was selected for cloning studies. The CYP2C subfamily is the most complex CYP subfamily thus far identified, and its members are generally constitutively expressed. At the commencement of this project, thirty-six members of the CYP2C subfamily had been isolated and characterised from a variety of mammalian species. A further ten mammalian *CYP2C* cDNAs have since been isolated during the course of thesis work. Sequence analysis has revealed that all members of the CYP2C subfamily are more than 60% similar in their amino acid sequence to each other.

CYP2C cloning studies were performed in representative Australian marsupial species, including the koala (*Phascolarctos cinereus*), tammar wallaby (*Macropus eugenii*) and western quoll (*Dasyurus geoffroii*). A number of different methodologies were employed, including RT-PCR, 5' and 3' RACE, and library screening. The results obtained from these techniques are discussed in the following sections.

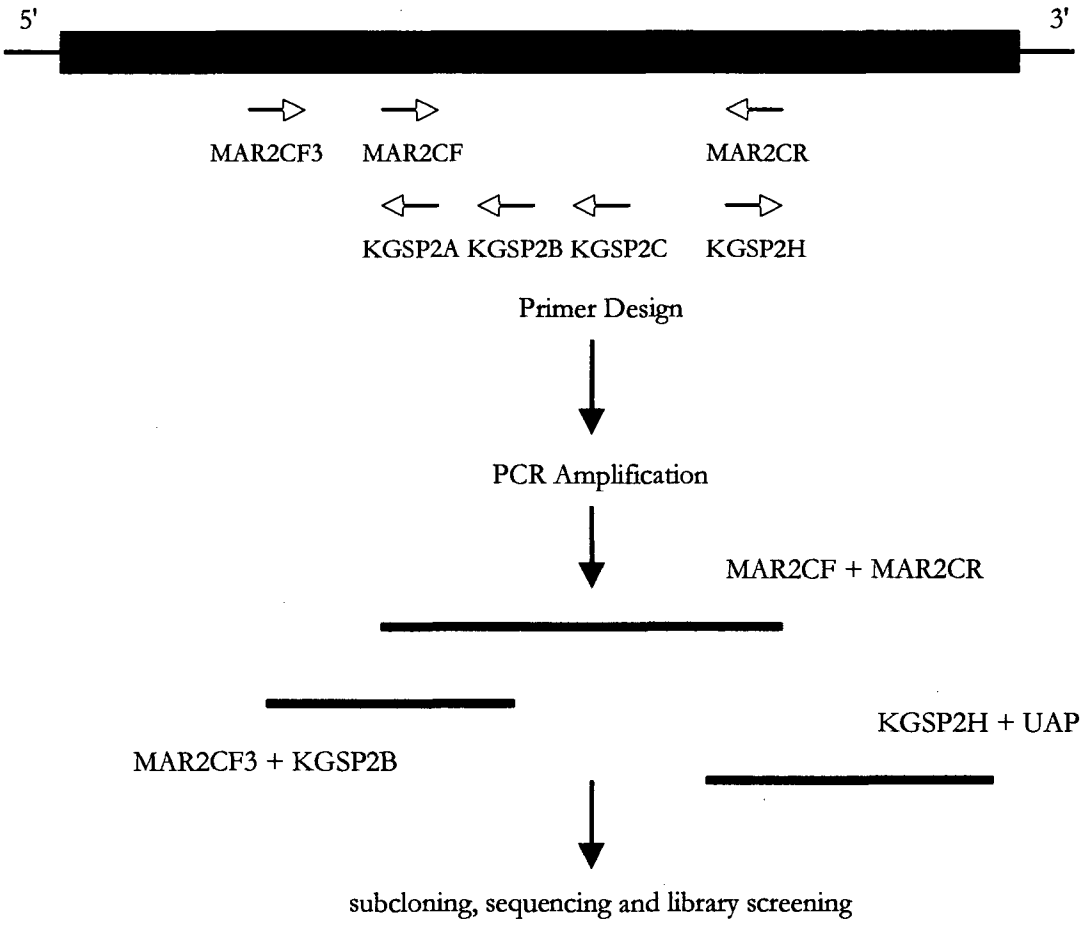
5.2. Research strategy

The research strategy, to define CYP2C multiplicity and isolate new *CYP2C* cDNAs from Australian marsupials, involved amplifying short cDNA fragments using conserved primers from previously isolated *CYP2C* cDNAs to use as DNA probes in the screening of a cDNA library. The primary methods used were RT-PCR from marsupial liver RNA samples and hybridisation screening of cDNAs from a liver library. A more detailed account of these techniques is provided in chapter 2.

An analysis of mammalian *CYP2C* cDNA sequences cloned to date identified several regions of conserved sequence. Two degenerate oligonucleotide primers, which were complementary to a

number of mammalian *CYP2C* cDNAs, were designed and used to amplify *CYP2C* sequences from marsupial liver RNA by RT-PCR. The PCR products were subcloned directly into pGEM®-T vector and transformed into *E.coli* DH5α competent cells. Plasmid DNA was purified and then sequenced to determine if it encoded a new member of the *CYP2C* subfamily. A schematic diagram of the research strategy is outlined in Figure 5.1.

Figure 5.1. Research strategy to isolate a marsupial *CYP2C* cDNA sequence



The positions of the primers used in PCR amplification experiments are indicated in relation to a consensus *CYP2C* cDNA. Conserved regions were identified from the alignment of multiple mammalian *CYP2C* cDNA sequences. Four primers (forward primers, MAR2CF, MAR2CF3 and KGSP2H, and reverse primers MAR2CR and KGSP2B) were designed to amplify *CYP2C* sequences from marsupial liver cDNA. Amplified PCR products were subcloned and individual clones were selected for sequencing.

5.3 Methods

5.3.1. Primer design

At the commencement of the project, two degenerate oligonucleotide primers, MAR2CF and MAR2CR, were designed against highly conserved regions in mammalian *CYP2C* cDNA sequences and used in RT-PCR amplification experiments to obtain the corresponding sequences in marsupial RNA. Where discrepancies existed between the base-pairs of the aligned mammalian *CYP2C* cDNA sequences, appropriate degeneracy was introduced. Two additional oligonucleotide primers, MAR2CF3 and KGSP2B, were subsequently designed to amplify a more 5' *CYP2C* region of marsupial cDNA. Each primer was designed with an approximate GC content of 50%, and incorporated no more than two G or C residues in the last five base pairs at the 3' end. All primers were checked against each other for complementarity, particularly at the 3' end. The binding locations of the primers are shown in relation to selected *CYP2C* sequences in Figures A.1-A.3 (Appendix A). The sequences of the forward and reverse primers are shown in Table 5.1.

Three additional koala specific primers, designated KGSP2C, KGSP2A and KGSP2H, were designed from the sequence analysis of an amplified koala *CYP2C* fragment in order to amplify the 5 and 3' ends of a koala *CYP2C* cDNA sequence. The sequences of the primers are also shown in Table 5.1.

Finally, the sequence of the UAP primer (Life Technologies, USA) utilised in section 5.4.3 is provided in Figure A.4 (Appendix A).

Table 5.1. CYP2C primer sequences. Sequence of the primers used to amplify the corresponding segments of approximately 700, 400 and 500 bp from marsupial liver cDNA samples. The letters denoting degeneracy include, Y= C+T, S= G+C, K= G+T, R= A+G, W= A+T, H= A+T+C, B= G+T+C, D= G+A+T.

NAME: COMPLEMENTARITY	PRIMER SEQUENCE
MAR2CF: degenerate CYP2C (forward primer)	5'- TGT GYT CCH KSC AAY GTS ATY TSY -3'
MAR2CR: degenerate CYP2C (reverse primer)	5'- ATY YAR RAA RTG NSC WGG GTC AA -3'
MAR2CF3: degenerate CYP2C (forward primer)	5'- CNA TGG ATS YNG TBD TGK TSY T -3'
KGSP2A: reverse primer specific (to Koala CYP2C#9)	5'- ACA AAT CAC ATT GCA TGG AGC ACA -3'
KGSP2B: reverse primer specific (to Koala CYP2C#9)	5'- GTT AAC GAG GCG GAA GTT TTC A -3'
KGSP2C: reverse primer specific (to Koala CYP2C#9)	5'- GGA TGT ATG TTC CGA ATG CT -3'
KGSP2H: forward primer specific (to Koala CYP2C#9)	5'- CCA GTT TGA CCC AGC ACA TTT CTT -3'

The MAR2CF-MAR2CR primer combination amplified a region of approximately 720 bp. The MAR2CF3 and KGSP2B primers were used to amplify a region of about 400 bp which was closer to the 5' end of the cDNA sequence.

5.4. Results

5.4.1. Amplification of marsupial *CYP2C* cDNA sequences by polymerase chain reaction

A PCR experiment was initially conducted to amplify a region of approximately 700 bp from western quoll liver cDNA at an annealing temperature of 52°C using the MAR2CF forward primer and MAR2CR reverse primer (see section 5.3.1. for primer details). To increase the probability of amplifying *CYP2C* sequences from quoll liver cDNA, the selected annealing temperature was significantly lower than the calculated T^m of the primers (~60°C). Electrophoretic analysis of the PCR products generated at an annealing temperature of 52°C revealed the presence of a 700 bp band in quoll liver cDNA samples. A representative gel is shown in Figure 5.2A. Negative controls devoid of template were included in all experiments. Positive controls using rat liver cDNA as template resulted in successful amplification of a 700bp fragment (data not shown).

Subsequent PCR experiments performed with koala and tammar wallaby liver cDNA using the same primer combination produced a comparable result to the western quoll. Agarose gel analysis of the products generated by PCR from tammar wallaby and koala cDNA at an annealing temperature of 52°C is shown in Figures 5.2B and 5.2C. A band at about 700 bp which corresponds in size to that expected for the MAR2CF forward primer and MAR2CR reverse primer was clearly visualised in each species (Figure 5.2B, lanes 4,5, and 5.2C, lanes 4-6).

Figure 5.2. Analysis of marsupial PCR products by agarose gel electrophoresis

Agarose gel analysis of PCR products generated from western quoll (A), tammar wallaby (B) and koala (C) liver cDNA using forward primer MAR2CF and reverse primer MAR2CR to amplify a region of approximately 700 bp at an annealing temperature of 52°C.

(A) PCR products generated from western quoll cDNA

Lane 1: 100 bp DNA ladder. Lanes 2-4: Negative control (PCR with no DNA template). Lanes 5-7: Sample of PCR products amplified from western quoll cDNA.

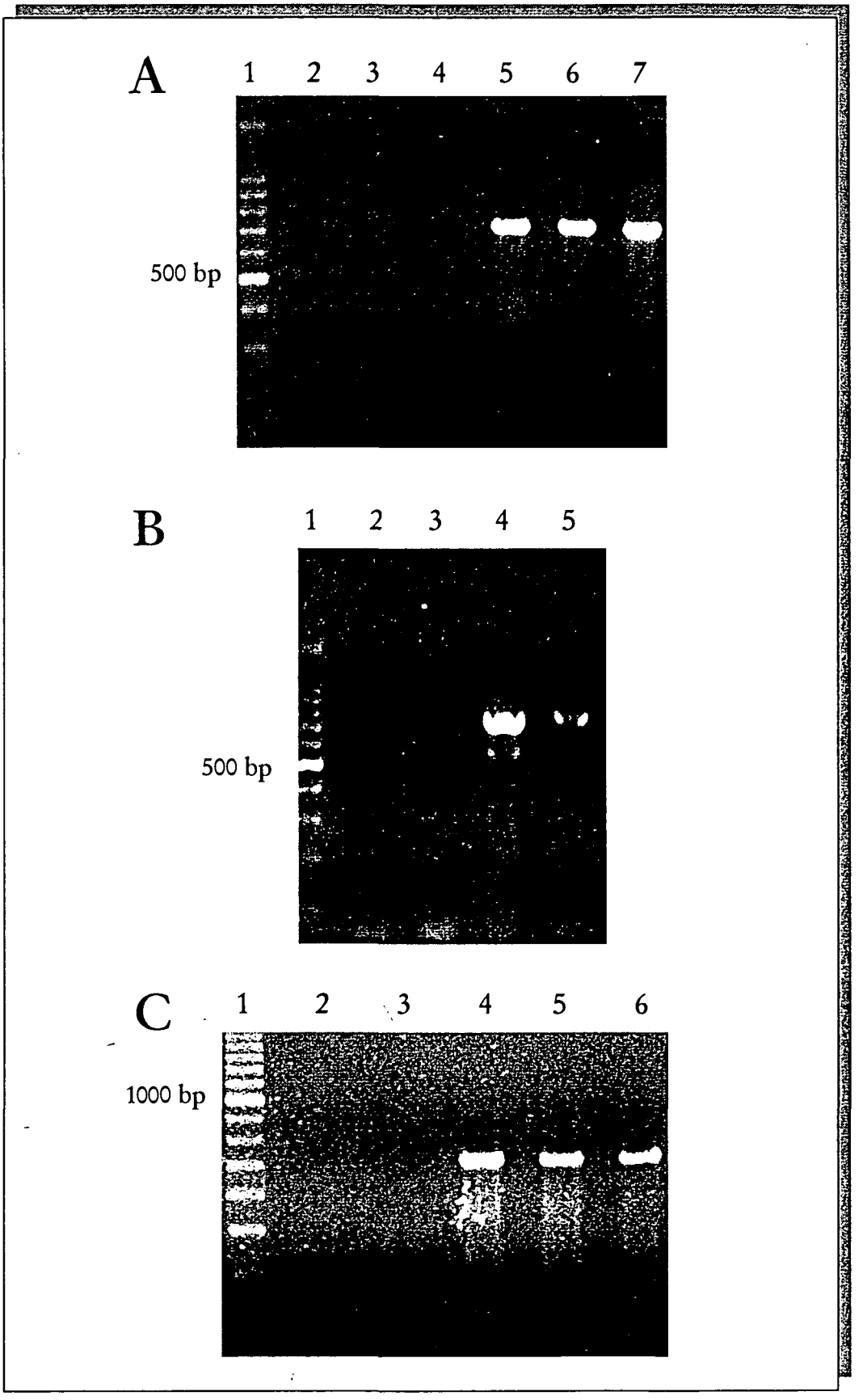
(B) PCR products generated from tammar wallaby cDNA

Lane 1: 100 bp DNA ladder. Lane 2: Negative control (PCR with no DNA template). Lane 3: Negative control (PCR with no DNA template). Lanes 4, 5: Sample of PCR products amplified from tammar wallaby cDNA.

(C) PCR products generated from koala cDNA

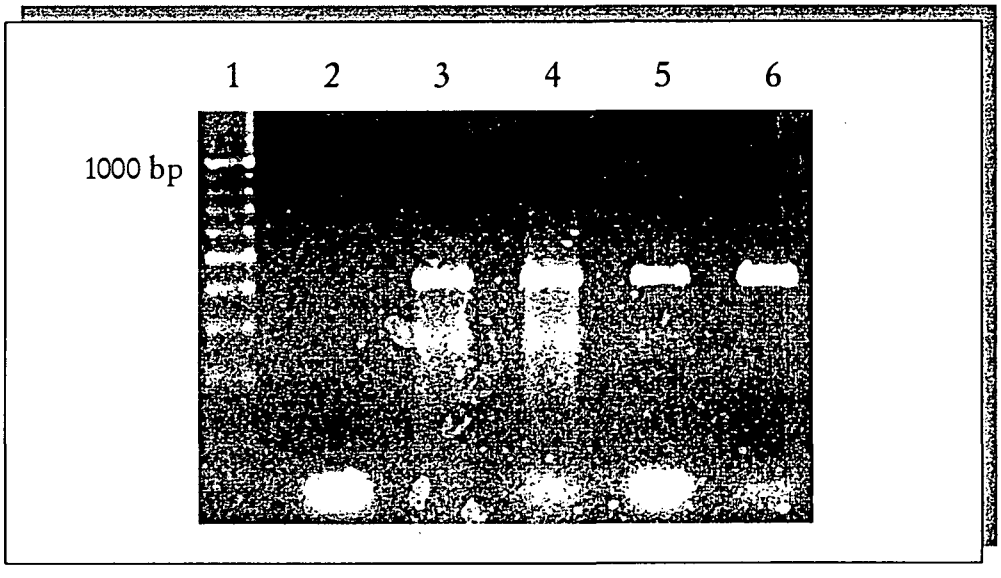
Lane 1: 100 bp DNA ladder. Lanes 2-3: Negative control (PCR with no DNA template). Lanes 4-6: Sample of PCR products amplified from koala cDNA.

Figure 5.2. Analysis of marsupial PCR products by agarose gel electrophoresis



A PCR experiment was also performed to amplify a more 5' region of about 400 bp from koala liver cDNA using the forward primer MAR2CF3 and reverse primer KGSP2B (see section 5.3.1. for primer details) at an annealing temperature of 56°C. In order to facilitate the successful amplification of additional *CYP2C* sequences from koala liver, gene-specific cDNA synthesised by 5' RACE was also utilised as template (see Chapter 2, section 2.2.15.1 for 5' RACE cDNA synthesis). A 400 bp fragment, which corresponded in size to that expected for the primer combination, was visualised in each experimental PCR sample (Figure 5.3, lanes 3-6). PCR products were successfully amplified using either first-strand cDNA or gene-specific 5' RACE cDNA as template.

Figure 5.3. Analysis of koala PCR products by agarose gel electrophoresis.



Analysis of the products generated by PCR from koala first-strand cDNA and 5' RACE koala cDNA using the forward primer MAR2CF3 and reverse primer KGSP2B by agarose gel electrophoresis (2%). An annealing temperature of 56°C was utilised.

Lane 1: 100 bp DNA ladder. **Lane 2:** Negative control (PCR with no DNA template). **Lanes 3, 4:** Sample of PCR products amplified from koala cDNA. **Lanes 5, 6:** Sample of PCR products amplified from koala 5' RACE cDNA.

Following the amplification of DNA fragments using both sets of primers, selected PCR samples were subcloned directly into pGEM®-T vector and sequenced in order to verify their identity as described in Chapter 2 (sections 2.2.8, 2.2.9, 2.2.10). It should be noted that during the screening for inserts (section 2.2.8.4), several bands ranging in size from 300-1000 bp were observed. A selection of these bands were also sequenced to analyse their identity.

5.4.2. Sequence analysis of amplified marsupial polymerase chain reaction products

5.4.2.1. Western quoll *CYP2C* cDNA sequences

Sequencing of cloned 700 bp PCR products generated from western quoll cDNA revealed multiple clones exhibiting similarity to known mammalian *CYP2C* sequences. Sequence analysis revealed the presence of three novel *CYP2C* cDNAs, designated quoll *CYP2C#1*, *#2* and *#4*. A nucleotide alignment of the three partial western quoll *CYP2C* cDNAs is shown in Figure 5.4. Quoll *CYP2C* clones *#1* and *#4* share a high degree of similarity, while quoll *CYP2C#2* is not as highly conserved. The amplified PCR products do not incorporate the haem-binding site which would confirm their identity as CYPs, however each deduced amino acid sequence exhibited a significant degree of similarity to other members of the CYP2C subfamily (60-70%) (see Figure 5.5). Sequence analysis of amplified PCR products of various sizes, other than the predicted size, demonstrated that they were PCR artefacts. The artefacts consisted of products resulting from the non-specific binding of the primers to various other regions of the cDNA template. This non-specific binding results from the use of low annealing temperatures in these experiments.

Figure 5.4. Sequence analysis of three partial quoll *CYP2C* cDNA clones

Three clones, designated Quoll *CYP2C#1*, *#2* and *#4*, amplified from western quoll cDNA were sequenced and aligned. Shaded areas represent conserved nucleotide sequences. The nucleotides are numbered in fifty base-pair intervals and a consensus sequence appears beneath each alignment block.

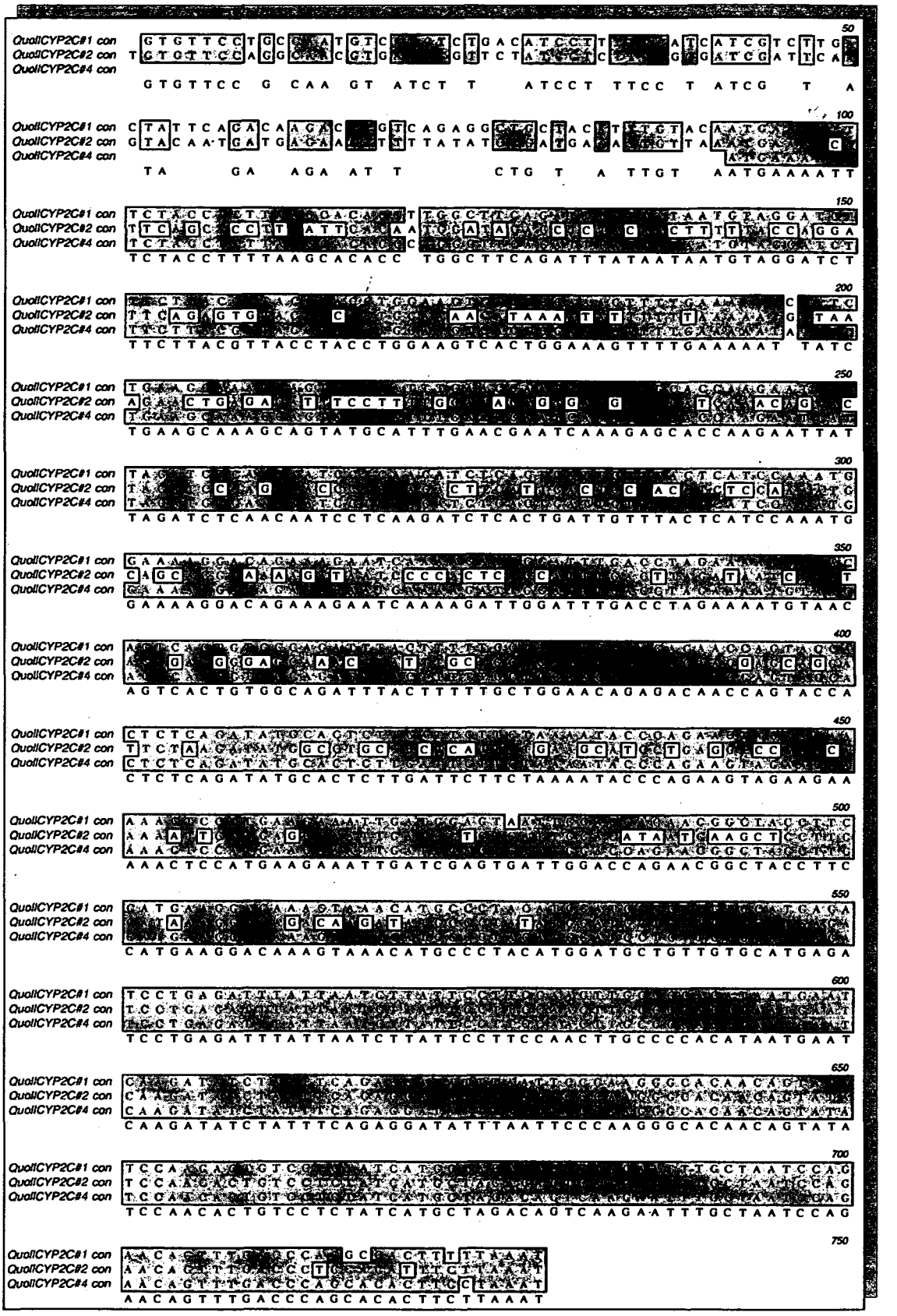
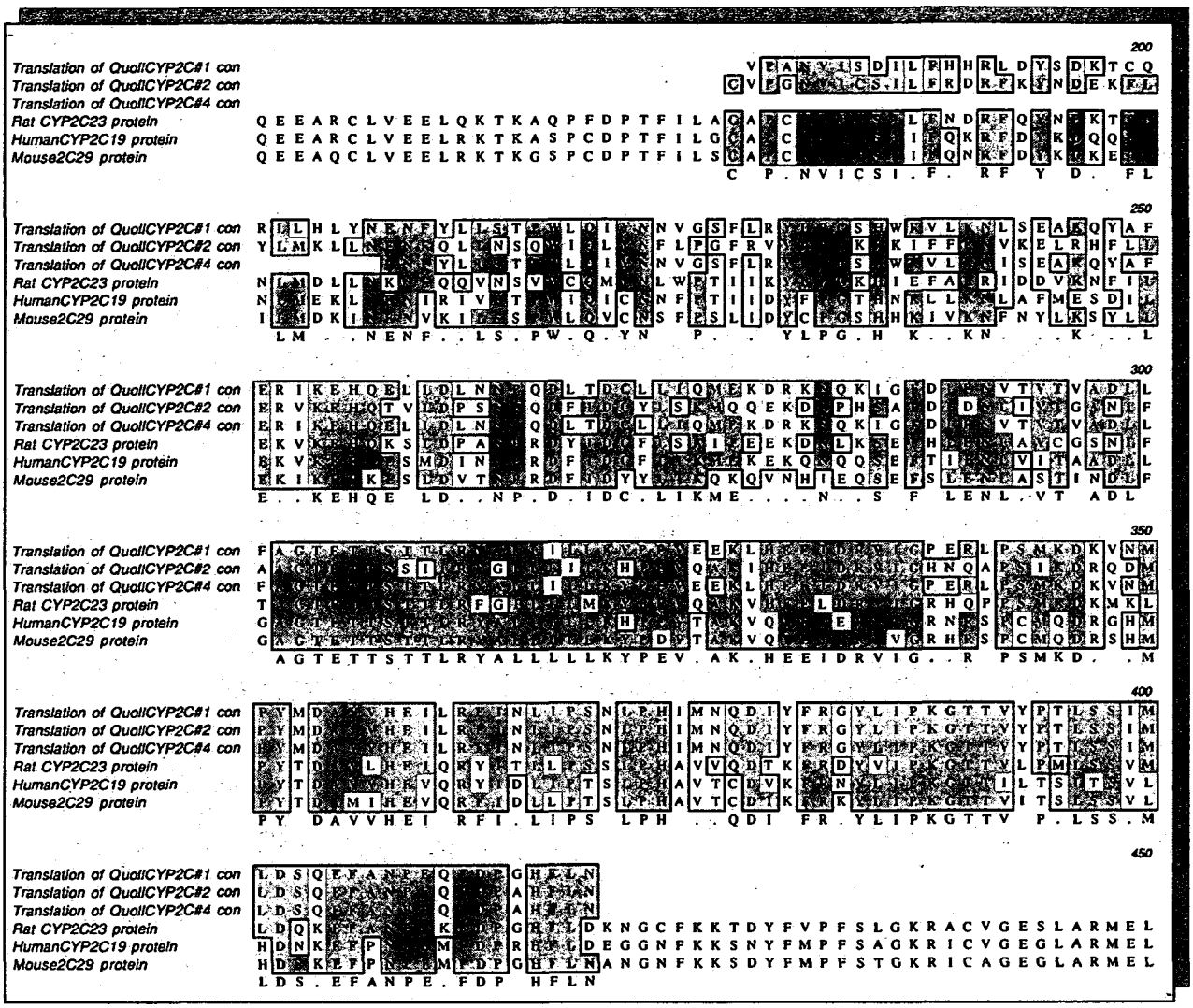

QuollCYP2C#1 con
QuollCYP2C#2 con
QuollCYP2C#4 con
50
100
150
200
250
300
350
400
450
500
550
600
650
700
750

Figure 5.5. Alignment of the deduced amino acid sequences of quoll clones *CYP2C#1*, *#2* and *#4* with mouse, rat and human CYP2C proteins



The deduced amino acid sequences of the three quoll *CYP2C* clones were aligned with mouse CYP2C29, rat CYP2C23 and human CYP2C19 protein sequences. The dark shaded areas represent conserved amino acid residues, while the lighter shaded areas indicate amino acid residues exhibiting structural similarity. The position of the residues in relation to the translational initiation sequence of one of the mammalian proteins is shown in parentheses at the end of each row. A consensus sequence appears beneath each alignment block.

5.4.2.2. Tammar wallaby *CYP2C* cDNA sequences

Sequence analysis of 700 bp products generated from tammar wallaby cDNA by PCR revealed the presence of three novel forms exhibiting similarity to known *CYP2C* sequences. The three clones, designated wallaby *CYP2C#2*, *#3* and *#4*, were aligned at the nucleotide level and varying regions of similarity were identified (Figure 5.6). The three wallaby clones were found to be approximately 60% similar to other members of the CYP2C subfamily at the amino acid level (Figure 5.7). Sequence analysis of PCR products other than the predicted size revealed the presence of PCR artefacts, most likely occurring as a result of non-specific binding of the primers to the template.

5.4.2.3. Koala *CYP2C* cDNA sequences

Sequence analysis of 700 bp products generated by PCR from koala cDNA using the MAR2CF forward primer and MAR2CR reverse primer revealed the presence of three different clones exhibiting similarity to known *CYP2C* sequences. A nucleotide alignment of the clones, designated Koala *CYP2C#4*, *#8* and *#9*, is provided in Figure 5.8. The three koala *CYP2C* clones, like the other two marsupial species studied, exhibited approximately 67% similarity to other known mammalian CYP2C sequences at the amino acid level (Figure 5.9). Sequence analysis of PCR products that were not of the predicted size revealed that they were PCR artefacts.

Figure 5.6: Sequence analysis of three partial tammar wallaby *CYP2C* cDNA clones

Three clones, designated wallaby *CYP2C#2*, *#3* and *#4*, of the 700 bp product amplified from tammar wallaby cDNA by PCR were sequenced and aligned. Shaded areas represent conserved nucleotide sequences. The nucleotides are numbered in fifty base-pair intervals and a consensus sequence appears beneath each alignment block.

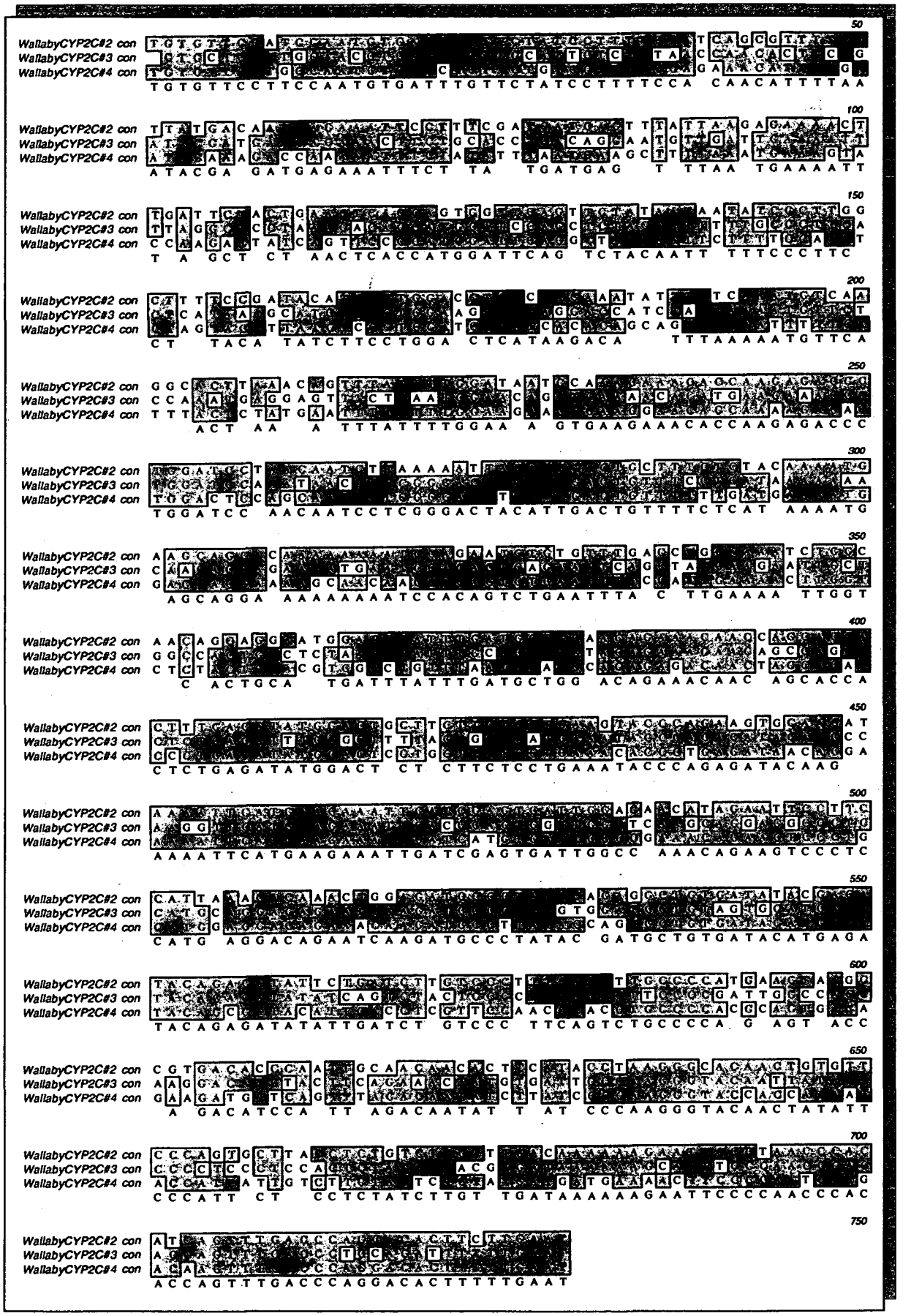
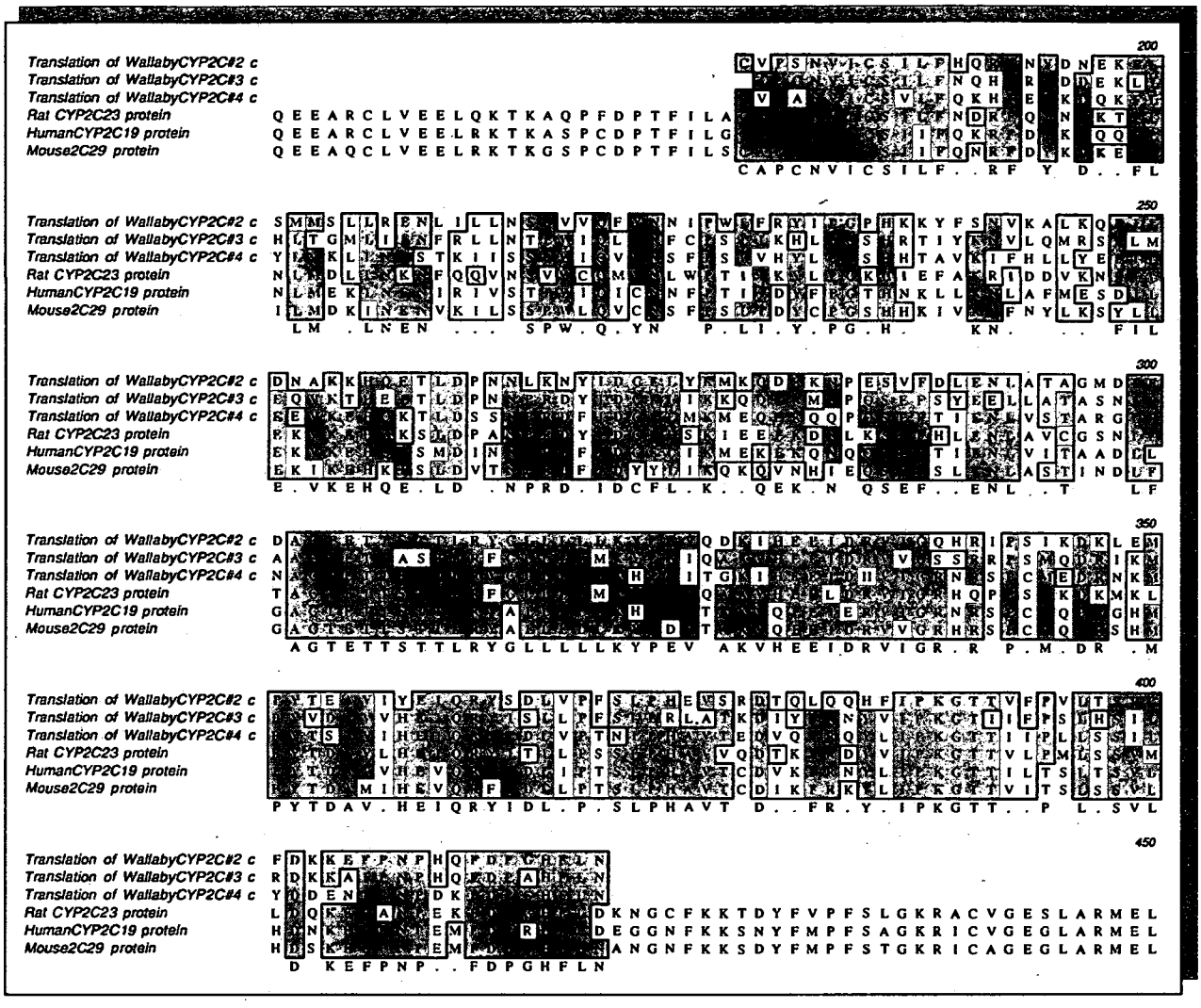

WallabyCYP2C#2 con
WallabyCYP2C#3 con
WallabyCYP2C#4 con
50
TGTGTTCCTTCCAAATGTGATTTGTTCTATCCTTTTCCA CAACATTTTAA
100
ATACGA GATGAGAAATTTCT TA TGATGAG T TTAA TGAAAATT
150
T A GCT CT AACTCACCATGGATTCAG TCTACAATT TTTCCCTTC
200
CT TACA TATCTTCCTGGA CTCATAAGACA TTTAAAAAATGTTCA
250
ACT AA A TTTATTTTGGAA A GTGAAGAAACACCAAGAGACCC
300
TGGATCC AACAATCCTCGGGACTACATTGACTGTTTTCTCAT AAAATG
350
AGCAGGA AAAAAAAAATCCACAGTCTGAATTTA C TTGAAAA TTGGT
400
C ACTGCA TGATTTATTTGATGCTGG ACAGAAACAAC AGCACCA
450
CTCTGAGATATGGACT CT CTTCTCCTGAAAATACCCAGAGATACAAG
500
AAAATTCATGAAGAAATTGATCGAGTGATTGGCC AAACAGAAGTCCCTC
550
CATG AGGACAGAATCAAGATGCCCTATAC GATGCTGTGATACATGAGA
600
TACAGAGATATATTGATCT GTCCC TTCAGTCTGCCCCA G AGT ACC
650
A GACATCCA TT AGACAATAT T AT CCCAAGGGTACAACTATATT
700
CCCATT CT CCTCTATCTTGT TGATAAAAAAGAATTCCCCAACCCAC
750
ACCAGTTTGACCCAGGACACTTTTTGAAT

Figure 5.7: Alignment of the deduced amino acid sequences of tammar wallaby clones *CYP2C#2*, *#3* and *#4* with mouse, rat and human CYP2C proteins



The deduced amino acid sequences of the three tammar wallaby *CYP2C* clones were aligned with mouse CYP2C29, rat CYP2C23 and human CYP2C19 protein sequences. The dark shaded areas represent conserved amino acid residues, while the lighter shaded areas indicate amino acid residues exhibiting structural similarity. The position of the residues in relation to the translational initiation sequence of one of the mammalian proteins is shown in parentheses at the end of each row. A consensus sequence appears beneath each alignment block.

Figure 5.8: Sequence analysis of three partial koala *CYP2C* cDNA clones

Three clones, designated koala *CYP2C#4*, *#8* and *#9*, of the 700 bp product amplified from koala cDNA by PCR were sequenced and aligned. Shaded areas represent conserved nucleotides. The nucleotides are numbered in fifty base-pair intervals and a consensus sequence appears beneath each alignment block.

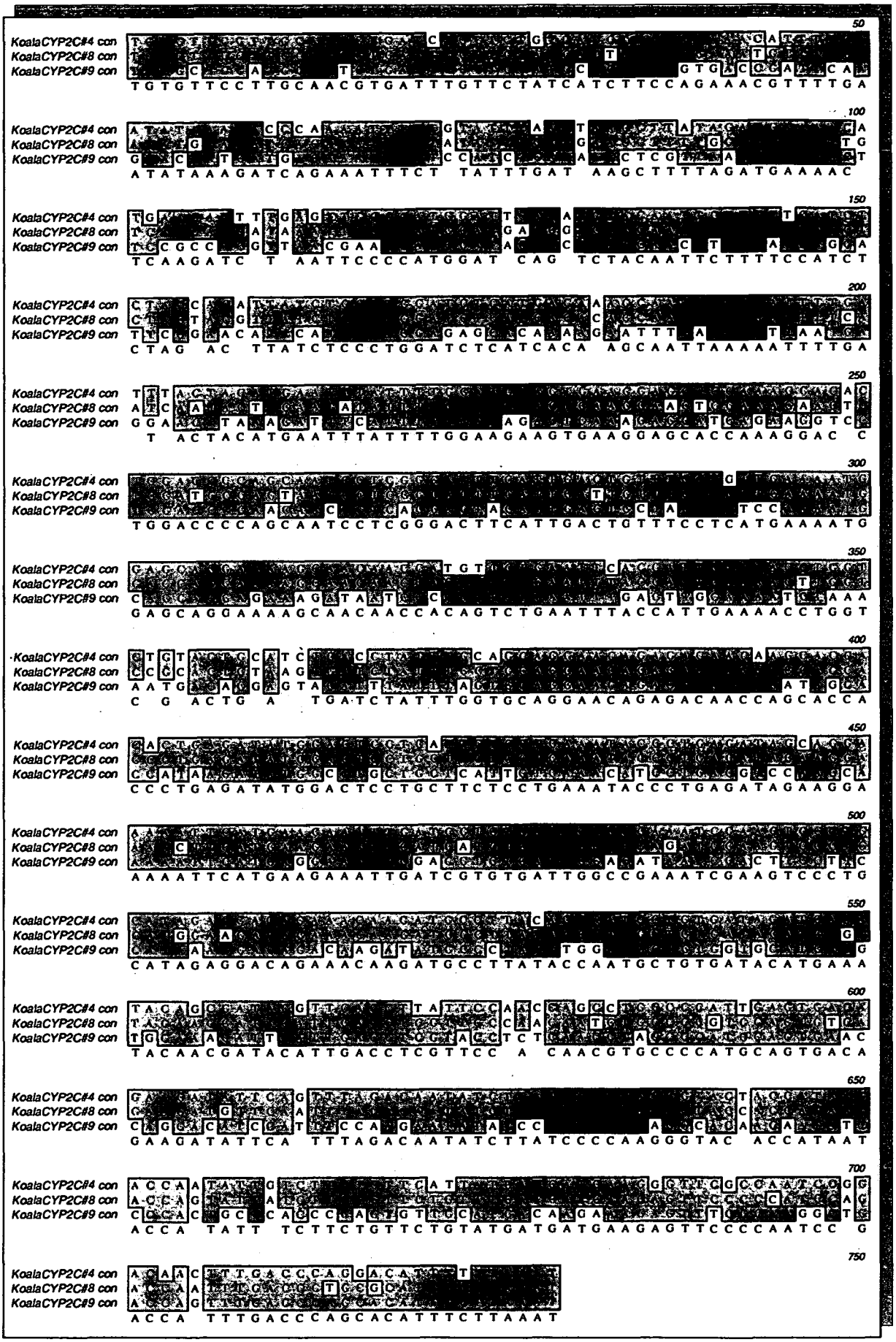
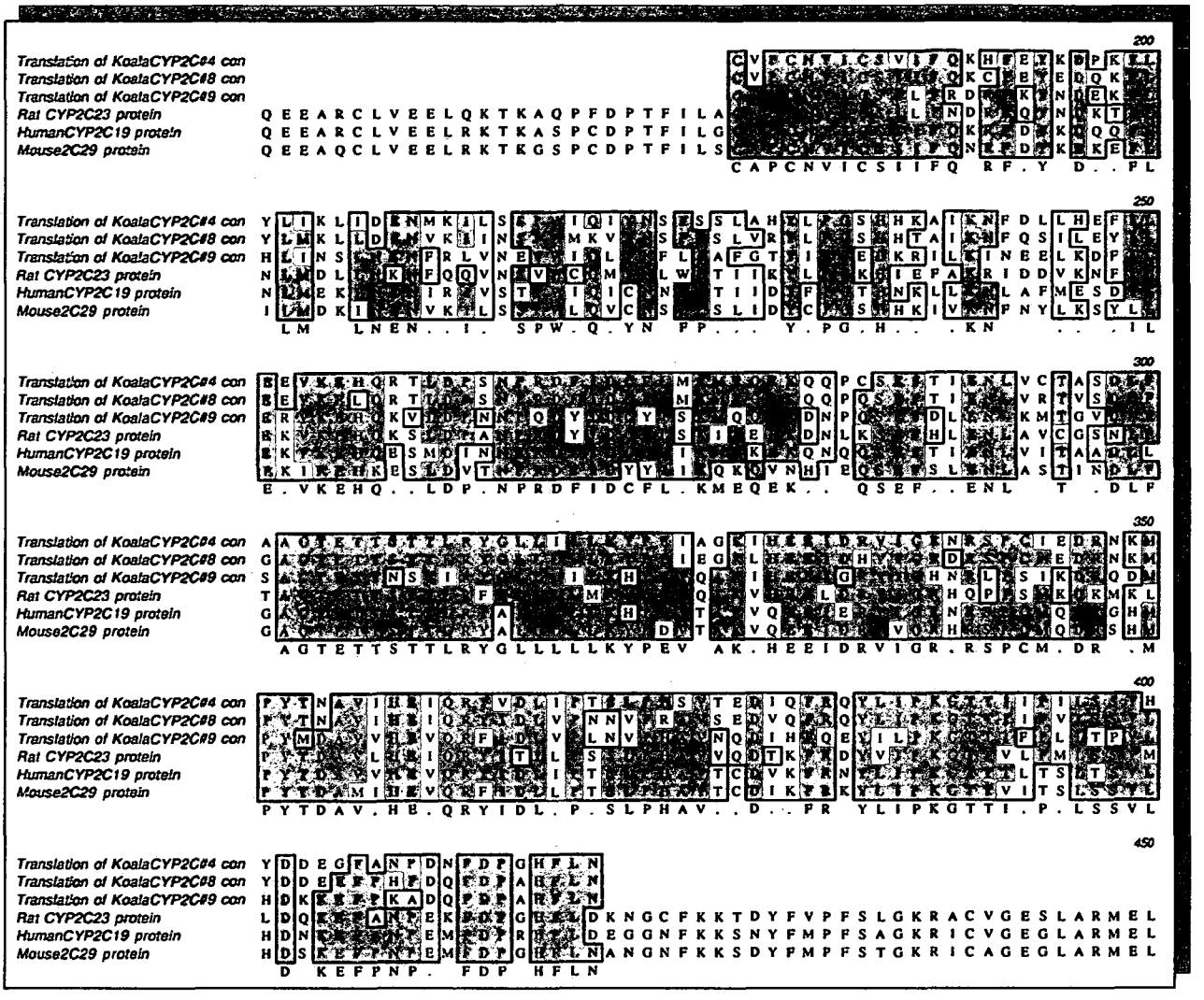

KoalaCYP2C#4 con
KoalaCYP2C#8 con
KoalaCYP2C#9 con
50
TGTGTTCCTTGCAACGTGATTTGTTCTATCATCTTCCAGAAACGTTTTGA
100
ATATAAAGATCAGAAATTTCT TATTTGAT AAGCTTTTAGATGAAAAC
150
TCAAGATC T AATTCCCCATGGAT CAG TCTACAATTCTTTTCCATCT
200
CTAG AC TTATCTCCCTGGATCTCATCACA AGCAATTAAAAATTTTGA
250
T ACTACATGAATTTATTTTGGAAGAAGTGAAGGAGCACCAAAGGAC C
300
TGGACCCCAGCAATCCTCGGGACTTCATTGACTGTTTCCTCATGAAAATG
350
GAGCAGGAAAAGCAACAACCACAGTCTGAATTTACCATTGAAAACCTGGT
400
C G ACTG A TGATCTATTTGGTGCAGGAACAGAGACAACCAGCACCA
450
CCCTGAGATATGGACTCCTGCTTCTCCTGAAATACCCTGAGATAGAAGGA
500
AAAATTCATGAAGAAATTGATCGTGTGATTGGCCGAAATCGAAGTCCCTG
550
CATAGAGGACAGAAACAAGATGCCTTATACCAATGCTGTGATACATGAAA
600
TACAACGATACATTGACCTCGTTCC A CAACGTGCCCCATGCAGTGACA
650
GAAGATATTCA TTTAGACAATATCTTATCCCCAAGGGTAC ACCATAAT
700
ACCA TATT TCTTCTGTTCTGTATGATGATGAAGAGTTCCCCAATCC G
750
ACCA TTTGACCCAGCACATTTCTTAAAT

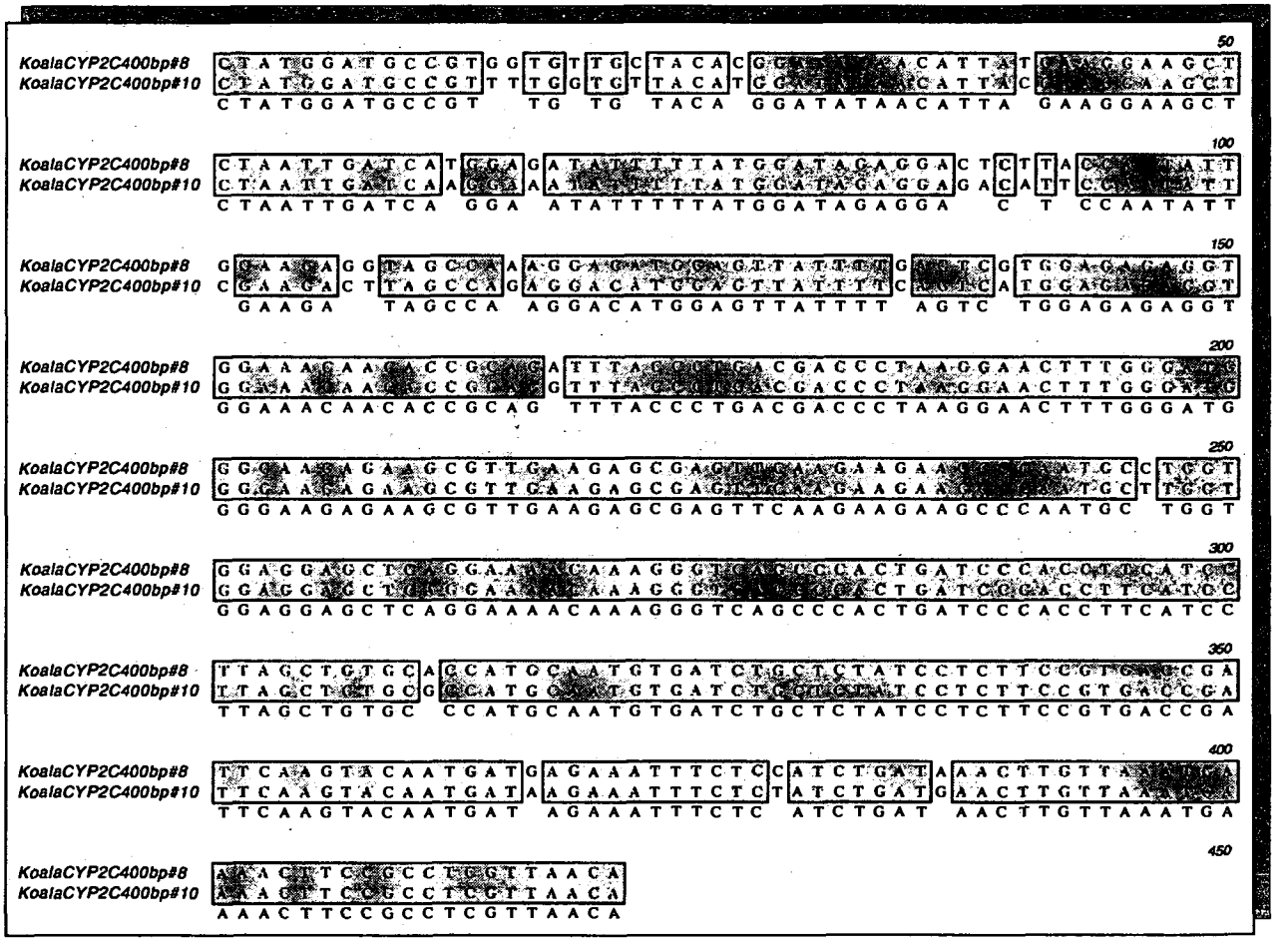
Figure 5.9: Alignment of the deduced amino acid sequences of koala clones *CYP2C#4*, *#8* and *#9* with mouse, rat and human CYP2C proteins



The deduced amino acid sequences of the three koala *CYP2C* clones were aligned with mouse CYP2C29, rat CYP2C23 and human CYP2C19 protein sequences. The dark shaded areas represent conserved amino acid residues, while the lighter shaded areas indicate amino acid residues exhibiting structural similarity. The position of the residues in relation to the translational initiation sequence of one of the mammalian proteins is shown in parentheses at the end of each row. A consensus sequence appears beneath each alignment block.

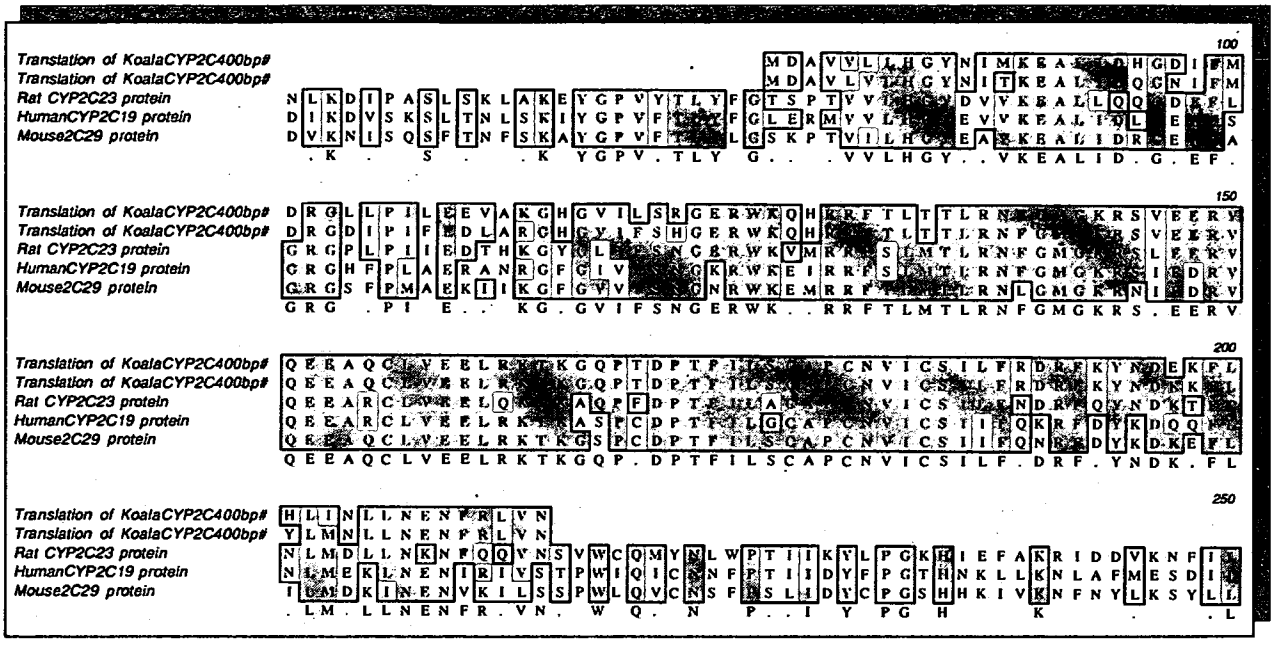
Sequence analysis of the 400 bp products amplified by PCR from koala cDNA using the MAR2CF3 forward primer and KGSP2B reverse primer revealed the presence of two different cDNA clones exhibiting similarity to known mammalian *CYP2C* sequences. A nucleotide alignment of the clones, designated Koala *CYP2C400bp#8* and *#10*, is provided in Figure 5.10. An alignment of the deduced amino acid sequences of the two clones with rat, human and mouse CYP2C proteins demonstrated that they were situated within 100 aa of the 5' ends of known mammalian CYP2C sequences (Figure 5.11).

Figure 5.10: Sequence analysis of two partial 400 bp koala *CYP2C* cDNA clones



Two clones, designated Koala *CYP2C400bp#8* and *#10*, of the 400 bp product amplified from koala cDNA by PCR were sequenced and aligned. Shaded areas represent conserved nucleotides. The nucleotides are numbered in fifty base-pair intervals and a consensus sequence appears beneath each alignment block.

Figure 5.11: Alignment of the deduced amino acid sequences of the 400 bp koala *CYP2C* clones with mouse, rat and human CYP2C proteins



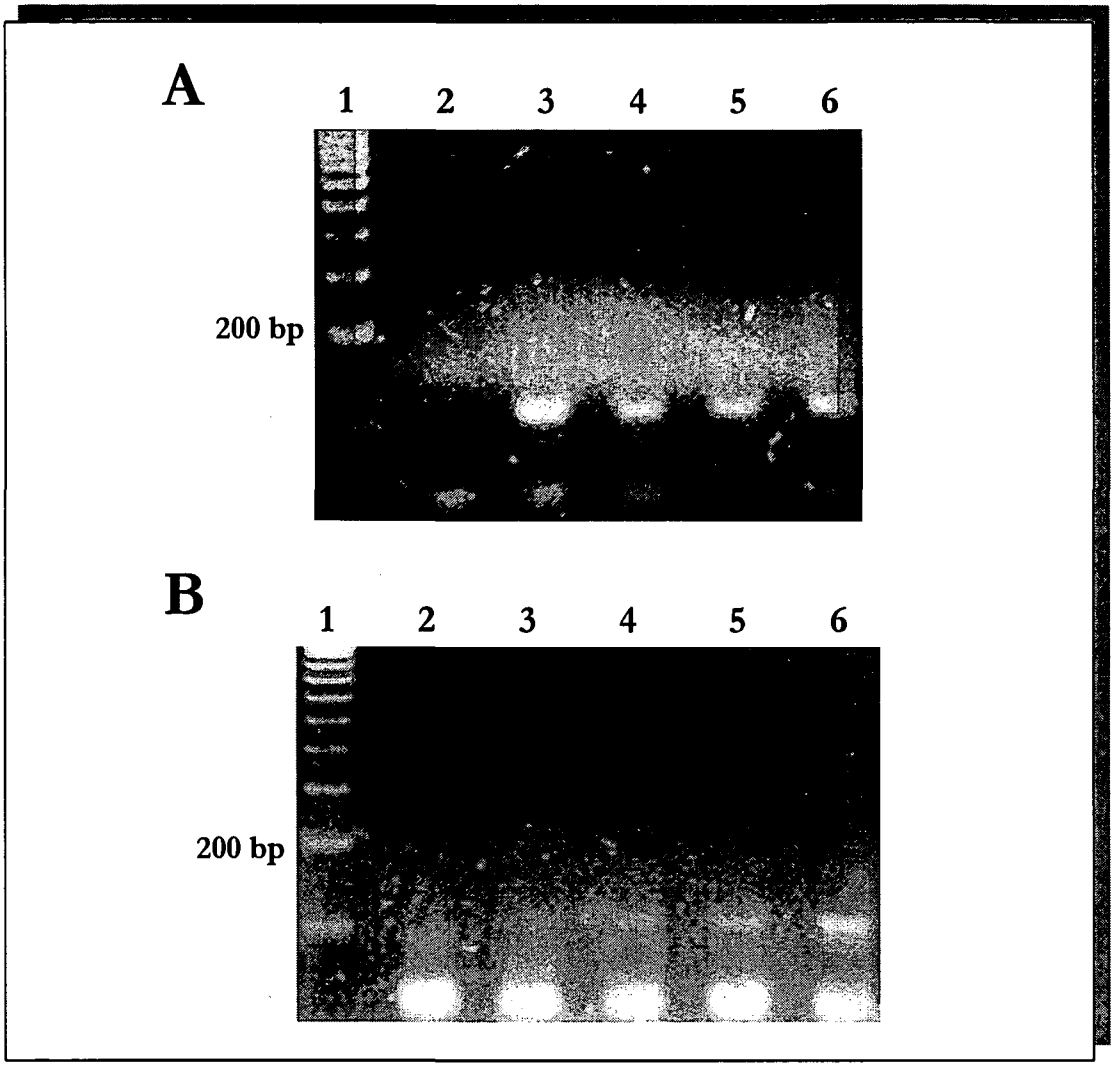
The deduced amino acid sequences of the two koala *CYP2C* clones were aligned with mouse CYP2C29, rat CYP2C23 and human CYP2C19 protein sequences. The dark shaded areas represent conserved amino acid residues, while the lighter shaded areas indicate amino acid residues exhibiting structural similarity. The position of the residues in relation to the translational initiation sequence of one of the mammalian proteins is shown in parentheses at the end of each row. A consensus sequence appears beneath each alignment block.

5.4.3. Rapid Amplification of cDNA ends (RACE) of a koala *CYP2C* cDNA sequence

RACE was attempted in order to try and amplify the 5' and 3' ends of a koala *CYP2C* cDNA sequence. For 5' RACE, following the synthesis and TdT-tailing of koala gene-specific cDNA using the KGSP2C primer, PCR experiments were conducted to try and amplify the 5' end of a koala *CYP2C* cDNA sequence using the KGSP2B and UAP primers (see section 5.3.1. of this chapter for primer details). A fragment of about 500-600 bp was expected for this primer combination. Agarose gel analysis of the products generated by PCR from TdT-tailed koala cDNA using the KGSP2B and UAP primers at an annealing temperature of 55°C revealed the absence of any amplified products (data not shown).

An analysis of the gene-specific koala cDNA template was performed to determine whether the KGSP primer had initiated successful first-strand cDNA synthesis. Analysis of the cDNA template was initially conducted using the forward primer MAR2CF and reverse primer KGSP2B, both located slightly 5' of the KGSP2C primer (see Figure 5.1 in section 5.2 for primer details), at an annealing temperature of 55°C. A small fragment of about 100 bp was expected for the primer combination. Agarose gel analysis of the products amplified by PCR using the MAR2CF and KGSP2B primers revealed the presence of a band at approximately 100 bp (Figure 5.12A, lanes 3-6). In addition, agarose gel analysis of the products generated by PCR from the gene-specific koala cDNA, purified through the glassmax column, using the same primer combination at an annealing temperature of 55°C also revealed the presence of a 100 bp fragment (Figure 5.12B, lanes 4-6).

Figure 5.12: Analysis of 5' RACE koala PCR products by agarose gel electrophoresis



Agarose gel analysis of the products generated by PCR from 5' RACE koala cDNA using the MAR2CF forward primer and KGSP2B reverse primer to amplify a region of approximately 100 bp at an annealing temperature of 55°C.

(A) **PCR products generated using 5' RACE koala cDNA. Lane 1:** 100 bp DNA marker. **Lane 2:** Negative control (PCR with no DNA template). **Lanes 3-6:** Sample of PCR products amplified from 5' RACE koala cDNA.

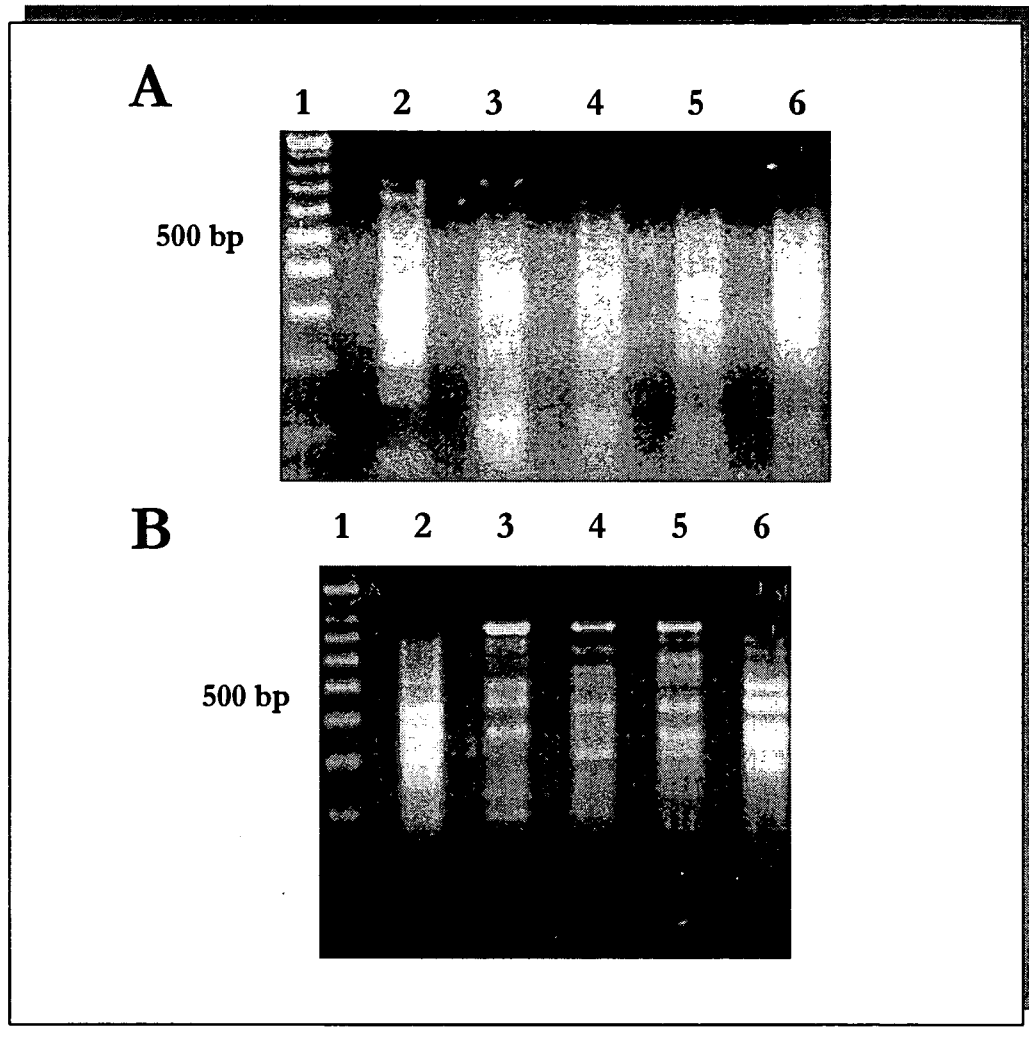
(B) **PCR products generated using column purified 5' RACE koala cDNA. Lane 1:** 100 bp DNA marker. **Lane 2:** Negative control (PCR with no DNA template). **Lanes 3-6:** Sample of PCR products amplified from column purified 5' RACE koala cDNA.

An additional analysis of the cDNA template was performed with the primers MAR2CF3 and KGSP2B, both located 5' of the KGSP2C primer (see Figure 5.1 in section 5.2 for primer details)

at an annealing temperature of 56°C (Figure 5.3). Agarose gel analysis of the products generated by PCR at each annealing temperature revealed the presence of a band at approximately 400 bp which corresponded in size to that expected for the MAR2CF3 and KGSP2B primer combination (Figure 5.3, lanes 5-6).

Following an alternative TdT-tailing protocol of the gene-specific koala cDNA (Chapter 2, section 2.2.15.3), PCR experiments were again conducted to amplify the 5' end of a koala *CYP2C* cDNA sequence using the KGSP2B and UAP primers. Agarose gel analysis of the products generated by PCR from 5' RACE koala cDNA using the two primers at an annealing temperature of 55°C revealed a smear of amplified products in all samples, including the negative control (Figure 5.13A, lanes 2-6). However, agarose gel analysis of the products generated by PCR from koala cDNA with the same primers at a slightly higher annealing temperature of 59°C revealed the presence of a band at approximately 700 bp (Figure 5.13B, lanes 3-5).

Figure 5.13: Analysis of TdT-tailed 5' RACE koala PCR products by agarose gel electrophoresis



Agarose gel analysis of the products generated by PCR from TdT-tailed 5' RACE koala cDNA using the KGSP2B and UAP primers to amplify a region of approximately 500-600 bp at annealing temperatures of 55°C and 59°C.

(A) 55°C annealing temperature.

Lane 1: 100 bp DNA marker. **Lane 2:** Negative control (PCR with no DNA template). **Lanes 3-6:** Sample of PCR products amplified from TdT-tailed 5' RACE koala cDNA.

(B) 59°C annealing temperature.

Lane 1: 100 bp DNA marker. **Lane 2:** Negative control (PCR with no DNA template). **Lanes 3-6:** Sample of PCR products amplified from TdT-tailed 5' RACE koala cDNA.

For 3' RACE, agarose gel analysis of the products generated by PCR from koala cDNA using the forward primer KGSP2H and universal amplification primer (UAP) at annealing temperatures of 54°C and 63°C revealed the absence of any amplified products of the correct size (Figure 5.14A, lanes 3-5; Figure 5.14B, lane 3). However, electrophoretic analysis of the PCR products amplified at an annealing temperature of 60°C revealed the presence of a band at approximately 500 bp which corresponded in size to that expected for the primer combination (Figure 5.14C, lane 3). The amplified PCR products were subcloned directly into pGEM®-T vector (Chapter 2, section 2.2.8) and sequenced to determine their identity (Chapter 2, section 2.2.10).

Figure 5.14. Analysis of 3' RACE koala PCR products by agarose gel electrophoresis. Agarose gel analysis of the products generated by PCR from 3' RACE koala cDNA using the KGSP2H and UAP primers to amplify a region of approximately 500 bp at annealing temperatures of 54°C (A), 63°C (B) and 60°C (C).

(A) **54°C annealing temperature.**

Lane 1: 100 bp DNA marker. **Lane 2:** Negative control (PCR with no DNA template). **Lanes 3-5:** Sample of PCR products amplified from 3' RACE koala cDNA.

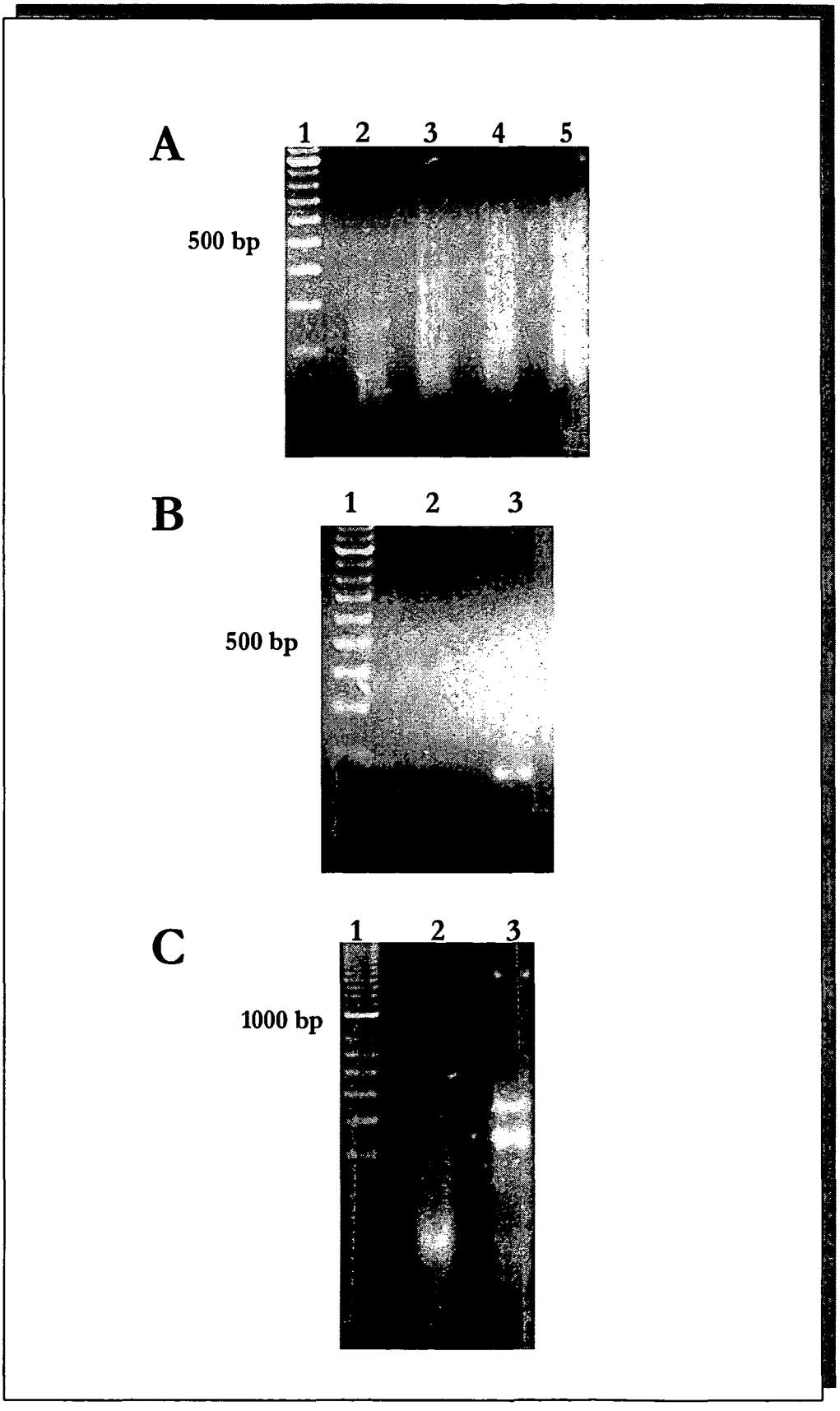
(B) **63°C annealing temperature.**

Lane 1: 100 bp DNA marker. **Lane 2:** Negative control (PCR with no DNA template). **Lanes 3:** Sample of PCR products amplified from 3' RACE koala cDNA.

(C) **60°C annealing temperature.**

Lane 1: 100 bp DNA marker. **Lane 2:** Negative control (PCR with no DNA template). **Lane 3:** Sample of PCR products amplified from 3' RACE koala cDNA.

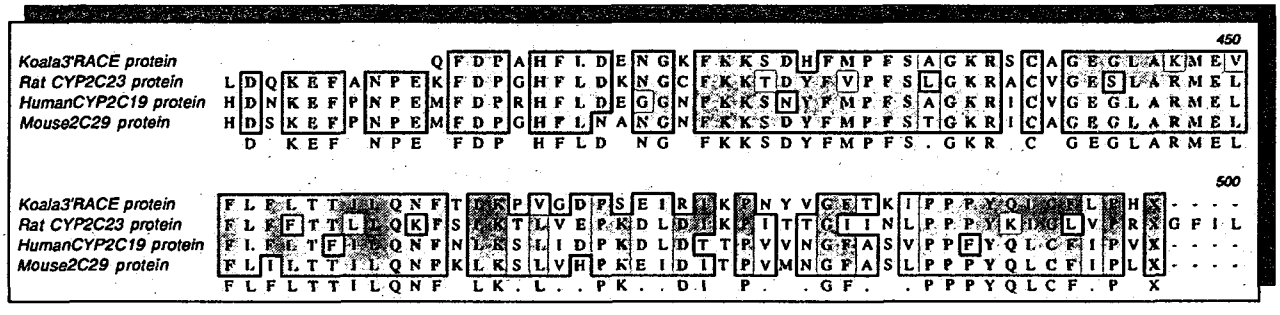
Figure 5.14. Analysis of 3' RACE koala PCR products by agarose gel electrophoresis



5.4.4. Sequence analysis of 3' RACE koala amplified products

Sequence analysis of cloned products of the predicted size generated by PCR using 3' RACE cDNA, revealed one sequence exhibiting similarity to the 3' end of known *CYP2C* sequences. An alignment of the deduced amino acid sequence of the 3' RACE koala *CYP2C* clone with other reported CYP2C proteins is shown in Figure 5.15. The stop codon is signified by an X, and is located at amino acid position 496 for all of the sequences shown. Sequence analysis of other cloned PCR products revealed that they were PCR artefacts.

Figure 5.15. Alignment of the deduced amino acid sequence of the 3' RACE koala *CYP2C* clone with mouse, rat and human CYP2C proteins



The deduced amino acid sequence of the 3' RACE koala *CYP2C* clone was aligned with mouse CYP2C29, rat CYP2C23 and human CYP2C19 protein sequences. The dark shaded areas represent conserved amino acid residues, while the lighter shaded areas indicate amino acid residues exhibiting structural similarity. The position of the residues in relation to the translational initiation sequence of one of the mammalian proteins is shown in parentheses at the end of each row. A consensus sequence appears beneath each alignment block.

5.4.5. Screening of a koala liver cDNA library with a 700 bp partial koala *CYP2C* sequence

In order to isolate full-length *CYP2C* cDNA sequences, a koala liver cDNA library was screened with a partial koala *CYP2C* cDNA sequence (Chapter 2, section 2.2.17). The koala *CYP2C#9* clone, which contained a 700 bp amplified PCR fragment (Chapter 5, section 5.4.2.3) was used as a cDNA probe to isolate full-length koala *CYP2C* cDNA sequences. Approximately 6 X 10^5 plaques were screened using this cDNA probe. Only those plaques appearing on both the master and duplicate filters were isolated. A number of positive clones were identified and purified through three rounds of screening. Single positive plaques were in vivo excised (Chapter 2, section 2.2.17.5) and the cDNA inserts excised by digestion with *Pst I* and *Apa I*. Agarose gel analysis of the cDNA inserts revealed they ranged in size from approximately 0.5-1.5 kbp. The average size of mammalian *CYP2C* cDNA sequences is approximately 1.8 kbp. Several of the largest clones were purified and sequenced using the T3 and T7 primers which flanked the 5' and 3' ends of the cDNA in the vector respectively.

Sequence analysis of a number of the larger positive clones revealed that each encoded a member of the *CYP2C* subfamily. The cDNA and deduced amino acid sequence comparison of these clones with other known mammalian CYP2C forms revealed that none of the isolated clones encoded an entire open reading frame.

5.4.6. Screening of a koala liver cDNA library with a 400 bp partial koala *CYP2C* sequence

In order to facilitate the successful isolation of a full-length koala *CYP2C* cDNA sequence, the koala liver cDNA library was re-probed with two 400 bp amplified koala PCR products (section 5.4.2.3) that were closer to the 5' end of known mammalian *CYP2C* sequences. Approximately 3 X 10^5 plaques were screened at a hybridisation temperature of 45°C. A number of positive clones were identified and purified through three rounds of screening. Agarose gel analysis of the excised cDNA inserts revealed they ranged from approximately 1.4-1.7 kbp in size. Several of the larger clones were purified and sequenced directly using the T3 and T7 primers.

5.4.7. Sequence analysis

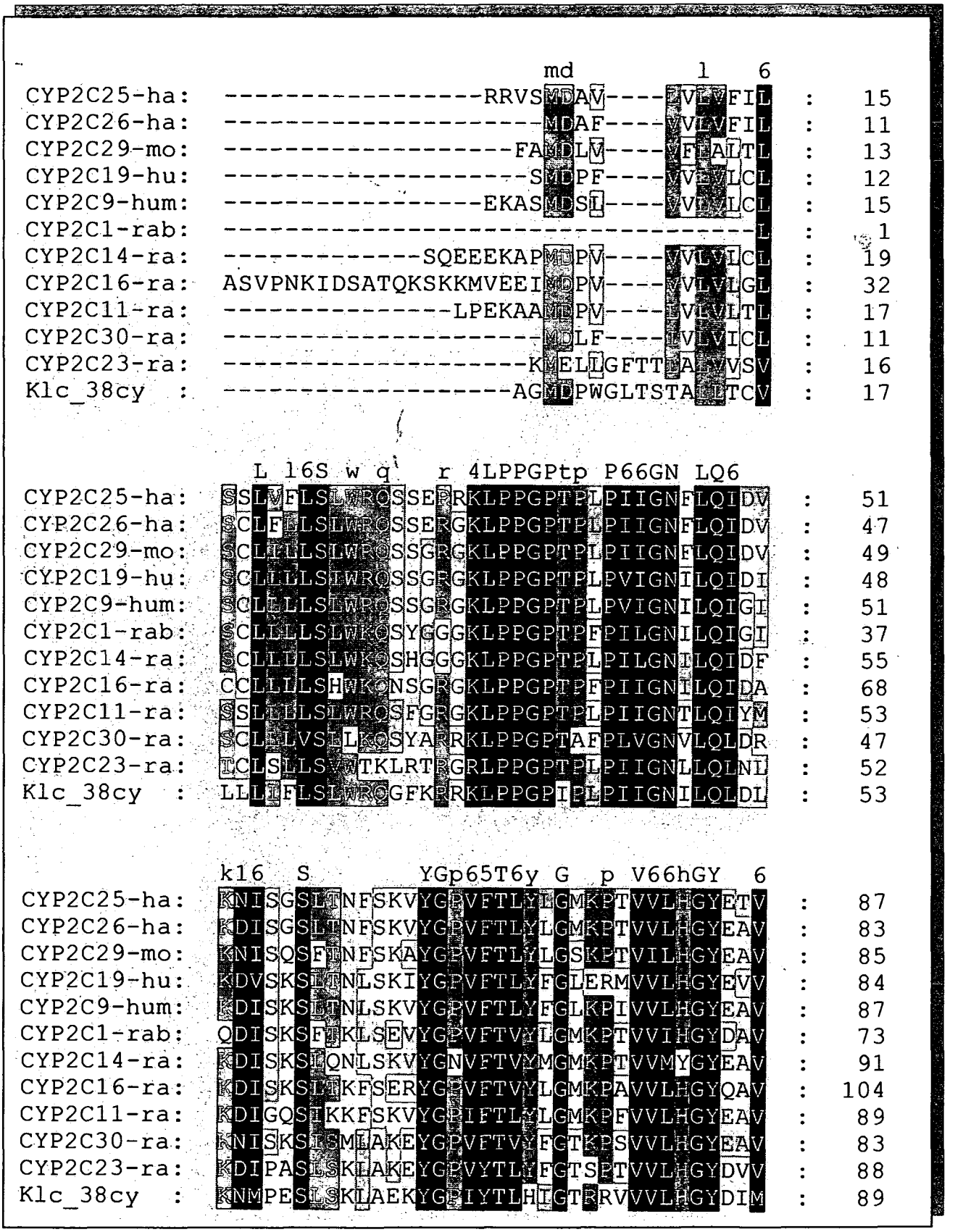
Sequence analysis of a number of clones identified four koala *CYP2C* cDNA sequences of full length or near full length. These have been designated *KLC#26*, *KLC#38*, *KLC#52* and *KLC#66*. An analysis of the deduced amino acid sequence of KLC#38 revealed it contained a translational start codon (methionine) and an open reading frame of 495 amino acids. This sequence is distinct from the previously isolated partial cDNAs, *CYP2C#4*, *CYP2C#8* and *CYP2C#9*. An alignment of the deduced amino acid sequence of the KLC#38 clone with a selection of published mammalian CYP2C proteins is shown in Figure 5.16. Analysis of clones *KLC#52* and *KLC#66* confirmed them as containing an identical open reading frame to the *KLC#38* clone. This sequence was submitted to the cytochrome P450 nomenclature committee for naming in May 2000 and was subsequently designated *CYP2C47* (http://drnelson.utmem.edu/CytochromeP450.html).

Analysis of the *KLC#26* clone identified it as being distinct from *KLC#38*, the deduced amino acid sequences of the two clones shared 92% identity. However, despite being longer than the KLC#38 clone, repeated sequencing of the KLC#26 clone confirmed the absence of a translational start codon. This clone was also distinct from the three previously isolated partial koala *CYP2C* cDNAs. This second clone was submitted to the CYP nomenclature committee and named *CYP2C48*.

Collectively, the cloning studies have identified five unique partial or full length hepatic *CYP2C* cDNAs present in the koala. These are designated *CYP2C #4* (partial), *CYP2C#8* (partial), *CYP2C#9* (partial), *KLC#26* (full length), and *KLC#38* (full length).

Figure 5.16: Alignment of the deduced amino acid sequence of the KLC#38 *CYP2C* cDNA clone with multiple CYP2C proteins

The deduced amino acid sequence of the KLC#38 *CYP2C* cDNA clone was aligned with the protein sequences of a number of mammalian CYP2C subfamily members. This alignment is shown over the next five pages. The darkness of shading emphasises the degree of conservation in each column of the alignment. This indicates which parts of the sequences are least tolerant of change, or where a change in the sequence is most likely to alter the structure or function of the molecule represented by the sequence. Three different shading levels of conservation have been used. Default levels have been set at or near 100% conserved, 80% or greater conserved and 60% or greater conserved. The top level is further distinguished by either upper case characters or numbers on the consensus line. The position of the residues in relation to its own translational initiation sequence is shown at the end of each row.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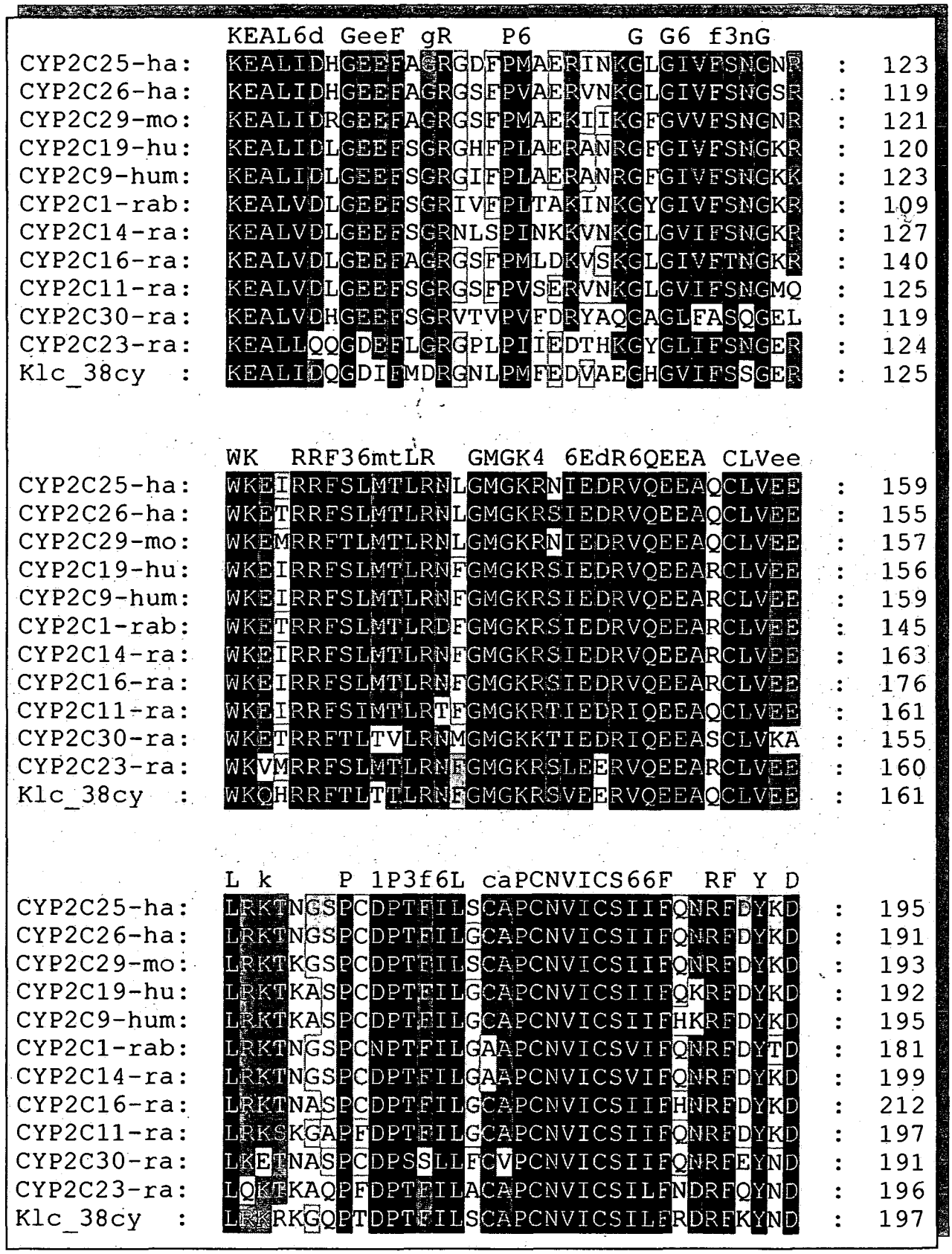
CYP2C26-ha: 11
CYP2C29-mo: 13
CYP2C19-hu: 12
CYP2C9-hum: 15
CYP2C1-rab: 1
CYP2C14-ra: 19
CYP2C16-ra: 32
CYP2C11-ra: 17
CYP2C30-ra: 11
CYP2C23-ra: 16
Klc_38cy : 17
CYP2C25-ha: 51
CYP2C26-ha: 47
CYP2C29-mo: 49
CYP2C19-hu: 48
CYP2C9-hum: 51
CYP2C1-rab: 37
CYP2C14-ra: 55
CYP2C16-ra: 68
CYP2C11-ra: 53
CYP2C30-ra: 47
CYP2C23-ra: 52
Klc_38cy : 53
CYP2C25-ha: 87
CYP2C26-ha: 83
CYP2C29-mo: 85
CYP2C19-hu: 84
CYP2C9-hum: 87
CYP2C1-rab: 73
CYP2C14-ra: 91
CYP2C16-ra: 104
CYP2C11-ra: 89
CYP2C30-ra: 83
CYP2C23-ra: 88
Klc_38cy : 89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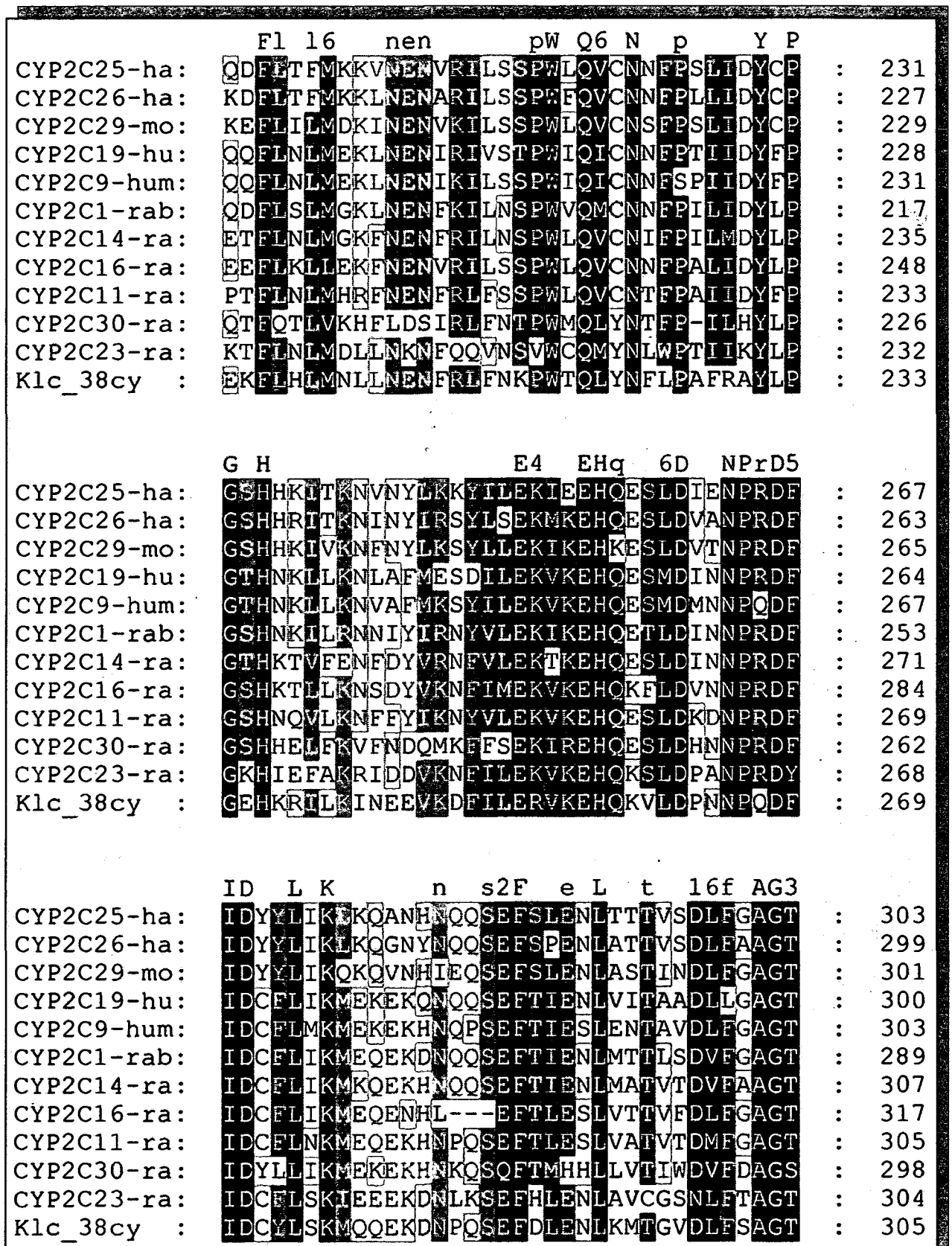
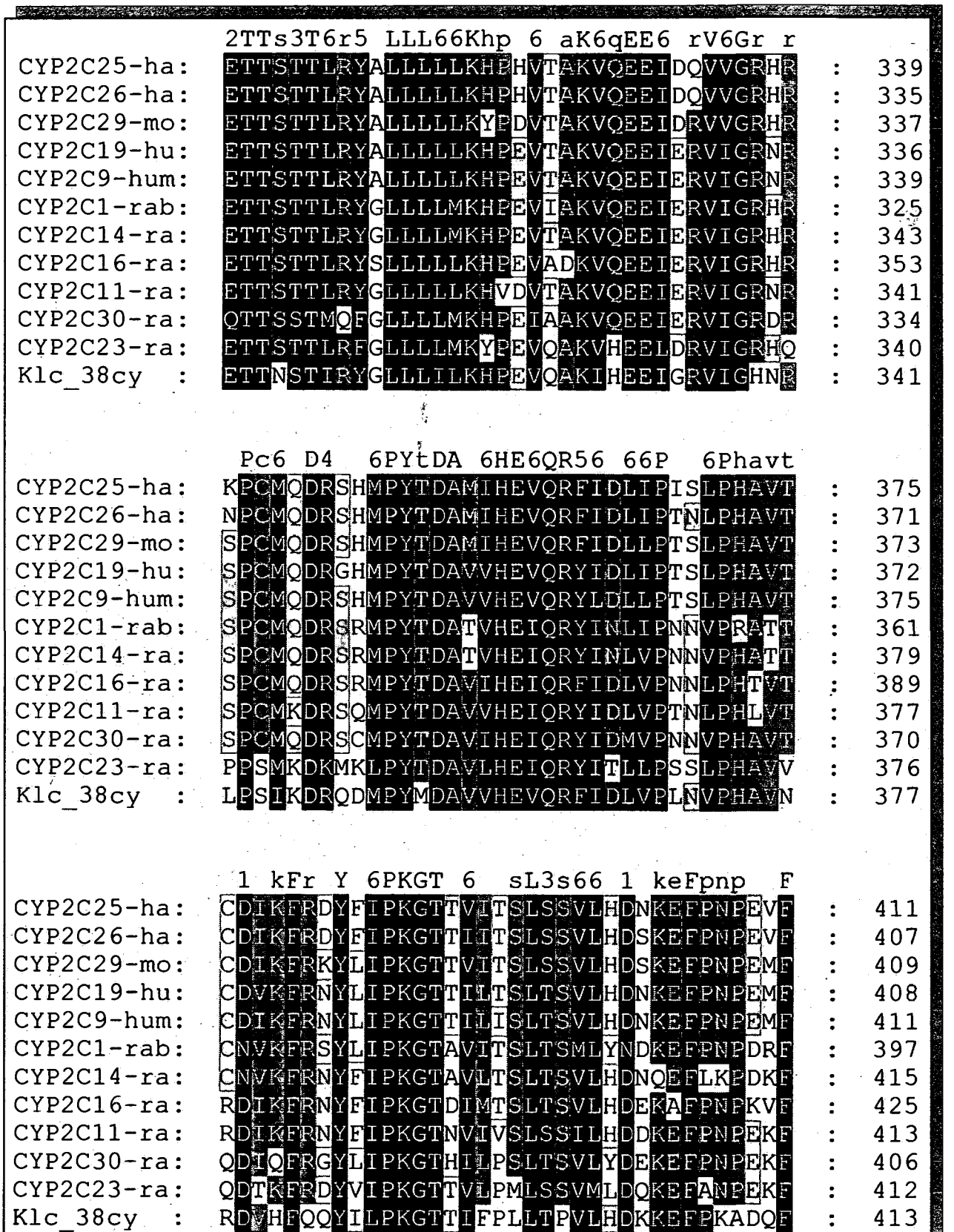
CYP2C26-ha: KEALIDHGEEFAGRGSFPVAERVNKGLGIVFSNGSR : 119
CYP2C29-mo: KEALIDRGEEFAGRGSFPMAEKIIKGFGVVFSNGNR : 121
CYP2C19-hu: KEALIDLGEEFSGRGHFPLAERANRGFGIVFSNGKR : 120
CYP2C9-hum: KEALIDLGEEFSGRGIFPLAERANRGFGIVFSNGKK : 123
CYP2C1-rab: KEALVDLGEEFSGRIVFPLTAKINKGYGIVFSNGKR : 109
CYP2C14-ra: KEALVDLGEEFSGRNLSPINKKVNKGLGVIFSNGKR : 127
CYP2C16-ra: KEALVDLGEEFAGRGSFPMLDKVSKGLGIVFTNGKR : 140
CYP2C11-ra: KEALVDLGEEFSGRGSFPVSERVNKGLGVIFSNGMQ : 125
CYP2C30-ra: KEALVDHGEEFSGRVTVPVFDRYAQCAGLFASQGEL : 119
CYP2C23-ra: KEALLQQGDEFLGRGPLPIIEDTHKGYGLIFSNGER : 124
Klc_38cy : KEALIDQGDIFMDRGNLPMFEDVAEGHGVIFSSGER : 125
WK RRF36mtLR GMGK4 6EdR6QEEA CLVee
CYP2C25-ha: WKEIRRFSLMTLRNLGMGKRNIEDRVQEEAQCLVEE : 159
CYP2C26-ha: WKETRRFSLMTLRNLGMGKRSIEDRVQEEAQCLVEE : 155
CYP2C29-mo: WKEMRRFTLMTLRNLGMGKRNIEDRVQEEAQCLVEE : 157
CYP2C19-hu: WKEIRRFSLMTLRNFGMGKRSIEDRVQEEARCLVEE : 156
CYP2C9-hum: WKEIRRFSLMTLRNFGMGKRSIEDRVQEEARCLVEE : 159
CYP2C1-rab: WKETRRFSLMTLRDFGMGKRSIEDRVQEEARCLVEE : 145
CYP2C14-ra: WKEIRRFSLMTLRNFGMGKRSIEDRVQEEARCLVEE : 163
CYP2C16-ra: WKEIRRFSLMTLRNFGMGKRSIEDRVQEEARCLVEE : 176
CYP2C11-ra: WKEIRRFSIMTLRTFGMGKRTIEDRIQEEAQCLVEE : 161
CYP2C30-ra: WKETRRFTLTVLRNMGMGKKTIEDRIQEEASCLVKA : 155
CYP2C23-ra: WKVMRRFSLMTLRNFGMGKRSLEERVQEEARCLVEE : 160
Klc_38cy : WKQHRRFTLTTLRNFGMGKRSVEERVQEEAQCLVEE : 161
L k P 1P3f6L caPCNVICS66F RF Y D
CYP2C25-ha: LRKTNGSPCDPTFILSCAPCNVICSIIFQNRFDYKD : 195
CYP2C26-ha: LRKTNGSPCDPTFILGCAPCNVICSIIFQNRFDYKD : 191
CYP2C29-mo: LRKTKGSPCDPTFILSCAPCNVICSIIFQNRFDYKD : 193
CYP2C19-hu: LRKTKASPCDPTFILGCAPCNVICSIIFQKRFDYKD : 192
CYP2C9-hum: LRKTKASPCDPTFILGCAPCNVICSIIFHKRFDYKD : 195
CYP2C1-rab: LRKTNGSPCNPTFILGAAPCNVICSVIFQNRFDYTD : 181
CYP2C14-ra: LRKTNGSPCDPTFILGAAPCNVICSVIFQNRFDYKD : 199
CYP2C16-ra: LRKTNASPCDPTFILGCAPCNVICSIIFHNRFDYKD : 212
CYP2C11-ra: LRKSKGAPFDPTFILGCAPCNVICSIIFQNRFDYKD : 197
CYP2C30-ra: LKETNASPCDPSSLLFCVPCNVICSIIFQNRFEYND : 191
CYP2C23-ra: LQKTKAQPFDPTFILACAPCNVICSILFNDRFQYND : 196
Klc_38cy : LRKRKGQPTDPTFILSCAPCNVICSILFRDRFKYND : 197


Fl 16 nen pW Q6 N p Y P
CYP2C25-ha: QDFLTFMKKVNENVRILSSPWLQVCNNFPSLIDYCP : 231
CYP2C26-ha: KDFLTFMKKLNENARILSSPWFQVCNNFPLLIDYCP : 227
CYP2C29-mo: KEFLILMDKINENVKILSSPWLQVCNSFPSLIDYCP : 229
CYP2C19-hu: QQFLNLMEKLNENIRIVSTPWIQICNNFPTIIDYFP : 228
CYP2C9-hum: QQFLNLMEKLNENIKILSSPWIQICNNFSPIIDYFP : 231
CYP2C1-rab: QDFLSLMGKLNENFKILNSPWVQMCNNFPILIDYLP : 217
CYP2C14-ra: ETFLNLMGKFNENFRILNSPWLQVCNIFPILMDYLP : 235
CYP2C16-ra: EEFLKLLEKFNENVRILSSPWLQVCNNFPALIDYLP : 248
CYP2C11-ra: PTFLNLMHRFNENFRLFSSPWLQVCNTFPAIIDYFP : 233
CYP2C30-ra: QTFQTLVKHFLDSIRLFNTPWMQLYNTFP-ILHYLP : 226
CYP2C23-ra: KTFLNLMDLLNKNFQQVNSVWCQMYNLWPTIIKYLP : 232
Klc_38cy : EKFLHLMNLLNENFRLFNKPWTQLYNFLPAFRAYLP : 233
G H E4 EHq 6D NPrD5
CYP2C25-ha: GSHHKITKNVNYLKKYILEKIEEHQESLDIENPRDF : 267
CYP2C26-ha: GSHHRITKNINYIRSYLSEKMKEHQESLDVANPRDF : 263
CYP2C29-mo: GSHHKIVKNFNYLKSYLLEKIKEHKESLDVTNPRDF : 265
CYP2C19-hu: GTHNKLLKNLAFMESDILEKVKEHQESMDINNPRDF : 264
CYP2C9-hum: GTHNKLLKNVAFMKSYILEKVKEHQESMDMNNPQDF : 267
CYP2C1-rab: GSHNKILRNNIYIRNYVLEKIKEHQETLDINNPRDF : 253
CYP2C14-ra: GTHKTVFENFDYVRNFVLEKTKEHQESLDINNPRDF : 271
CYP2C16-ra: GSHKTLLKNSDYVKNFIMEKVKEHQKFLDVNNPRDF : 284
CYP2C11-ra: GSHNQVLKNFFYIKNYVLEKVKEHQESLDKDNPRDF : 269
CYP2C30-ra: GSHHELFKVFNDQMKFFSEKIREHQESLDHNNPRDF : 262
CYP2C23-ra: GKHIEFAKRIDDVKNFILEKVKEHQKSLDPANPRDY : 268
Klc_38cy : GEHKRILKINEEVKDFILERVKEHQKVLDPNNPQDF : 269
ID L K n s2F e L t 16f AG3
CYP2C25-ha: IDYYLIKLKQANHNQQSEFSLENLTTTVSDLFGAGT : 303
CYP2C26-ha: IDYYLIKLKQGNYNQQSEFSPENLATTVSDLFAAGT : 299
CYP2C29-mo: IDYYLIKQKQVNHIEQSEFSLENLASTINDLFGAGT : 301
CYP2C19-hu: IDCFLIKMEKEKQNQQSEFTIENLVITAADLLGAGT : 300
CYP2C9-hum: IDCFLMKMEKEKHNQPSEFTIESLENTAVDLFGAGT : 303
CYP2C1-rab: IDCFLIKMEQEKDNQQSEFTIENLMTTLSDVFGAGT : 289
CYP2C14-ra: IDCFLIKMKQEKHNQQSEFTIENLMATVTDVFAAGT : 307
CYP2C16-ra: IDCFLIKMEQENHL---EFTLESLVTTVFDLFGAGT : 317
CYP2C11-ra: IDCFLNKMEQEKHNPQSEFTLESLVATVTDMFGAGT : 305
CYP2C30-ra: IDYLLIKMEKEKHNKQSQFTMHHLLVTIWDVFDAGS : 298
CYP2C23-ra: IDCFLSKIEEEKDNLKSEFHLENLAVCGSNLFTAGT : 304
Klc_38cy : IDCYLSKMQQEKDNPQSEFDLENLKMTGVDLFSAGT : 305


2TTs3T6r5 LLL66Khp 6 aK6qEE6 rV6Gr r
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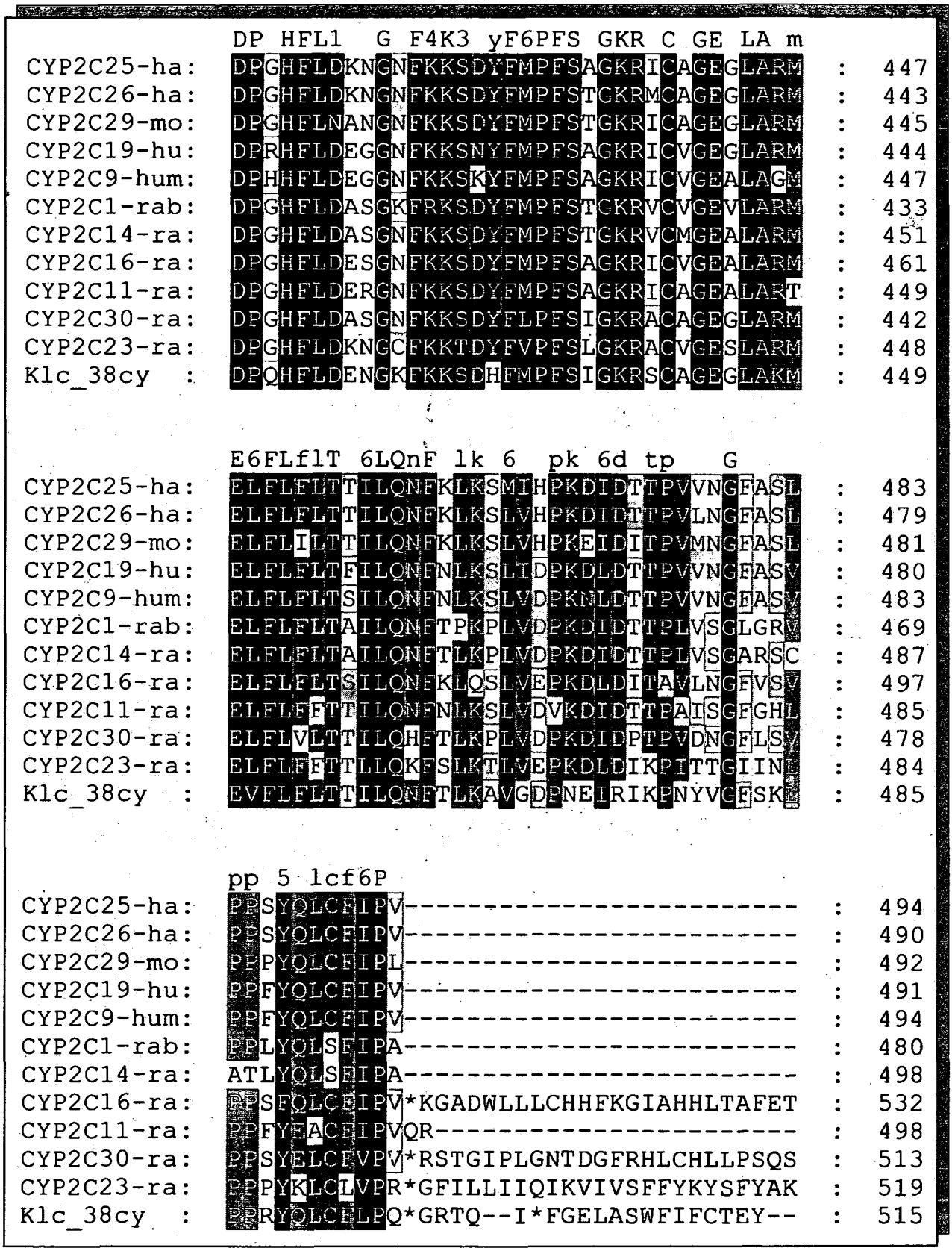
CYP2C26-ha: ETTSTTLRYALLLLLKHPHVTAKVQEEIDQVVGRHR : 335
CYP2C29-mo: ETTSTTLRYALLLLLKYPDVTAKVQEEIDRVVGRHR : 337
CYP2C19-hu: ETTSTTLRYALLLLLKHPEVTAKVQEEIERVIGRNR : 336
CYP2C9-hum: ETTSTTLRYALLLLLKHPEVTAKVQEEIERVIGRNR : 339
CYP2C1-rab: ETTSTTLRYGLLLLMKHPEVIAKVQEEIERVIGRHR : 325
CYP2C14-ra: ETTSTTLRYGLLLLMKHPEVTAKVQEEIERVIGRHR : 343
CYP2C16-ra: ETTSTTLRYSLLLLLKHPEVADKVQEEIERVIGRHR : 353
CYP2C11-ra: ETTSTTLRYGLLLLLKHVDVTAKVQEEIERVIGRNR : 341
CYP2C30-ra: QTTSSTMQFGLLLLMKHPEIAAKVQEEIERVIGRDR : 334
CYP2C23-ra: ETTSTTLRFGLLLLMKYPEVQAKVHEELDRVIGRHQ : 340
Klc_38cy : ETTNSTIRYGLLLILKHPEVQAKIHEEIGRVIGHNR : 341
Pc6 D4 6PYtDA 6HE6QR56 66P 6Phavt
CYP2C25-ha: KPCMQDRSHMPYTDAMIHEVQRFIDLIPISLPHAVT : 375
CYP2C26-ha: NPCMQDRSHMPYTDAMIHEVQRFIDLIPTNLPHAVT : 371
CYP2C29-mo: SPCMQDRSHMPYTDAMIHEVQRFIDLLPTSLPHAVT : 373
CYP2C19-hu: SPCMQDRGHMPYTDAVVHEVQRYIDLIPTSLPHAVT : 372
CYP2C9-hum: SPCMQDRSHMPYTDAVVHEVQRYLDLLPTSLPHAVT : 375
CYP2C1-rab: SPCMQDRSRMPYTDATVHEIQRYINLIPNNVPRATT : 361
CYP2C14-ra: SPCMQDRSRMPYTDATVHEIQRYINLVPNNVPHATT : 379
CYP2C16-ra: SPCMQDRSRMPYTDAVIHEIQRFIDLVPNNLPHTVT : 389
CYP2C11-ra: SPCMKDRSQMPYTDAVVHEIQRYIDLVPTNLPHLVT : 377
CYP2C30-ra: SPCMQDRSCMPYTDAVIHEIQRYIDMVPNNVPHAVT : 370
CYP2C23-ra: PPSMKDKMKLPYTDAVLHEIQRYITLLPSSLPHAVV : 376
Klc_38cy : LPSIKDRQDMPYMDAVVHEVQRFIDLVPLNVPHAVN : 377
1 kFr Y 6PKGT 6 sL3s66 1 keFpnp F
CYP2C25-ha: CDIKFRDYFIPKGTTVITSLSSVLHDNKEFPNPEVF : 411
CYP2C26-ha: CDIKFRDYFIPKGTTIITSLSSVLHDSKEFPNPEVF : 407
CYP2C29-mo: CDIKFRKYLIPKGTTVITSLSSVLHDSKEFPNPEMF : 409
CYP2C19-hu: CDVKFRNYLIPKGTTILTSLTSVLHDNKEFPNPEMF : 408
CYP2C9-hum: CDIKFRNYLIPKGTTILISLTSVLHDNKEFPNPEMF : 411
CYP2C1-rab: CNVKFRSYLIPKGTAVITSLTSMLYNDKEFPNPDRF : 397
CYP2C14-ra: CNVKFRNYFIPKGTAVLTSLTSVLHDNQEFLKPDKF : 415
CYP2C16-ra: RDIKFRNYFIPKGTDIMTSLTSVLHDEKAFPNPKVF : 425
CYP2C11-ra: RDIKFRNYFIPKGTNVIVSLSSILHDDKEFPNPEKF : 413
CYP2C30-ra: QDIQFRGYLIPKGTHILPSLTSVLYDEKEFPNPEKF : 406
CYP2C23-ra: QDTKFRDYVIPKGTTVLPMLSSVMLDQKEFANPEKF : 412
Klc_38cy : RDVHFQQYILPKGTTIFPLLTPVLHDKKEFPKADQF : 413


DP HFL1 G F4K3 yF6PFS GKR C GE LA m
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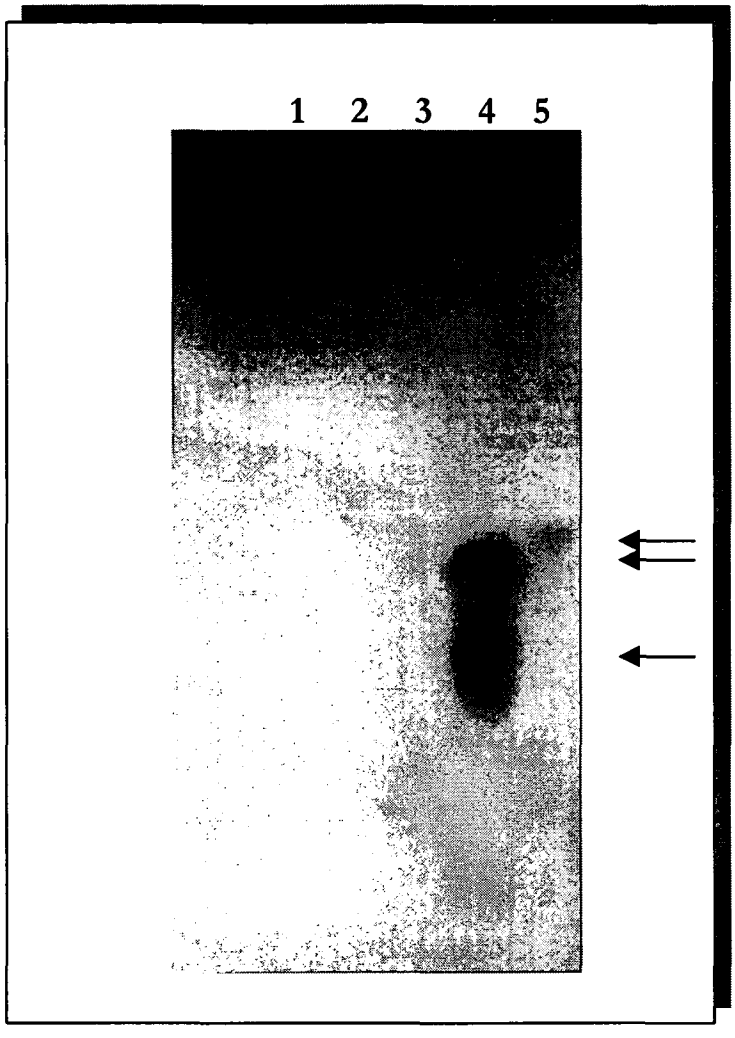
CYP2C26-ha: DPGHFLDKNGNFKKSDYFMPFSTGKRMCAGEGLARM : 443
CYP2C29-mo: DPGHFLNANGNFKKSDYFMPFSTGKRICAGEGLARM : 445
CYP2C19-hu: DPRHFLDEGGNFKKSNYFMPFSAGKRICVGEGLARM : 444
CYP2C9-hum: DPHHFLDEGGNFKKSKYFMPFSAGKRICVGEALAGM : 447
CYP2C1-rab: DPGHFLDASGKFRKSDYFMPFSTGKRVCVGEVLARM : 433
CYP2C14-ra: DPGHFLDASGNFKKSDYFMPFSTGKRVCMGEALARM : 451
CYP2C16-ra: DPGHFLDESGNFKKSDYFMPFSAGKRICVGEALARM : 461
CYP2C11-ra: DPGHFLDERGNFKKSDYFMPFSAGKRICAGEALART : 449
CYP2C30-ra: DPGHFLDASGNFKKSDYFLPFSIGKRACAGEGLARM : 442
CYP2C23-ra: DPGHFLDKNGCFKKTDYFVPFSLGKRACVGESLARM : 448
Klc_38cy : DPQHFLDENGKFKKSDHFMPFSIGKRSCAGEGLAKM : 449
E6FLflT 6LQnF lk 6 pk 6d tp G
CYP2C25-ha: ELFLFLTTILQNFKLKSMIHPKDIDTTPVVNGFASL : 483
CYP2C26-ha: ELFLFLTTILQNFKLKSLVHPKDIDTTPVLNGFASL : 479
CYP2C29-mo: ELFLILTTILQNFKLKSLVHPKEIDITPVMNGFASL : 481
CYP2C19-hu: ELFLFLTFILQNFNLKSLIDPKDLDTTPVVNGFASV : 480
CYP2C9-hum: ELFLFLTSILQNFNLKSLVDPKNLDTTPVVNGFASV : 483
CYP2C1-rab: ELFLFLTAILQNFTPKPLVDPKDIDTTPLVSGLGRV : 469
CYP2C14-ra: ELFLFLTAILQNFTLKPLVDPKDIDTTPLVSGARSC : 487
CYP2C16-ra: ELFLFLTSILQNFKLQSLVEPKDLDITAVLNGFVSV : 497
CYP2C11-ra: ELFLFFTTILQNFNLKSLVDVKDIDTTPAISGFGHL : 485
CYP2C30-ra: ELFLVLTTILQHFTLKPLVDPKDIDPTPVDNGFLSV : 478
CYP2C23-ra: ELFLFFTTLLQKFSLKTLVEPKDLDIKPITTGIINL : 484
Klc_38cy : EVFLFLTTILQNFTLKAVGDPNEIRIKPNYVGFSKL : 485
pp 5 1cf6P
CYP2C25-ha: PPSYQLCFIPV---------- : 494
CYP2C26-ha: PPSYQLCFIPV---------- : 490
CYP2C29-mo: PPPYQLCFIPL---------- : 492
CYP2C19-hu: PPFYQLCFIPV---------- : 491
CYP2C9-hum: PPFYQLCFIPV---------- : 494
CYP2C1-rab: PPLYQLSFIPA---------- : 480
CYP2C14-ra: ATLYQLSFIPA---------- : 498
CYP2C16-ra: PPSFQLCFIPV*KGADWLLLCHHFKGIAHHLTAFET : 532
CYP2C11-ra: PPFYEACFIPVQR---------- : 498
CYP2C30-ra: PPSYELCFVPV*RSTGIPLGNTDGFRHLCHLLPSQS : 513
CYP2C23-ra: PPPYKLCLVPR*GFILLIIQIKVIVSFFYKYSFYAK : 519
Klc_38cy : PPRYQLCFLPQ*GRTQ--I*FGELASWFIFCTEY-- : 515

5.4.8. RNA blot analysis

In order to assess expression of the marsupial CYP2C subfamily at the mRNA level, RNA blot analysis was performed using koala, tammar wallaby and rat mRNA samples. Total RNA from the koala and rat was included on the blot for control purposes. Equal amounts of hepatic RNA from each species (5μg for mRNA and 10μg for total RNA) was probed with a 700 bp *CYP2C* cDNA (*CYP2C #9*) cloned from the koala. This fragment was obtained by PCR (section 5.4.2.3).

Probing of each species with the koala *CYP2C* cDNA sequence revealed a number of intense bands in the koala mRNA sample and much fainter bands in the koala total RNA preparation, as indicated by the arrows (Figure 5.17, lanes 3,4). A faint band was also detected in the tammar wallaby mRNA sample (Figure 5.17, lane 5). No bands were detected in either of the rat RNA samples (Figure 5.17, lanes 1,2). The size of the largest koala and tammar wallaby bands was estimated at ~1.8 kbp, based on the migration of the 18s and 28s bands of the rat total RNA sample.

Figure 5.17. RNA blot analysis of koala, tammar wallaby and rat liver mRNA and total RNA probed with a 700 bp partial koala *CYP2C* sequence



Total RNA and mRNA prepared from koala, tammar wallaby and rat liver samples was probed with a specific 700 bp koala *CYP2C* cDNA sequence at a hybridisation temperature of 45°C. **Lane 1:** rat total RNA. **Lane 2:** rat mRNA. **Lane 3:** koala total RNA. **Lane 4:** koala mRNA. **Lane 5:** tammar wallaby mRNA.

5.5. Discussion

At the commencement of these studies, there was a lack of information concerning cytochrome P450 multiplicity and expression in all Australian marsupial species. Such studies have been hindered by the lack of available marsupial cDNA libraries and tissue resources. As outlined in Chapter 3, the establishment of a tissue bank at the School of Pharmacy and Medical Sciences has facilitated cloning studies in several Australian marsupial species. This enabled the cloning of partial *CYP* cDNAs from several species using a PCR based approach. Such an approach has been applied extensively for cloning *CYP* sequences from a wide variety of species in the absence of any sequence data. Koala and tammar wallaby liver tissue from this bank was subsequently used for the commercial construction of cDNA libraries enabling full length sequences to be obtained. The studies presented in this chapter provide the first cloned *CYP* cDNA sequences from any Australian marsupial species and confirm the multiplicity of the CYP2C subfamily in the koala and other marsupial species.

5.5.1. Isolation of *CYP2C* cDNAs from Australian native marsupial species

PCR amplification experiments were conducted using primers designed from highly conserved regions in previously reported mammalian *CYP2C* cDNA sequences. This approach successfully amplified multiple *CYP2C* cDNAs from the western quoll, tammar wallaby and koala.

5.5.1.1. Analysis of western quoll *CYP2C* cDNA sequences

Sequence analysis of the PCR products amplified from western quoll cDNA revealed three clones that were 728 bp, 729 bp and 638 bp in length. The clones, designated Quoll *CYP2C#1*, *#2* and *#4* encoded open reading frames of 242 aa, 243 aa and 212 aa respectively. Quoll *CYP2C#1* and Quoll *CYP2C#4* are essentially identical with only a few amino acid differences. It is therefore likely that the two clones represent allelic variants of a single quoll *CYP2C* gene. However, an analysis of the full-length cDNA sequences from both clones would be required to confirm this hypothesis. The remaining clone, Quoll *CYP2C#2*, is significantly different to the other two clones and encodes a different member of the western quoll *CYP2C* subfamily.

A comparison of the deduced amino acid sequences of the three western quoll *CYP2C* clones with rat CYP2C23, human CYP2C19 and mouse CYP2C29 proteins showed that the marsupial clones were 60% similar to other mammalian sequences. The full coding regions of these new marsupial *CYP2C* cDNAs have not been isolated to date.

Due to the lack of available tissue, immunoblot analyses were unable to be performed with western quoll liver microsomes using anti-CYP2C6 IgG. Immunoblotting experiments would have provided a more general indication of the number of CYP2C members for this species, and complemented the cloning studies. However, the experiments presented in this chapter have confirmed the presence of at least two new *CYP2C* cDNAs in the western quoll (*Dasyurus geoffroii*). Based on the immunoblotting and cloning data obtained for the other marsupial species it is likely that this species possesses additional, and as yet unidentified, *CYP2C* cDNAs.

5.5.1.2. Analysis of tammar wallaby *CYP2C* cDNA sequences

Sequence analysis of the PCR products amplified from tammar wallaby cDNA revealed two clones that were 729 bp and a third of 728 bp. The clones, designated Wallaby *CYP2C#2*, *#3* and *#4* encoded open reading frames of 243 aa, 242 aa and 243 aa, respectively. The clones incorporate approximate amino acid positions 177-419, relative to previously reported mammalian CYP2C proteins, and do not incorporate the haem-binding region indicative of all CYPs. Analysis of the deduced amino acid sequences of the three partial tammar wallaby clones indicates that each encodes a different member of the *CYP2C* subfamily, and are 60% similar to other mammalian CYP2C proteins. The full coding regions of the new tammar wallaby *CYP2C* cDNAs have not been isolated to date.

Immunoblotting experiments performed with tammar wallaby liver microsomes using anti-rat CYP2C6 IgG detected the presence of a least two CYP2C forms (Chapter 4, section 4.3.3.1.1). The cloning studies presented in this chapter have further confirmed that the tammar wallaby (*Macropus eugenii*) possesses at least three unique *CYP2C* cDNAs. The possibility of the tammar wallaby encoding additional, and as yet unidentified, *CYP2C* cDNAs also cannot be discounted. The *CYP2C* clones and sequencing data generated during these studies combined with the

availability of a tammar wallaby liver cDNA library should ensure the isolation of multiple full-length tammar wallaby *CYP2C* cDNA sequences in the near future.

5.5.1.3. Analysis of koala *CYP2C* cDNA sequences

Sequence analysis of the PCR products amplified from koala cDNA revealed three clones that were all 729 bp in length. The clones, designated Koala *CYP2C#4*, *#8* and *#9*, each encoded open reading frames of 243 aa. Analysis of the deduced amino acid sequences of the partial koala clones demonstrated that each encodes a different member of the CYP2C subfamily, and each shows approximately 60% amino acid similarity to other mammalian CYP2C proteins.

To complement the PCR and cloning studies, 5' and 3' RACE experiments were conducted in order to try and amplify the corresponding cDNA ends of a koala *CYP2C* cDNA sequence. Specific oligonucleotide primers, designed from sequencing information previously obtained for Koala *CYP2C#9*, were initially used in PCR experiments with gene-specific koala cDNA to amplify the 5' end of a koala *CYP2C* cDNA sequence. PCR experiments conducted at a number of different annealing temperatures failed to amplify any products corresponding to the predicted size. PCR analysis of the gene-specific koala cDNA template (prior to TdT-tailing) using internal primers confirmed that it had been successfully reverse-transcribed. A variation of the TdT-tailing procedure of the 5' end, the final step prior to PCR amplification, was therefore conducted as per the manufacturers protocols. PCR experiments were performed at a range of annealing temperatures using standard PCR conditions. A multitude of products were amplified (including in the negative control) at each annealing temperature. Sequence analysis of PCR products which corresponded to the expected size revealed that they were not *CYP2C* sequences. Time constraints prevented any further investigation of the 5' RACE procedure.

3' RACE was also conducted using koala cDNA as template in an attempt to isolate the 3' end of a koala *CYP2C* cDNA sequence. A specific oligonucleotide primer, designed from the sequencing information previously obtained for Koala *CYP2C#9*, was utilised in PCR experiments with koala liver cDNA. Sequence analysis of one clone containing an amplified fragment of 500 bp revealed that it was a *CYP2C* cDNA sequence. The amplified product

contained a stop codon at amino acid position 496 and also possessed a poly-A tail of 28 bp. All of the other sequenced clones were found to be PCR artefacts.

The 5' and 3' RACE experiments to isolate the cDNA ends of a koala *CYP2C* cDNA sequence, and ultimately a full coding region, proved to be successful for the 3' end only. Complications arising from the TdT-tailing procedure of the 5' end of a gene-specific koala cDNA inhibited the subsequent PCR amplification step. Therefore, even though the 3' RACE procedure proved to be successful and a koala *CYP2C* 3' cDNA end was amplified, the failure to isolate the corresponding 5' cDNA end meant the ultimate goal of obtaining a full-length *CYP2C* cDNA sequence was not achieved using this methodology.

The 5' RACE kit (Life Technologies, Sydney) has since been found to be unsuccessful in amplifying the 5' end of any CYP sequence for each member of our laboratory. Similar complications involving the TdT-tailing procedure and subsequent PCR amplification were encountered. Due to the absence of marsupial cDNA libraries at the time, the kit was viewed as the only option in obtaining a full-length marsupial *CYP2C* cDNA sequence. In retrospect, the utilisation of a different 5' RACE kit (Stratagene, Australia) may have produced more favourable results using this methodology. The acquisition of a koala liver cDNA library thankfully provided an alternative to the 5' RACE procedure.

To obtain a full length koala *CYP2C* cDNA sequence, a commercially prepared koala liver cDNA library was screened with various probes obtained in previous experiments. Screening of the koala liver cDNA library with a 5' 400 bp koala *CYP2C* clone yielded several full and near full length *CYP2C* cDNA sequences. Subsequent analysis confirmed the cloning of two full length CYPs, *KLC#38* and *KLC#26*.

The first full length clone, KLC#38, contains an initiation codon and was found to encode an entire open reading frame of 495 aa. The KLC#38 clone contains the conserved signature sequence motif indicative of all CYP sequences (see Figure 5.18). An amino acid sequence comparison of the KLC#38 clone with a number of mammalian CYP2C proteins is provided in

Table 5.2. The full-length KLC#38 clone exhibited 50-60% amino acid similarity to each of the selected mammalian CYP2C proteins, and has therefore been classified into the CYP2C subfamily. The clone has since been named *CYP2C47* by the CYP nomenclature committee. This sequence was the first marsupial full length CYP sequence to appear in the CYP sequence database, which now comprises several thousand sequences from a diverse array of species (Nelson, 2000).

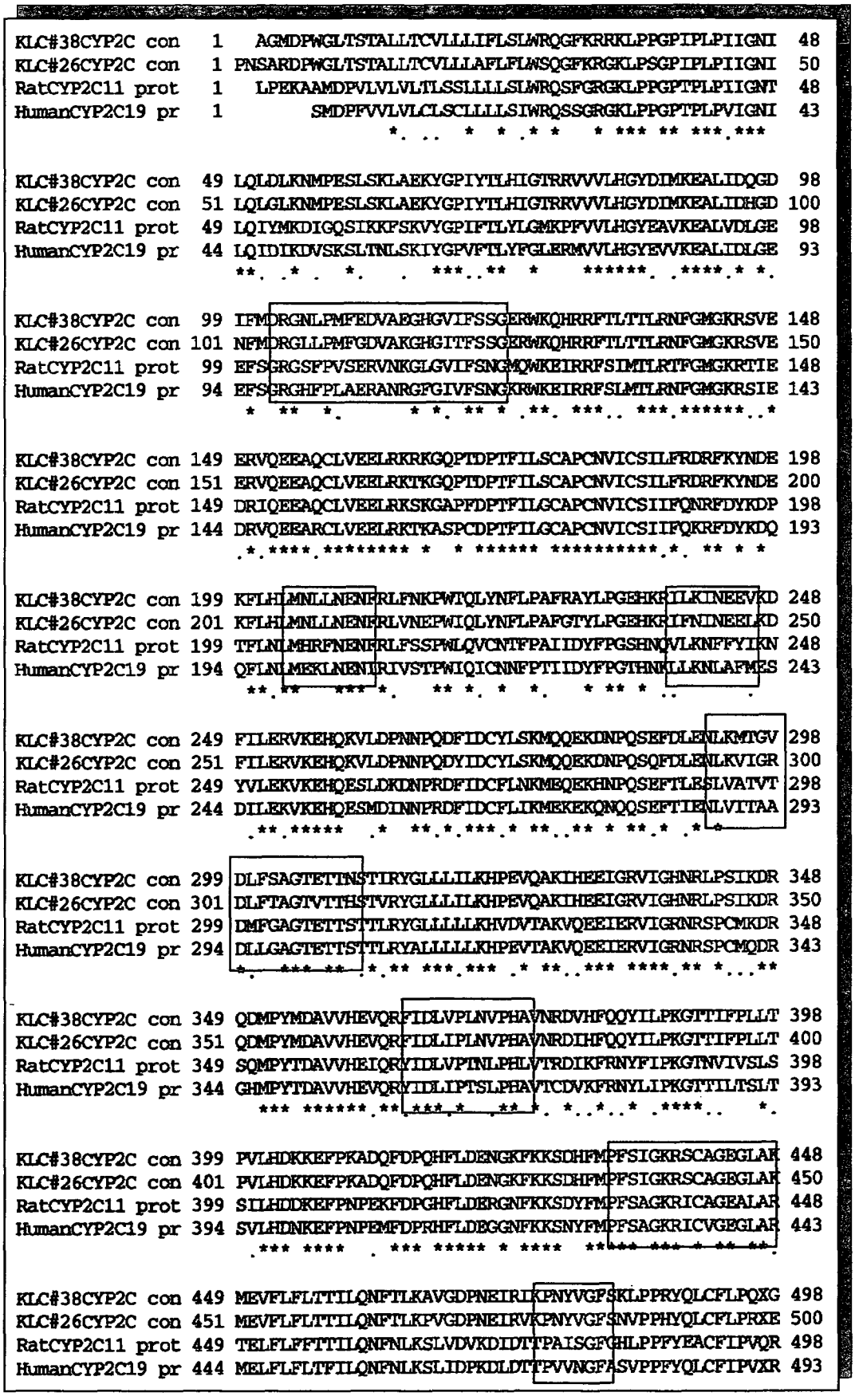
Table 5.2. Amino acid sequence comparison of the KLC#38 and KLC#26 clones with selected mammalian CYP2C proteins

	Rabbit 2C1	Rabbit 2C16	Hamster 2C25	Human 2C9	Mouse 2C29	Rat 2C23	Rat 2C11	Human 2C19
KLC#26	**58**	**56**	**58**	**58**	**57**	**60**	**56**	**60**
KLC#38	**57**	**60**	**58**	**59**	**58**	**61**	**59**	**60**

The *KLC#26* is 1653bp in length and encodes an open reading frame of 499 aa. Multiple sequencing analyses of the 5' region of the clone revealed that it did not contain an initiation codon, even though it was larger than the KLC#38 sequence. The Met➡Arg amino acid substitution (ATG➡AGG) in the KLC#26 clone may represent an error in the reverse-transcription of mRNA during library construction. An alignment of the deduced amino acid sequences of the KL#26 and KLC#38 clones revealed they were 92% similar (Figure 5.18). The clone was submitted to the CYP nomenclature committee and subsequently named *CYP2C48*. This clone represents the second marsupial full-length CYP sequence to appear in the CYP database (Nelson, 2000). In conclusion, it is anticipated that an Arg➡Met amino acid substitution in the KLC#26 clone, through site-directed mutagenesis or PCR, would facilitate the successful expression of protein and enable a determination of its catalytic properties towards dietary compounds.

As mentioned previously, CYPs can exhibit partially overlapping as well as distinct substrate specificities (Gonzalez, 1990). Consequently, numerous studies have investigated which parts of the CYP are involved in the recognition or binding of the substrates, and hence determining its substrate specificity (Gotoh, 1992). The CYP2 family has been a major focus of such studies as it consists of a number of related genes suitable for comparative analyses of nucleotide substitution patterns (Gotoh, 1992). Gotoh (1992) identified six putative substrate recognition sites by comparative sequence analysis methodology in the CYP2 family. Furthermore, an analysis of all the known point mutations and chimeric fragments that alter substrate specificities of CYP2 family members has shown that they are located within, or close to, these substrate recognition sites (Gotoh, 1992). The location of the substrate recognition sites (SRS) and the cytochrome P450 haem-binding domain for the KLC#26, KLC#38, rat and human CYP2C proteins is shown in Figure 5.18. The importance of SRS-1, SRS-4 and SRS-5 for binding substrates is well documented (Gotoh, 1992). Analysis of the SRS sites of the isolated koala CYP2C clones with rat CYP2C11 and human CYP2C19 proteins revealed several subtle differences which may potentially affect the binding of substrates and thus alter the substrate specificity of the two koala CYP2C enzymes. For example, the Gly→Asp single amino acid substitution in both koala CYP2C clones at the beginning of SRS-1 (residue 102) represents a significant increase in the molecular weight of the protein at this position for the koala CYP2C enzymes, in relation to other mammalian CY2C proteins (Figure 5.18, also see Figure 5.16). The Gly residue is highly conserved across the majority of CYP2 family members, thereby confirming its relative importance (Gotoh, 1992). An additional amino acid substitution, Ser→Asn (residue 309) detected in the KLC#38 CYP2C protein sequence may also affect the catalytic activity of the expressed enzyme (Figure 5.18). The Ser residue is conserved in each mammalian CYP2C isoform isolated to date, as well as in multiple CYP2 family members (Gotoh, 1992; Nelson, 2000). In conclusion, the koala CYP2C isoforms exhibit unique amino acid differences in comparison to other mammalian CYP2C proteins, at several locations important in determining its substrate specificity. Subsequent analyses of the catalytic activities of the expressed koala CYP2C protein toward known CYP2C substrates will provide important data as to the properties of these new koala CYP2C isoforms.

Figure 5.18. Alignment of the deduced amino acid sequences of the two koala library clones with rat and human CYP2C proteins. The six SRS sites (red) and the cytochrome P450 haem-binding site (blue) are boxed (Gotoh, 1992).



In summary, an immunoblot analysis of koala liver microsomes with anti-rat CYP2C6 IgG (Chapter 4, section 4.3.3.1.1) provided the initial insight into the multiplicity and possible importance of this CYP subfamily in the metabolism of dietary xenobiotic compounds. Subsequent RT-PCR studies confirmed the presence of at least three unique *CYP2C* forms in this species. The screening of a koala liver cDNA library with a specific koala *CYP2C* cDNA probe culminated in the successful isolation of two full-length *CYP2C* cDNA clones. The newly acquired koala liver cDNA library represents an ideal tool in which to isolate further *CYP2C* sequences. The studies presented in this chapter have demonstrated that the library contains full-length *CYP2C* cDNA sequences, which can be isolated if a suitable 5' cDNA fragment is utilised as a probe. The sequence information provided in this chapter for the remaining *CYP2C* cDNA clones should facilitate the isolation of the entire-coding regions for these *CYP2C* sequences from the koala cDNA library.

In conclusion, a total of five unique *CYP2C* cDNA sequences have been cloned from the koala. These sequences confirm the apparent multiplicity of the *CYP2C* subfamily in this species, and represent an ideal opportunity for new researchers to isolate the full-length cDNA sequences of the remaining members. The first full-length *CYP2C* cDNA sequences isolated from the koala, *CYP2C47* and *CYP2C48*, represents an initial opportunity to investigate the catalytic activity of expressed koala CYP2C enzymes against xenobiotic compounds of dietary significance. In addition, the activity of the expressed protein can be further investigated against endogenous substrates and man-made environmental chemicals. These studies would enable a more accurate understanding of the response of Australian marsupials which have been exposed to herbicides and pesticides used to control native flora and fauna in the environment. Such information is currently unavailable for any CYP family in any Australian marsupial species at the molecular level.

Chapter 6

Identification and cloning of UDP glucuronosyltransferases from representative Australian marsupial species

6.1. Introduction

UDP glucuronosyltransferases (UGTs) comprise a family of enzymes responsible for glucuronidating a variety of endogenous and exogenous substrates. Initial investigations into UGTs, as with cytochrome P450 enzymes, centred exclusively upon protein purification experiments. The advent of recombinant DNA techniques during the early 1980's enabled the isolation of the first *UGT* cDNA sequences. To date, more than 100 *UGT* cDNAs have been isolated and characterised from a variety of plant, bacterial and mammalian species (Mackenzie *et al.*, 1997). UGTs are divided into two families, UGT1 and UGT2, based on their amino acid sequences. UGT enzymes within a given family exhibit at least 50% homology, and are less than 50% similar to members of the other family. *UGT1* family members are involved in the glucuronidation of bilirubin, quinones and phenol's, while *UGT2* genes glucuronidate endogenous steroids and biogenic amines.

At the commencement of this work, no *UGT* cDNA sequences, either partial or full-length, had been isolated from any Australian marsupial species. As UGTs play a critical role in glucuronidating a variety of xenobiotic and physiologically relevant compounds, it was also important to investigate and characterise UGTs in representative Australian marsupial species.

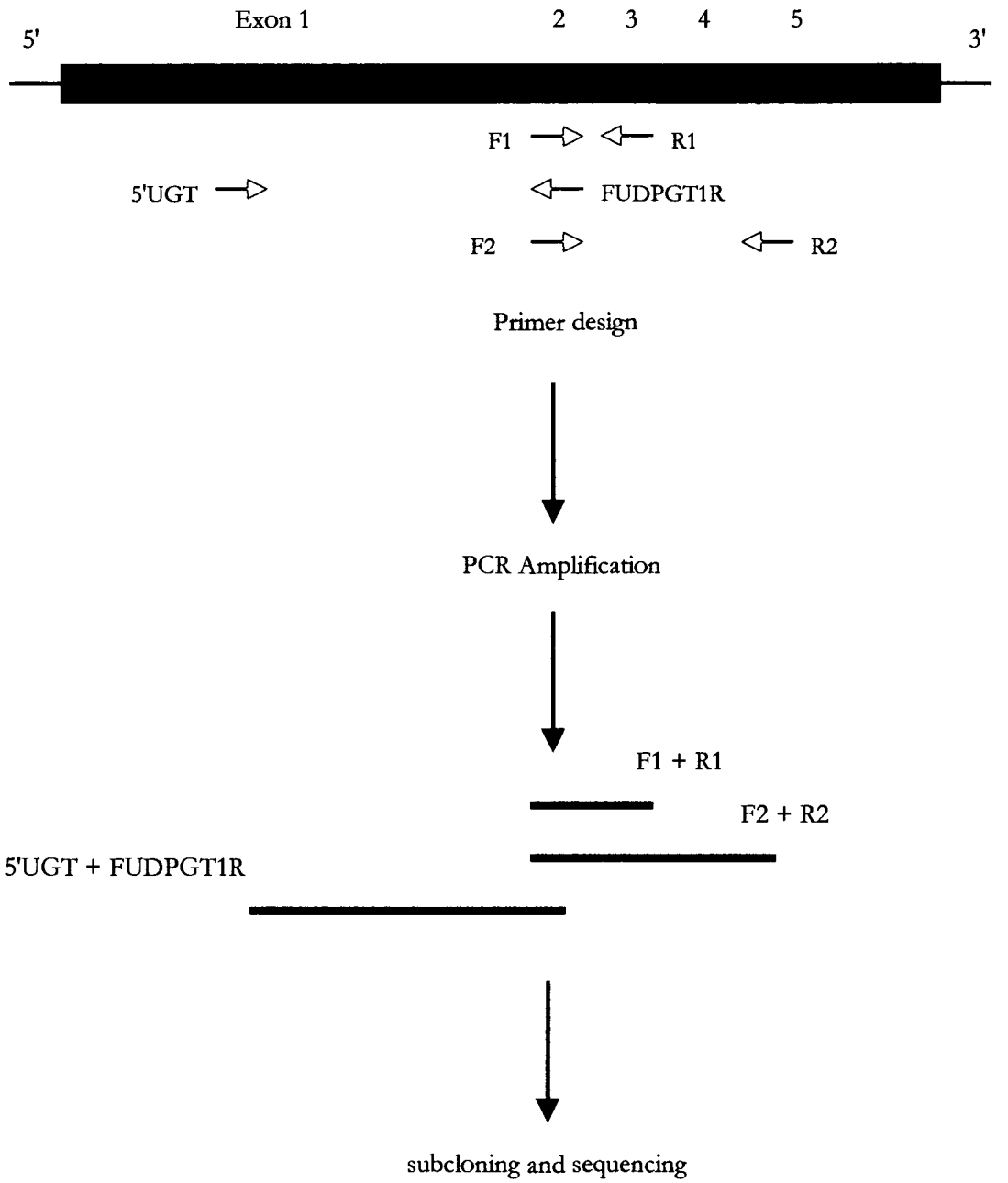
A primary aim of this project was to isolate and clone new *UGT* cDNA sequences present in a selection of Australian marsupial species. Several different methodologies were employed, including immunoblotting, RT-PCR and cDNA library screening. The results obtained from these approaches are discussed in the following sections.

6.2. Research strategy

The research strategy, to isolate new members belonging to UGT families 1 and 2 from Australian marsupials, initially involved performing immunoblot analyses of marsupial liver microsomes with anti-mouse UGT2 IgG to examine the extent and multiplicity of marsupial UGT enzyme expression. From the results of these studies, PCR experiments were conducted to amplify short cDNA fragments using conserved primers designed from known UGT cDNA sequences to use as DNA probes in the screening of a cDNA library. The primary methods used were RT-PCR from marsupial liver RNA samples and hybridisation screening of clones from marsupial cDNA libraries. A more detailed account of these techniques is provided in Chapter 2.

For this project, PCR amplification experiments were performed to amplify unique *UGT* cDNA sequences from marsupial first strand cDNA. The cloned partial sequences could then be isolated and used as cDNA probes to screen a koala liver cDNA library, in an attempt to isolate full coding sequences. Four oligonucleotide primers, which were complementary to human *UGT* families 1 and 2 were used to amplify the corresponding segments of approximately 200 bp (*UGT1* family) and 600 bp (*UGT2* family) in marsupial *UGT* sequences. Although the primers contained *Xba I* sites (TCT AGA) to facilitate subcloning, amplified PCR products were subcloned directly into pGEM®-T vector and transformed into *E.coli* DH5α competent cells. Plasmid DNA was purified and then sequenced to determine if it encoded a new member of the UGT family. A schematic diagram of the research strategy is outlined in Figure 6.1.

Figure 6.1. Research strategy to isolate a marsupial *UGT* cDNA sequence



The positions of the primers used in PCR amplification experiments are indicated in relation to a consensus *UGT* cDNA. Four oligonucleotide primers (forward primers, F1-2 and reverse primers, R1-2) previously designed to conserved regions of human *UGT* sequences were used to amplify *UGT1* and *UGT2* cDNA sequences from marsupial liver cDNA. Amplified PCR products were subcloned and individual clones were selected for sequencing.

6.3. Methods

6.3.1. Primer design

Four oligonucleotide primers, previously designed by Dr Behnaz Mojarrabi, Flinders University of South Australia, against highly conserved regions of the eight human liver *UGT* cDNA sequences identified to date, were used to amplify the corresponding *UGT* segments in marsupial liver cDNA. The primers contained a GC content of at least 50% and were checked against each other for complementarity, particularly at the 3' ends.

Two additional oligonucleotide primers, designated 5'UGT and FUDPGT1R, were subsequently designed to highly conserved regions of mammalian *UGT1* cDNA sequences and were used in RT-PCR amplification experiments to obtain the corresponding regions in marsupial cDNA. Where discrepancies existed between the base-pairs of the aligned mammalian *UGT1* sequences, appropriate degeneracy was introduced. Each primer was designed with an approximate GC content of 50%, and incorporated no more than two G or C residues in the last five base pairs at the 3' end.

A schematic diagram indicating primer direction and location is provided in Figure 6.1. The binding location of the primers is also shown in relation to selected *UGT* sequences in Figure B.1-B.2 (Appendix B). The sequences of the forward and reverse primers are shown in Table 6.1.

Table 6.1. UGT Primer sequences. Sequence of the primers used to amplify the corresponding segments of approximately 200 and 600 bp from marsupial liver cDNA samples. The letters denoting degeneracy include, Y= C+T, K= G+T, R= A+G, S= G+C.

NAME: COMPLEMENTARITY	PRIMER SEQUENCE
Forward primer 1 (F1): specific to the UGT1 family	5'- GCC TCT AGA AAT GCT TCT GGA GAA CAT -3'
Reverse primer 1 (R1): recognises UGT1, UGT2 families	5'- CCA TCT AGA TCG TTY CGG GGT AKC CAC TT -3'
Forward primer 2 (F2): specific to the UGT2 family	5'- GCC TCT AGA CAG AGC TCT GGA GAA AAT GG -3'
Reverse primer (R2): recognises UGT1, UGT2 families	5'- CTG TCT AGA GTG AGG TCG GTG GGC TG -3'
5'UGT: recognises the UGT1 family	5'- ATG GAT GGS AGC CAC TGG YTS ASC A -3'
FUDPGT1R: recognises the UGT1 family	5'- GTT CTC CAG AAG CAT TRA YRT AGG CTT C -3'

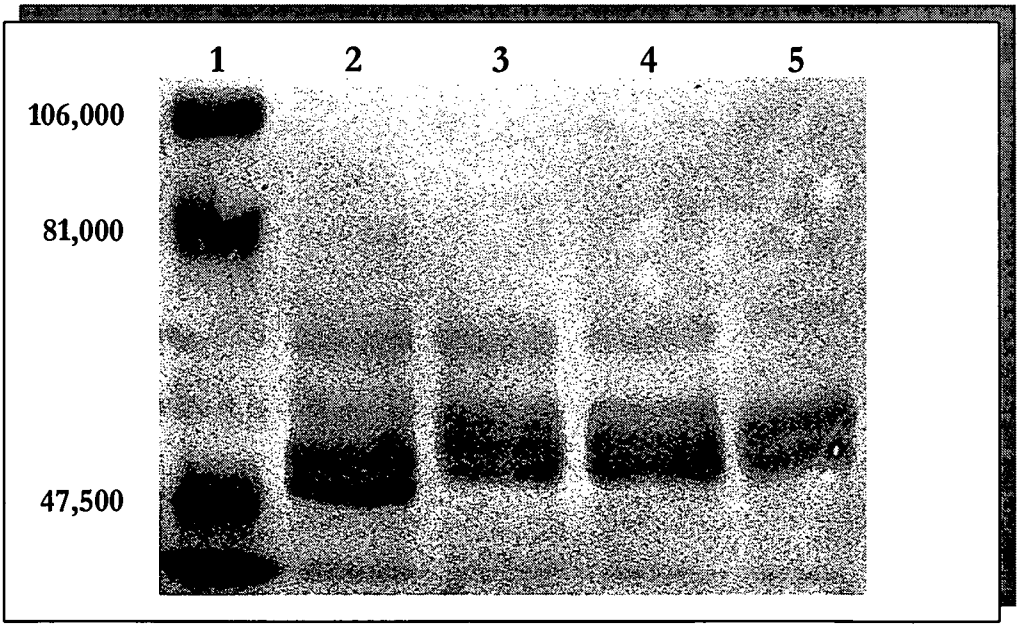
Forward primer 1 and reverse primer 1 were used to amplify a region of approximately 200 bp from the *UGT1* family. In addition, the 5'UGT forward primer and FUDPGT1R reverse primer were used to amplify a larger cDNA fragment from the *UGT1* family of approximately 800 bp. Forward primer 2 and reverse primer 2 were used to amplify a cDNA fragment of about 600 bp from the *UGT2* family.

6.4. Results

6.4.1. Immunoblot analysis of marsupial liver microsomes with anti-mouse UGT2 IgG

An immunoblotting experiment performed with 10μg of koala, tammar wallaby, wombat and rat liver microsomes using a goat anti-mouse UGT2 IgG revealed the detection of multiple immunoreactive bands for each of the tested species (Figure 6.2, lanes 2-5). The UGT antibody was raised against a single band excised from a SDS-PAGE gel and recognises multiple UGT2 enzymes in rat and mouse (Mackenzie *et al.*, 1984). At least two immunoreactive bands were detected in rat liver microsomes, at approximately 48-50 kDa (Figure 6.2, lane 2). As the rat UGT2 family comprises multiple forms, it appears likely that the two bands represent a number of isoforms. The tested marsupial liver microsomal samples each exhibited multiple intense immunoreactive bands of the appropriate size (Figure 6.2, lanes 3-5). At least three bands were detected for each species.

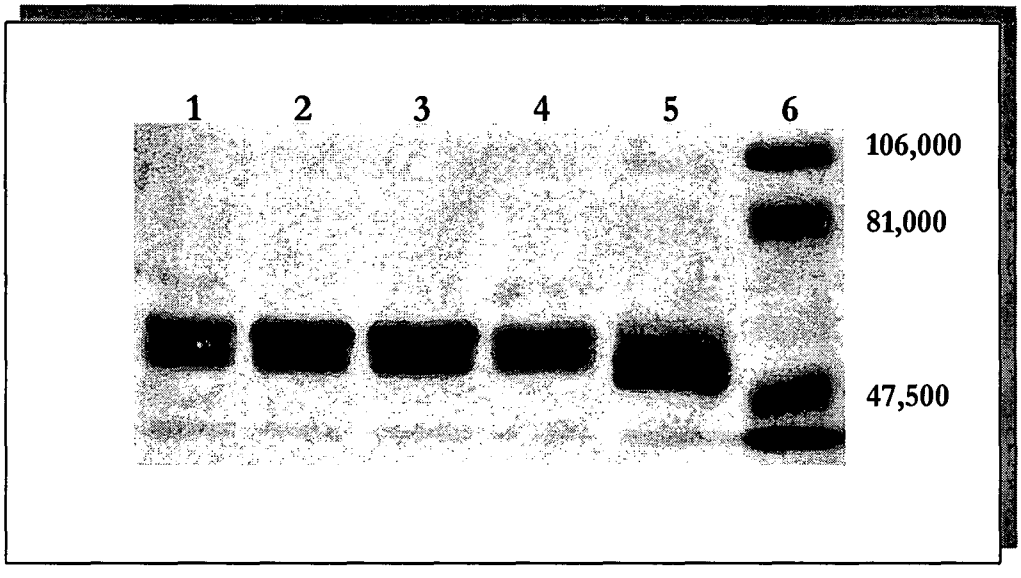
Figure 6.2. Immunoblot analysis of marsupial liver microsomes with anti-mouse UGT2 IgG



Hepatic microsomal fractions (10μg) from the koala, tammar wallaby, wombat and rat were separated by SDS PAGE (7.5% gel), transferred to a nitrocellulose membrane, and probed with goat anti-mouse UGT2 IgG. **Lane 1:** pre-stained marker (Life Technologies). **Lane 2:** rat hepatic microsomes. **Lane 3:** koala hepatic microsomes. **Lane 4:** tammar wallaby hepatic microsomes. **Lane 5:** wombat hepatic microsomes.

Immunoblot analysis of 20μg of koala pouch young liver microsomes with the anti-mouse UGT2 IgG detected multiple immunoreactive bands in each sample (Figure 6.3, lanes 1-4). As seen in the previous immunoblot with adult koala liver microsomes, at least two immunoreactive bands at about 49-52 kDa were detected in each sex and developmental stage of the pouch young microsomes (Figure 6.3, lanes 1-4). The rat hepatic microsomal sample produced an identical result to the previous immunoblot (Figure 6.3, lane 5).

Figure 6.3. Immunoblot analysis of koala pouch young liver microsomes with anti-mouse UGT2 IgG



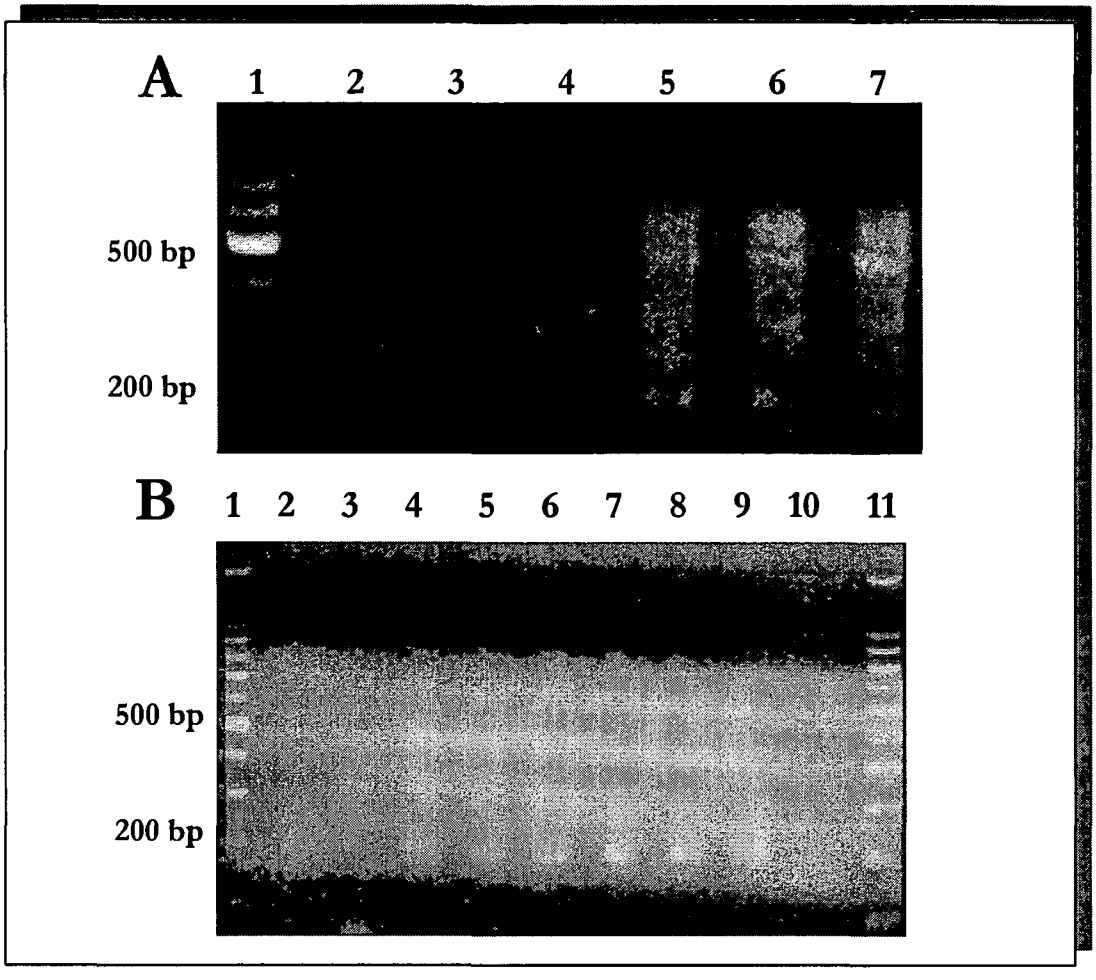
20μg of hepatic microsomes purified from male and female pouch young koalas aged between 180-400 days were separated by SDS PAGE (7.5% gel), transferred to a nitrocellulose membrane, and probed with goat anti-mouse UGT2 IgG. **Lane 1:** male koala (180-250 days old, 240 gms) hepatic microsomes. **Lane 2:** male koala (180-250 days old, 390 gms) hepatic microsomes. **Lane 3:** female koala (180-250 days, 370 gms) hepatic microsomes. **Lane 4:** male koala (250-400 days, 430 gms) hepatic microsomes. **Lane 5:** rat hepatic microsomes (control). **Lane 6:** pre-stained marker.

6.4.2. Amplification of UDP glucuronosyltransferase sequences from marsupial cDNA by polymerase chain reaction

A PCR experiment was initially conducted to amplify a region of about 200 bp from western quoll liver cDNA at an annealing temperature of 50°C using the forward primer 1 and reverse primer 1 (see section 6.3.1. for primer details). Agarose gel analysis of the products generated by PCR revealed the presence of 200, 300 and 500 bp bands in all quoll liver cDNA samples. The 200 bp band corresponds in size to that expected for the primer combination. A representative gel is shown in Figure 6.4A. Negative controls devoid of template were included in all experiments.

A subsequent PCR experiment performed with tammar wallaby cDNA using forward and reverse primer 1 at the same annealing temperature (50°C), also revealed the presence of a 200 bp band which corresponded to the expected size. A representative gel is shown in Figure 6.4B. Amplified fragments at approximately 400 and 800 bp were also visualised. PCR experiments were not conducted with koala cDNA using forward and reverse primers 1.

Figure 6.4. Analysis of western quoll and tammar wallaby PCR products by agarose gel electrophoresis



Analysis of the products generated by PCR from western quoll (A) and tammar wallaby cDNA (B) using forward primer 1 and reverse primer 1 to amplify a region of approximately 200 bp at an annealing temperature of 50°C.

(A) PCR products generated from western quoll cDNA

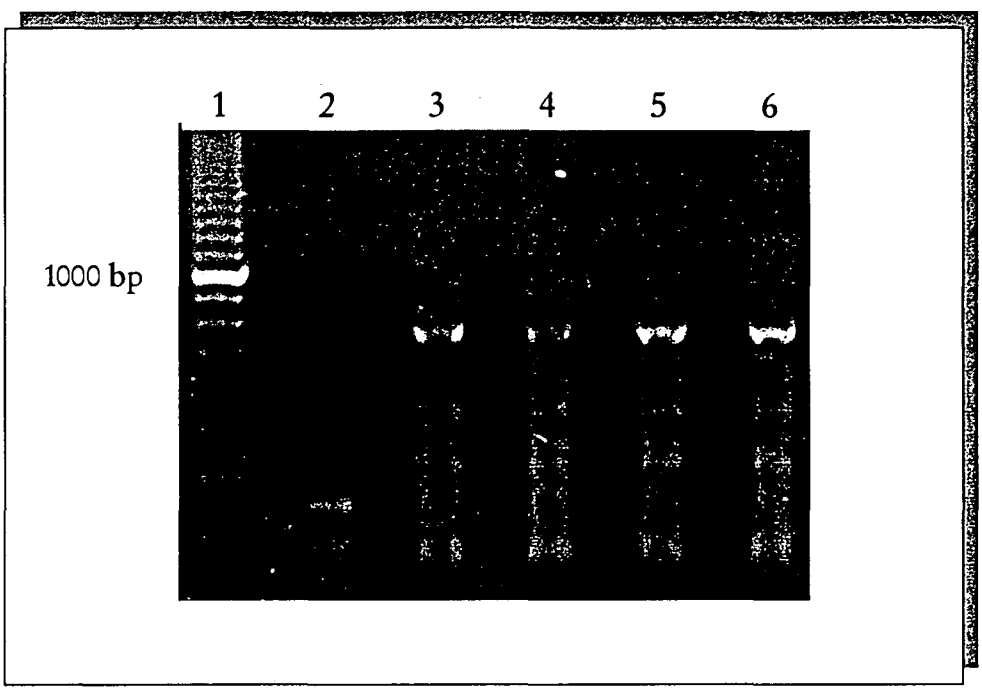
Lane 1: 100 bp DNA ladder, relative sizes of bands are shown to the left of the figure. **Lane 2:** blank. **Lane 3:** Negative control (PCR with no DNA template). **Lanes 4-7:** Sample of PCR products amplified from western quoll cDNA.

(B) PCR products generated from tammar wallaby cDNA

Lane 1: 100 bp DNA ladder. **Lane 2:** blank. **Lane 3:** Negative control (PCR with no DNA template). **Lanes 4-9:** Sample of PCR products amplified from tammar wallaby cDNA. **Lane 10:** blank. **Lane 11:** 100 bp DNA ladder.

PCR experiments were performed with koala cDNA and forward primer 5'UGT and reverse primer FUDPGT1R (see section 6.3.1. for primer details). Agarose gel analysis of the products generated by PCR at an annealing temperature of 48°C revealed the presence of a band at about 800 bp which corresponded in size to that expected for the primer combination. A representative gel is shown in Figure 6.5.

Figure 6.5. Analysis of koala PCR products by agarose gel electrophoresis



Analysis of the products generated by PCR from koala cDNA using the 5'UGT forward primer and FUDPGT1R reverse primer to amplify a region of approximately 800 bp at an annealing temperature of 48°C.

Lane 1: 100 bp DNA ladder, relative sizes of bands are shown to the left of the figure. **Lane 2:** Negative control (PCR with no DNA template). **Lanes 3-6:** Sample of PCR products amplified from koala cDNA.

Agarose gel analysis of the products generated by PCR from western quoll liver cDNA using forward primer 2 and reverse primer 2 at an annealing temperature of 50°C revealed a number of amplified bands at approximately 600, 400, 350 and 200 bp (Figure 6.6A, lanes 4-7). The 600 bp band corresponded to the expected size for the primer combination. Agarose gel analysis of the products amplified by PCR from quoll liver cDNA using forward and reverse primers 2 at a slightly higher annealing temperature of 52°C revealed a similar result as that obtained at the 50°C annealing temperature (data not shown).

A PCR experiment performed with tammar wallaby liver cDNA using the same primers at an annealing temperature of 55°C is shown in Figure 6.6B. Agarose gel analysis of the PCR products revealed the presence of a 600 bp product in all samples (Figure 6.6B, lanes 4-6). Finally, agarose gel analysis of the products amplified by PCR from koala liver cDNA using forward primer 2 and reverse primer 2 at an annealing temperature of 50°C is illustrated in Figure 6.6C. A band at about 600 bp was visualised, which corresponded to the expected size (Figure 6.6C, lanes 3-5).

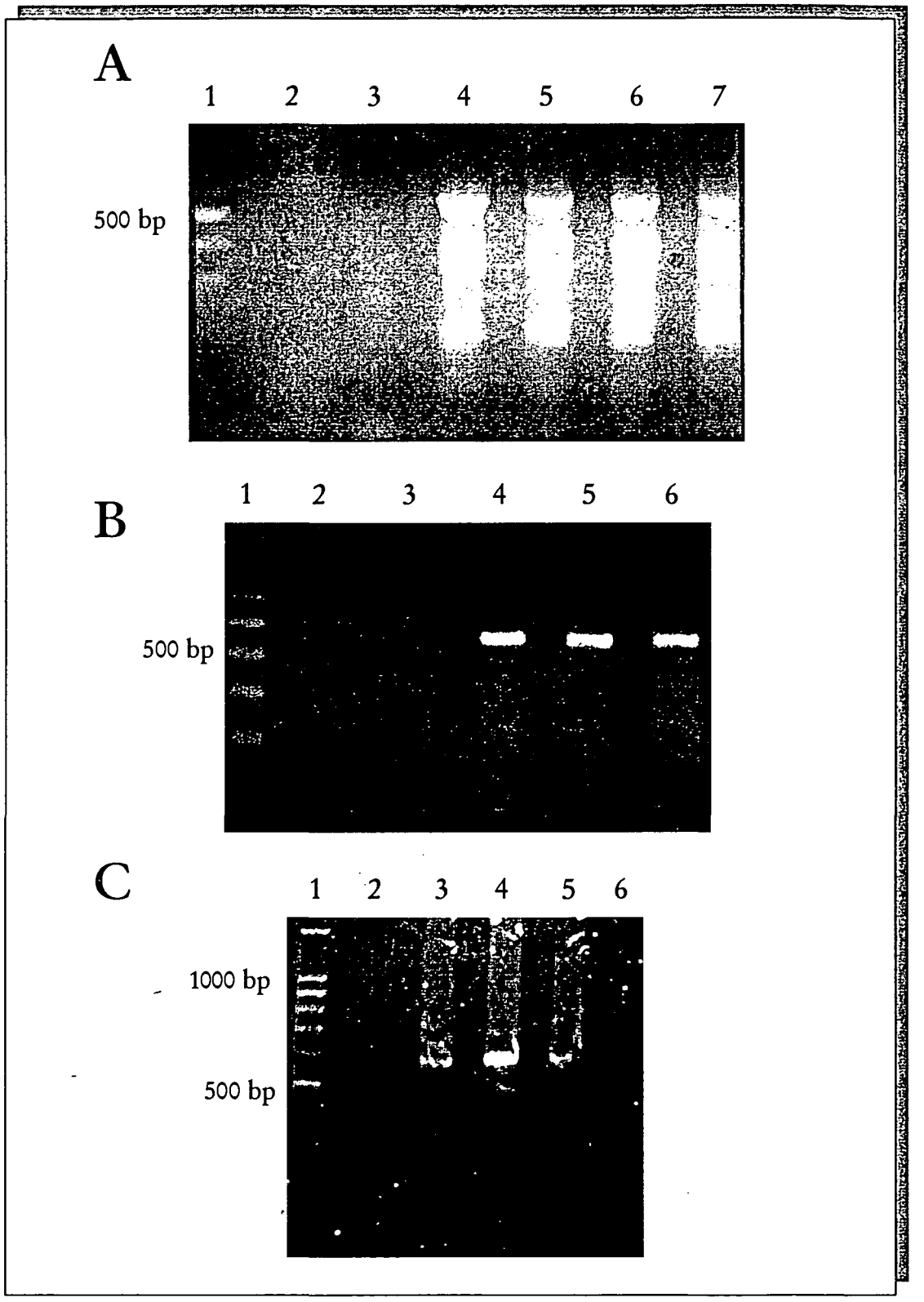
Figure 6.6. Analysis of marsupial *UGT2* PCR products by agarose gel electrophoresis. Analysis of the PCR products generated from western quoll cDNA (A), tammar wallaby (B) and koala cDNA (C) using forward primer 2 and reverse primer 2 to amplify a region of approximately 600 bp at annealing temperatures of 50°C (A,C) and 55°C (B).

(A) **PCR products generated from western quoll cDNA at 50°C annealing temperature**
Lane 1: 100 bp DNA ladder. **Lane 2:** blank. **Lane 3:** Negative control (PCR with no DNA template). **Lanes 4-7:** Sample of PCR products amplified from western quoll cDNA.

(B) **PCR products generated from tammar wallaby cDNA at 55°C annealing temperature**
Lane 1: 100 bp DNA ladder. **Lane 2:** blank. **Lane 3:** Negative control (PCR with no DNA template). **Lanes 4-6:** Sample of PCR products amplified from tammar wallaby cDNA.

(C) **PCR products generated from koala cDNA at 50°C annealing temperature**
Lane 1: 100 bp DNA ladder. **Lane 2:** Negative control (PCR with no DNA template). **Lanes 3-6:** Sample of PCR products amplified from koala cDNA.

Figure 6.6. Analysis of marsupial *UGT2* PCR products by agarose gel electrophoresis

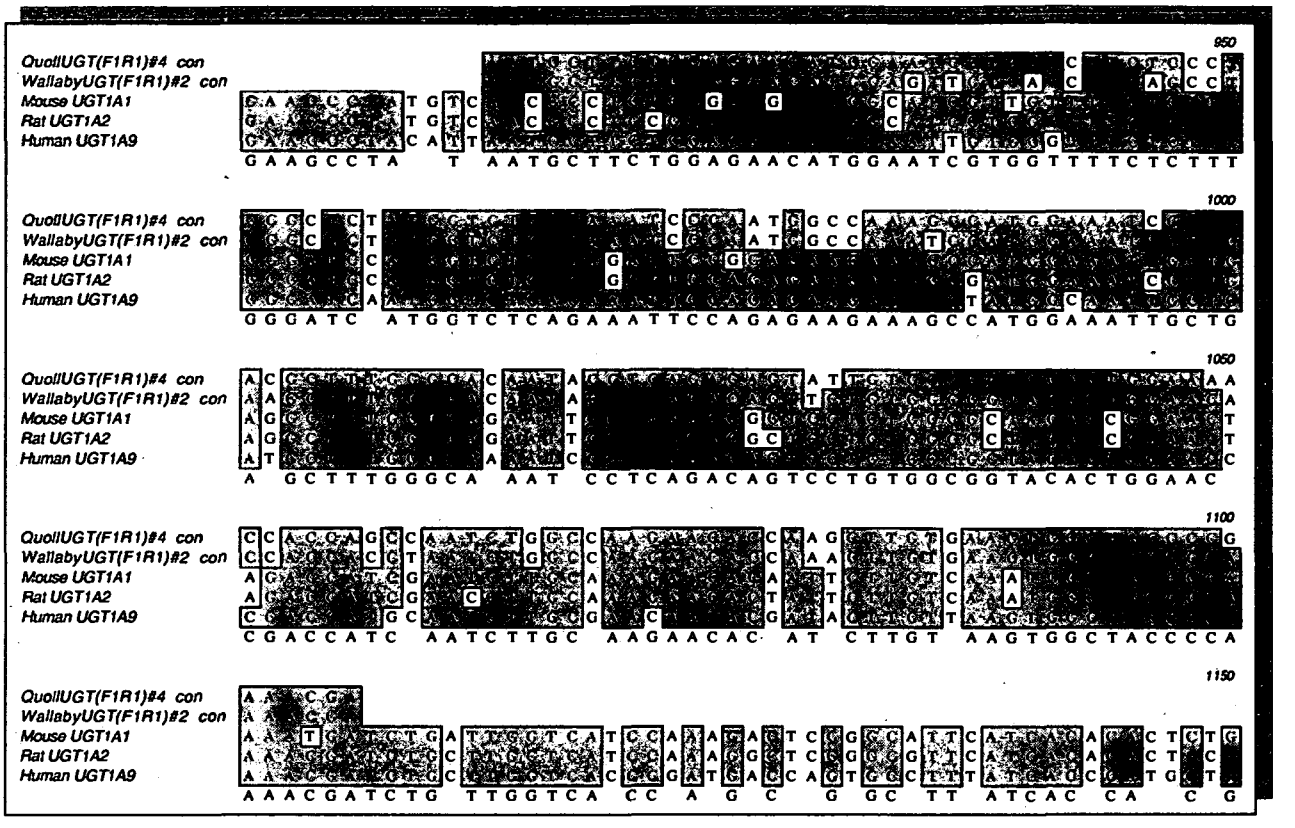


Following the amplification of DNA fragments using both sets of primers, selected PCR samples were subcloned directly into pGEM®-T vector and sequenced in order to verify their identity as described in Chapter 2 (sections 2.2.8. and 2.2.10). It should be noted that during the screening for inserts (section 2.2.8.4), several bands ranging in size from 100-800 bp were observed. A selection of these bands were also sequenced to analyse their identity.

6.4.3. Sequence analysis of amplified marsupial polymerase chain reaction products

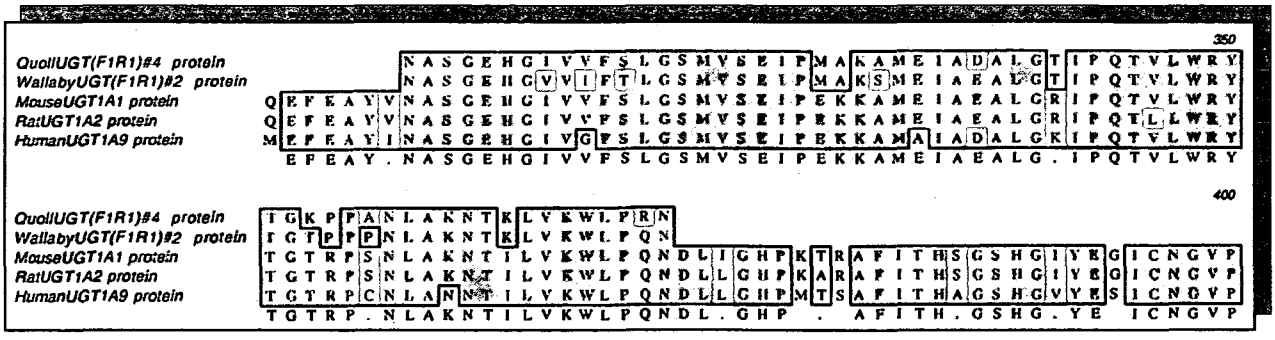
Sequencing of cloned PCR products generated from marsupial liver cDNA which were of the predicted size, revealed sequences that were homologous to known *UGT* sequences. In the western quoll and tammar wallaby, sequence analysis of the 200 bp product generated using forward and reverse primers 1 revealed that both cDNAs encoded new members of the UGT1 family. A nucleotide alignment of the western quoll and tammar wallaby *UGT1* cDNA sequences with other mammalian *UGT1* cDNAs is shown in Figure 6.7. Both sequences also exhibited a high degree of homology to other mammalian UGT1 members at the amino acid level (82%) (Figure 6.8). Sequence analysis of amplified PCR products other than the predicted size, demonstrated that they were PCR artefacts. The artefacts consisted of products resulting from the non-specific binding of the primers to various other regions of the cDNA template.

Figure 6.7. Sequence analysis of the western quoll and tammar wallaby *UGT1* clones with selected mammalian *UGT1* sequences



The western quoll and tammar wallaby clones of the 200 bp PCR products amplified from the respective cDNA were sequenced and aligned against mouse *UGT1A1*, rat *UGT1A2* and human *UGT1A9*. Shaded areas represent conserved nucleotides. The nucleotides are numbered in fifty base-pair intervals and a consensus sequence appears beneath each alignment block.

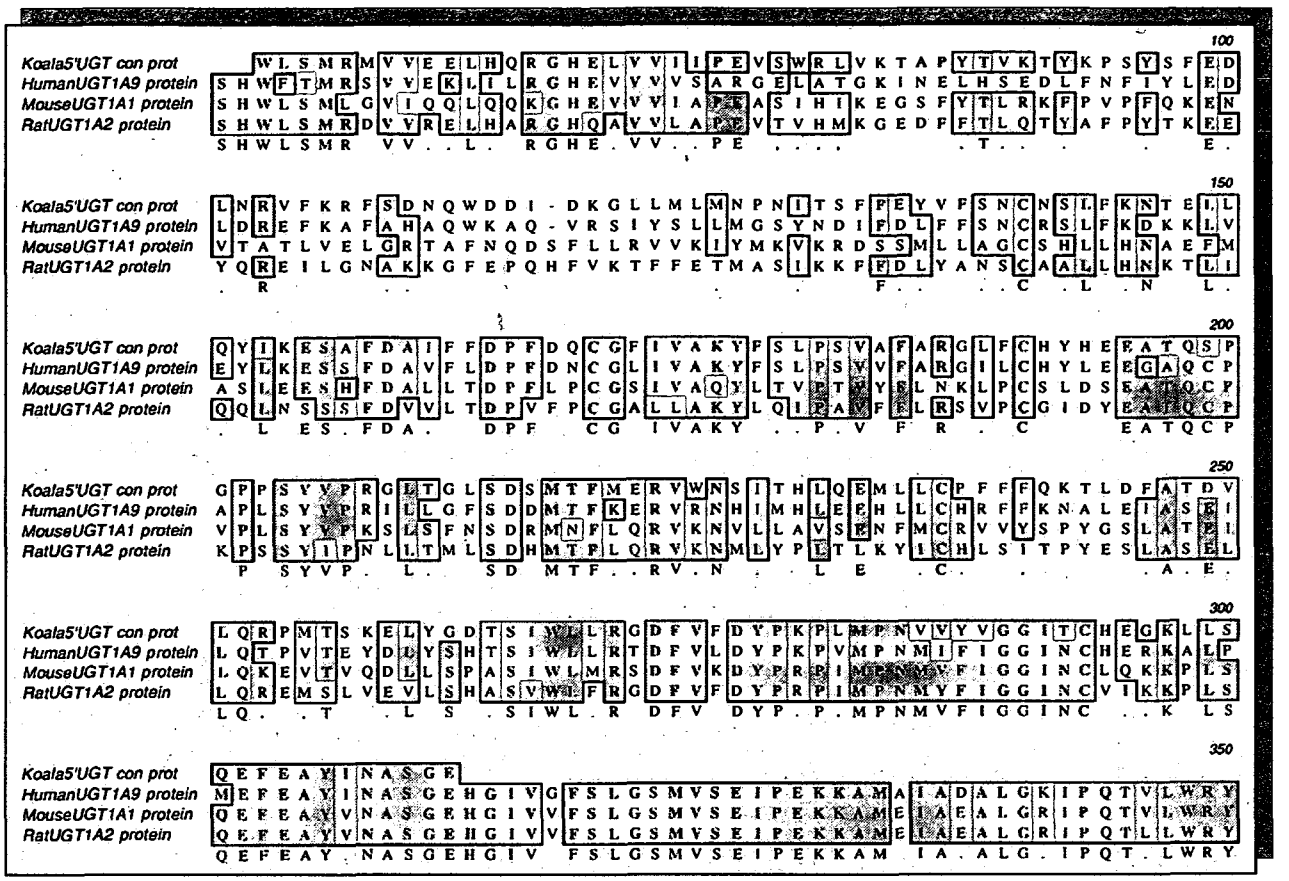
Figure 6.8. Alignment of the deduced amino acid sequences of the western quoll and tammar wallaby *UGT1* clones with mouse, rat and human UGT1 proteins



The deduced amino acid sequences of the western quoll and tammar wallaby *UGT1* clones were aligned with mouse UGT1A1, rat UGT1A2 and human UGT1A9 protein sequences. The dark shaded areas represent conserved amino acid residues, while the lighter shaded areas indicate amino acid residues exhibiting structural similarity. The position of the residues in relation to the translational initiation sequence of one of the mammalian proteins is shown in parentheses at the end of each row. A consensus sequence appears beneath each alignment block.

In the koala, sequence analysis of the 800 bp products generated by the forward primer UGT5'F and reverse primer FUDPGT1R2 revealed the presence of one cDNA sequence exhibiting similarity to previously reported mammalian *UGTs*. Unlike the previously cloned *UGT* PCR products which amplified segments of common exons 2-5, the primer combination amplified a region corresponding to the first exon in the *UGT1* gene family. The *UGT1* gene family comprises at least 12 exons, each possessing the ability to splice with common exons 2-5, resulting in the formation of gene products with different *N*-terminal halves but identical *C*-terminal halves (Mackenzie *et al.*, 1997). The cloned koala *UGT1* sequence would therefore be expected to exhibit significantly lower similarity to other mammalian *UGT1* sequences, as the N-terminal halves are not as conserved across mammalian species. An alignment of the deduced amino acid sequence of the koala *UGT1* cDNA with mouse, rat and human UGT1 proteins is illustrated in Figure 6.9.

Figure 6.9. Alignment of the deduced amino acid sequence of the koala *UGT1* clone with mouse, rat and human UGT1 proteins

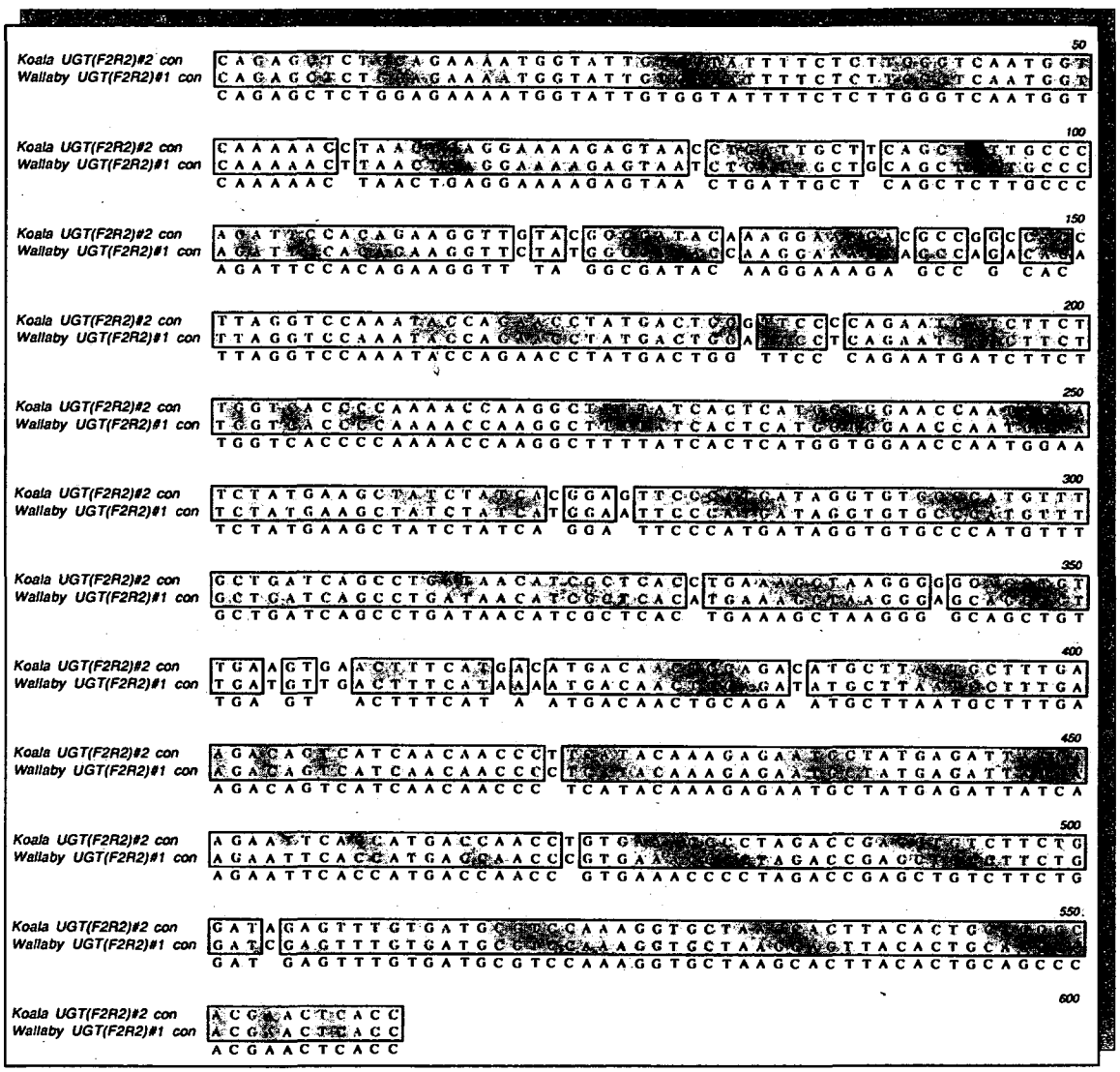


The deduced amino acid sequence of the koala *UGT1* clone was aligned with mouse UGT1A1, rat UGT1A2 and human UGT1A9 protein sequences. The dark shaded areas represent conserved amino acid residues, while the lighter shaded areas indicate amino acid residues exhibiting structural similarity. The position of the residues in relation to the translational initiation sequence of one of the mammalian proteins is shown in parentheses at the end of each row. A consensus sequence appears beneath each alignment block.

In the tammar wallaby and koala, sequence analysis of the 600 bp products amplified by forward and reverse primers 2 revealed that both encoded new members of the *UGT2* family. A nucleotide alignment of the tammar wallaby and koala *UGT2* cDNAs is illustrated in Figure 6.10. The two new marsupial *UGT* cDNAs exhibited 95% homology to each other at the nucleotide level (25 base pair mismatches in 561 nucleotides). As expected, the koala and tammar wallaby UGT2 forms also exhibited a significant degree of similarity to other members of the UGT2

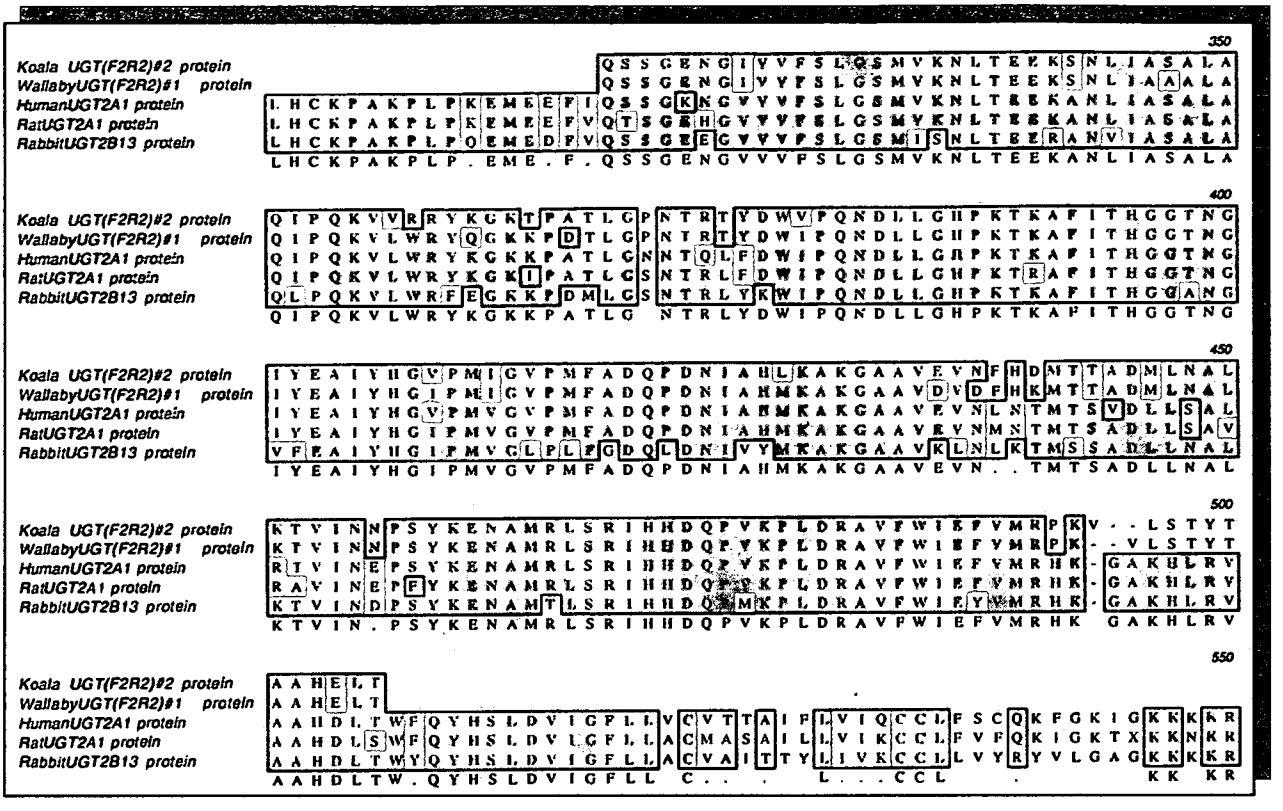
family at the amino acid level (84%) (Figure 6.11). Sequence analysis of amplified western quoll 600 bp PCR products did not reveal any clones exhibiting homology to other mammalian *UGT2* cDNA sequences. Sequence analysis of amplified products other than the predicted size revealed the presence of PCR artefacts.

Figure 6.10. Sequence analysis of the tammar wallaby and koala *UGT2* clones



The tammar wallaby and koala clones of the 600 bp PCR products amplified from the respective cDNA were sequenced and aligned. Shaded areas represent conserved nucleotides. The nucleotides are numbered in fifty base-pair intervals and a consensus sequence appears beneath each alignment block.

Figure 6.11. Alignment of the deduced amino acid sequences of the tammar wallaby and koala *UGT2* clones with human, rat and rabbit UGT2 proteins



The deduced amino acid sequences of the tammar wallaby and koala *UGT2* clones were aligned with human UGT2A1, rat UGT2A1 and rabbit UGT2B13 protein sequences. The dark shaded areas represent conserved amino acid residues, while the lighter shaded areas indicate amino acid residues exhibiting structural similarity. The position of the residues in relation to the translational initiation sequence of one of the mammalian proteins is shown in parentheses at the end of each row. A consensus sequence appears beneath each alignment block.

6.4.4. Screening of a koala liver cDNA library with a 600 bp partial koala *UGT2* sequence

To try and isolate full-length *UGT* cDNA sequences a koala liver cDNA library was screened with a partial koala *UGT2* cDNA sequence (Chapter 2, section 2.2.17). The koala *F2R2UGT* clone, which contained a 600 bp amplified PCR fragment homologous to the *UGT2* family (Chapter 6, section 6.4.3), was used as a cDNA probe to isolate full-length *UGT* sequences. 6 X 10^5 plaques were screened with the probe at a hybridisation temperature of 45°C. A number of

positive clones were identified and purified. Agarose gel analysis of the excised cDNA inserts revealed they ranged in size from 0.6-1.0 kbp. The average size of a mammalian *UGT2* coding region is approximately 1.7 kbp. Several of the largest clones were purified and sequenced using the T3 and T7 primers which flanked the 5' and 3' ends of the cDNA in the vector respectively.

6.4.5. Sequence analysis

Sequence analysis of a number of the larger positive clones revealed that each encoded a member of the UGT2 family. The cDNA and deduced amino acid sequence comparison of these clones with other known mammalian UGT2 sequences revealed that each of the clones corresponded to highly conserved sequences, exons 2-5, at the 3' end of the open reading frame of UGT2. Consequently, none of them encoded an entire open reading frame.

6.4.6. Screening of a koala liver cDNA library with a partial koala 800 bp *UGT1* sequence

In a further attempt to isolate a full-length koala *UGT* cDNA sequence, the koala liver cDNA library was re-screened with the 800 bp koala *UGT1* PCR fragment which corresponded to a segment of exon 1. It was thought that the screening of the library with a cDNA probe located significantly closer to the 5' end of the sequence would increase the likelihood of isolating a *UGT* cDNA sequence encoding an entire open reading frame. In the initial screening experiment, 3 X 10^5 plaques were screened with the probe at a hybridisation temperature of 45°C. No positive plaques were detected under these conditions.

6.4.7. Screening of a tammar wallaby liver cDNA library with a partial koala 800 bp *UGT1* sequence

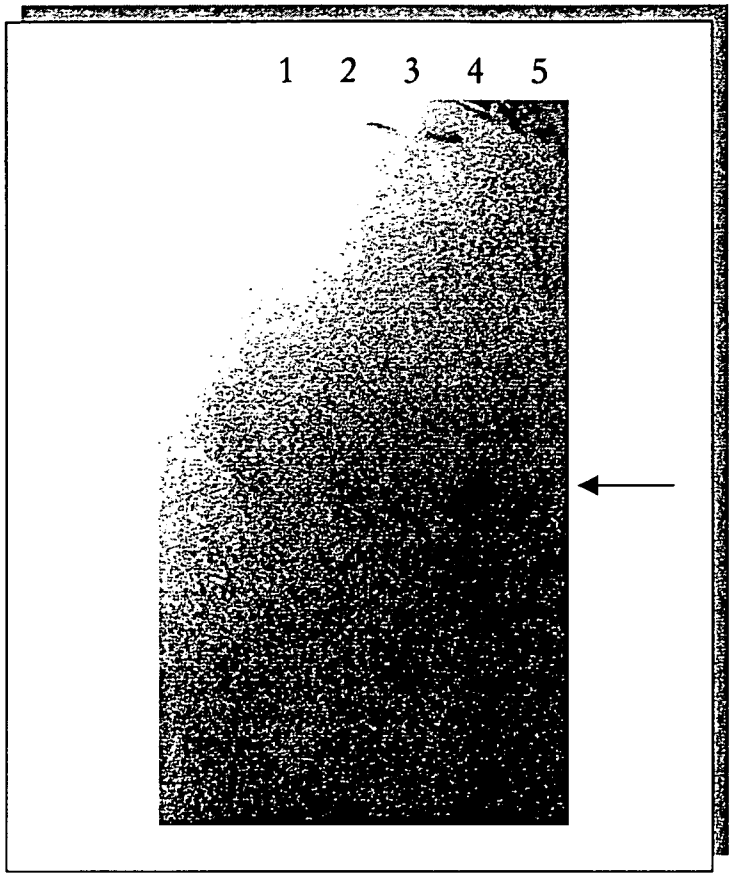
A subsequent attempt to isolate a full-length marsupial *UGT1* cDNA sequence using the 800 bp partial koala *UGT1* PCR fragment to screen a tammar wallaby liver cDNA library also proved to be unsuccessful. 3 X 10^5 plaques were initially screened with the probe at a hybridisation temperature of 43°C.

6.4.8. RNA blot analysis

To assess expression of the marsupial UGT1 family at the mRNA level, RNA blot analysis was performed using koala, tammar wallaby and rat mRNA samples. Total RNA from the koala and rat was included on the blot for control purposes. Equal amounts of hepatic RNA from each species (5μg for mRNA and 10μg for total RNA) was probed with a 800 bp *UGT1* fragment (5'UGT) cloned from the koala. This fragment was obtained by PCR (section 6.4.2).

Probing of each species with the koala *UGT1* sequence revealed an intense band in the koala mRNA sample, and a smear of much fainter bands in the koala total RNA preparation (Figure 6.12, lanes 3,4). A very faint band was also detected in the tammar wallaby mRNA sample (Figure 6.12, lane 5). No bands were detected in either of the rat RNA samples (Figure 6.12, lanes 1,2).

Figure 6.12. RNA blot analysis of koala, tammar wallaby and rat liver mRNA and total RNA probed with a 800 bp partial koala *UGT1* sequence



Total RNA and mRNA prepared from koala, tammar wallaby and rat liver samples was probed with a specific 800 bp koala *UGT1* cDNA sequence at a hybridisation temperature of 45°C.

Lane 1: rat total RNA. **Lane 2:** rat mRNA. **Lane 3:** koala total RNA. **Lane 4:** koala mRNA. **Lane 5:** tammar wallaby mRNA.

6.5 Discussion

Prior to the commencement of this project, no studies had investigated UGT expression and multiplicity in any of Australia's marsupial species. The availability of marsupial tissues and cDNA libraries, as outlined in Chapter 3, has enabled an analysis of UGT enzyme expression and the subsequent cloning of partial *UGT* cDNAs in a range of Australian marsupials. The studies reviewed in this chapter provide the first data for UGT enzyme expression in any marsupial species, and confirm the presence of genes from both *UGT* families in the koala, tammar wallaby and western quoll.

6.5.1. Immunoblot analysis of adult and pouch young marsupial liver microsomes

Immunoblot analysis of marsupial liver microsomes with anti-mouse UGT2 IgG detected a multitude of immunoreactive bands for each marsupial species examined. At least two, distinct immunoreactive bands were exhibited in each species, however the exact number of isoforms these bands represent is unable to be determined from the blot. Immunoblot analysis of koala pouch young liver microsomes with anti-mouse UGT2 IgG also detected multiple, intense immunoreactive bands at each developmental stage tested.

UGTs comprise a family of enzymes that catalyse the covalent addition of glucuronic acid to a diverse array of lipophilic chemicals (Tukey and Strassburg, 2000). They play a major role in the detoxication of numerous endogenous and exogenous compounds leading to the generation of more polar, water-soluble products that can be more readily excreted in bile or urine (Tukey and Strassburg, 2000). The identification of a genetic polymorphism for the *UGT1A* gene has been shown to confer a reduction in bilirubin glucuronidation, leading to genetic hyperbilirubinaemia (Crigler-Najjar and Gilbert's syndromes) (de Wildt *et al.*, 1999a). The ontogeny of the UGT2B subfamily has been characterised in humans and rats (Patel *et al.*, 1998; de Wildt *et al.*, 1999a).

The immunoblotting studies outlined in this chapter have provided the first evidence for UGT expression in any Australian marsupial species. The experiments further confirmed the apparent multiplicity of the UGT2 family in three marsupial species, the koala, tammar wallaby and wombat. In addition, the extent and multiplicity of UGT expression in developmentally

immature koalas may reflect the importance of UGTs in the metabolism of dietary compounds. However, an alternate hypothesis that the presence of UGTs in pouch young koalas may be representative of their role in the metabolism of endogenous substrates cannot be discounted.

In conclusion, the detection of multiple UGT2 bands in each of the marsupials examined indicates that several UGT2 protein sequences may exist in these species.

6.5.2. Isolation of *UGT1* sequences from Australian native marsupial species

PCR amplification experiments were initially performed using four oligonucleotide primers designed from highly conserved regions in previously reported human liver *UGT* cDNAs. The primers successfully amplified *UGT1* and *UGT2* cDNAs in the quoll, tammar wallaby and koala.

6.5.2.1. Analysis of *UGT1* sequences from Australian marsupial species

Sequence analysis of the PCR products amplified from western quoll and tammar wallaby cDNA revealed two clones, 194 bp in length, exhibiting similarity to other mammalian *UGT1* sequences. The clones, designated Quoll *UGT(F1R1)#4* and Wallaby *UGT(F1R1)#2*, each encoded open reading frames of 64 aa. The clones exhibited 92% similarity to each other at the nucleotide level (15 base pair mismatches in 194 nucleotides), as they spanned the region across highly conserved common exons 2 and 3. An alignment of the deduced amino acid sequences of the quoll and tammar wallaby *UGT1* clones with mouse UGT1A1, rat UGT1A2 and human UGT1A9 proteins demonstrated that both clones were approximately 80% similar to other reported mammalian sequences. The full coding regions of these sequences have not been isolated to date.

In addition, sequence analysis of PCR products amplified from koala cDNA using an additional set of primers revealed one clone, 792 bp in length, exhibiting similarity to known *UGT1* sequences. The clone, designated Koala *5'UGT*, encoded an open reading frame of 263 aa and spans the region incorporating unique exon 1. As the first exons of the UGT1 family can splice with common exons 2-5, and as such are regarded as distinct genes, the clone exhibits significantly less amino acid similarity (56%) to other reported mammalian UGT1 proteins. An alignment of the deduced amino acid sequence of the koala *5'UGT* clone with all previously

reported mammalian UGT1 proteins revealed that it most likely represents an orthologue of human UGT1A7. However, an analysis of the full-length cDNA sequence from the koala *5'UGT* clone would be required to confirm this hypothesis.

To complement the PCR studies, a koala liver cDNA library was screened in an attempt to isolate a full length koala *UGT1* cDNA sequence. Two screening experiments performed with the koala liver cDNA library using the koala 5'UGT cDNA as a probe failed to detect any positive clones. The reasons behind these negative outcomes remain unclear, however the possibility that the koala library may not contain the particular cDNA that the *UGT* probe was directed towards cannot be discounted.

A subsequent screening of a tammar wallaby liver cDNA library using the same koala probe also failed to detect any positive clones.

6.5.2.2. Analysis of *UGT2* sequences from Australian marsupial species

Sequence analysis of the PCR products amplified from koala and tammar wallaby cDNA revealed the isolation of two clones, 561 bp in length, exhibiting similarity to other mammalian *UGT2* sequences. The clones, designated Koala *UGT(F2R2)#2* and Wallaby *UGT(F2R2)#1*, both encode open reading frames of 187 aa and incorporate regions spanning common exons 2-5. An alignment of the deduced amino acid sequences of both clones with human UGT2A1 and rat UGT2A1 proteins demonstrated that each was approximately 85% similar to other representative UGT2A proteins.

To isolate a full length koala *UGT2* cDNA sequence, a koala liver cDNA library was screened with the partial koala *UGT(F2R2)* cDNA clone obtained in previous PCR experiments. A number of positive clones were isolated and sequenced. All of the sequenced clones encoded UGT proteins, however none of them incorporated an entire open reading frame. In retrospect, the attempt at isolating a full length *UGT* cDNA sequence from the library using a *UGT* probe incorporating common exon regions 2-5, at the 3' end of the sequence, presented a significant challenge. As explained in the previous chapter (section 5.4.5), an initial attempt at isolating a full

length koala *CYP2C* cDNA sequence from the library using a cDNA probe located approximately 150 aa from the 5' end, detected multiple partial and no full length sequences. The successful isolation of a *CYP2C* cDNA sequence was only achieved using a probe situated approximately 80 aa from the 5' end of known CYP2C proteins. In comparison, the koala *UGT2* cDNA probe is situated over 300 aa from the 5' end of known mammalian UGT2 proteins. Therefore, while this probe may extend the sequencing knowledge of a koala *UGT2* cDNA from a subsequent screening procedure, it is highly unlikely that it can facilitate the isolation of a full length *UGT2* sequence. A PCR-based approach utilising degenerate oligonucleotide primers to amplify a new koala *UGT2* cDNA probe situated closer to the 5' end of known mammalian UGT2 sequences, represents a more viable option. Time constraints prevented my undertaking of this option. The sequencing information presented here should facilitate the eventual isolation of a full length *UGT2* sequence from the koala cDNA library.

In summary, an immunoblot analysis of adult marsupial liver microsomes with anti-mouse UGT2 IgG revealed the potential multiplicity of UGT2 forms in koala, tammar wallaby and wombat liver. Subsequent RT-PCR experiments using primers specific for human UGT2 cDNAs, confirmed the presence of at least one *UGT2* cDNA sequence in both the koala and tammar wallaby. PCR experiments also culminated in the cloning of a *UGT1* cDNA sequence from koala, tammar wallaby and western quoll. In conclusion, the koala cDNA library has already been shown to contain relatively large insert sizes, which can encode an entire open reading frame. Therefore, by utilising the *UGT* cDNA sequencing knowledge already obtained for the koala to generate a more suitable cDNA probe, the isolation of a full length koala *UGT1* or *UGT2* cDNA sequence from the koala library seems likely in the near future. Finally, the expression of a marsupial UGT protein and subsequent analysis of its activity towards both physiological and xenobiotic compounds would provide a better understanding of the response of Australian marsupials to environmental chemical exposure.

Chapter 7

General Discussion

Scientific research into Australia's native fauna, within the field of molecular toxicology, has been largely neglected in the past. The relative lack of data concerning the response of marsupials to environmental chemical contamination has important toxicological implications. The information presented in this thesis has significantly enhanced our understanding of important components of the detoxication processes of representative Australian marsupials. Furthermore, these studies provide a stimulus for future investigations into our native fauna, which should enable a more critical appreciation of these unique mammals from both evolutionary and toxicological perspectives, at a molecular level.

The Australian continent, with its collection of unique native marsupials, is a vivid example of the detrimental effects man can have on the species that live in co-existence. Prior to European settlement, native animals within Australia inhabited varying extremes of climatic regions. From dense, tropical regions to dry arid ecosystems, Australian marsupial species had successfully adapted to every environment. The geographical isolation of Australia and absence of intrusive competitors provided the ideal setting for this extraordinarily successful colonisation. However, the arrival of Europeans to Australian shores forever changed this faunal menagerie. Australia now has the worst modern mammalian extinction rate in the world (Threatened Species Network, 1998). Since settlement by Europeans, 30 species of mammals and birds and about 100 species of plants have become extinct in Australia (Threatened Species Network, 1998). At least another 57 species of mammals, birds, reptiles, frogs and fish are also now nationally endangered (Threatened Species Network, 1998). All of these species could become extinct within the next 10-20 years.

European settlement of Australia during the mid 19th century sparked the beginning of a catastrophic period for the resident marsupial species. While there are undoubtedly many factors introduced by humans that have adversely affected their survival, the wholesale clearing of bushland and deliberate introduction of exotic mammals have been the most deleterious.

One of the most significant factors affecting the survival of marsupials has unquestionably been the destruction of their native habitat. The clearing of natural vegetation for agricultural

farmland, mining, and urban development has, and is continuing to remove the habitats of innumerable marsupial species. Further land degradation resulting from exotic weed infestation, pollution and changes in fire frequency has also impacted negatively on various marsupial populations.

The economic survival of rural communities in Australia relies almost exclusively on European agricultural farming techniques, practices that include the wholesale removal of native trees and scrubland, increased soil erosion and the diversion of water supplies. Until it becomes economically beneficial for primary producers to re-vegetate their farms with native trees and shrubs or even to farm native marsupials as a food source, the remaining marsupial species in this country will continue to be sacrificed at the expense of human agricultural production.

In summary, the future of a number of Australian marsupial species appears decidedly bleak. The destruction of vast areas of environment through agricultural farming practices and urban development, combined with the effects of introduced mammalian predatory species has, and is continuing to cause the extinction of much of our indigenous fauna. To address this problem, many of Australia's prominent national wildlife parks are now focusing on stocking and breeding endangered native marsupial species, for example the Adelaide Zoo and the Healesville and Warrawong Sanctuaries. While these breeding strategies are important in raising the numbers of seriously endangered marsupial populations, they can only be considered a short-term remedy.

Another strategy, however, which involves the introduction of a selected marsupial species to an isolated island landmass has considerably more potential to produce a viable, long-term solution. The relative success achieved with the introduction of the koala (*Phascolarctos cinereus*) to Kangaroo Island, a relatively undeveloped landmass off the South Australian coast containing virtually no introduced pest species, clearly illustrates how well marsupials can colonise an environment with minimal human interference. In fact, a sterilisation and relocation programme has only recently been implemented on the island due to the high number of koalas exceeding the available food source. If koalas had not been introduced to Kangaroo Island some fifty years ago, South Australia would currently support only a few, small colonies (Strahan, 1983). Other successful

examples of introducing native species to relatively uninhabited islands include the quokka (*Setonix brachyurus*) on Rottnest Island and more recently, the Bilby (*Macrotis lagotis*) on Thistle Island.

In conclusion, unless concerted efforts are made towards re-vegetating the native flora on mainland Australia, and changing the way the existing land is utilised, the indigenous fauna will continue to rapidly head towards extinction. Until such efforts begin, the most viable solution for the survival of all Australia's marsupial species is to introduce small colonies to island landmasses that contain minimal human activity, but more importantly, no introduced pest species.

The future of Australia's remaining marsupial species will ultimately be determined by the action of state and federal governments combined with inputs from associated environmental groups. However, scientific investigations into Australian marsupials can provide vital knowledge which can re-shape Australia's dated environmental regulations regarding its native fauna. Due to the lack of relevant information concerning marsupial metabolism, Australia's current environmental regulations have been extrapolated from other mammalian sources. A comprehensive and comparative analysis of representative Australian marsupial species, specifically targeting the molecular identification of xenobiotic-metabolising enzymes, would therefore provide a greater understanding of the impact of agricultural and industrial environmental chemicals on their survival. Such information would facilitate the implementation of relevant environmental regulations specific for Australia's indigenous fauna.

Scientific research into Australia's native marsupials has in the past predominantly focused upon various aspects of their unique reproductive and developmental strategies. Indeed, the ease of accessibility of the developing organ systems has facilitated numerous investigations into marsupial fertilisation, sexual determination and hormonal control of sexual differentiation. Such studies have elucidated a number of intriguing differences existing between marsupial mammals and their eutherian counterparts.

Relatively few studies have investigated the biochemical and molecular regulatory pathways of Australian marsupials. While the reasons for this are varied, and include political, moral and funding issues, information regarding marsupial detoxication processes is paramount if we are to understand if man-made chemicals are significantly contributing to their endangered status. Taking into consideration the evolution and geographical isolation of Australia's native marsupials with their subsequent adaptation to various aspects of the continent's challenging flora, it is reasonable to assume they may possess slightly different detoxication processes than that reported for most mammalian species. One prime example is the evolutionary genetic tolerance exhibited by selected marsupial species to fluoroacetate, a chemical previously used for the control of predators, rabbits and pigs in Australia. It is likely that marsupials possess additional, but as yet unidentified, detoxication anomalies.

The relative lack of information regarding the identification of xenobiotic-metabolising enzymes in Australian marsupial species, is most likely reflective of the absence of suitable starting material. The most critical and determining step of molecular biology techniques is the purification of high quality RNA that can be utilised in subsequent cloning studies. To isolate suitable RNA, it is necessary to snap-freeze the original tissue with liquid N_2 almost immediately upon expiration of the animal. A delay of a few minutes generally results in the subsequent degradation of RNA within the tissue, making it unsuitable to use in molecular biology experiments. Consequently, it is always necessary to be present before the animal is euthanased.

The most easily accessible and localised sources of marsupial species in this country are undoubtedly the major zoos and wildlife parks. From my experiences, these establishments have been quite willing to provide marsupial species excess to their needs, and to accommodate our research procedures. Discussions with rangers and staff have confirmed their awareness of wildlife parks in the promotion of marsupial scientific research, while not impacting negatively on their social, moral or economic positions. A successful relationship with such establishments would therefore ensure a constant supply of tissues from a diverse array of Australian marsupial species, for which cloning studies could be initiated.

In the present studies, tissues from a variety of Australian marsupial species have been successfully procured for the purpose of molecular biology experiments. Indeed, the marsupial tissue bank, and acquired knowledge, at the University of South Australia provides an opportunity to initiate investigations into the expression and isolation of virtually any gene or enzyme for a number of Australian native marsupials.

Cytochromes P450 represent a logical enzyme family to investigate in Australian marsupials due to their critical importance in catalysing the oxidative metabolism of innumerable xenobiotic compounds and endogenous substrates. The major phase I xenobiotic-metabolising enzyme, cytochromes P450 are an animals' primary defence in the metabolism and subsequent detoxification of pollutants and chemical carcinogens. The advent of modern recombinant biology techniques over the last two decades has seen cytochrome P450 research proceed at a frenetic pace. The cytochrome P450 system has since been extensively characterised in a host of mammalian species, including humans, mice, rats and rabbits. A significant lack of information exists regarding cytochromes P450 and UGTs in a unique class of mammals, marsupials.

The studies outlined in this thesis have provided an initial understanding of the expression of the major xenobiotic-metabolising CYPs and UGT families in three unique Australian marsupials, the koala, tammar wallaby and wombat. The acquired information has enabled an appreciation of the extent and multiplicity of CYP and UGT expression in these marsupials, and provided a direction for subsequent cloning studies. Furthermore, a preliminary developmental analysis of CYP and UGT expression in juvenile koala samples of varying ages provided data confirming the multiplicity and extent of expression in a pouch young Australian marsupial species. The detection of a number of the major xenobiotic-metabolising CYPs in relatively immature koalas may indicate that dietary xenobiotics influence the developmental expression of these enzymes in the koala. However, an alternate hypothesis that the presence of these enzymes in pouch young koalas is reflective of their role in the metabolism of endogenous compounds can also be put forward.

The expression and multiplicity of one xenobiotic-metabolising CYP subfamily, CYP2C, in Australian marsupials was confirmed by cloning experiments. These studies, which identified five unique *CYP2C* cDNAs and culminated in the isolation of the first two full length *CYP* cDNA sequences from a marsupial species, the koala, ensured the *CYP2C* subfamily as a future avenue of study in marsupials. While the isolation of a full length marsupial *UGT* cDNA sequence proved to be unsuccessful, studies confirmed the presence of both UGT families in the koala and have provided a direction for the continuing study of an important phase II xenobiotic-metabolising enzyme family in Australian marsupials.

In conclusion, koala *CYP2C47* provides a tool for the first studies into the catalytic activity and substrate specificity of an expressed marsupial CYP2C protein towards both xenobiotic compounds (dietary, agricultural) and endogenous substrates. The outcome of such investigations would, apart from providing a new and unique insight into the CYP activity of this unknown mammal, also enable a greater understanding of the response of Australian marsupials exposed to man-made chemicals. It is hopeful that such information, in conjunction with the work of other researchers in the field, can facilitate the introduction of regulatory controls for Australia's native marsupial species to ensure their protection from environmental chemical contamination.

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Appendix A

The oligonucleotide primers utilised in PCR amplification experiments to isolate marsupial *CYP2C* cDNA sequences, are aligned with other selected mammalian *CYP2C* sequences in Figures A.1-A.3. The MAR2CF and MAR2CR primers, which incorporate redundancies to account for the base differences between mammalian *CYP2C* cDNA sequences, were used to amplify a region of approximately 700 bp. The MAR2CF3 and KGSP2B primers were used to amplify another region of about 400 bp. The use of these sets of primers maximized the likelihood of amplifying unique *CYP2C* sequences in marsupials (Chapter 4).

The letters denoting degeneracy are underlined in the primer sequences and include, Y= C+T, S= G+C, K= G+T, R= A+G, W= A+T, H= A+T+C, B= G+T+C, D= G+A+T. Primer length is indicated in parenthesis, and the position of the primers (3' end) in relation to the translational initiation sequences are also indicated.

MAR2CF	5'- TGT GYT CCH KSC AAY GTS ATY TSY -3'	(24)
CYP2C29 (mouse)	TGT GCT CCA TGC AAT GTC ATC TGC	(544)
CYP2C19 (human)	TGT GCT CCC TGC AAT GTG ATC TGC	(542)
CYP2C23 (rat)	TGT GCT CCC TGC AAC GTG ATC TGC	(554)

Figure A.1. Sequence alignment of MAR2CF (24 mer) with mammalian *CYP2C* cDNAs.

MAR2CR	5'- ATY YAR RAA RTG NSC WGG GTC AA -3'	(23)
CYP2C29 (mouse)	ATT TAG AAA GTG CCC AGG GTC AA	(1249)
CYP2C19 (human)	ATC CAG AAA GTG ACG AGG GTC AA	(1247)
CYP2C23 (rat)	ATC CAG AAA GTG TCC TGG ATC AA	(1259)

Figure A.2. Sequence alignment of MAR2CR (23 mer) with mammalian *CYP2C* cDNAs.

MAR2CF3	5'- CNA TGG ATS YNG TBD TGK TSY T -3'	(22)
CYP2C29 (mouse)	AAG CAC AGT GTC TTG TGG AAG A	(471)
CYP2C19 (human)	AAG CCC GCT GCC TTG TGG AGG A	(469)
CYP2C23 (rat)	GAA CCT CTC CTA CTG TGG TCC T	(229)

Figure A.3. Sequence alignment of MAR2CF3 (22 mer) with mammalian CYP2C cDNA sequences.

5'- CUA CUA CUA CUA GGC CAC GCG TCG ACT AGT AC -3'

Figure A.4. Sequence of the 5' RACE UAP primer

Appendix B

The oligonucleotide primers used in PCR experiments to isolate marsupial *UGT1* cDNA sequences, are aligned with selected mammalian *UGT* sequences. The 5'UGT and FUDPGT1R primers, which contained degeneracy to account for the base differences between mammalian *UGT* sequences, were used to amplify a region of approximately 800 bp.

The letters denoting degeneracy are underlined in the primer sequences and include, Y= C+T, S= G+C, K= G+T, R= A+G, W= A+T, H= A+T+C, B= G+T+C, D= G+A+T. Primer length is indicated in parenthesis, and the position of the primers (3' end) in relation to the translational initiation sequences are also indicated.

5'UGT	5'- ATG GAT GGS AGC CAC TGG YTS ASC A -3'	(25)
UGT1A9 (human)	ATG GAT GGG AGC CAC TGG TTC ACC A	(156)
UGT1A7 (human)	ATG GAT GGG AGC CAC TGG TTC ACC A	(261)
UGT1A5 (rat)	ATG GAG GGC AGC CAC TGG CTG AGC A	(97)

Figure B.1. Sequence alignment of 5'UGT (25 mer) with mammalian *UGT1* cDNAs.

FUDPGT1R	5'- GTT CTC CAG AAG CAT TRA YRT AGG CTT C -3'	(28)
UGT1A9 (human)	GTT CTC CAG AAG CAT TAA TGT AGG CTT C	(896)
UGT1A1 (mouse)	GCC ACA CAG ATG CAT GGC TGA GAA CCT C	(892)
UGT1A5 (rat)	GCT CCC CAG AGG CGT TGA CAT AGG CTT C	(730)

Figure B.2. Sequence alignment of FUDPGT1R (28 mer) with mammalian *UGT1* cDNAs.

Appendix C

Table C.1. Oligonucleotide primers used to sequence the entire coding region of the KLC#38 cDNA clone (Chapter 4).

Name: complementarity	Primer sequence
KGSPseq1: forward primer specific to KLC#38	**5'- TGT GCA CCA TGC AAT GTG ATC TGC -3'**
KGSPseq2: forward primer specific to KLC#38	**5'- CGT GTG ATT GGA CAT AAT CGA C -3'**
MAR2CF2R: reverse primer specific to KLC#38	**5'- TAC ATG GAT ATG ACA TTA TGA AGG A -3'**

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Comparative Biochemistry and Physiology Part C 123 (1999) 67–73

CBP

Hepatic microsomal enzyme activity in the koala and tammar wallaby: high 17β-hydroxysteroid oxidoreductase activity in koala liver microsomes

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Abstract

We have studied the hepatic microsomal xenobiotic metabolising capacity of koala (*Phascolarctos cinereus*) and tammar wallaby (*Macropus eugenii*). Total cytochrome P450 content in hepatic microsomes from koala (0.87 ± 0.18 nmol/mg protein, $n = 4$, mean (S.D.) and rat were comparable while tammar wallaby displayed reduced P450 content (0.24 ± 0.04 nmol/mg protein). Associated microsomal activities (NADPH cytochrome P450 reductase, aminopyrine *N*-demethylation, aniline hydroxylation, and androstenedione 6β- and 16α-hydroxylation) in koala liver were similar to or reduced relative to rat. Hepatic microsomal NADPH-supported 17β-hydroxysteroid oxidoreductase (17β-HSOR) activity was significantly higher in koala (9.99 ± 3.08 nmol/mg protein/min) than in tammar wallaby liver (0.86 ± 0.16 nmol/mg protein/min). However, when NADH was utilised as cofactor the activity was similar in both marsupial species (koala, 1.44 ± 0.84 nmol/mg protein/min; tammar wallaby, 1.52 ± 0.44 nmol/mg protein/min). Michaelis–Menten parameters for the kinetics of 17β-HSOR androstenedione reduction by NADPH and NADH were determined in the koala. The K_m for androstenedione was of the order of 1.9–4 μM ($n = 4$) irrespective of the cofactor used, whilst the K_m for NADPH was 0.04–0.05 μM ($n = 2$) and for NADH was 134–430 μM ($n = 2$). Potential inhibitors were evaluated for their effects on NADPH-mediated 17β-HSOR activity with menadione and, to lesser extents, menthone, benzaldehyde and metyrapone eliciting significant inhibition. From detailed kinetic studies menthone was found to be an uncompetitive inhibitor of the activity in koala liver (K_i 220 μM). © 1999 Elsevier Science Inc. All rights reserved.

Keywords: Androstenedione; Cytochrome P450; 17β-Hydroxysteroid dehydrogenase; 17β-Hydroxysteroid oxidoreductase; Koala; Tammar wallaby

1. Introduction

Hepatic microsomal enzymes, in particular the cytochromes P450 (CYP), are significant in the metabolism of xenobiotic and endogenous substrates. Metabolism of these substrates can potentially alter their activity and toxicity. Most information in the literature about this very important group of enzymes comes from information about rats, humans, mice and rabbits. Few studies have examined xenobiotic metabolism in Australian marsupials [3,12–14] and to date no studies have reported data obtained in koala liver. The present study was undertaken to examine the hepatic microsomal xenobiotic metabolising capacity of the koala (*Phascolarctos cinereus*) and of the tammar wallaby (*Macropus eugenii*). The diet of the koala is restricted to the leaves of certain *Eucalyptus* species which contain a high proportion of potentially toxic

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plant secondary metabolites such as phenolic compounds and terpenoids [4]. The tammar wallaby is a grazing herbivore.

Aside from the cytochrome P450s there are many other enzymes which participate in xenobiotic and endobiotic metabolism. Enzymes that catalyse the reduction of carbonyl compounds of endogenous or exogenous origin may also participate in the oxidoreduction of hydroxy- or keto-substituted steroids (reviewed in [10]). The metabolism of ketosteroids and other carbonyl compounds by common enzymes has been demonstrated in a number of species [1,9,18–20] with the enzymes being localised to both microsomal [1,9] and cytosolic fractions [9,18–20].

The enzyme 17β-hydroxysteroid oxidoreductase (17β-HSOR), also known as 17β-hydroxysteroid dehydrogenase (17β-HSD), controls the important final step in the biosynthesis of androgens and estrogens. At least five isoenzymes, that exhibit different patterns of expression in cells, substrate specificity and regulatory mechanisms, have been described [7]. Reduction (e.g. androstenedione to testosterone) utilises NADPH and NADH as cofactors whereas NADP and NAD are cofactors for the oxidative (dehydrogenation) pathway. The 17β-HSOR enzymes are considered to be members of both the aldo–keto reductase and short-chain alcohol dehydrogenase protein families [17].

2. Materials and methods

2.1. Materials

NADP, NADPH, NADH, bovine serum albumin, isocitrate dehydrogenase, unlabelled androst-4-ene-3,17-dione (androstenedione), 17β(-hydroxy-4-androsten-3-one (testosterone), 3β-hydroxysteroid dehydrogenase, cytochrome *c*, benzaldehyde, acetaldehyde, cyclohexanone, acetophenone, menadione, 4-pyridinecarbox aldehyde and metyrapone were purchased from the Sigma Chemical Co. (St. Louis, MO, USA. Menthone was purchased from TCI (Tokyo, Japan). [4-^{14}C] Androstenedione (spec. act. 59 mCi/mmol) was purchased from Amersham Australia (Sydney). 16β-Hydroxyandrostenedione was prepared enzymatically by the action of 3β-hydroxysteroid dehydrogenase on 3β,16β-dihdroxyandrost-5-ene-17-one (Prof. D.N. Kirk and MRC Steroid Reference Collection, Queen Mary's College, London, UK) as described [22]. Other hydroxylated testosterone and androstenedione standards were obtained from the MRC Steroid Collection or Steraloids, Inc. (Wilton, NH, USA). All solvents and other miscellaneous chemicals were at least analytical reagent grade.

2.2. Animals

Livers were obtained from four road-injured koalas (two male and two female) ultimately killed using pentobarbital. Livers were also scavenged from six tammar wallabies (five male and one female) which were destroyed as part of an animal control program. Male Hooded Wistar rats were used as the source of rat liver tissue. All animals (koalas, wallabies and rats) were sexually mature. Livers from all species were quickly removed and rapidly chilled after death of the animal and frozen in liquid nitrogen before storage at −70°C. Hepatic microsomes were prepared from these livers by homogenisation. The homogenate was centrifuged at 10,000 × *g* for 20 min and the resulting supernatant was then centrifuged at 105,000 × *g* for 50 min. The microsomal pellet was resuspended and recentrifuged at 105,000 × *g* for 50 min prior to resuspension and storage at −70°C and subsequent use.

2.3. Assays

Protein concentrations were determined by the method of Lowry [8]. CYP content and NADPH CYP reductase activity were quantified as described previously [11]. Aminopyrine demethylase activity was measured using a substrate concentration of 25 mM and the formaldehyde that was produced was quantitated by the method of Nash [16]. Aniline hydroxylase activity was measured as previously described [6].

Microsomal androstenedione hydroxylase and 17β-HSOR activity was assayed essentially by the procedure of Gustafsson and Ingelman–Sundberg [5] and Waxman [24]. For incubations utilising NADPH, microsomal fractions were incubated in a 1-ml reaction mixture containing isocitrate (4 mM), isocitrate dehydrogenase (0.4 IU), $MgCl_2$ (8 mM), 1 mM NADP and androstenedione (3.5–350 μM, 130 μCi/mmol) for 10 min at 37°C, after which the incubation mixtures were extracted twice with ethyl acetate. In some incubations 1 mM NADH was used in place of the NADPH-generating system and in kinetic studies the NADPH and NADH concentrations were varied whilst other components were held constant. Inhibitors, where used, were added in ethanol and appropriate control incubations were performed concurrently. Application to, and development of, thin-layer chromatography (TLC) plates was carried out as previously described [23]. Zones corresponding to hydroxylated androstenedione standards and to testosterone were visualised under UV light and scraped into vials for scintillation spectrometry (ACS, Amersham).

All assay conditions were optimised with respect to time, protein concentration and substrate concentrations to ensure linearity.

2.4. Data analysis

For each experiment, data are expressed as the mean of triplicate observations. Variability of the population is indicated by the S.D. An iterative non-linear least squares regression program (Multifit 2.0, Cambridge, UK) was used to fit untransformed kinetic data. Statistical analysis was carried out using one-way analysis of variance (STATVIEW, Berkeley, USA) followed by a Scheffe F-test to detect differences.

3. Results

Data for CYP content, NADPH CYP reductase, aminopyrine demethylase, aniline hydroxylase, androstenedione 6β- and 16α-hydroxylase activities are shown in Table 1. The assay of androstenedione hydroxylase activity also enabled the measurement of 17β-HSOR activity, as reflected by the production of testosterone from androstenedione.

The data in Table 1 clearly indicate that CYP content in hepatic microsomes from the koala (0.87 ± 0.18 nmol/mg protein) is similar to that in rat liver (0.65 ± 0.07 nmol/mg protein) and higher than that measured in tammar wallaby (0.24 ± 0.04 nmol/mg protein), whilst the NADPH CYP reductase activity is similar in all three species. Aminopyrine demethylase activity did not vary greatly in hepatic microsomes from the three species, whereas aniline hydroxylase activity was somewhat higher in the wallaby than in the other species (Table 1; $P < 0.05$). Androstenedione 6β- and 16α-hydroxylase activities were significantly lower ($P < 0.05$) in the koala than in rat. The most striking finding to emerge from this study was that NADPH-mediated 17β-HSOR activity was much greater in microsomal fractions from koala liver than in wallaby or rat. Interestingly, the approximate five-fold species difference in NADPH-dependent 17β-HSOR activity was not observed when NADH was utilised.

Microsomes from koala, wallaby and rat liver were incubated with a range of androstenedione concentrations and testosterone formation determined utilising either NADPH or NADH as cofactors. The data were used to construct Lineweaver–Burk plots (representative plots shown in Fig. 1). In rat hepatic microsomes the plots were linear ($r^2 = 0.98$) with either cofactor. Apparent K_m and V_{max} values for the NADPH- and NADH-supported reactions were, respectively, 8.4 and 7.9 μM and 2.0 and 14.1 nmol/mg protein/min.

Plots using data obtained from koala and wallaby liver microsomes were not linear and therefore were consistent with at least a two-enzyme system for the formation of testosterone. The non transformed data from koala microsomes were fitted to a reduced two-enzyme system, using iterative non-linear least squares regression. The data were fitted to a reduced two enzyme system. The equation employed was:

$$\text{Velocity of testosterone formation} = \frac{V_{max}S}{K_m} + Cl_{int2}S$$

where V_{max} and K_m are the maximal velocity and Michaelis–Menten constant, respectively, for the high-affinity enzyme, Cl_{int} is the intrinsic clearance of the low-affinity enzyme in the reduced two-enzyme system, and S is substrate concentration. Apparent values of K_m, V_{max} and Cl_{int2} are presented in Table 2. As displayed in Table 2 the K_m with respect to androstenedione did not vary with the cofactor used (K_m 1.86–4.78 μM); however, the affinity of the enzyme for

Table 1
CYP content, CYP associated activities and 17β-hydroxysteroid oxidoreductase activities in hepatic microsomes prepared from koala, wallaby and rat[a]

Content or activity	Koala ($n = 4$)	Koala S.D.	Tammar wallaby ($n = 6$)	Tammar wallaby S.D.	Rat ($n = 4$)	Rat S.D.
Cytochrome P450 (nmol/mg protein)	0.87	0.18	0.24[b]	0.04	0.65	0.07
NADPH cytochrome P450 reductase (nmol/mg protein/min)	150.5	34	167.0	34.8	158	18.5
Aminopyrine demethylase (nmol/mg protein/min)	4.23	0.30	4.86	0.57	4.37	0.17
Aniline hydroxylase (nmol/mg protein/min)	0.38	0.02	0.55[b]	0.08	0.33	0.05
Androstenedione 6β-hydroxylase (nmol/mg protein/min)	0.19	0.10	0.32[b]	0.21	0.98[b]	0.15
Androstenedione 16α-hydroxylase (nmol/mg protein/min)	0.14	0.17	0.29	0.26	1.90[b]	0.08
17β-Hydroxysteroid oxidoreductase (NADPH cofactor) (nmol/mg protein/min)	9.99	3.08	0.86[b]	0.16	2.01[b]	0.49
17β-Hydroxysteroid oxidoreductase (NADH cofactor) (nmol/mg protein/min)	1.44	0.84	1.52	0.44	ND	ND

[a] Androstenedione concentration was 175 μM. S.D. for each activity or content is tabulated separately.
[b] Different from koala microsomes at $P < 0.05$. ND not done.

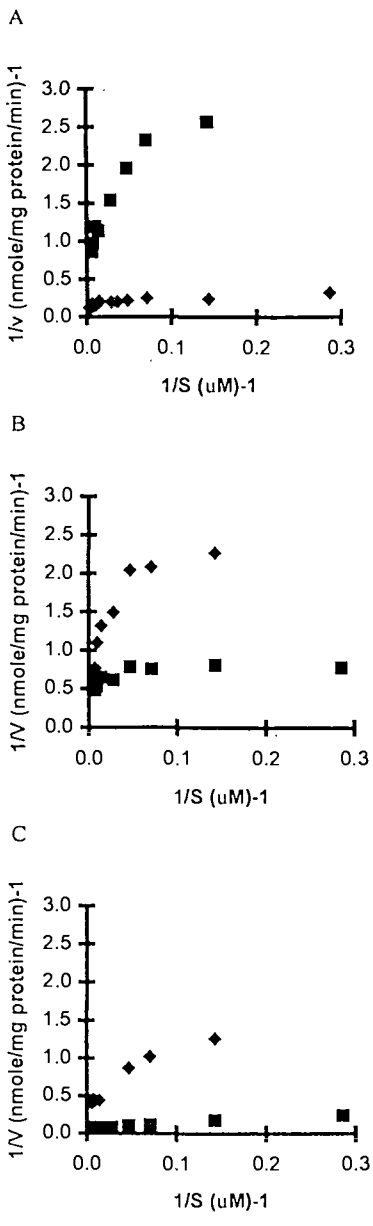

Fig. 1. Representative Lineweaver–Burk plots of individual animal hepatic microsomal 17β-HSOR activity utilising both NADPH (◆) and NADH (◇) as cofactor and androstenedione as second substrate. NADPH or NADH concentration was 1 mM. These are saturating concentrations of cofactor. (A) Koala, (B) wallaby, (C) rat liver microsomal metabolism.

the two cofactors varied by more than three orders of magnitude (K_m, NADPH cofactor 0.04–0.05 μM compared to K_m, NADH cofactor 134–430 μM. Data from wallaby microsomes could not be fitted by this approach.

A range of potential inhibitors of 17β-HSOR activity (NADPH mediated) were examined (Table 3). Of the compounds, menadione, benzaldehyde, menthone, metyrapone and cyclohexanone inhibited the conversion of androstenedione to testosterone by at least 30% at a test concentration of 0.5 mM (Table 3). 4-Pyridinecarboxaldehyde, acetaldehyde and acetophenone were essentially non-inhibitory. More extensive experimentation was undertaken with menthone, a eucalyptus constituent, in order to characterise the nature and affinity of inhibition. Initial experiments were conducted at a range of menthone concentrations (0.125–1 mM) at relatively low concentrations of androstenedione (21–70 μM) and an NADPH concentration of 1 μM. Activation of 17β-HSOR activity was observed at the higher concentrations of menthone (Fig. 2). Subsequent experiments were conducted at relatively low menthone concentrations (0.0156–0.125 mM). Dixon and Cornish–Bowden plots (Fig. 3) indicate that menthone is an uncompetitive inhibitor of koala hepatic microsomal 17β-HSOR (K_i 220 μM).

4. Discussion

Few studies have examined xenobiotic metabolism in Australian marsupials. To our knowledge this study is the first which reports data obtained in koala liver. The koala's diet is unique and consists of large amounts of eucalyptus leaves which contain a range of potentially toxic chemicals. Enzymes that mediate the biotransformation of the toxic constituents of this unique diet have yet to be identified.

In the koala and rat liver microsomes total CYP contents were similar and were somewhat higher than in hepatic microsomes from the wallaby. None of the typical substrates for rat CYPs were subject to unusually extensive oxidation in koala liver. Thus, rates of aminopyrine demethylation and aniline hydroxylation in microsomes from koala and wallaby liver were similar to those observed in male rat liver. In contrast, rates of androstenedione 6β- and 16α-hydroxylation were much lower in hepatic microsomes from both marsupial species than in the rat. These findings are consistent with earlier reports that *in vitro* xenobiotic metabolism in hepatic microsomes from Australian marsupials are generally lower than in the corresponding fractions from laboratory rodents [3,12–14]. The test substrates are good catalytic indicators of CYPs from the 2A, 2B, 2C, 2E and 3A subfamilies. It is feasible, therefore, that the koala possesses hepatic CYPs that correspond to several CYPs from rat liver.

The most noteworthy finding to arise from the present study was the approximate five fold more rapid rate of NADPH-supported androstenedione reduction to testosterone in koala liver than in wallaby or rat. The microsomal conversion of androstenedione to testosterone, with NADPH as the preferred cofactor in the koala therefore conforms to the general observations made for the type 1 and 3 17β-HSORs, and type 1 11β-HSD (reviewed in [7]). NADPH-supported testosterone formation was similar in microsomes from

Table 2
Apparent kinetic parameters for 17β-hydroxysteroid oxidoreductase activities in hepatic microsomes prepared from koala[a]

Animal	Variable	Constant	K_m (μM)	V_{max} (nmol/mg protein/min)	V_{max}/K_m (ml/mg protein/min)	Cl_{int2} (ml/mg protein/min)
1	A	NADPH	1.86 ± 0.37	4.76 ± 0.30	2.55	0.010 ± 0.002
2			3.47 ± 0.44	4.50 ± 0.37	1.30	0.004 ± 0.003
1	A	NADH	2.71 ± 0.23	0.51 ± 0.08	0.19	0.004 ± 0.001
2			4.78 ± 0.31	0.89 ± 0.07	0.19	0.007 ± 0.002
1	NADPH	A	0.04 ± 0.01	2.78 ± 0.34	69.5	0.45 ± 0.11
2			0.05 ± 0.01	2.10 ± 0.28	42.0	0.48 ± 0.18
1	NADH	A	134 ± 18	0.89 ± 0.12	0.007	0.74 ± 0.16
2			430 ± 27	1.97 ± 0.32	0.005	0.36 ± 0.13

[a] Data are shown for two individual animals for both substrate (A, androstenedione) and cofactor (NADH or NADPH). Androstenedione concentration was 350 μM or 175 μM when NADPH or NADH concentrations were varied respectively, and NADPH or NADH was 1 mM when androstenedione concentration was varied. These are saturating concentrations of substrate. Untransformed data were fitted to a reduced two-enzyme Michaelis–Menten equation. Data are shown ± S.E.

male and female koala liver (two animals of both genders were included in the present study). However, the assertion that the activity may be gender-dependence could only be made after further measurements in a larger sample size.

Kinetic analysis of 17β-HSOR activity is not straightforward. The activity in rat liver followed Michaelis–Menten kinetics, but those in hepatic microsomes from the marsupial species were more complex. Kinetic plots were non-linear so that the data was fitted to a two-enzyme system. Apparent K_m and V_{max} values for the high-affinity enzymes from each species are presented in Table 2. The affinity of the enzyme for substrate (androstenedione) is similar, regardless of the cofactor employed, and NADPH supports more efficient substrate turnover. Thus, it is quite possible that the same enzyme mediates androstenedione reduction by both cofactors. However, the affinity of the enzyme for NADPH is approximately 3000-fold greater than for NADH.

It has been suggested that 17β-HSOR enzymes may also contribute to the reduction of aldehydes and ketones (reviewed in [10]). We evaluated a range of aldehydes and ketones as potential inhibitors of 17β-HSOR activity. As might have been expected, menadione, a widely used two electron acceptor, effectively inhibited 17β-HSOR activity. Acetaldehyde, a frequently used aldehyde dehydrogenase substrate also inhibited the activity. Cyclohexanone, benzaldehyde and metyrapone are also established substrates for aldo–keto reductases [9,18]. Our finding that these three compounds inhibited 17β-HSOR activity suggests that they may be alternate substrates of the enzyme. Interestingly, 4-pyridinecarboxaldehyde which has been identified as being a substrate of several purified cytosolic 17β-HSORs [19] showed only poor inhibition of the koala microsomal 17β-HSOR.

Alicyclic ketones such as menthone and piperitone are found in eucalyptus leaves [2] and are therefore candidates for reduction and conjugation, perhaps by glucuronidation, prior to excretion. In the present study menthone elicited inhibition and activation of 17β-HSOR activity in a concentration-dependent manner.

Table 3
Inhibition of 17β-hydroxysteroid oxidoreductase activities in hepatic microsomes ($n = 3$) prepared from koala[a]

Inhibitor	% control activity	S.D.
4-Pyridinecarboxaldehyde	94	5
Acetaldehyde	87	7
Acetophenone	80	7
Cyclohexanone	69	12
Metyropone	65	17
Menthone	63	11
Benzaldehyde	63	6
Menadione	29	7

[a] Reactions utilised 87.5 μM androstenedione and NADPH as cofactor. The concentration of potential inhibitors was 0.5 mM.Control activity was determined in the presence of appropriate solvent concentrations. S.D. of % control activity for each inhibitor is tabulated separately.

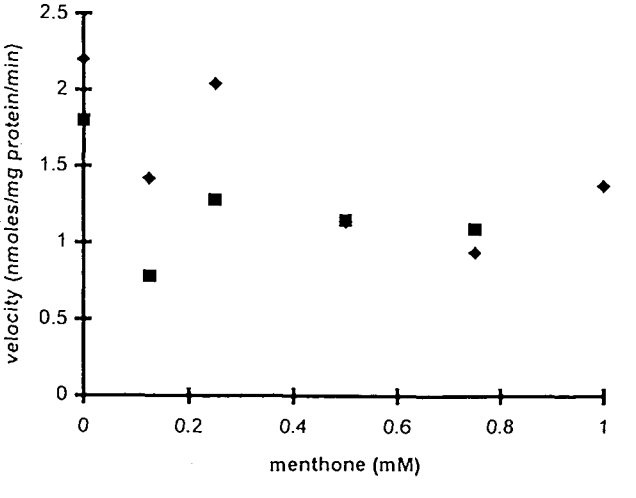

Fig. 2. Representative plot of hepatic microsomal 17β-HSOR activity versus menthone concentration. Androstendione concentrations were 21 (■) and 35 (◆) μM.

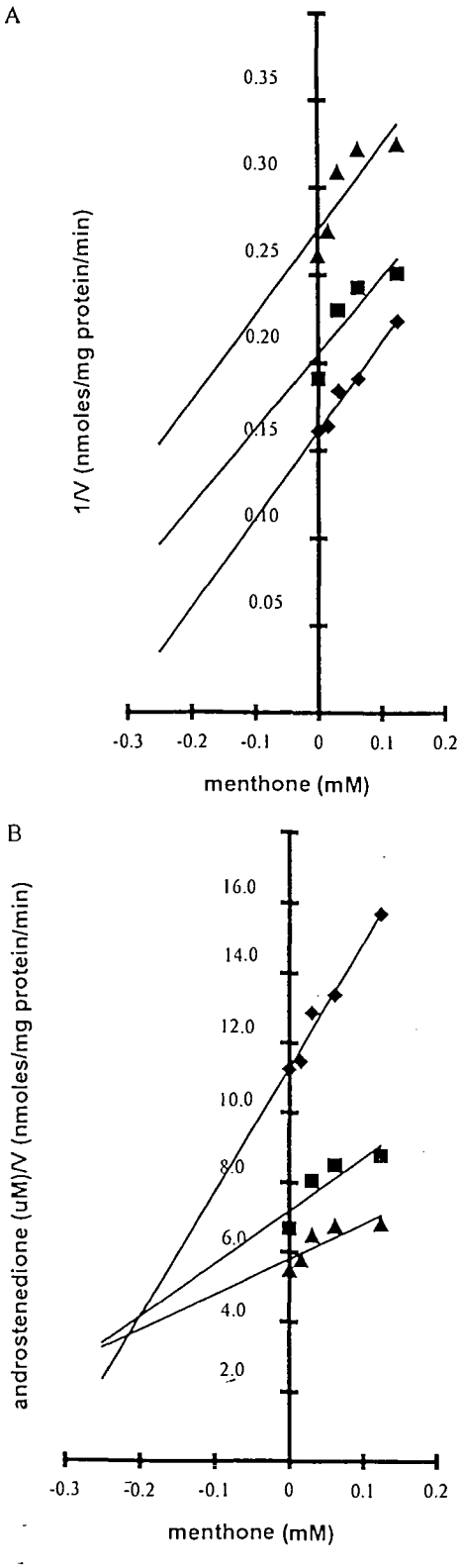

Fig. 3. (A) Representative Dixon plot, (B) representative Cornish-Bowden plot for inhibition of individual koala hepatic microsomal 17β-HSOR activity (NADPH cofactor) by menthone. Concentrations of androstenedione were 21 (▲), 35 (■) and 70 (◆) μM. Regression lines of best fit are shown.

At high substrate concentrations, menthone stimulated the rate of androstenedione reduction to testosterone in microsomal fractions from koala liver in vitro. Inhibition was observed at low concentrations of the ketone. Product activation of the reduction of ketosteroids to hydroxysteroids has been observed in rat hepatic microsomes [15], although the present situation appears more closely related to substrate activation. Inhibition data were subjected to graphical analysis and indicated that menthone was an uncompetitive inhibitor of koala microsomal 17β-HSOR activity. This analysis would be consistent with the binding of menthone to the 17β-HSOR–androstenedione enzyme–substrate complex to yield an inactive ternary complex [21]. In uncompetitive inhibition substrate (here, androstenedione) is required for the binding of the inhibitor (menthone).

It is important to remember that the eucalyptus leaf is an obligatory component of the diet of the koala. Thus, it is likely that the detoxification system of this species has adapted to the presence of chemicals in the diet that may otherwise precipitate serious adverse effects. Although a physiological role for the highly active NADPH-mediated 17β-HSOR activity in koala liver remains unclear, the findings of the present study implicate this enzyme in the overall enzymic defence of the koala against plant chemicals.

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