

AN ABSTRACT OF THE THESIS OF

Yea-Huey Yang for the degree of Doctor of Philosophy in Genetics presented on December 12, 1997. Title: Cloning, Heterologous Expression, and Characterization of the Cytochrome P450 Monooxygenases in Rainbow Trout (*Oncorhynchus mykiss*).

Abstract approved: Redacted for Privacy

Donald R. Buhler

Rainbow trout (*Oncorhynchus mykiss*) possess complex constitutive cytochrome P450 monooxygenase systems that have not been well characterized. The major constitutive cytochrome P450 LMC2 has been cloned previously and assigned the name CYP2K1 by the P450 Nomenclature Committee. To further characterize CYP2K1 and other P450s in rainbow trout, cDNAs encoding trout P450s were cloned, sequenced and designated as CYP2K3, CYP2K4 and CYP2M1, respectively. Sequence comparison between CYP2K1 and CYP2K3 showed 96.5% identity in the deduced amino acid sequences and a 39-bp deletion in close proximity to the conserved heme-binding domain in CYP2K3. CYP2K4 also shared high homology (94.2%) with CYP2K1 in the deduced amino acid sequence. The deduced N-terminal amino sequence of CYP2K4 matches closely to that of KM2, a reported male-specific P450 purified from trunk kidney of sexually mature male rainbow trout.

The catalytic activity of CYP2M1 toward lauric acid was examined by cDNA-directed expression in the COS-7 and baculovirus/insect cell expression systems. This P450 has lauric acid ( $\omega$ -6)-hydroxylase activity not observed in other P450s. The baculovirus-expressed CYP2K1 catalyzed the regiospecific ( $\omega$ -1)-hydroxylation of lauric acid and the activation of aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) to AFB<sub>1</sub>-8,9-epoxide. The AFB<sub>1</sub>-epoxide was identified as the AFB<sub>1</sub>-exo-epoxide which is more mutagenic than the *endo*-epoxide. CYP2K3 protein was expressed in recombinant baculovirus/insect cells; however, this P450 did not display the CO-difference spectrum and exhibited no enzymatic activity toward lauric acid or AFB<sub>1</sub>.

Northern blot analysis exhibited pronounced differences in the sex- and tissue-specific expression of CYP2M1 and CYP2K4 in the liver and trunk kidney. The expression of CYP2M1 was detected in the liver from juvenile and mature trout of both sexes. Negligible amounts of CYP2M1 mRNA were found in the juvenile and sexually mature male trunk kidney. A 2.8 Kb CYP2K4 cross-hybridized mRNA band was detected in the trunk kidney and liver of 3-yr-old male trout but not in females of the same age, or juvenile liver and trunk kidney of both sexes.

Rainbow trout P450s have been shown to metabolize a number of endogenous and xenobiotic compounds. Knowledge of these P450 will contribute to the understanding of trout as a model for xenobiotic metabolism.

© Copyright by Yea-Huey Yang  
December 12, 1997  
All Rights Reserved

Cloning, Heterologous Expression, and Characterization of  
the Cytochrome P450 Monooxygenases in Rainbow Trout  
(*Oncorhynchus mykiss*).

by

Yea-Huey Yang

A THESIS

submitted to

Oregon State University

in partial fulfillment of  
the requirements for the  
degree of

Doctor of Philosophy

December 12, 1997  
Commencement June 1998

Doctor of Philosophy thesis of Yea-Huey Yang presented on  
December 12, 1997

APPROVED:

Redacted for Privacy

---

Major Professor, representing Genetics Program

Redacted for Privacy

---

Chair of Genetics Program

Redacted for Privacy

---

Dean of Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Redacted for Privacy

---

Yea-Huey Yang, Author

## ACKNOWLEDGMENTS

I would like to thank God for giving me the strength to accomplish this degree. I would like to thank Dr. Donald R. Buhler for giving me the opportunity to conduct research in his laboratory, and for his guidance, encouragement, patience, understanding and financial support. I would also like to thank Dr. George Bailey, Dr. Jo-Ann Leong, and Dr. Henry Schaup for serving on my advisory committee, and Dr. James Baggett for serving as my graduate school representative.

There have been so many others over the years that have helped me in accomplished this goal. I would like to thank especially Dr. Cristobal Miranda for his resourceful guidance and assistance in my research, and Dr. Jun-Lan Wang for her help both in research and daily life, and Ms. Marilyn Henderson for her advice and technical support.

Special thanks also go to the National Institute of Environmental Health Sciences for funding this research.

I would like to dedicate this thesis to my parents, brothers and sisters, without their unhesitating support, this would not be possible.

## CONTRIBUTION OF AUTHORS

Dr. Cristobal Miranda and Dr. Jun-Lan Wang were involved in the design, analysis and writing of each manuscript. Dr. Tommy Andersson has provided N-terminal amino acid sequence of P450 KM2. Dr. Byung-Woo Ryu has assisted in sequencing of CYP2K4.

## Table of Contents

<u>CHAPTER</u>	<u>PAGE</u>
1. Introduction and Literature Review	1
Introduction-----	1
Literature Review-----	4
History-----	4
The P450 superfamily-----	5
Evolution-----	5
P450 nomenclature-----	6
General aspects of cytochrome P450 monooxygenase reaction-----	8
Reaction mechanism-----	8
Reactions catalyzed by cytochrome P450-----	11
Metabolism of endogenous compounds--	14
Metabolism of xenobiotics-----	16
Cytochrome P450 in rainbow trout-----	17
CYP1 family-----	19
CYP2 family-----	22
CYP3 family-----	25
CYP4 family-----	27
Steroidogenic cytochrome P450s-----	27
Other cytochrome P450s in rainbow trout-----	29
cDNA-directed expression of cytochromes P450 -----	32
Transient expression systems-----	33
Stable expression systems-----	35

## Table of Contents (cont)

Continued

<u>CHAPTER</u>	<u>PAGE</u>
2. CYP2M1: Cloning, Sequencing and Expression of a New Cytochrome P450 from Rainbow Trout Liver with Fatty Acid ( $\omega$ -6)-Hydroxylation Activity	38
Abstract-----	39
Introduction-----	40
Materials and Methods-----	43
Experimental animals-----	43
Materials-----	43
Poly(A) <sup>+</sup> RNA isolation and cDNA library construction-----	44
Screening of cDNA library-----	45
cDNA sequencing and analysis-----	45
N-terminal amino acid determination-----	46
Northern blot analysis-----	46
Southern blot analysis-----	48
Mass spectrometric analysis-----	48
Expression of CYP2M1 in COS-7 cells-----	49
Expression of CYP2M1 in baculovirus-insect cells-----	50
Western blot analysis-----	51
Enzyme assays-----	52
Results and Discussion-----	55
Identification and sequence analysis of cloned cDNA-----	55
Analysis of CYP2M1 gene expression-----	56
Southern blot analysis-----	57
cDNA-mediated CYP2M1 expression in COS-7 cells and recombinant baculovirus infected Sf9 cells-----	58
Acknowledgement-----	64

Table of Contents (cont)

Continued

<u>CHAPTER</u>	<u>PAGE</u>
3. Cloning, Sequencing and Heterologous Expression of CYP2K1 and CYP2K3 from Sexually Mature Rainbow Trout Liver, and Their Roles in Aflatoxin B <sub>1</sub> Activation	79
Abstract-----	80
Introduction-----	82
Materials and Methods-----	84
Experimental animals-----	84
Materials-----	84
Total RNA preparation-----	85
cDNA library construction for 5'- and 3'-RACE polymerase chain reaction-----	85
cDNA-directed expression of CYP2K1 and CYP2K3 in Sf9 insect cells-----	87
Western blot analysis-----	90
Enzyme assays-----	91
Results-----	93
CYP2K3 cDNA cloning and sequence analysis-----	93
CYP2K1 and CYP2K3 expression in Sf9 cells-----	94
Catalytic properties of cDNA-expressed P450s and purified P450 LMC2-----	95
Discussion-----	98
Acknowledgement-----	103

## Table of Contents (cont)

Continued

<u>CHAPTER</u>	<u>PAGE</u>
4. Primary Structure and Tissue Expression of CYP2K4, a New Cytochrome P450 from Trunk Kidney of Post-Spawning Male Rainbow Trout	115
Abstract-----	116
Introduction-----	118
Materials and Methods-----	121
Experimental animals-----	121
Total and poly(A) <sup>+</sup> RNA isolation-----	121
Construction and screening of $\lambda$ -gt11 cDNA library-----	121
Rapid amplification of cDNA ends by polymerase chain reaction (5'- and 3'-RACE)-----	122
cDNA sequencing -----	124
Northern blot analysis-----	124
Results-----	126
Cloning and sequencing analysis-----	126
Northern blot analysis-----	127
Discussion-----	128
Acknowledgement-----	131
5. Conclusion-----	137
Bibliography-----	141

## List of Figures

<u>Figure</u>	<u>PAGE</u>
1.1 Catalytic cycle of cytochrome P450 associated with monooxygenation reactions-----	10
1.2 Cytochrome P450-dependent monooxygenase systems of mitochondria, bacteria and endoplasmic reticulum-----	12
2.1 Nucleotide and deduced amino acid sequences for rainbow trout CYP2M1 cDNA-----	69
2.2 MALDI-TOF mass spectrum of rainbow trout P450 LMC1-----	71
2.3 Northern blot analysis of CYP2M1-----	72
2.4 Southern blot analysis of CYP2M1-----	74
2.5 CO-difference spectrum of baculovirus-expressed CYP2M1-----	75
2.6 Western blotting of purified P450 LMC1 and expressed CYP2M1-----	76
2.7 Representative reverse-phase HPLC elution profile of lauric acid and ( $\omega$ -6)-hydroxylauric acid produced by BV-CYP2M1-----	77
2.8 Characteristic mass spectrum of ( $\omega$ -6)-hydroxylauric acid produced by BV-CYP2M1-----	78
3.1 Nucleotide and deduced amino acid sequences of CYP2K3 cDNA-----	105
3.2 Amino acid sequence alignment between CYP2K1 and CYP2K3-----	107
3.3 CO-reduced difference spectrum of cDNA-directed expression of CYP2K1-----	108
3.4 Immunoblots of baculovirus expressed CYP2K1 and CYP2K3-----	109

## List of Figures (cont)

Continued

<u>Figure</u>	<u>PAGE</u>
3.5 HPLC chromatograms of [ <sup>14</sup> C]-lauric acid hydroxylation metabolites produced by BV-CYP2K1 and purified LMC2-----	110
3.6 HPLC chromatograms of AFB <sub>1</sub> -GSH conjugates produced by BV-CYP2K1, purified LMC2, human CYP3A4 or human CYP1A2 in the presence of mouse liver cytosol-----	111
3.7 HPLC chromatograms of AFB <sub>1</sub> -GSH conjugates produced by BV-CYP2K1, purified LMC2, human CYP3A4 or human CYP1A2 in the presence of purified rat GSTs and GSH-----	113
4.1 RACE-PCR products-----	132
4.2 Assembly strategy for the cloned CYP2K4 cDNA--	133
4.3 Nucleotide and deduced amino acid sequences Of rainbow trout CYP2K4-----	134
4.4 Amino acid sequence alignment between CYP2K4, CYP2K1, CYP2K3 and the N-terminal region of P450 KM2-----	135
4.5 Northern blot analysis of CYP2K4-----	136

## List of Tables

<u>Table</u>	<u>PAGE</u>
1.1 General overview of cytochrome P450-----	7
1.2 Types of P450 catalyzed reactions and representative substrates-----	13
1.3 Isoforms of cytochrome P450 involved in steroid hormone synthesis-----	15
1.4 Representative chemical carcinogens metabolized by cytochrome P450-----	18
2.1 Proximal cysteine-containing peptide involves in heme binding in P450s of rainbow trout-----	65
2.2 Quantitation of CYP2M1 mRNA present in the liver-----	66
2.3 Quantitation of CYP2M1 mRNA present in the kidney-----	67
2.4 Catalytic activities of purified rainbow trout P450 LMC1, COS-7 and baculovirus- expressed CYP2M1-----	68
3.1 Comparison of the catalytic activities of BV-CYP2K1, BV-CYP2K3 and purified LMC2-----	104

# **Cloning, Heterologous Expression, and Characterization of the Cytochrome P450 Mono- oxygenases in Rainbow Trout (*Oncorhynchus mykiss*)**

## **CHAPTER 1**

### **Introduction and Literature Review**

#### *Introduction*

The cytochromes P450 consist of a large and expanding superfamily of heme-thiolate enzymes that are responsible for the oxidative, peroxidative and reductive metabolism of both endogenous and exogenous compounds. The name cytochrome P450 was given because of the characteristic CO-bound, reduced absorption peak at 450 nm discovered by Omura and Sato in 1962 (Omura and Sato, 1962). The membrane-bound P450s are expressed mainly in liver, with generally lesser expression in extrahepatic tissues.

The rainbow trout (*Oncorhynchus mykiss*) has been used extensively as an alternative animal model in toxicity research and carcinogenesis studies because of its high sensitivity to various xenobiotics (Bailey et al., 1996). Many of these xenobiotics are detoxified and/or bioactivated by cytochromes P450.

Rainbow trout possess a complex P450 monooxygenase system and several different isoforms have been isolated,

purified and their catalytic activities analyzed. The polycyclic aromatic hydrocarbon (PAH)- and  $\beta$ -naphthoflavone (BNF)-inducible CYP1A1 was first purified from BNF-treated rainbow trout liver microsomes (Williams and Buhler, 1984). The cDNA encoding for CYP1A1 was isolated from liver of 3-methylcholanthrene (3-MC)-treated rainbow trout (Heilmann et al., 1988). Subsequently, two CYP1A genes were isolated from a rainbow trout genomic DNA library (Berndtson and Chen, 1994) and designated as CYP1A1 and CYP1A3 (Nelson et al., 1996). The catalytic activities of cDNA-expressed trout CYP1As were characterized in the yeast expression system (You et al., 1997).

The steroidogenic P450s (CYP11A1, CYP17, CYP19) have been cloned from rainbow trout ovary, their cDNAs expressed and the catalytic activities of the expressed P450s then determined in COS cells (Takahashi, et al., 1993; Sakai, et al., 1992; Tanaka, et al., 1992).

Five constitutive isoforms of cytochrome P450 also were purified from liver of untreated rainbow trout (Miranda et al., 1989). Based on the MW observed in SDS-PAGE, these latter P450s were named P450 LMC1 to LMC5. The P450 LMC2 has been cloned, sequenced (Buhler et al., 1994) and designated as CYP2K1 by the P450 Nomenclature Committee (Nelson et al., 1996). This P450 LMC2 is immunologically and catalytically indistinguishable from the P450 LM2 (Williams and Buhler, 1984) purified from liver of BNF-treated trout. However, the catalytic activity of the cDNA-expressed CYP2K1 has not been characterized. Southern blotting analysis has revealed the existence of multiple P450 genes that are closely

related to CYP2K1. Northern blotting of liver and trunk kidney from sexually mature male trout showed the presence of more than one CYP2K1 cross-reacting mRNA. Western blot analysis of liver and trunk kidney from sexually mature male trout exhibited multiple bands that can be recognized by antibodies raised against P450 LMC2 (Buhler et al., 1994). These data suggest the presence of multiple copy genes related to CYP2K1.

To further understand the cytochromes P450 in rainbow trout, the goals of this research were as follows:

1. Prepare a cDNA clone of the constitutive P450 LMC1 isolated from liver of rainbow trout. Determine the catalytic activity toward lauric acid by cDNA-directed expression of P450 LMC1 produced in COS-7 and baculovirus expression systems.
2. Prepare cDNA clones of other CYP2K1-like genes from liver and undertake cDNA-directed expression of the corresponding proteins in baculovirus. Determine the catalytic activities of cDNA-expressed CYP2K1 and related P450 clones toward lauric acid and aflatoxin B<sub>1</sub>.
3. Prepare a cDNA clone of CYP2K1-related P450 from the trunk kidney of sexually mature male rainbow trout.

## *Literature Review*

### **History**

Studies on the cellular pigments have been described since the early 1800s. The small membrane vesicles isolated after disruption of liver tissue and differential centrifugation were named microsomes (Claude, 1946). These microsomes were later shown to be derived from the endoplasmic reticulum. In 1955, when liver microsomes from rat and pig were treated with the reducing agent sodium dithionite and flushed with carbon monoxide, a new pigment absorbing light at 450 nm was observed by Klingenberg (1958) and Garfinkel (1958). This novel pigment was further identified by Omura and Sato (1962) as a hemoprotein and denoted as "P450" meaning "a pigment with absorption at 450 nm". This P450 could be solubilized from microsomal membrane and converted to P420 (an inactive form of P450) by snake venom or detergent (Omura and Sato, 1962; 1964). The physiological function of cytochrome P450 was first demonstrated by showing its involvement in the 21-hydroxylation of 17 $\alpha$ -hydroxyprogesterone in the microsomal fraction of the adrenal cortex (Estabrook et al., 1963).

Progress in the past three decades has revealed that the P450 superfamily has immense diversity in its functions, with hundreds of isoforms in many species catalyzing many types of chemical reactions. It is postulated that the members of the P450 superfamily are

able to metabolize a million or more substrates, thus the name "DIVERSOZYMES" has been proposed for this remarkable family of hemoproteins. (Coon et al., 1996).

P450s are ubiquitous occurring in almost every phylum, from bacteria to plants and animals (Nelson et al., 1996). Initially, it was thought that fish did not contain cytochrome P450 monooxygenases (Brodie and Maickel, 1962). Further studies in the late 1960's (Buhler, 1966; Buhler and Rasmusson, 1968; Dewaide and Henderson, 1968), however, showed that P450s were present in the livers of rainbow trout and other fishes.

### **The P450 superfamily**

#### *Evolution*

It is now widely accepted that the cytochrome P450 gene superfamily is very old and evolved from a single ancestral gene which existed before the time of prokaryote/eukaryote divergence, probably 1.4 billion years ago (Nebert and Gonzalez, 1985, 1987, 1990; Nelson and Strobel, 1987; Nebert and Nelson, 1991). Based on the functions of P450s in animals and the metabolism of steroids and xenobiotics, steroid metabolizing P450s are thought to be the most ancient. Some mitochondrial P450s involved in steroid metabolism are related to bacterial P450s as inferred from sequence data as well as from localization and the usage of similar electron transfer

system (iron-sulfur adrenodoxin). To date, there are almost 500 P450 genes and 22 pseudogenes reported in 85 eukaryote (including vertebrates, invertebrates, plants and fungi) and 20 prokaryote species. The diversity of cytochromes P450 found in various species is shown in Table 1.1 (Nelson et al., 1996).

### *P450 nomenclature*

It was not until 1987 that a nomenclature system was developed for naming individual cytochromes P450 based on the proposed divergent evolution of the cytochrome P450 superfamily, as inferred from the degree of amino acid sequence identity between P450s (Nebert, et al., 1987). The gene is named with the italicized root symbol "CYP" for human ("Cyp" for mouse and *Drosophila*) representing "cytochrome P450" followed by an Arabic numeral designating gene family, a capital letter denoting subfamily, and a second number for the individual genes with numbers assigned according to the order of discovery within a subfamily. The cDNAs, mRNAs and enzymes in all species (including mouse) include all capital letters with no italics or hyphens (Nelson et al., 1996). P450s with more than 40% identity in amino acid sequence are grouped into one family, and with more than 55% identity are assigned to the same subfamily.

Table 1.1

General overview of cytochrome P450 families/subfamilies and functions in various species (Nelson et al., 1996)

P450	Organism; biochemical function
CYP1	Vertebrates; dioxin inducible; metabolism of polycyclic hydrocarbon, halogenated and heterocyclic hydrocarbons, and aromatic amines
CYP2	Vertebrates and invertebrates; metabolism of environmental chemicals
CYP3	Vertebrates; metabolism of drugs and environmental chemicals
CYP4	Vertebrates, fatty acid hydroxylases; invertebrates, unknown function(s)
CYP5	Vertebrates; thromboxane synthase
CYP6	Insects; metabolism of plant products and pesticides
CYP7A	Vertebrates; cholesterol 7 $\alpha$ -hydroxylase
CYP7B	Vertebrates; unknown function(s)
CYP8	Vertebrates; prostacyclin synthase
CYP9	Insects
CYP10	Mollusks (mitochondria enzyme)
CYP11	Vertebrates; cholesterol side chain cleavage, steroid 11 $\beta$ -hydroxylase, and androsterone synthase (mitochondria enzyme)
CYP12	Insects (mitochondria enzyme)
CYP13	Nematodes
CYP14	Nematodes
CYP15	Insects
CYP16	Nematodes
CYP17	Vertebrates; steroid 17 $\alpha$ -hydroxylase
CYP18	Insects
CYP19	Vertebrates; aromatization of androgens
CYP21	Vertebrates; steroid 21-hydroxylase
CYP24	Vertebrates; steroid 24-hydroxylase (mitochondria enzyme)
CYP27	Vertebrates; steroid 27-hydroxylase (mitochondria enzyme)
CYP51	Animals, filamentous fungi, yeast and plants; sterol synthesis
CYP52	Yeast; alkane hydroxylase
CYP53-62	Fungi
CYP71-92	Plants
CYP73	Plants, cinnamic acid hydroxylase
CYP101-118	Bacteria

## General aspects of cytochrome P450 monooxygenase reaction

Studies on the catalytic activities of P450-dependent monooxygenase in microsomes have revealed that three components are essential to the enzymatic activities: cytochrome P450, NADPH-cytochrome P450 reductase, and phospholipid (Lu and Coon, 1968; Strobel et al., 1970).

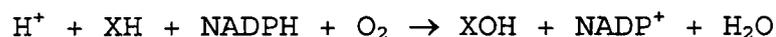
### *Reaction mechanism*

The presence of highly conserved sequences in the functional domains of bacterial and eukaryotic P450s suggests similarities in their monooxygenation activity. The reaction mechanism of P450-dependent monooxygenase activity has been described based on years of studies primarily with the camphor-metabolizing P450 from bacteria called P450cam. The catalytic events for P450 monooxygenase reactions involve the following steps (Mesnil et al., 1984; Guengerich and MacDonald, 1990; Poulos and Raag, 1992):

1. binding of a substrate (XH) to P450 in  $\text{Fe}^{3+}$  state,
2. reduction of NADPH-cytochrome P450 reductase ( $\text{F}_{\text{P1}}$ ) by NADPH,
3.  $\text{Fe}^{3+}$  is reduced to  $\text{Fe}^{2+}$  by accepting an electron from NADPH-cytochrome P450 reductase,
4. binding of molecular oxygen to give  $\text{Fe}^{2+}$  P450-dioxygen complex,

5. acceptance of a second electron from NADPH-cytochrome P450 reductase, or from cytochrome b5, to form a peroxide,
6. cleavage of the O-O bond with addition of the distal oxygen atom into a newly formed water molecule,
7. transfer of the second oxygen atom to the substrate to form the final product (XOH),
8. release of the final product and return of cytochrome P450 to the resting  $\text{Fe}^{3+}$  state.

The overall stoichiometry of the monooxygenase reaction is



A schematic representation of the steps in P450 catalysis is illustrated in Fig. 1.1.

Alternative catalytic mechanisms have been described involving peroxide activation (Guengerich, 1991; Porter and Coon, 1991; Ortiz de Motellano, 1989). In the peroxide shunt, a peroxy compound (ROOH) such as an alkyl hydroperoxide or peracid donates oxygen atom to hydroxylate the substrate with no requirement for  $\text{O}_2$  or for NADPH as an electron donor. The reaction is presented as follows:  $(\text{XH})\text{Fe}^{3+} + \text{ROOH} \rightarrow (\text{XOH})\text{Fe}^{3+} + \text{ROH}$ . Another mechanism is the reductive cleavage of xenobiotic hydroperoxides and lipid peroxide ( $\text{R}'\text{LOOH}$ ) with hydrocarbon formation ( $\text{R}'\text{H}$ ). The reaction is believed to involve stepwise one-electron transfer, resulting in homolysis of the peroxide oxygen-oxygen bond and generation of an alkoxy radical with  $\beta$ -scission of the radical followed by reduction of the second radical to the hydrocarbon. The reaction is summarized as  $\text{R}'\text{LOOH} + \text{XH} +$

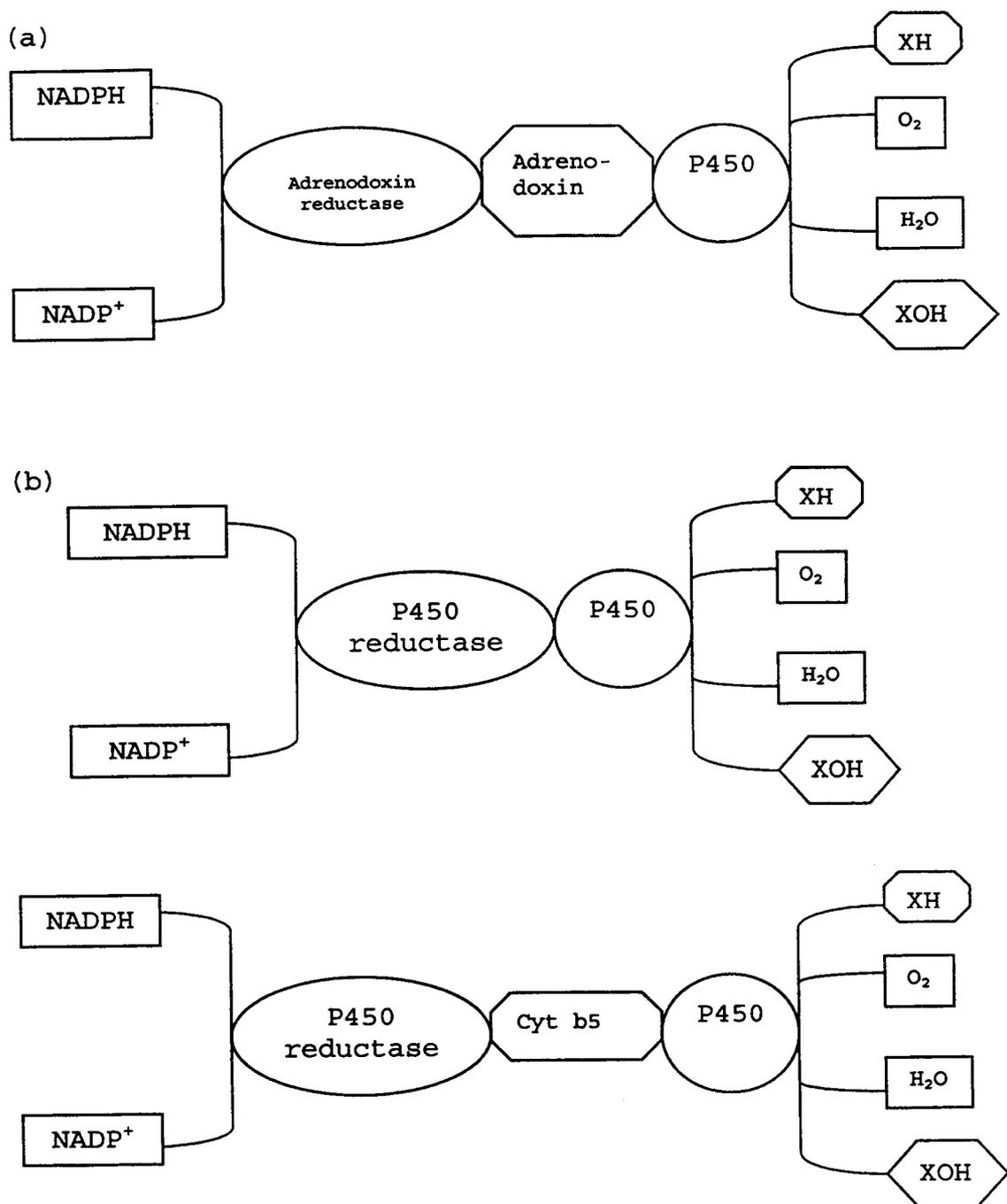


$2e^- \rightarrow \text{XOH} + \text{R}'\text{H} + \text{LO}$ . The reductive cleavage of lipid hydroperoxide has been linked to the loss of membrane integrity (Vaz, et al., 1990).

It is well known that P450s are present in both mitochondria and endoplasmic reticulum (microsomes). In the microsomes, an FMN- and FAD- containing membrane-bound flavoprotein, NADPH-cytochrome P450 reductase, transfers an electron directly from NADPH to P450s, whereas in mitochondria, as well as in most bacteria, a soluble ferredoxin type iron-sulfur protein, adrenodoxin, acts as an electron carrier between the adrenodoxin reductase and P450s. In addition, cytochrome b5 has been shown to participate in transferring electrons from NADPH-cytochrome P450 reductase to certain isoforms of P450 (Imai, 1981; Morgan and Coon, 1984; Jansson et al., 1985). The relationship between NADPH, adrenodoxin and P450 reductases, cytochrome b5, and cytochrome P450 are presented in Fig. 1.2.

#### *Reactions catalyzed by cytochrome P450*

Cytochrome P450 is the most versatile biological catalyst. The major types of reactions and representative substrates are listed in Table 1.2. Although P450s are recognized to be oxygenases, some isoforms also function as reducing catalysts. The biological function of P450 reactions can be grouped according to the types of substrate they metabolize: (1) the synthesis and



**Fig. 1.2** Cytochrome P450-dependent monooxygenase systems of mitochondria, bacteria, (a) and endoplasmic reticulum (b)

Table 1.2

Types of P450 catalyzed reactions and representative substrates (Modified from Stegeman and Hahn, 1994)

Types of Reaction	Representative substrates
Aromatic hydroxylation	benzo[a]pyrene and other polyaromatic hydrocarbons
Aliphatic hydroxylation	n-hexane; n-propylbenzene
Epoxidation	aflatoxin B <sub>1</sub> ; arachidonic acid
N-Dealkylation	aminopyrine; ethylmorphine
O-Dealkylation	7-ethoxyresorufin
S-Dealkylation	methylmercaptan
N-Oxidation	aniline; amphetamine
S-Oxidation	aldicarb
P-Oxidation	parathion
Desulfuration	parathion
Oxidative deamination	arginine
Oxidative dehalogenation	halothane
Reductive dehalogenation	carbon tetrachloride; hexachlorobenzene

degradation of endogenous compounds, and (2) biotransformation of foreign chemicals (xenobiotics). The metabolism of both types of substrate will be discussed below.

### *Metabolism of endogenous compounds*

Cytochromes P450 are involved in many physiologically important pathways. In eukaryotes, the endogenous compounds metabolized by P450s include fatty acid and fatty acid derivatives (lauric acid, arachidonic acid, prostaglandins) (Capdevila et al., 1992), steroids and steroid-derived compounds (cholesterol, corticosteroids, estrogens, androgens, vitamin D, bile acid, ecdysteroids) (Jefcoate, 1986; Waterman et al., 1986), and plant constituents (cinnamic acid, flavonoids) (Kochs and Grisebach, 1989; Kochs et al., 1992). Most P450s can catalyze the oxidation of a large number of naturally occurring and xenobiotic compounds; however, some isoforms of cytochrome P450 involved in steroidogenic pathways are highly selective in their substrates. These steroidogenic P450s include CYP7, CYP11A1, CYP11B1, CYP17, CYP19, CYP21, CYP24 and CYP27. A list of P450 isoforms involved in steroid hormone synthesis is given in Table 1.3.

Saturated (lauric acid) and polyunsaturated fatty acids (arachidonic acid) and prostaglandins have been used as model substrates for many different isoforms of P450. In mammals, hydroxylation of prostaglandin, lauric acid and arachidonic acid at the terminal methyl ( $\omega$ )-position

Table 1.3

Isoforms of cytochrome P450 involved in steroid hormone synthesis

Enzyme	Cell location	Organ location	Reaction catalyzed
CYP11A1 (P450 <sub>scc</sub> )	Mitochondria	Adrenal, gonads, liver	Cholesterol side chain cleavage to yield pregnenolone
CYP11B (P450 <sub>c11</sub> )	Mitochondria	Adrenal	11-Hydroxylase, 18-hydroxylase, and 18-methyloxidase; essential for glucocorticoid and mineralocorticoid synthesis
CYP17 (P450 <sub>c17</sub> )	Endoplasmic reticulum	Adrenal, gonads	17 $\alpha$ -Hydroxylase and 17,20-lyase necessary for androgen and cortisol synthesis
CYP19 (P450 <sub>arom</sub> )	Endoplasmic reticulum	Gonads	Aromatization of testosterone to yield estrogen
CYP21 (P450 <sub>c21</sub> )	Endoplasmic reticulum	Adrenal	21-Hydroxylation necessary for cortisol synthesis

are primarily catalyzed by P450s in the CYP4A subfamily (Aoyama et al., 1990). Hydroxylation at the ( $\omega$ -1)- and ( $\omega$ -2)-positions were also observed (Romano et al., 1988; Roman et al., 1993). Mammalian isoforms in family 1 and 2 such as CYP1A1, CYP1A2, CYP2C2, CYP2C6, CYP2C9, CYP2C11 and CYP2E1 (Falck, et al., 1990; Imai, et al., 1990; Fukuda, et al., 1994; Imaoka, et al., 1996) hydroxylate lauric acid at the ( $\omega$ -1)-position. Microsomes from liver of rainbow trout and sea bass hydroxylate lauric acid at several subterminal positions, ( $\omega$ ), ( $\omega$ -1) to ( $\omega$ -6) for rainbow trout (Buhler et al., 1997), and ( $\omega$ ), ( $\omega$ -1) to ( $\omega$ -4) for sea bass (Lemaire et al., 1992). A recent report also showed that CYP2N1, CYP2N2 and CYP2P3 from killifish (*Fundulus heteroclitus*) metabolize arachidonic acid to produce epoxyeicosatrienoic acids (EETs) and hydroxyeicosatrienoic acids (HETEs) (Oleksiak et al., 1997).

### *Metabolism of xenobiotics*

Four families of cytochrome P450 (CYP1 to CYP4) are the major enzymes for the catalysis of xenobiotic metabolism (Gonzalez, 1990). These P450s can metabolize a wide variety of structurally diverse xenobiotic compounds. Substrates include drugs, industrial chemicals and byproducts, carcinogens, pesticides, and fungal- and plant-derived compounds. The P450 superfamily exhibits broad and overlapping substrate specificities and regioselectivities (multiple sites of oxidation) and thus

can metabolize almost any compound (mostly lipophilic) to which they are exposed (Coon et al., 1996).

The functional properties of cytochrome P450 in metabolizing foreign compounds can be grouped into three categories: 1. toxification and detoxification of environmental chemicals; 2. drug metabolism which includes termination of drug action (e.g., warfarin), facilitation of drug elimination, prodrug activation (e.g., cyclophosphamide) and drug toxication; and 3. carcinogen activation. Many biologically-inert carcinogens require bioactivation by cellular enzymes prior to exerting their damaging effects. The exposure to these reactive electrophilic metabolites results in cell toxicity and death during high dose exposure or causes cell transformation resulting from mutations in genes encoding proteins for controlling cell growth during sublethal dose exposure. It is generally believed that metabolism of chemical carcinogens play an important role in the development of cancer in animals. Representative chemical carcinogens metabolized by cytochromes P450 are listed in Table 1.4. (Guengerich, 1988, 1993; Gonzalez and Gelboin, 1994; Buhler, 1995; Gooneratne et al., 1997; You et al., 1997).

### **Cytochrome P450 in rainbow trout**

As in mammals, cytochromes P450 are the major enzymes involved in the metabolism of both endogenous and exogenous compounds in rainbow trout and other aquatic

Table 1.4

Representative chemical carcinogens metabolized by cytochromes P450

Chemicals	Representative chemical carcinogen	P450
Polycyclic aromatic hydrocarbons (PAH)	Benzo(a)pyrene (BP) Dimethylbez(a)anthracene (DMBA)	CYP1A1, CYP2B1, CYP1A3
Aromatic amines	2-Aminofluorene, 4-Aminobiphenyl	CYP1A2
Nitroaromatic hydrocarbons	N,N-Dimethylnitrosamine and its derivatives	CYP2E1
Hydrazine	Procarbazine	CYP1A1, CYP2C6
Halogenated hydrocarbons	Chloroform, Ethylene dibromide, 1,1,1-Trichloroethane	CYP2E1
Miscellaneous chemical carcinogens	Aflatoxin B <sub>1</sub>	CYP1A*, 1A2, 2A6, 2B1, 2B6, 2B7, 2C11, 2C12, 2K1, 3A4
	Senecionine and other certain pyrrolizidine alkaloids	CYP3A4
	Benzene	CYP2E1

\* CYP1A from rainbow trout.

species (Buhler and Williams, 1989; Goksøyr and Förlin, 1992, Guengerich, 1992, Stageman and Hahn, 1994). The catalytic activities of fish P450s are generally lower than in mammals and the temperature optima is about 25°C instead of 37°C.

### *CYP1 family*

**CYP1A subfamily.** Members of the CYP1A subfamily have been the most intensively investigated P450s in mammalian and fish species because of their induction by many environmental carcinogens such as polycyclic aromatic hydrocarbons (PAHs) and planar halogenated aromatic hydrocarbons, including polychlorinated biphenyls (PCBs) and 2,3,7,8-tetrachlorodibenzodioxin (TCDD). Many CYP1A-like proteins have been purified from several fish species, including rainbow trout (Williams and Buhler, 1982, 1984; Gooneratne et al., 1997), scup (Klotz et al., 1983), cod (Goksøyr, 1985), and perch (Zhang et al., 1991). CYP1A cDNAs have been cloned from rainbow trout (Heilmann et al., 1988; You et al., 1997), plaice (Leaver et al., 1993), red sea bream (Mizukami et al., 1994), toadfish, scup (Morrison et al., 1995), 4-eye butterfly fish (Roy et al., 1995), tomcod, sea bass, and ice cod (Nelson et al., 1996).

Two CYP1A P450s, CYP1A1 and CYP1A2, have been isolated from mammalian species. Based on the P450 phylogenetic tree, mammalian CYP1A1 and CYP1A2 originated by gene duplication and evolved after fish diverged from

land animals (Nelson et al., 1996). It was originally hypothesized that only one CYP1A gene exists in fish (Nebert and Gonzalez, 1987). This theory was supported by Heilmann et al. (1988) who cloned one CYP1A cDNA from rainbow trout and designated it as CYP1A1. The dispute over the existence of a second CYP1A gene in rainbow trout was not resolved until two CYP1A genes were cloned from rainbow trout by Berndtson and Chen (1994). A recent revision by the P450 Nomenclature Committee has designated CYP1A genes from Berndtson and Chen as CYP1A1 and CYP1A3 (Nelson et al., 1996) and Heilmann's CYP1A cDNA as CYP1A1/CYP1A3 chimera, possibly a cloning artifact. CYP1A1 and CYP1A3 shared 96% identity in the coding region; however, their 5'flanking sequences are significantly different, only 37% homologous. The presence of CYP1A1 and CYP1A3 gene products was confirmed in a recent report by Gooneratne et al. (1997) who isolated two CYP1A proteins HAP 1 and HAP 2 from liver of BNF-treated rainbow trout. The N-terminal amino acid sequences (determined by Edman degradation) of these purified proteins were compared with those predicted from the CYP1A1 and CYP1A3 reported by Berndtson and Chen. CYP1A1 and CYP1A3 are identical in the first 24 amino acid residues, except for the residues 14 and 21. The N-terminal region of HAP 1 is identical to that of CYP1A1 in the first 24 amino acids and HAP 2 is identical to that of CYP1A3 in the first 14 amino acids. The authors concluded that HAP 1 and HAP 2 correspond to CYP1A1 and CYP1A3, respectively. Based on the elution order obtained by DEAE-Sepharose chromatography and the estimate of amino

acid composition, two previously purified CYP1As from BNF-treated rainbow trout, LM4a and LM4b (Williams and Buhler, 1984) probably correspond to CYP1A3 and CYP1A1, respectively (Gooneratne et al., 1997).

Enzymatic activities of rainbow trout CYP1As have been characterized using purified or cDNA-directed yeast-expressed proteins to metabolize various substrates. LM4a and LM4b purified from BNF-treated rainbow trout did not show differential activities in metabolizing benzo(a)pyrene and acetanilide (Williams and Buhler, 1984). Similarly, HAP 1 and HAP 2 had identically high activities toward 7,12-dimethylbenz[a]anthracene (DMBA), 7-ethoxyresorufin, methoxyresorufin, and phenacetin (Gooneratne et al., 1997). Further investigation showed that no qualitative difference in the DMBA metabolite profile was obtained from the two trout CYP1A forms (Miranda et al., unpublished). Trout CYP1A1 variants (v2 and v3) derived from cDNA-directed expression in yeast exhibited distinct but similar ethoxyresorufin O-deethylase (EROD) activity and metabolized aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) to AFM<sub>1</sub> with similar but not identical K<sub>m</sub> value, 20 ± 3 μM for v2 and 13 ± 2 μM for v3 (You et al., 1997). In mammals, AFB<sub>1</sub>, methoxyresorufin, and phenacetin are used as selective diagnostic substrates for CYP1A2 (Nerurkar et al., 1993; Xiaodong et al., 1994). On the basis of amino acid comparison and substrate selectivity of trout and mammalian CYP1As, trout CYP1As appear to have overlapping substrate specificities, exhibiting the characteristics of both mammalian CYP1A1 and CYP1A2. These observations imply that these CYP1As evolved from a common ancestral

gene. A comparison between P450 phylogenetic tree and the evolutionary timescale revealed that CYP1A2 arose from gene duplication in land animals around 135 million years ago (Lewis, 1996) and the CYP1A3 gene diverged from trout CYP1A1 very recently, about 12-14 million years ago (Nelson et al., 1996).

### *CYP2 family*

**CYP2K subfamily.** The major constitutive cytochrome P450 (LM2) was purified from liver of BNF-treated rainbow trout (Williams and Buhler, 1984). Subsequently, a P450 isoform (LMC2) was isolated from livers of untreated rainbow trout (Miranda et al., 1989). These two proteins are indistinguishable biochemically and immunologically as shown by the same electrophoretic mobility on SDS-PAGE gel (54,000 Da) and Western blot analysis. They also exhibited identical CO-difference spectra with maximum absorption at 450 nm.

LMC2 was highly active toward various exogenous and endogenous compounds. This isoform hydroxylates lauric acid mainly at the ( $\omega$ -1)-position with a small amount of ( $\omega$ -2)-hydroxylation also produced (Buhler et al., 1997). It also exhibits progesterone 16 $\alpha$ -hydroxylase, testosterone 16 $\beta$ -hydroxylase, 17 $\beta$ -estradiol 2-hydroxylase and benzphetamine N-demethylase (BND) activities (Miranda et al., 1989). P450 LMC2 also catalyzed the activation of AFB<sub>1</sub> to its electrophilic carcinogenic form, AFB<sub>1</sub>-8,9-epoxide, which then binds covalently to DNA (Williams and

Buhler, 1983a). The DNA adduct formed by AFB<sub>1</sub> in trout was further identified as 8,9-dihydro-8-(N<sup>7</sup>-guanyl)-9-hydroxyafatoxin B<sub>1</sub> (AFB<sub>1</sub>-N<sup>7</sup>-GUA) the same as produced in mammals (Croy et al., 1980; Bailey et al., 1988). This is important because it may help confirm the utility of rainbow trout as a model for AFB<sub>1</sub>-induced human carcinogenesis. The (ω-1)-hydroxylase activity of lauric acid, BND activity and AFB<sub>1</sub>-DNA adduct formation mediated by trout microsomes or purified LMC2 were significantly inhibited by rabbit polyclonal antibodies raised against P450 LMC2 (Miranda, et al., 1989; Buhler et al., 1997). In a previous study, CYP1A-specific inhibitors (ANF and BNF) and the CYP1A1 peptide antibodies also inhibited trout microsomal lauric acid (ω-1)-hydroxylase activity and AFB<sub>1</sub>-DNA binding catalyzed by P450 LMC2 (Takahashi et al., 1995). The configuration similarity at, or near the active sites for the trout LMC2 and CYP1A has been suggested to be responsible for the antibody cross-reactivity and the loss of the inhibitor specificity observed in mammalian CYP1A.

To further characterize this P450, a full-length cDNA clone has been isolated by screening a 3-yr old male rainbow trout liver cDNA library with rabbit anti-LMC2 polyclonal and anti-LMC2 monoclonal antibodies (3M1 and 3M3). This 1,859 bp cDNA contained an open reading frame encoding a protein of 504 amino acids with a calculated molecular weight of 56,795 Da. Based on amino acid sequence comparison, this trout P450 has been assigned by the P450 Nomenclature Committee to a new cytochrome P450 subfamily designated as CYP2K1 (Buhler et al., 1994).

Northern and Western blot analysis revealed marked differences in the sex- and organ-specific expression of CYP2K1 at both the transcriptional and translational levels in the liver and trunk kidney of sexually mature trout. A 440-bp 3'-terminal cDNA probe (2K1,7c) was used in Northern blot analysis. Trunk kidney from sexually mature male trout expressed 2K1,7c hybridizable mRNA to a significantly greater level than did trunk kidney from females, with multiple mRNA transcripts appearing in the 1.9- and 2.8-Kb region. Bands of 2.8 Kb were detected in the livers of sexually mature male trout and were generally undetectable in the female liver samples. By contrast, 1.9-Kb mRNA bands were expressed at similar level in the liver of juvenile trout from both sexes.

Western blot analysis of microsomal protein from liver and trunk kidney of sexually mature rainbow trout probed with polyclonal and two different monoclonal anti-LMC2 antibodies exhibited similar sex- and tissue-related differences. Furthermore, Southern blotting of rainbow trout genomic DNA suggested the existence of multiple copies of CYP2K1-related genes.

The hepatic expression of CYP2K1 mRNA and protein at the early developmental stages was also investigated (Buhler-Wang et al., 1997). Total RNA and protein from liver of 30-day embryos (one day before hatching), 2-day and 10-day (51 days after fertilization) sac-fry, demonstrated a progressive increase in CYP2K1 expression indicated by Northern and Western blot analysis. The levels of CYP2K1 protein expressed in 30-day embryo, 2-day sac fry, 10-day sac fry and 1-yr old trout liver were

0.035 pmole/ $\mu$ g DNA, 0.08 pmole/ $\mu$ g DNA, 0.12 pmole/ $\mu$ g DNA and 0.9 pmole/ $\mu$ g DNA, respectively.

### *CYP3 family*

**CYP3A subfamily.** P450 LMC5 was purified from livers of untreated rainbow trout to near homogeneity with a specific content of 14.9 nmole/mg protein (Miranda et al., 1989). This P450 isoform had a relative molecular weight of 59,000 Da on SDS-PAGE gel and a maximum absorption in the CO-reduced difference spectrum at 448 nm. In the reconstituted P450 LMC5 system, this P450 exhibited significant activity for 6 $\beta$ -hydroxylation of testosterone and progesterone and for the N-demethylation of benzphetamine (Miranda et al., 1989).

The 6 $\beta$ -hydroxylation activity of steroids is associated with CYP3A enzymes in mammals. To further determine the relationship between P450 LMC5 and mammalian CYP3A isoforms, Western blot analysis using rabbit anti-LMC5 polyclonal antibodies showed strong cross-reactivity toward CYP3A1 from rat liver microsomes and CYP3A4 from human liver microsomes (Miranda et al., 1991). Reciprocal immunoblot analysis showed that anti-human CYP3A4 IgG reacted strongly with P450 LMC5; however, anti-rat CYP3A1 antibody did not recognize P450 LMC5. Anti-CYP3A4 antibody and a mechanism-based inhibitor of CYP3A4, gestodene, significantly inhibited progesterone 6 $\beta$ -hydroxylase activity of trout liver microsomes. These

data suggest the similarity in the structure and function between P450 LMC5 and human CYP3A4.

A 1,802-bp cDNA clone was isolated from a female liver cDNA library using anti-LMC5 polyclonal antibody (Lee et al., 1997). This cDNA contained a complete open reading frame encoding a protein of 518 amino acids with a calculated molecular weight of 59,206 Da. Based on amino acid sequence comparison, this cDNA has been assigned to CYP3A subfamily as CYP3A27 by the P450 Nomenclature Committee. The deduced N-terminal sequence of CYP3A27 agreed closely with that of purified P450 LMC5. Northern blot analysis exhibited marked differences in the age-, sex- and tissue-specific expression of this gene. Two major CYP3A27 cDNA hybridizable bands were detected at 2.8 and 4 Kb. The 2.8 Kb transcript was expressed in the small intestine, liver, ovary, stomach, and trunk kidney of 2-yr old female trout and in the liver, testes, stomach and trunk kidney of 2-yr old male trout. Expression of 2.8 Kb mRNA increased with age in the male rainbow trout, reaching maximum in 3-yr sexually mature fish. However, this 2.8 Kb mRNA was barely detectable in the sexually mature female trout. The 4 Kb CYP3A27 hybridizable mRNA was mainly expressed in liver of sexually immature males and had very weak expression in the liver of sexually mature males.

*CYP4 family*

**CYP4T subfamily.** A new hepatic rainbow trout cytochrome P450 has been isolated recently by RT-PCR using degenerate primers designed from the highly conserved regions of P450s in the CYP4 family (Falckh et al., 1997). The resulting PCR product was 390 bp encoding a peptide of 130 amino acid residues. Sequence comparison showed 55.4% identity to rat CYP4B2 and 54.6% identity to rabbit CYP4B1, respectively. Based on the homology of this peptide to other members in the CYP4 family, this P450 gene was designated as CYP4T1 by the P450 Nomenclature Committee. This cytochrome P450 represents the first identified as a CYP4 family member in an aquatic vertebrate; however, the full-length cDNA sequence and catalytic activity of CYP4T1 remain to be determined.

*Steroidogenic cytochrome P450s*

**CYP11A1 (P450scc).** The cholesterol side-chain cleavage P450 (P450scc or CYP11A1), one of the steroidogenic enzymes, has been cloned from a rainbow trout ovary cDNA library using human P450scc cDNA as a probe (Takahashi et al., 1993). The cDNA contained an open reading frame encoding a protein of 514 amino acids. The predicted amino acid sequence of trout P450scc showed 48% homology with that of human P450scc, and 46% with that of isoforms from rat, bovine and pig. cDNA-directed expression of trout P450scc in COS-1 cells (monkey kidney

cells) produced a protein converting cholesterol to pregnenolone which is a characteristic catalytic activity of P450<sub>scc</sub> (CYP11A1). The trout CYP11A1 cDNA only hybridized to a single 1.8 Kb mRNA transcript expressed most abundantly in postovulatory follicles.

**CYP17 (P45017c).** A cDNA clone encoding P45017c was isolated from a rainbow trout ovarian follicle cDNA library using human CYP17c cDNA as a probe (Sakai et al., 1992). The CYP17c cDNA from rainbow trout contained an open reading frame of 1,542 bp encoding a protein of 512 amino acids. The deduced amino acid sequence showed the highest homology to that of chicken CYP17c isoform with 64% identity. Heterologous expression of CYP17 in COS-1 cells exhibited 17 $\alpha$ -hydroxylase and 17,20-lyase activities, as observed in mammalian CYP17 isoforms which are involved in the synthesis of steroid hormones. Northern blot analysis showed that only one 2.4 Kb mRNA transcript was detected in the early- and mid-vitellogenic stages of female maturation while abundant levels of CYP17c were expressed in the post-vitellogenic stage and after ovulation.

**CYP19 (P450arom).** A cDNA encoding CYP19 (P450arom) was isolated from a rainbow trout ovary cDNA library using a full-length human CYP19 cDNA as a probe (Tanaka et al., 1992). The cDNA clone contained an open reading frame encoding a protein of 522 amino acids. The predicted amino acid sequence shared 52% homology with human, mouse and rat CYP19 and 53% with that of the chicken isoform.

The characteristic enzyme activity of CYP19, conversion of testosterone to  $17\beta$ -estradiol, was demonstrated by expressing trout CYP19 in COS-1 cells. Northern blot analysis of total RNA from trout ovaries at various stages revealed that a 2.6 Kb CYP19 hybridizable mRNA was detected at the early and late vitellogenic stages. No CYP19 RNA transcript was expressed in the later stage of ovulation.

#### *Other cytochrome P450s in rainbow trout*

**P450 LMC1.** P450 LMC1 was purified from liver of untreated rainbow trout to a specific content of 11.6 nmole/mg protein (Miranda et al., 1989). This protein exhibited a characteristic CO-difference spectrum with maximum absorption at 450 nm. Based on the electrophoretic mobility on SDS-PAGE compared to molecular weight standards, the apparent molecular weight of P450 LMC1 was estimated to be 50,000 Da. Rabbit anti-LMC1 and anti-LMC2 polyclonal antibodies cross-reacted to one another and anti-LMC1 IgG reacted strongly to rat CYP2B1 (Miranda, et al, 1990). The catalytic activity of P450 LMC1 toward various substrates was examined in reconstitution systems supplemented with rat NADPH-cytochrome P450 reductase, rabbit cytochrome b5 and DLPC. This P450 isoform had modest activity toward  $17\beta$ -estradiol, progesterone and testosterone, but activity toward benzo(a)pyrene and benzphetamine was not detected (Miranda et al., 1989). However, P450 LMC1 showed high lauric acid

hydroxylase activity resulting in a hydroxylation product identified as ( $\omega$ -6)-hydroxylauric acid (Buhler et al., 1997). Anti-LMC1 antibodies strongly inhibited the metabolism of lauric acid in microsomes and reconstitution systems.

**P450 LMC3.** P450 LMC3 was partially purified from the liver of untreated rainbow trout with a specific content of 2.8 nmole/mg protein (Miranda et al., 1989). This P450 isoform had a reduced CO-difference maximum at 450 nm and an estimated molecular weight of 56,000 Da as determined by SDS-PAGE. P450 LMC3 was highly unstable, rapidly being converted to P420 during the P450 spectrum measurement and the reconstitution assay was impossible to perform. Rabbit polyclonal antibodies raised against P450 LMC3 failed to show any inhibition effects on the metabolism of lauric acid, testosterone or  $17\beta$ -estradiol in microsomal incubations. However, anti-P450 LMC3 IgG inhibited the formation of an unidentified highly polar metabolite of progesterone in the incubation mixture containing untreated trout liver microsomes (Miranda and Buhler, unpublished data). The substrate specificity of this enzyme remains to be determined by cloning the P450 LMC3 cDNA and subsequent heterologous expression.

**P450 LMC4.** P450 LMC4 was isolated from liver of untreated rainbow trout with a specific content of 9.0 nmole/mg protein and an apparent molecular weight of 58,000 Da (Miranda et al., 1989). P450 LMC4 was recognized by anti-LMC3 IgG in the ELISA assay (Miranda, et al.,

1990). Rabbit polyclonal antibodies raised against P450 LMC4 did not inhibit the enzymatic activities of trout liver microsomes toward lauric acid, testosterone, or  $17\beta$ -estradiol but inhibited the formation of an unidentified polar metabolite of progesterone (Miranda and Buhler, unpublished data).

**P450con.** P450con was partially purified from liver of rainbow trout treated with various inducers with an apparent molecular weight of 54,000 Da (Celander et al., 1989) Immunological analysis showed that P450con is very similar if not identical to the P450 LMC5 which belongs to the CYP3A subfamily (Celander et al., 1996).

**P450 KM1 and KM2.** Two cytochrome P450s have been isolated from trunk kidney of sexually mature male rainbow trout designated as P450 KM1 and KM2 with estimated molecular weights of 54,000 and 52,000 Da, respectively (Andersson, 1992).

Rabbit polyclonal antibodies raised against P450 KM2 did not recognize the P450 KM1 isoform (Andersson, 1992). Western blot analysis revealed that this KM2 constituted about 66% of total P450 in the kidney of sexually mature male trout and 15% of total P450 in sexually mature female kidney. P450 KM2 was inducible by 11-ketotestosterone but not by testosterone. Polyclonal antibodies raised against LMC1 and LMC2 cross-reacted with KM2 (Miranda et al., unpublished observation).

## **cDNA-directed expression of cytochromes P450**

The cDNA-directed expression of cytochrome P450 in various prokaryote and eukaryote systems has provided a powerful tool in the study of structure and function of the cytochrome P450 superfamily. The catalytic activity and other properties of expressed P450 can be studied under conditions in which the activities of other enzymes do not interfere. It is especially important when closely related proteins are present and can not be detected or discriminated. Reliable expression is essential for the study of structure and function through site-directed mutagenesis and it also provides large quantity of protein for biophysical studies (e.g. NMR, crystallization and X-ray diffraction). For some proteins that are difficult to obtain by conventional isolation and purification methods, cDNA-directed expression provides the means to an unlimited source of that protein. Furthermore, expression systems can be used to add the protein of interest into a particular type of cell to study the effect of that protein on the cells.

Two types of expression systems have been used to study P450s. Transient expression systems involve transfecting or infecting cells with recombinant plasmid or virus, respectively; and eventually the cells die. Stable expression systems have a fixed or integrated plasmid that does not kill the cells.

### *Transient expression system*

**P450 expression in COS cells.** The African green monkey kidney cells were the first mammalian cells used for P450 expression (Zuber et al., 1986). COS cells express the SV40 T antigen and are capable of supporting replication of expression vectors containing the SV40 origin of replication. This system is fast and easy to use. The cDNA is cloned to an expression vector under the control of an appropriate promoter and no modification is necessary. The enzyme activity can be determined 2-3 days after transfection. COS cells contain adequate NADPH-cytochrome P450 reductase and cytochrome b5 to support the P450 activity. A disadvantage of this system is the low level of expression, on the order of a few pmole per mg of microsomal protein. Additionally, the total P450 content is not easily determined using the CO-difference spectral method. However, it does provide a good screening tool for P450 enzyme assays.

**P450 expression in vaccinia virus.** Vaccinia virus has been used to express several mammalian P450s (Gonzalez, et al., 1991). Vaccinia virus contains a large genome of almost 200,000 bp and requires homologous recombination for the generation of recombinant virus. Once the P450 cDNA-containing virus has been established, it can be used to infect mammalian cell lines. HepG2 cells are the preferred choice because they have ample NADPH-P450 reductase, cytochrome b5 and endoplasmic reticulum. Modification of the cDNA is not required since

5'- and 3'-untranslated regions do not interfere with the levels of expression. Fifteen to twenty-five pmole/mg total cellular protein have been obtained for different P450s (Gonzalez and Korzekwa, 1995). The disadvantage of this system is the length of time required to generate a recombinant virus, ranging from one to several months.

**P450 expression in baculovirus-insect cells.** The baculovirus-insect cell expression system has gained widespread popularity in the expression of P450 and other proteins (Lee et al., 1996). Recombinant virus generation is similar to that for vaccinia virus due to the large genome size. The DNAs of two commonly used baculoviruses, *Autographa californica* nuclear polyhedrosis virus (AcMNPV) and *Bombyx mori* nuclear polyhedrosis virus (BmNPV) are both approximately 130,000 bp. The expression of insert cDNA is driven by the strong viral polyhedrin or p10 gene promoter in recombinant virus. Vectors have been developed that contain multiple cloning sites and multiple promoters allowing the simultaneous expression of two or more proteins in a single recombinant virus. cDNA modification is not required. The time and effort involved in recombinant virus generation have been reduced by the use of linearized viral DNA (Kitts and Possee, 1993) and the bacmid system (Luckow et al., 1993). Several insect cell lines have been developed, Sf9 and Sf21 from fall armyworm, and BTI-TN-5BI-4 (High Five) from cabbage looper.

The biggest advantage of the baculovirus expression system is the high level of expression (100-1000 pmole/mg

total cell lysate) obtained with many recombinant proteins when hemin or 5-aminolevulinic acid/iron-citrate is used as a supplement. The percentage of apoproteins that have proper heme incorporation is quite variable, ranging from 40% to over 90%. The reason for this variability is unknown (Gonzalez and Korzekwa, 1995). A disadvantage of this system is the lack of endogenous NADPH-cytochrome P450 reductase and cytochrome b5 in the host cells. Reconstitution of P450 with reductase and sometimes cytochrome b5 is necessary in the enzyme assays. These two enzymes (NADPH-cytochrome P45 reductase and cytochrome b5) can also be efficiently expressed using baculovirus (Lee et al., 1995).

#### *Stable expression systems*

**P450 expression in bacteria.** Bacteria have been used to produce several P450s (Gonzalez and Korzekwa, 1995). High-level expression of P450 can not be achieved without modifications of the N-terminal region of the enzyme (Li and Chaing, 1991). The second codon of the P450 is changed to that encoding an alanine along with a series of silent base changes in the first eight codons to make the 5'-end of the mRNA AT rich, similar to other *E. coli* mRNAs (Stormo et al., 1982). The N-terminal modification would not be expected to alter catalytic activity, since one can remove the N-terminal hydrophobic region of P450s and maintain enzymatic activities (Larson et al., 1991; Li and Chaing, 1991; Barnes et al., 1991). Yields of modified

P450s ranging from 20 to 700 nmole of P450 per liter of bacteria culture have been reported (Sandhu et al., 1993; Fisher et al., 1992). P450 expressed in *E. coli* must be reconstituted with NADPH-cytochrome P450 reductase and cytochrome b5 (for certain P450s) for the measurement of enzymatic activity.

**P450 expression in yeast.** A yeast system was first used to stably carry out the cDNA-directed expression of P450 (Oeda et al., 1985; Sakaki et al., 1985). The levels of expression reported range from subnanomole to hundreds of nanomole per liter (Gonzalez and Korzekwa, 1995). The disadvantage of yeast systems is that certain P450s may not be expressed as well as others and proper expression vector design is important. For example, nontranslated regions of cDNA could inhibit proper transcription and translation (Pompon 1988). In addition, the microsomal environment might not precisely reflect that of higher eukaryote cells, although most data suggest that yeast-expressed mammalian P450s have activities predicted from studies with microsomal and purified enzymes (Gonzalez and Gelboin, 1994).

**P450 expression in B lymphoblastoid cells.** The B lymphoblastoid cell line has been very useful in expressing human P450s. This Epstein-Barr virus (EBV) transformed cell line allows the extrachromosomal replication of a plasmid vector containing the origin of replication of EBV (Gonzalez, et al., 1991). Multiple P450 cDNAs can be expressed simultaneously and used to

determine mutagenicity of potential carcinogens (Crespi et al., 1991). The disadvantage of this system is the variability in levels of the expression of different P450 isoforms.

**P450 expression in V79 cell.** Chinese hamster V79 cells have been developed to stably expressed P450s because of their widespread use in genetic toxicology and eukaryote mutagenesis analysis (Doehmer and Oesch, 1991; Doehmer, 1993). The cells are transformed using standard SV40-derived expression vector containing a selectable marker. Spontaneous integration occurs and positive clones are selected. V79 cells also contain an ample amount of NADPH-cytochrome P450 reductase. The disadvantage of this system is the low levels of expression and the difficulty of spectral quantitation of P450.

**P450 expression in retrovirus.** Only a limited number of P450s have been expressed using retroviruses (Battula, 1989). The cDNA is stably integrated into the genome of the host cells. The recombinant virus can be used to infect most mammalian cell lines. In most cases, a single copy of the cDNA is inserted to the chromosome, and its integration site probably determines the level of P450 expression. The amount of expression is low, a value of 10 pmole/mg microsomal protein has been reported (Dai et al., 1993). This system has not been used widely because of the complexity and the time required for isolating an expressing clone.

## CHAPTER 2

**CYP2M1: Cloning, Sequencing and Expression of a New  
Cytochrome P450 from Rainbow Trout Liver with Fatty Acid  
( $\omega$ -6)-Hydroxylation Activity**

Yea-Huey Yang<sup>1,2</sup>, Jun-Lan Wang<sup>2</sup>, Cristobal L. Miranda<sup>2</sup> and  
Donald R. Buhler<sup>1,2,3</sup>

<sup>1</sup>Genetics Program, <sup>2</sup>Department of Agricultural Chemistry,  
and <sup>3</sup>Marine/Freshwater Biomedical Sciences Center  
Oregon State University  
Corvallis OR 97331

Abbreviated Title: Cloning of Rainbow Trout CYP2M1

Correspondence to:

Dr. Donald R. Buhler  
Department of Agricultural Chemistry  
Oregon State University  
ALS 1007  
Corvallis OR 97331-7301

Submitted to Archives of Biochemistry and Biophysics,  
11/97

### Abstract

A cDNA clone was isolated from a female rainbow trout liver  $\lambda$ -gt11 library using polyclonal antibodies raised against rainbow trout cytochrome P450 LMC1. This clone consisted of 2,149 nucleotides and contained an open reading frame encoding a protein of 499 amino acids with a calculated MW of 56,850 Da. On the basis of the P450 sequence comparisons, this rainbow trout P450 was assigned by the P450 Nomenclature Committee to a new P450 subfamily designated as CYP2M1. Northern blot results suggest that the expression of CYP2M1 at the transcriptional level was generally sex, tissue and age specific. By use of a full-length CYP2M1 cDNA probe, it was observed that this cDNA hybridized strongly to a single 2.2 Kb transcript in juvenile female rainbow trout trunk kidney, and to liver from juvenile and sexually mature trout from both sexes. Negligible amounts of mRNA hybridizable to CYP2M1 cDNA were found in the juvenile and sexually mature male trunk kidney. cDNA-directed expression in COS-7 cells and of recombinant baculovirus in insect cells produced a protein that was reactive with rabbit anti-trout P450 LMC1 polyclonal antibody and exhibited the unique ( $\omega$ -6)-hydroxylation toward lauric acid previously observed with rainbow trout P450 LMC1.

## Introduction

Cytochromes P450 constitute a superfamily of heme-thiolate proteins that play important roles in oxidative metabolism of endogenous and exogenous compounds. As seen in mammals, multiple forms of P450 were found in fish (Miranda et al., 1989). At least five constitutive forms (P450 LMC1 to LMC5) and two  $\beta$ -naphthoflavone (BNF)-inducible forms (a CYP1A1 ortholog and CYP1A3) from the liver of juvenile rainbow trout (*Oncorhynchus mykiss*) have been isolated and characterized (Williams and Buhler 1983, 1984; Miranda et al., 1989; Gooneratne et al., 1997). One of the constitutive cytochrome P450s, P450 LMC2, was subsequently cloned, sequenced and assigned as CYP2K1 (Buhler et al., 1994). Three steroidogenic cytochrome P450s cDNA also have been cloned from the ovary of rainbow trout and their catalytic activities determined (Sakai et al., 1992; Tanaka et al., 1992; Takahashi et al., 1993).

Purified rainbow trout P450 LMC1 had an apparent molecular weight of 50,000 Da based on SDS-PAGE mobility and molecular weight standards (Miranda et al., 1989). Reconstitution experiments with purified LMC1 exhibited modest activity toward  $17\beta$ -estradiol, progesterone, and testosterone. However, reconstituted LMC1 showed higher activity toward lauric acid (Miranda et al., 1989, 1990b). Lauric acid has been used widely as a diagnostic model substrate in studies of the regioselectivity of P450-mediated fatty acid hydroxylation (Straub et al., 1993). Thus, different P450s have shown distinct regiospecificity in the hydroxylation of lauric acid. Cytochrome P450s from

human and rat liver microsomes are involved in the hydroxylation of lauric acid and other fatty acids at the ( $\omega$ -) and ( $\omega$ -1) positions (Cajacob et al., 1988; Castle et al., 1995). Human and rat CYP2E1 have high lauric acid ( $\omega$ -1)-hydroxylation activity (Imaoka et al., 1996). In mammals, members of the CYP4A subfamily are responsible for the  $\omega$ -hydroxylation of lauric acid (Aoyama et al., 1990). Microsomes from the liver of sea bass (*Dicentrarchus labrax*), however, metabolized lauric acid at five different sites: ( $\omega$ -), ( $\omega$ -1), ( $\omega$ -2), ( $\omega$ -3), and ( $\omega$ -4) (Lemaire et al., 1992). Recently, hepatic microsomes from yearling juvenile rainbow trout were shown to form ( $\omega$ -), ( $\omega$ -1)-, ( $\omega$ -2)-, ( $\omega$ -3)-, ( $\omega$ -4)-, ( $\omega$ -5)- and ( $\omega$ -6)-OH lauric acid oxidation products (12-OH, 11-OH, 10-OH, 9-OH, 8-OH, 7-OH and 6-OH lauric acid, respectively) as verified by GC/MS analysis (Buhler et al., 1997). Reconstituted systems were also used to show that rainbow trout P450 LMC1 (CYP2M1, as shown in this study) was responsible for the oxidation of lauric acid at the ( $\omega$ -6)-position while CYP2K1 (P450 LMC2) mainly formed ( $\omega$ -1)-OH lauric acid together with a smaller quantity of the ( $\omega$ -2)-hydroxylation product.

To further investigate the nature of rainbow trout P450 LMC1 and its expression in liver and trunk kidney, we have isolated and cloned a full-length cDNA encoding for this protein. Based on the deduced amino acid sequence, rainbow trout hepatic cytochrome P450 LMC1 has been assigned to a new P450 subfamily, designated cytochrome P450 2M1 or CYP2M1. A functional recombinant CYP2M1 enzyme was successfully expressed in COS-7 cells and also in insect

cells (*Spodoptera frugiperda*, Sf9) that exhibited the same properties as purified P450 LMC1 including identical regiospecific catalytic activity toward lauric acid.

## Materials and Methods

### Experimental animals

Juvenile (1-yr old and weighed 100-300 g) and sexually mature (3-yr old and weighed 3-4 Kg) post-spawning male and female rainbow trout (*Oncorhynchus mykiss*) of the Mt. Shasta strain were raised in the Marine/Freshwater Biomedical Sciences Center aquatic facility of Oregon State University and used in this study.

### Materials

Restriction enzymes (BamHI, HindIII, KpnI, SphI, SpeI, NotI, SstI, EcoRI) were from Stratagene (La Jolla, CA) or New England Biolab (Beverly, MA). The Gigapack lambda package system, random primer labeling kit (Prime-it II), and QuikHyb Rapid Hybridization Solution, were from Stratagene (La Jolla, CA). Quick Prep micro mRNA purification kit was from Pharmacia (Uppsala, Sweden). The TRI Reagent, FORMazol and methylene blue staining solution were purchased from Molecular Research Center (Cincinnati, OH). SuperScript choice cDNA synthesis system, goat anti-rabbit IgG (H+L) (human adsorbed), horseradish peroxidase, ampicillin (sodium salt, lyophilized), T4 DNA ligase, T4 DNA polymerase, T4 polynucleotide kinase, ImmunoSelect Immunoblotting System, Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), LIPOFECTAMINE Transfection

Reagent, SF-900 II serum free medium, *Trichoplusia ni* Medium-Formulation Hink (TNM-FH), and dithiothreitol (DTT) were all from GIBCO BRL (Grand Island, NY). COS-7 cells (derived from kidney cells of Africa green monkey) were a kind gift from Dr. David W. Barnes, Cell Culture Facility, Environmental Health Sciences Center, Oregon State University. Hybond-N<sup>+</sup> nylon membrane, Hybond-C extra-supported nitrocellulose, [ $\alpha$ -<sup>32</sup>P]-dATP, [ $\gamma$ -<sup>32</sup>P]-ATP and [1-<sup>14</sup>C]-lauric acid were from Amersham (Amersham, UK). Erase-a-Base DNA deletion system and Wizard Miniprep DNA purification system were from Promega (Madison, WI). Acetonitrile (HPLC grade) was from J.T. Baker Chemical (Phillipsburg, NJ). The pBlueBacIII and Sf9 cells are the kind gifts from Dr. George F. Rohrmann, Department of Agriculture Chemistry, Oregon State University. Linear AcMNPV (*Autographa californica* nuclear polyhedrosis virus) DNA and liposomes were purchased from Invitrogen (San Diego, CA.). Lauric acid (sodium salt), NADPH, ethylenediamine tetraacetic acid (EDTA) and CsCl were purchased from Sigma Chemical (St. Louis, MO).

#### **Poly(A)<sup>+</sup> RNA isolation and cDNA library construction**

Poly (A)<sup>+</sup> RNA was prepared using the Quick Prep micro mRNA purification kit. Poly (A)<sup>+</sup> RNA was isolated from the livers of one year, two year and three year female rainbow trout and equal amounts were mixed and used for cDNA synthesis. A cDNA library was prepared in the  $\lambda$ -gt11 vector using BRL SuperScript Choice System and Gigapack *in vitro*

lambda packaging system according to the manufacturer's instructions. The library was amplified by growing in *E.coli* Y1088.

### **Screening of cDNA library**

Approximately  $1 \times 10^6$  pfu from the amplified library were screened with rabbit anti-P450 LMC1 polyclonal antibodies (Miranda et al., 1989) according to the instruction manual of ImmunoSelect Immunoblotting System. Positive clones were plaque-purified through three rounds of repeated screening. The cDNA insert was excised from  $\lambda$ -phage DNA by EcoRI restriction enzyme and then ligated to EcoRI digested pSPORT1 plasmid for sequencing. Various restriction enzymes (HindIII, PstI, SstI, KpnI, and BamHI) were used to establish the restriction map of this cDNA.

### **cDNA sequencing and analysis**

Partially deleted pSPORT1-cDNA recombinant subclones were prepared by Erase-a-Base System (Promega). Briefly, pSPORT1 containing the cDNA insert was digested with SphI and EcoRI restriction enzymes to generate 5'-protruding and 3'-overhang ends. Exonuclease III was used to digest the insert cDNA specifically from 5'-protruding end. The nested unidirectional deletion subclones from both directions were prepared and sequenced using fluorescence labeled oligonucleotide primer on an automated sequencer (ABI Model

373A, version 1.0.2) serviced by the Center for Gene Research and Biotechnology (CGRB), Oregon State University, Corvallis, OR. Each base was determined at least once from each strand. Sequence data were analyzed using GCG program (Madison, WI).

### **N-terminal amino acid determination**

Cytochrome P450 LMC1 from rainbow trout liver was purified as described (Miranda et al., 1989). The purified LMC1 protein was separated from impurities by SDS-PAGE and transferred onto Immobilon P neutral nylon membrane by electroblotting, and 14 residues of the N-terminal amino acid sequences were determined by Edman degradation method using an automated protein sequencer (ABI Model 477A, Utah State University).

### **Northern blot analysis**

Total RNA from trout liver and trunk kidney were isolated by TRI Reagent, precipitated with isopropanol, washed with 70% ethanol and then dissolved in FORMazol. For CYP2M1 mRNA quantitation, synthesized CYP2M1 RNA was used to establish a standard curve. Briefly, CYP2M1 cDNA-containing pSPORT1 was linearized by HindIII digestion, T7 RNA polymerase was used to produce CYP2M1 RNA from T7 promoter within the pSPORT1 plasmid. The template DNA was removed by DNase I digestion followed by a Sephadex G-50 column

chromatography. The synthesized RNA was ethanol precipitated, resuspended in FORMazol and stored at  $-80^{\circ}\text{C}$  until use.

The RNA samples were glyoxylated, size separated by 0.8% agarose gel electrophoresis, and transferred to the Hybond-N<sup>+</sup> nylon membrane (Sambrook et al., 1989a). The RNA containing membrane was stained with methylene blue to verify the integrity of RNA samples. The immobilized RNA was hybridized in Quikhyb hybridization solution with random-primed [<sup>32</sup>P]-labeled full-length CYP2M1 cDNA probe at  $68^{\circ}\text{C}$  for 2 hours. Hybridized blots were washed twice with 2X SSC/0.1% SDS at room temperature for 15 min each and a more stringent wash with 0.1X SSC/0.1% SDS at  $50^{\circ}\text{C}$  for 20 min. The washed blots were exposed to Kodak XAR-5 X-ray film. The 18S rRNA was probed by a universal 15-mer oligonucleotide probe (5'-ACGGGCGGTGTGTRC-3', 1406R) (Lane et al., 1985) corresponding to a small subunit of the 18S ribosomal RNA which then was used as an internal standard for Northern blotting analysis. The oligonucleotide probe was end-labeled with  $\gamma$ -[<sup>32</sup>P]-ATP using T4 polynucleotide kinase. Hybridization was performed exactly the same as described for the cDNA probe except that hybridization temperature was reduced to  $37^{\circ}\text{C}$ . The hybridized blots were washed with 0.2X SSPE/0.1% SDS at room temperature for twice and once at  $37^{\circ}\text{C}$ . The resulting X-ray films were analyzed using Personal Densitometer SI (PDSI-PC, Molecular Dynamics, Sunnyvale, CA) provided by the CGRB. The integrated values were analyzed by Power Point software by Microsoft (Redmond, WA). The standard curve was generated and linear equation was obtained by adding trendline not forced through zero.

The quantitative values of the mRNA were normalized with 18S rRNA and calculated based on the linear curve equation obtained by Microsoft Excel program.

### **Southern blot analysis**

Genomic DNA from the liver of one 3-yr old sexually mature male rainbow trout was prepared as described by Buhler et al. (1994). BamHI, EcoRI, XbaI, and XhoI were used to digest the purified genomic DNA. The digested DNA was separated by 0.7% agarose gel electrophoresis and then capillary transferred to a nylon membrane (Hybond-N<sup>+</sup>) with 10x SSC buffer (Southern 1975). The transferred DNA on the membrane was hybridized with 3'-untranslated region (3'-UTR, from 1,750 to 2,149 bp) of CYP2M1 cDNA and washed following the same procedure described for RNA analysis.

### **Mass spectrometric analysis**

Matrix-assisted laser desorption-time of flight (MALDI-TOF) mass spectrometry of the purified rainbow trout P450 LMC1 was performed as described by Lewis et al. (Lewis et al., 1993) using sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) as the matrix and bovine serum albumin as the internal standard.

### **Expression of CYP2M1 in COS-7 cells**

For expression of CYP2M1 protein from the cloned cDNA, the full-length cDNA insert was removed from pSPORT1 plasmid with SalI and BamHI restriction digestion. The insert DNA was ligated to the compatible BamHI and XhoI ends of pSVL expression vector that contains the SV40 and pBR322 origins of replication. The expression of insert cDNA in COS-7 is driven by the SV40 late promoter, which is a strong virus promoter recognized by the transcription machinery of many mammalian cells. The recombinant pSVL construct was amplified in *E.coli* DH5 $\alpha$  and isolated by alkaline lysis and purified by banding twice in CsCl gradient centrifugation (Sambrook et al., 1989b).

COS-7 cells were grown in D-MEM supplemented with 10% fetal bovine serum at 37°C under 5% CO<sub>2</sub>. Cells were transfected with the recombinant plasmid by LIPOFECTAMINE Transfection Reagent according to the manufacturer's protocol. Forty-eight hours after transfection, the cells were harvested for preparation of microsomes.

The cells from each plate were washed once with phosphate-buffered saline and then dislodged from the plate by a rubber policeman. The cells were disrupted at power setting of 2 with a sonicator (SONICATOR, model W-10, Heat Systems-Ultrasonics Inc. Plainview, NY) in homogenization buffer (0.25 mM sucrose, 1 mM EDTA) until more than 80% of cells have broken as determined microscopically. Cell debris was removed by centrifugation at 10,000 x g for 5 min at 4°C. The microsomes were collected from the supernatant by centrifugation at 150,000 x g for 20 min. The microsomal

pellet was homogenized in the resuspension buffer (100 mM potassium phosphate, pH 7.4, 0.1 mM EDTA, 20% glycerol) and stored at  $-80^{\circ}\text{C}$ .

### **Expression of CYP2M1 in baculovirus-insect cells**

The cDNA containing the complete coding region of CYP2M1 was removed from pSPORT1 plasmid by BamHI and HindIII digestion. The resulting fragment was then ligated to the baculovirus transfer vector pBlueBacIII. The Sf9 cells were maintained in TNM-HF/10% FBS at  $27^{\circ}\text{C}$ . The recombinant virus was constructed by co-transfecting cDNA containing pBlueBacIII and linear AcMNPV DNA into Sf9 cells using Insectin liposomes. The recombinant virus, a homologous recombination product of the transfer vector and the AcMNPV DNA, places CYP2M1 expression under the control of virus polyhedrin promoter and  $\beta$ -galactosidase under the virus early-to-late promoter. The recombinant virus was isolated using plaque assay and X-gal for color selection (O'Reilly et al., 1992). Identification of recombinant virus was first done by visualization of blue and occlusion body negative plaques. The purity of the plaque and the presence of cDNA insert were then confirmed by polymerase chain reaction (PCR) using recombinant baculovirus PCR forward (5'-TTTACTGTTTTTCGTAACAGTTTTG-3') and reverse (5'-CAACAACGCACAGAATCTAGC-3') primers. The PCR product was further analyzed by diagnostic restriction digestion. Due to the expression level differences between each recombinant virus, four different recombinant viruses were selected to

check the protein yield by Western blotting. The most productive virus was amplified to prepare high titer stock for expression. The presence of cDNA and the absence of contaminating wild type virus in the high titer stock were checked again using PCR. To express CYP2M1, Sf9 cells were grown in SF-900 serum-free medium to a density of  $1.8-2 \times 10^6$  cells/ml in spinner flasks (Bellco Glass) at room temperature (24-25°C). Cells were infected at multiplicity of infection (MOI) of 1 to 5. Equal molar mixture of BSA and hemin stock solution, prepared as described previously (Gragan et al., 1995), were added at the time of infection to the final concentration of 1.5 µg/ml of hemin to compensate for low endogenous levels of hemin in the insect cells. Cells were harvested 72-84 hours after infection, washed once with PBS, and resuspended in 250 mM sucrose, 1 mM EDTA, 1 mM DTT and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). Cell lysates were prepared by sonication and total P450 content was measured using CO-difference spectrometry (Omura and Sato 1964). Solubilization of CYP2M1 from Sf9 cell membrane was performed with 1.4% of sodium cholate in homogenization buffer (100 mM potassium phosphate, 20% glycerol, 0.1 mM EDTA) incubating overnight at 4°C (Sigle et al., 1994). The protein concentration was determined by Coomassie Plus Protein Assay Reagent (Pierce Chemical).

#### **Western blot analysis**

Proteins from the microsomes of COS-7 cells and from recombinant baculovirus-infected Sf9 cell lysate were

separated by electrophoresis in SDS-7.5% polyacrylamide gel. The separated proteins were electro-blotted to a nitrocellulose membrane. The protein containing membrane was blocked with 5% nonfat dry milk (Lucerne) in TBS/T (20 mM Tris-HCl pH 7.5, 0.5M NaCl, 0.05% Tween 20). After blocking, the membrane was incubated with rabbit anti-trout LMC1 polyclonal antibodies (Miranda et al., 1989) for 30 min. The unbound antibodies were washed away in TBS/T and further incubated with 1:12000 dilution of secondary antibody (goat anti-rabbit antibodies conjugated to horseradish peroxidase) for 45 min. After washing with TBS/T, the immunoreactive proteins were detected with ECL Western blotting detection reagents.

### **Enzyme assays**

To determine the catalytic activity of expressed CYP2M1, microsomes from pSVL-CYP2M1 cDNA-transfected and pSVL vector only transfected COS-7 cells were used in the enzyme reactions. A 250  $\mu$ l reaction mixture containing 100 mM potassium phosphate, pH 7.4, 5 mM MgCl<sub>2</sub>, 1 mM EDTA, 250  $\mu$ g microsomal protein, 50 nmole [1-<sup>14</sup>C]-lauric acid (0.25  $\mu$ Ci), and 1 mM NADPH was incubated at 30°C or 37°C for 1 hr.

Enzyme assays for the baculovirus expressed CYP2M1 (BV-CYP2M1) were carried out in the presence of 0.4 nmole purified rat liver NADPH-cytochrome P450 reductase, and 0.2 nmole rabbit cytochrome b5. The Sf9 cell homogenate containing 0.1 nmole of CYP2M1, P450 reductase and cytochrome b5 were preincubated at room temperature for 10

min before the buffer (50 mM Tris-HCl pH 7.6) and 100 nmole of lauric acid ( $C^{14}$ -labeled + cold) were added. Reactions were initiated by adding 1 mM of NADPH. The total reaction volume was 500  $\mu$ l. Sample mixtures were incubated for 1 hr in a 27°C water bath with vigorous shaking.

Reactions were terminated by addition of 10%  $H_2SO_4$  and extracted twice with 3 ml of ethyl ether. The ether was evaporated under  $N_2$  and the dried samples were stored at -20°C until analyzed by reverse-phase HPLC (rp-HPLC) or gas chromatography/mass spectroscopy (GC/MS).

The metabolites of each reaction were separated by rp-HPLC on a 4.6 mm (ID) x 25 cm ZORBAX (ODS) column (DuPont) connected to a Radiomatic FLO-ONE  $\beta$  Radioactive Flow Detector. Lauric acid and its metabolites were eluted from the column with a gradient - 25% acetonitrile/0.2% acetic acid for 35 min followed by a 2-min linear gradient to 80% acetonitrile, and then 80% acetonitrile/0.2% acetic acid for 10 min. Retention times for ( $\omega$ -6)-hydroxylauric acid and lauric acid were 40 min and 45 min, respectively as reported earlier (Buhler et al., 1997). Based on the conversion efficiency of lauric acid to ( $\omega$ -6)-hydroxylauric acid in the HPLC profile, the catalytic activity (nmole/min/nmole P450) of CYP2M1 was calculated.

Because the standards for lauric acid metabolites are not available for HPLC, GC/MS was used to further analyze these oxidation products. Briefly, the dried extract samples were redissolved in 50  $\mu$ l of diethyl ether and methylated with diazomethane for 1 hr at room temperature. The remaining diethyl ether and diazomethane were removed under a stream of nitrogen. The methylated products were

then derivatized in equal volume of dry pyridine and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), and heated for 1 hr at 60°C. The samples were analyzed on a Finnigan 4023 Quadrupole GC/MS.

## *Results and Discussion*

### **Identification and sequence analysis of cloned cDNA**

A  $\lambda$ -gt11 cDNA library was constructed from female rainbow trout liver and screened with rabbit anti-LMC1 polyclonal antibodies. One of the clones selected was subcloned to pSPORT1 for sequencing analysis and restriction mapping. This cDNA was 2,149 bp long with an open reading frame of 1,497 bp and contained a 36 bp 5' flanking region and a 616 bp 3'-untranslated region. The putative polyadenylation signal AATAAA was located 17 bp upstream from the poly(A)<sup>+</sup> tail (Fig. 2.1). The deduced N-terminal amino acid sequence for this cDNA was identical to that of 12 of 14 amino acid residues as determined by Edman degradation of P450 LMC1 purified from microsomes of rainbow trout liver. The open reading frame encoded a 499-amino acid protein with calculated  $M_r$  of 56,850 Da. The deduced  $M_r$  of the cloned P450 agreed well with the measured molecular weight for purified P450 LMC1 of 57,114  $\pm$  55 as determined by MALDI-TOF mass spectrometry (Fig. 2.2). Based on the deduced amino acid sequence of the cloned trout cytochrome P450 LMC1, it was assigned as CYP2M1 or P450 2M1 to a new cytochrome P450 subfamily by Dr. David R. Nelson of the P450 Nomenclature Committee (Nelson et al., 1996). Sequence comparisons between rainbow trout CYP2M1 and rainbow trout CYP2K1 showed 57% homology in nucleotide sequences and 42% identity in amino acid sequences. Sequence alignment of hemin-binding region between rainbow

trout CYP2M1 (Fig. 2.1), CYP2K1 (Buhler et al., 1994), CYP1A1 (Heilmann et al., 1988), rainbow trout steroidogenic P450s, CYP17 (Sakai et al., 1992), CYP19 (Tanaka et al., 1992) CYP11A1 (Takahashi et al., 1993), and consensus sequences of other P450s (Mansuy and Renaud 1995) suggested conserved evolution of this functional domain during development (Table 2.1).

### **Analysis of CYP2M1 gene expression**

Expression of CYP2M1 at the transcription level was investigated by Northern blot hybridization of total RNA from rainbow trout liver and trunk kidney using [<sup>32</sup>P]-labeled full-length CYP2M1 cDNA as a probe. CYP2M1 cDNA hybridized to a single 2.2 Kb transcript in liver and kidney. A synthesized CYP2M1 RNA was used to establish a standard curve for estimation of the extent of CYP2M1 mRNA expression. Differences in sample loading also were normalized by densitometry scans of the 18S rRNA. Expression of CYP2M1 mRNA in rainbow trout liver was not significantly different with respect to sex in juveniles. The highest CYP2M1 expression was in liver of the sexually mature males compared to mature male or juvenile fish of both sexes (Fig. 2.3A). Mean values for the expressed CYP2M1 mRNA in liver were 13.8, 10.6, 31.2 and 12.2 pg/μg total RNA for juvenile male and female and sexually mature male and female trout, respectively with standard deviation of up to 65% (Table 2.2). Hence, there was substantial variability in CYP2M1 expression between individual fish. However,

trunk kidney from juvenile female rainbow trout produced 20-fold more CYP2M1-hybridizable mRNA than did juvenile male trunk kidney (Table 2.3), as shown by the values of 13.2 pg CYP2M1 mRNA/ $\mu$ g total RNA for female and 0.6 pg/ $\mu$ g for male trunk kidney, respectively. The expression level of this mRNA was decreased in trunk kidney from sexually mature rainbow trout of both sexes as compared to juvenile rainbow trout. In fact, CYP2M1 expression in sexually mature male trunk kidney was barely detectable (Fig. 2.3B). As seen previously with rainbow trout CYP2K1 (Buhler et al., 1994), sexual dimorphism of CYP2M1 expression, therefore, was more pronounced in the kidney than in the liver.

#### **Southern blot analysis**

The complexity of the CYP2M1 gene was analyzed by Southern blot hybridization. A shorter probe (~400 bp) containing part of 3'-UTR of CYP2M1 cDNA was used. The advantage of using a short probe was that introns and restriction sites were less likely to fall within the small region to be probed, and thus only one single band was usually detected per gene copy (Spoerel and Kafatos 1987). All the vertebrate genes for a given P450 family that have been examined thus far, contain the same number of exons and similar intron-exon boundaries (Nelson et al., 1996). If the CYP2M1 gene shared the same gene organization as seen in the CYP2 family, the 3'-UTR probe corresponds to the last exon (exon 9) of CYP2M1 gene. Although the banding pattern of Southern blot and Northern blot analysis suggest that

CYP2M1 is a single copy gene (Fig. 2.3 and 2.4), the presence of a second highly homologous gene can not be excluded.

#### **cDNA-mediated CYP2M1 expression in COS-7 cells and recombinant baculovirus infected Sf9 cells**

In order to ascertain whether the CYP2M1 cDNA encoded for a protein exhibiting the same lauric acid ( $\omega$ -6)-hydroxylation activity as did purified rainbow trout LMC1, CYP2M1 cDNA was ligated to the expression vector pSVL for transient expression in COS-7 cells. Although COS cells provided a rapid way to express the CYP2M1 protein for measurement of enzyme activity, the expression level was so low that the CO-difference spectrum was too weak to be detected. Accordingly, we made use of baculovirus infected insect cells to obtain an improved expression of rainbow trout CYP2M1. Baculovirus, in the presence of hemin supplement, gave good expression routinely producing 60-90 nmole spectrally active CYP2M1 P450/liter cell culture under our expression conditions. Based on Western blotting, the yield of apoprotein was about 240 pmole/mg of insect cell homogenate; however, the spectrally active P450 content indicated that only 40% of apoprotein incorporated hemin properly. The incomplete hemin incorporation in the baculovirus system is seen with other P450s (Buters et al., 1995; Paine et al., 1996). Based on preliminary studies, we routinely used hemin at 1.5  $\mu$ g/ml. Increasing the hemin concentration over 2  $\mu$ g/ml did not improve P450 content, but

produced large amounts of nonspecific non-P450 hemin conjugates which resulted in strong absorption at 420 nm (Gragan et al., 1995). The nonspecific absorption was decreased as less hemin supplement was added. When hemin concentration was reduced to 0.5  $\mu\text{g/ml}$ , P450 could not be detected spectrally, giving results comparable to that seen in the control without hemin supplementation. The typical CO-difference spectrum produced by baculovirus-Sf9-expressed CYP2M1 is shown in Fig. 2.5. Expression of CYP2M1 was greatest between 72-84 hr post-infection as monitored by P450 CO-difference spectrum.

Microsomes from CYP2M1 cDNA transfected COS-7 cells and recombinant baculovirus infected Sf9 cell homogenate expressed protein that was recognized by anti-P450 LMC1 polyclonal antibodies (Fig. 2.6). No immunoreactive proteins were detected in microsomes from cells transfected with pSVL vector alone, uninfected Sf9 cells and Sf9 cells infected with wild type baculovirus (data not shown). The purified rainbow trout LMC1 protein and the expressed CYP2M1 showed the same electrophoretic mobility in SDS-PAGE (Fig. 2.6).

The enzymatic activity of expressed protein from COS-7 and baculovirus was examined using  $[1-^{14}\text{C}]$ -lauric acid as a substrate. The lauric acid and its metabolites from the reactions were analyzed by reverse-phase HPLC and GC/MS. Two different temperatures, 30°C and 37°C, were used in the incubation for COS-7 cell microsomes. A temperature of 30°C was used for measuring rainbow trout microsomal lauric acid hydroxylase activities and that of reconstituted trout P450s (1) while 37°C is generally employed for determination of

P450-mediated activity in mammals. In the present study, lauric acid ( $\omega$ -6)-hydroxylase activity of CYP2M1 expressed in COS-7 cells was found only in the 37°C incubation. A single, major metabolite corresponding to ( $\omega$ -6)-hydroxylauric acid was produced by microsomes isolated from CYP2M1 cDNA transfected COS-7 cells (data not shown). The lauric acid metabolites were not seen in microsomes from untransfected COS-7 cells and COS-7 cells transfected with pSVL vector alone at both 30 and 37°C. The structural organization of P450, NADPH-cytochrome P450 reductase and lipid within rainbow trout microsomes have been found responsible for lower temperature optimum of mixed-function oxidase enzymes in rainbow trout as compared to rat microsomes (Williams et al., 1983). The lack of activity of COS-7 expressed CYP2M1 at lower temperature (30°C) may be due to different environment of the CYP2M1 in the COS-7 cells. Therefore, a fish P450, such as CYP2M1, which has a low temperature optimum for catalytic activity in a reconstituted system, could behave like mammalian P450s that have higher temperature optima when the enzyme is expressed in cultured mammalian COS-7 cells. The rainbow trout steroidogenic cytochromes P450 (CYP11A1, CYP17, and CYP19) expressed in COS cells also showed catalytic activity toward their specific substrates at 37°C (Sakai et al., 1992; Tanaka et al., 1992; Takahashi et al., 1993).

The catalytic activity of BV-CYP2M1 toward lauric acid was also determined. The incubations were done at 27°C, the temperature optimum for growing Sf-9 cells. The catalytic activity of BV-CYP2M1 was first measured using total cell homogenate supplemented with NADPH-cytochrome P450 reductase

and cytochrome b5, but the activity was low, only 1/10 that of the reconstituted LMC1. Solubilization of BV-CYP2M1 from Sf9 cell membranes with 1.4% sodium cholate overnight as described by Sigle et al. (1994), however, dramatically increased the lauric acid ( $\omega$ -6)-hydroxylase activity by 10-fold with a turnover number of 1.24 nmole/min/nmole P450, an activity level that was comparable to that of the purified P450 LMC1 (1.30 nmole/min/nmole P450) (Table 2.4). The increase in activity of cholate-treated CYP2M1-containing homogenate may be due to the conformational change induced by detergent, possibly increasing the interaction between P450 and NADPH-P450 reductase (Yun et al., 1997). The HPLC profile and mass spectrum of ( $\omega$ -6)-hydroxylauric acid produced by BV-CYP2M1 are shown in Fig. 2.7 and Fig. 2.8, respectively. The lauric acid hydroxylation products were not detected by the homogenate from uninfected Sf9 cells and Sf9 cells infected with wild type baculovirus.

Unlike most mammalian P450s which hydroxylate lauric acid primarily in the terminal methyl ( $\omega$ )- or subterminal ( $\omega$ -1)-position, juvenile rainbow trout hepatic microsomes metabolize lauric acid in several positions, ranging from ( $\omega$ ), ( $\omega$ -1), ( $\omega$ -2), ( $\omega$ -3), ( $\omega$ -4), ( $\omega$ -5), to ( $\omega$ -6) (Buhler et al., 1997). Our observation (Fig. 2.7 and 2.8) suggested that CYP2M1 was the P450 responsible for the ( $\omega$ -6)-hydroxylation of lauric acid an activity apparently not found with mammalian liver microsomes. However, the physiological function of this P450 is unknown and no ortholog in other species has been discovered. The CYP2M1 P450 may be unique to fish species and may be responsible

for metabolism of fatty acids and other fatty acid derivatives. Additional studies are needed to determine the catalytic activity of CYP2M1 toward other physiologically important fatty acids (linoleic acid, arachidonic acid, eicosapentaenoic acid, docosapentaenoic acid and docosahexaenoic acid), prostaglandins and other substrates. Prostaglandins were identified in many fish species and they are involved in ovulation and spawning behavior changes (Bell et al., 1986). Fish are poikilothermic animals and the fatty acid composition of the cell membrane is involved in an adaptive mechanism for maintaining membrane homeoviscosity and physiological function in response to temperature changes in environment of these species (March 1993).

In summary, we have isolated a cDNA clone designated as CYP2M1. The expression of CYP2M1 cDNA in COS-7 cells and baculovirus produced a protein with lauric acid ( $\omega$ -6)-hydroxylase activity. The CYP2M1 protein from both expression systems was indistinguishable immunologically and biochemically to the P450 LMC1 we have purified from rainbow trout liver microsomes. The overexpression of CYP2M1 in the baculovirus-insect cell produced high level of expression (60-90 nmole/liter) comparable with yeast and *E.coli* expression systems. The lipid environment of insect cells can support the catalytic and substrate specific activities supplemented with P450 NADPH-cytochrome P450 reductase, and NADPH. The turnover number of lauric acid hydroxylation with the baculovirus expressed CYP2M1 was comparable to that of rainbow trout liver microsomes, allowing the use of the membrane fraction for enzymatic studies without purifying

the P450. This is the first published report of cDNA-directed expression of a functional fish P450 using the baculovirus-insect cell (Sf9) expression system.

### *Acknowledgement*

The authors would like to thank Ms. Marilyn C. Henderson and Xine Zhao for their valuable assistance, and Dr. Jerry D. Hendricks, Ted Will and Dan Arbogast of the Marine/Freshwater Biomedical Research Center, Oregon State University, for their help in the care and maintenance of the rainbow trout used in this study. The authors also would like to thank Ms. Elisabeth Barofsky and Mr. Don Griffin of the Mass Spectrometry Core Unit of the Oregon State University Environmental Health Sciences Center for running the MALDI-TOF mass spectrometry on P450 LMC1 and for running the GC/MS, respectively. The authors thank Dr. David W. Barnes, Dr. George F. Rohrmann and their associates for the help in cell culture and cell culture facilities. Appreciation also goes to Mr. Maciej Kedzierski for the computer quantitation and graphing. Support for this study came from the National Institute of Health, grant Nos. ES000210, ES03850 and ES04766. This manuscript was issued as Technical Paper No. 11,251 from Oregon Agricultural Experiment Station, Oregon State University, Corvallis, Oregon.

Table 2.1

Proximal Cysteine-Containing Peptide Involves in Heme  
Binding in P450s of Rainbow Trout

	1	2	3	4	5	6	7	8	9	10
P450 (consensus)	F	G/S	X	G	X	R/H	X	C	hy	G
CYP2M1	F	G	V	G	K	R	A	C	P	G
CYP2K1	F	S	A	G	R	R	V	C	L	G
CYP1A1	F	G	M	D	K	R	R	C	I	G
P45019 (P450 <sub>arom</sub> )	F	G	S	G	P	R	S	C	V	G
P45017 (P450 <sub>c17</sub> )	F	G	A	G	P	R	S	C	I	G
P45011A1 (P450 <sub>scc</sub> )	F	G	F	G	P	R	Q	C	L	G

The heme-binding region consensus sequences of P450 was from (Mansuy and Renaud, 1995). hy refers to a hydrophobic amino acid, such as V, I, or L.

Table 2.2

Quantitation of CYP2M1 mRNA present in the liver

	Male (juvenile)			Female (juvenile)			Male (mature)			Female (mature)		
	1	2	3	4	5	6	7	8	9	10	11	12
CYP2M1 mRNA pg/ $\mu$ g total RNA	16.1	8.4	17.2	10.6	10.1	11.0	26.2	25.8	41.6	21.3	6.4	9.1
Mean pg/ $\mu$ g total RNA	13.8 $\pm$ 4.7			10.6 $\pm$ 0.5			31.2 $\pm$ 9.0			12.2 $\pm$ 7.9		

Table 2.3

Quantitation of CYP2M1 mRNA present in kidney

	Male (juvenile)			Female (juvenile)			Male (mature)			Female (mature)		
	1	2	3	4	5	6	7	8	9	10	11	12
CYP2M1 mRNA pg/ $\mu$ g total RNA	0.5	1.0	0.5	16.6	14.1	8.9	0*	0*	0*	1.0	4.4	0.45
Mean pg/ $\mu$ g total RNA	0.6 $\pm$ 0.3			13.2 $\pm$ 4.0			0*			2.0 $\pm$ 2.1		

\* Beyond the detection limits of the densitometer

Table 2.4

Catalytic activities of purified rainbow trout P450 LMC1,  
COS-7 and baculovirus-expressed CYP2M1

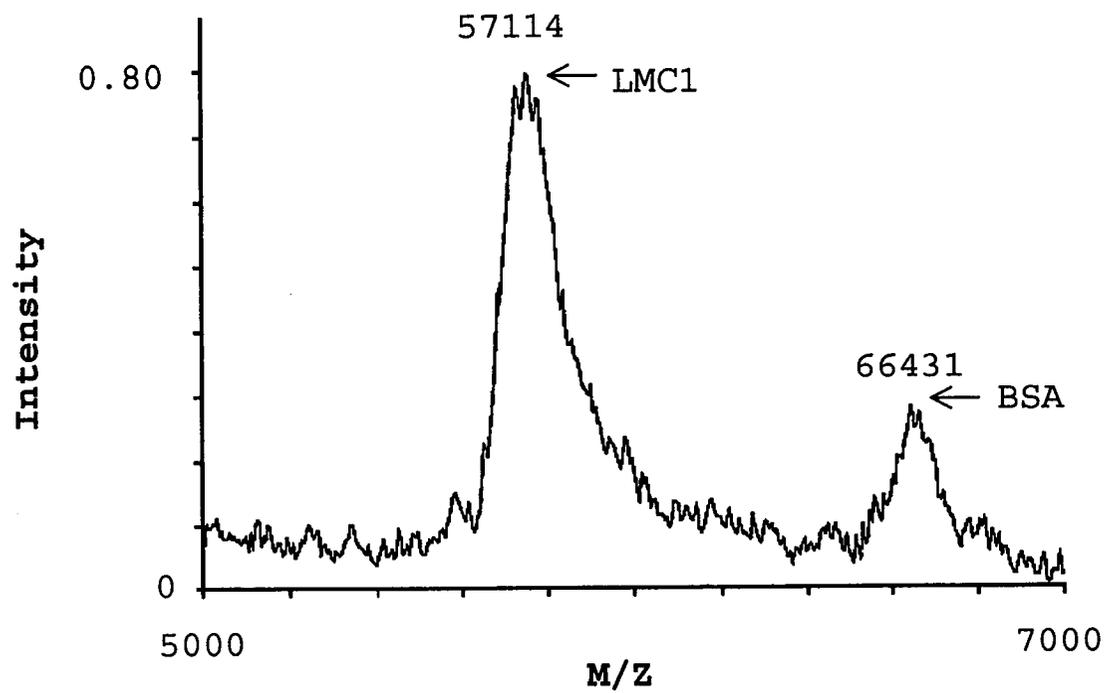
$\omega$ -6 Hydroxylation of lauric acid	Turnover Numbers (nmole/min/nmole P450)
LMC1 reconstitution	1.30
COS-CYP2M1	0.18
BV-CYP2M1 homogenate	0.12
BV-CYP2M1 cholate solubilized	1.24

**Fig. 2.1** Nucleotide and deduced amino acid sequences for rainbow trout CYP2M1 cDNA. The translation start site is underlined and the putative polyadenylation signal is in bold and underlined. The boxed area shows the putative heme-binding domain of this P450.

```

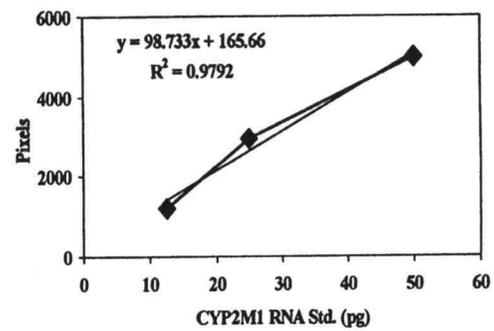
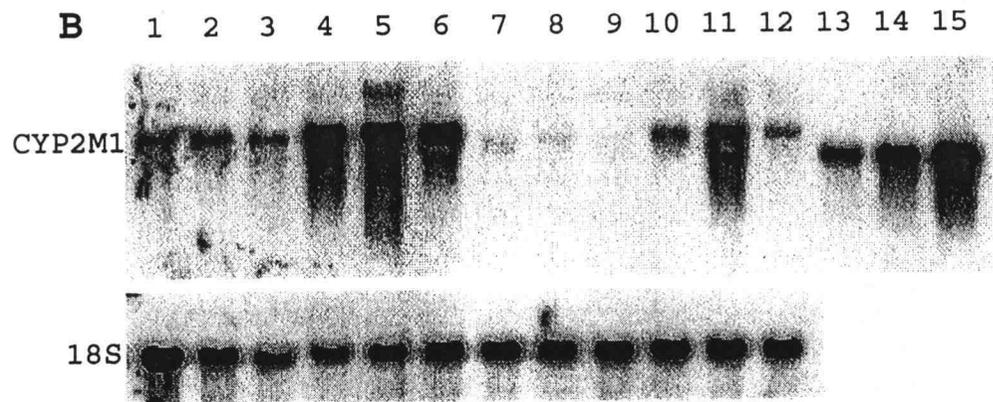
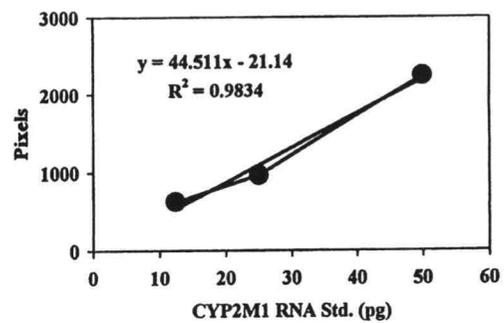
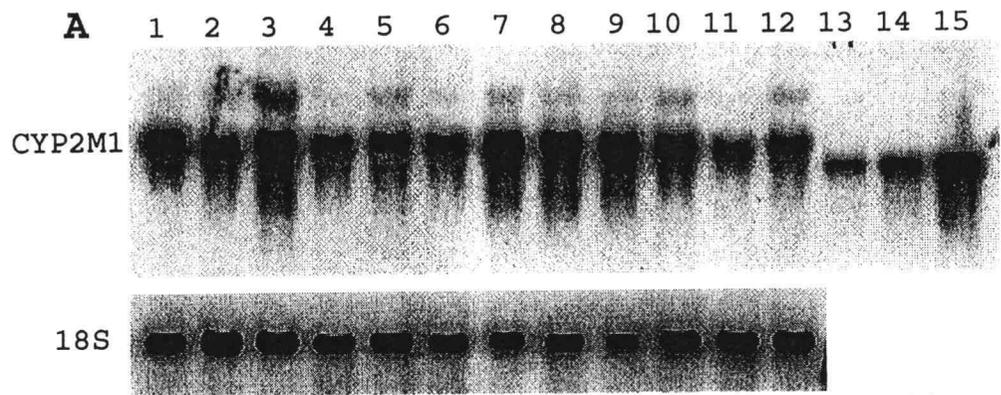
ctc cgt agg cga tca atg tcc tcg act cta ctg aag ATG GAT GTA CTT CAT ATT TTG CAG ACG AAC TTT GTG TCT ATT ATA ATC GGA 51
M D V L H I L Q T N F V S I I I G 17
TTT GTC GTT ATT ATT TTA TTG TGG ATG AAT CGA GGC AAA CAA AGC AAC TCA CGT TTG CCT CCT GGT CCT GCA CCG ATT CCT CTG CTT 138
F V V I I L L W M N R G K Q S N S R L P P G P A P I P L L 46
GGT AAC TTG TTA CGA ATG GAT GTG AAA GCG CCA TAC AAG CTC TAC ATG GAG CTR AGT AAG AAG TAT GGC TCA GTG TTT ACT GTA TGG 225
G N L L R M D V K A P Y K L Y M E L S K K Y G S V F T V W 75
CTG GGC TCC AAA CCT GTA GTG GTG ATC TCT GGC TAT CAG GCT ATC AAG GAT GCT TTT GTC ACA CAA GGA GAA GAG TTC AGC GGC AGA 312
L G S K P V V V I S G Y Q A I K D A F V T Q G E E F S G R 104
GCC AAC TAC CCC GTG ATC ATG ACA GTC TCT AAA GGA TAT GGG GTG TTG GTG AGC AGT GGG AAG AGA TCA AAA GAT CTT CGC AGG TTC 399
A N Y P V I M T V S K G Y G V L V S S G K R S K D L R R F 133
TCG CTG ATG ACC CTT AAA ACC TTT GGG ATG GGG CGC AGG AGC ATC GAA GAA CGT GTC CAA GAA GAG GCT AAG ATG CTG GTG AAA GCC 486
S L M T L K T F G M G R R S I E E R V Q E E A K M L V K A 162
TTC GGT GAA TAT AGA GAT TCA GTT GTC AAT CCC AAA GAA TTG CTT TGC AAT TGT GTT GGC AAT GTG ATC TGC TCC ATA GTC TTT GGG 573
F G E Y R D S V V N P K E L L C N C V G N V I C S I V F G 191
CAC AGG TTT GAA AAT GAC GAC CCA ATG TTT CAG CTC ATC CAA AAG GCT GTT GAT GCC TAC TTC AAT GTC CTC AGC AGC CCT ATT GGA 660
H R F E N D D P M F Q L I Q K A V D A Y F N V L S S P I G 220
GCG ATG TAC AAC ATG TTC CCT AGA ATC GTT TGG TGC TTT CCA GGC AAT CAC CAT GAG ATG TTT GCT ATC GTA AAC AAG GCC AAA GAT 747
A M Y N M F P R I V W C F P G N H H E M F A I V N K A K D 249
TAC ATA CAA GAG CAA GCA GAA ATC CGT CTT AAA ACC CTT AAC ATC TCT GAA CCT CAG GAC TTC ATC GAG GCC TTC CTA GTT AAA ATG 834
Y I Q E Q A E I R L K T L N I S E P Q D F I E A F L V K M 278
CTG GAG GAG AAA GAT GAT CCC AAC ACT GAG TTC AAC AAT GAT AAT ATG GTG ATG ACT GCA TGG AGC CTG TTT GCT GCT GGA ACA GAA 921
L E E K D D P N T E F N N D N M V M T A W S L F A A G T E 307
ACC ACA TCA TCC ACC CTA AGA CAA TCA TTT CTG ATG ATG ATA AAG TAC CCT CAC ATT CAA GAG AGT GTC CAG AAG GAG ATA GAC GAG 1008
T T S S T L R Q S F L M M I K Y P H I Q E S V Q K E I D E 336
GTG ATT GGC TCA AGA GTG CCC ACG GTT GAC GAC AGA GTT AAA ATG CCC TAC ACG GAT GCT GTC ATT CAC GAA GTC CAG CGA TAC ATG 1095
V I G S R V P T V D R V K M P Y T D A V I H E V Q R Y M 365
GAC CTC AGT CCC ACC TCT GTA CCA CAC AAA GTG ATG AGA GAT ACA GAG TTC TAC AAC TAC CAC ATT CCA GAG GGT ACC ATG GTC CTG 1182
D L S P T S V P H K V M R D T E F Y N Y H I P E G T M V L 394
CCT CTG CTG TCC TCT GTG CTT GTT GAT CCC AAA CTT TTT AAA AAC CCA GAT GAG TTT GAC CCA GAG AAC TTC CTG GAT GAA AAT GGA 1269
P L L S S V L V D P K L F K N P D E F D P E N F L D E N G 423
GTC TTT AAG AAA AAT GAT GGA TTC TTT GCT TTT GGA GTG GGG AAG CCG GCG TGT CCT GGG GAA GCT CTG GCC AGA GTG GAG CTC TTT 1356
V F K K N D G F F A F G V G K R A C P G E A L A R V E L F 452
CTC TTC TTT ACC TCC GTC CTG CAG CGC TTC ACT TTC ACC GGC ACC AAA CCT CCA GAG GAG ATC AAC ATC GAG CCA GCG TGC TCC AGC 1443
L F F T S V L Q R F T F T G T K P P E E I N I E P A C S S 481
TTT GGC CGC CTT CCG CGT TCC TAT GAT TGC TAC ATC AAA CTC AGG ACT GAG AAG TGA cca ctt tac tgt gga gag gtc aca ggc tca 1530
F G R L P R S Y D C Y I K L R T E K *
gct tca cag gca gct gct tgt caa ttt aca gta acc ata gcc tta cat tta cct cct ttt ttc ttt aga caa cta tat aag cgt cag 1617
tga gtt gta tgt att ttg cta tta tcc tca ggt agg atc tct aag gta ttg gtt gat att tca agg cta att act gtc tac att cat 1704
gtc ttt taa agc ttg ttt ttg gtt agg ttt caa gaa ttg cct tca gag atg ttg gtc ccc ata tct cta tgc tgt ata tca cac tca 1791
cct gct ttg tgc cct tgt aaa ata ggc cta tag gtt cat att aaa tcc tct att gaa aca tta tgg tga aag gac caa aag ttt cag 1878
gtg tta taa tgt aag caa ctg aaa ttg gtc ttg ttt tgt agg ttg cta cgt ggt tag gac tgt aca tct ata cat gca aag cac tta 1965
ggg gta ctg tac act atg tat act ggt ggg gtc act atg tta ttc tgc ctt tgt ttt caa cat gat tga tgt att gta aca caa cat 2052
ttg att gta act ttt gct tca act aat aaa taa cac tgc aca ctt caa aaa aaa aaa a 2113

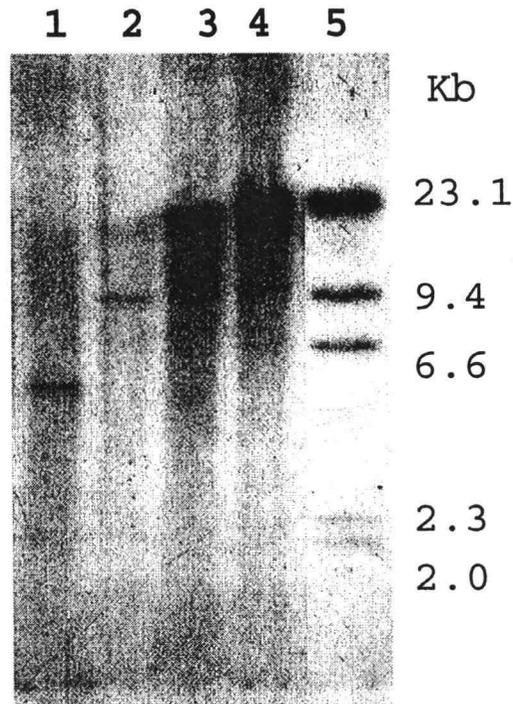
```



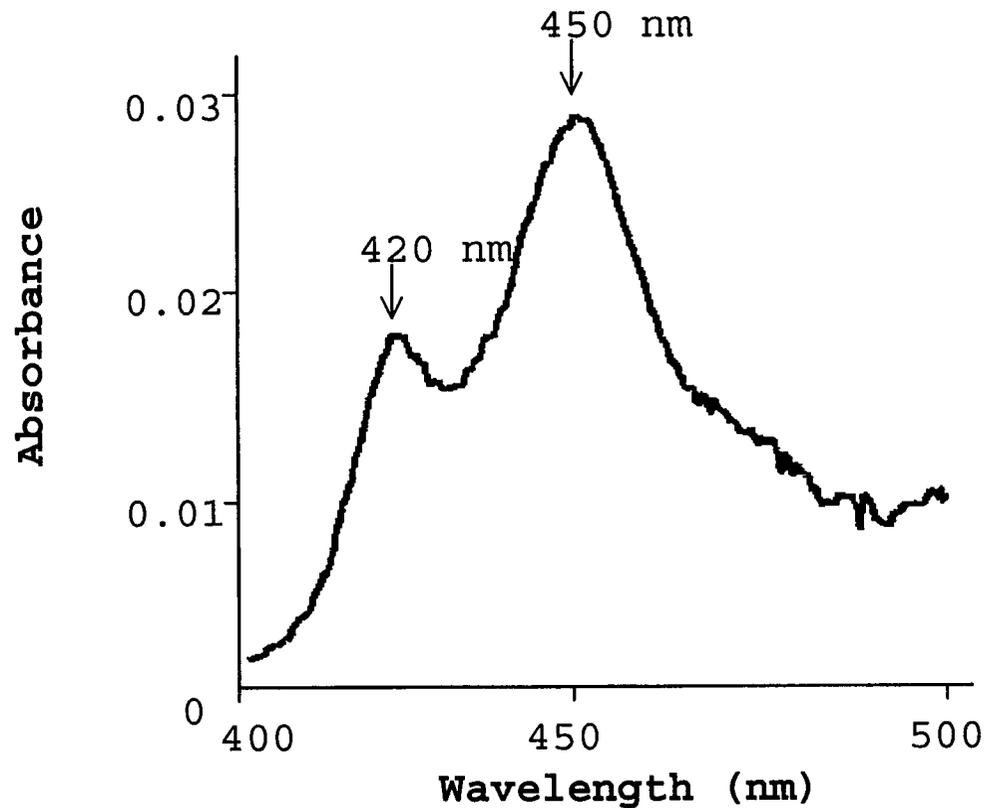
**Fig. 2.2** MALDI-TOF mass spectrum of rainbow trout P450 LMC1. Purified LMC1 was loaded with BSA (66,431 Da) as the internal standard. Sinapinic acid was used as the matrix.

**Fig. 2.3** Northern blot analysis of total RNA for the transcriptional expression of CYP2M1 in liver (A) and trunk kidney (B). 5  $\mu$ g of total RNA was loaded into each lane. Lanes 1-3: juvenile male; lanes 4-6: juvenile female; lanes 7-9: sexually mature male; lanes 10-12: sexually mature female; Lanes 13-15: synthesized CYP2M1 RNA, 12.5 pg, 25 pg, and 50 pg, respectively (the size of this synthesized RNA was 400 bp smaller because part of the 3'-untranslated region was not included). The blots were reprobbed with 18S oligonucleotide as an internal standard. The standard curve was plotted as described in methods.

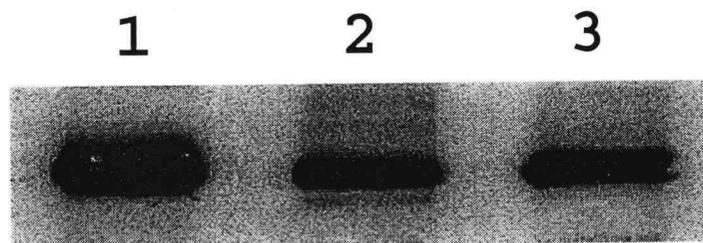




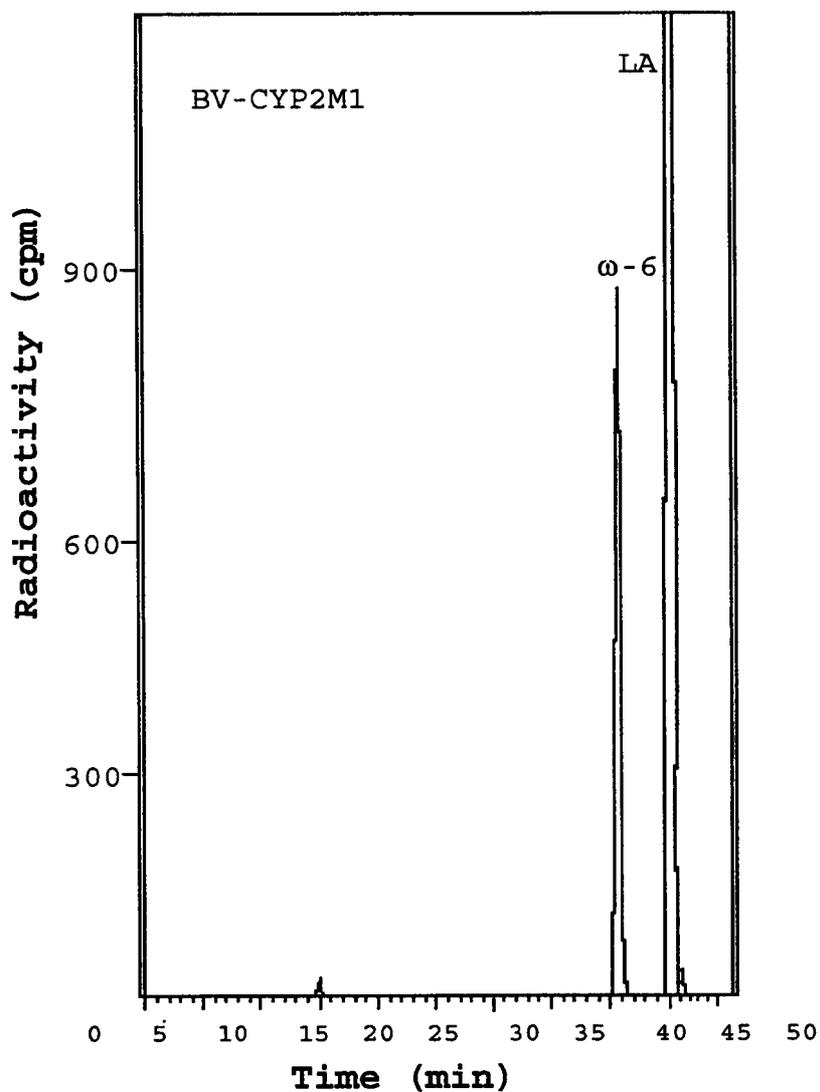
**Fig. 2.4** Southern blot analysis of CYP2M1. The genomic DNA from liver of rainbow trout was probed with 3'-untranslated region of CYP2M1 cDNA. Genomic DNA was digested with four different restriction enzymes. Each lane contained 15  $\mu$ g DNA. Lane 1: XhoI; lane 2: XbaI; lane 3: EcoRI; lane 4: BamHI; lane 5:  $\lambda$ /HindIII DNA marker.



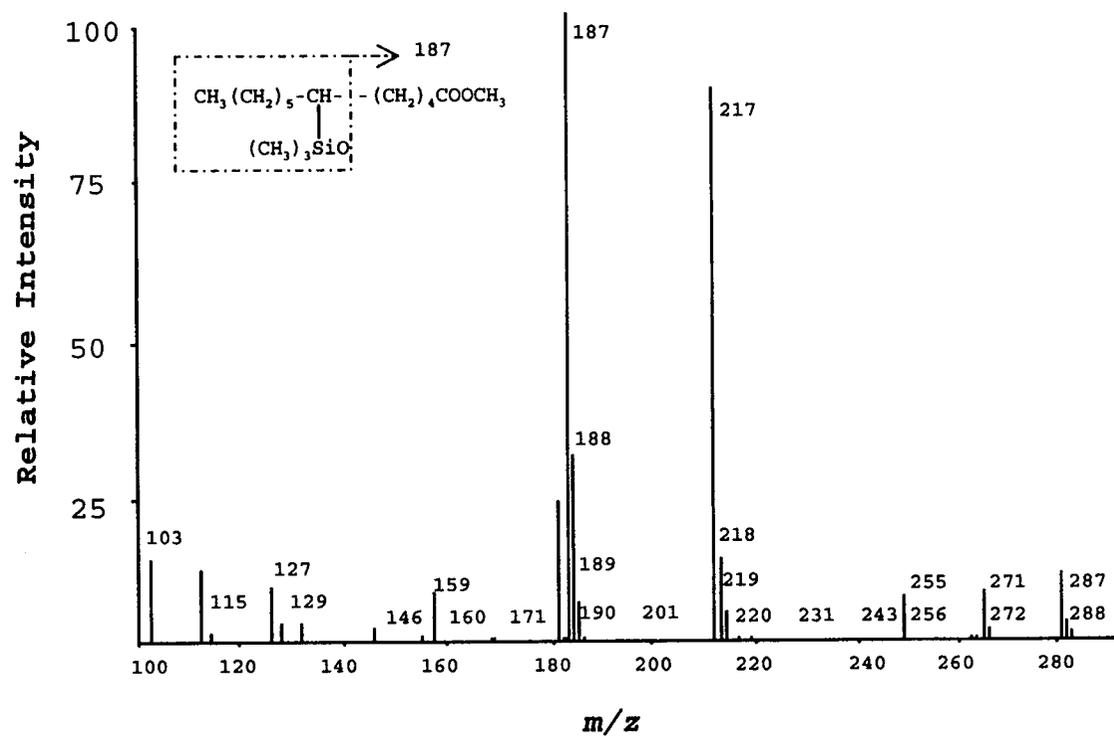
**Fig. 2.5** CO-difference spectrum of baculovirus-expressed CYP2M1. Homogenate (2.5 mg) from infected Sf9 cells was diluted in 2 ml of buffer (100 mM potassium phosphate buffer pH 7.5, 0.1 mM EDTA, 1 mM DTT and 20% glycerol). The difference spectrum was recorded between 400 and 500 nm



**Fig. 2.6** Western blotting of purified P450 LMC1 and expressed CYP2M1. Lane 1: purified P450 LMC1 (1 pmole); lane 2: microsomal protein of CYP2M1 cDNA transfected COS-7 (25  $\mu$ g); lane 3: total cell lysate of CYP2M1 recombinant baculovirus infected Sf9 cells (2.5  $\mu$ g).



**Fig. 2.7** Representative reverse-phase HPLC elution profile of lauric acid (LA) and ( $\omega$ -6)-hydroxylauric acid produced by BV-CYP2M1. The ( $\omega$ -6)-hydroxylauric acid was eluted at 40 min and the parent compound, lauric acid was eluted at 45 min.



**Fig. 2.8** Characteristic mass spectrum of ( $\omega$ -6)-hydroxylauric acid produced by BV-CYP2M1.

## CHAPTER 3

Cloning, Sequencing and Heterologous Expression of CYP2K1  
and CYP2K3 from Sexually Mature Rainbow Trout Liver, and  
Their Roles in Aflatoxin B<sub>1</sub> Activation

Yea-Huey Yang <sup>1,2</sup>, Cristobal L. Miranda <sup>2</sup>, Jun-Lan Wang<sup>2</sup>, and  
Donald R. Buhler <sup>1,2,3</sup>

<sup>1</sup>Genetics Program, <sup>2</sup>Department of Agricultural Chemistry,  
and <sup>3</sup>Marine/Freshwater Biochemical Sciences Center  
Oregon State University  
Corvallis, OR 97331

Correspondence to:

Dr. Donald R. Buhler  
Department of Agricultural Chemistry  
Oregon State University  
ALS 1007  
Corvallis OR 97331-7301

Formatted for submission

### Abstract

CYP2K1 is the most abundant P450 present in the liver of untreated rainbow trout (*Oncorhynchus mykiss*). Our previous study indicated the presence of P450s closely related to CYP2K1 in liver of sexually mature male rainbow trout. In this report, we have cloned and sequenced a cDNA from liver of sexually mature male trout with an open reading frame encoding for a protein of 491 amino acids and a deduced MW of 55,284 Da. Based on the P450 sequence analysis, this trout P450 was designated as CYP2K3 by the P450 Nomenclature Committee. Sequence comparison of CYP2K3 and CYP2K1 revealed 96.5% identity in the amino acid sequences and a 39-bp deletion in close proximity to the heme-binding region of CYP2K3. The coding regions of CYP2K1 and CYP2K3 cDNAs have been cloned to a baculovirus transfer vector pBlueBacIII to make recombinant baculovirus which was subsequently expressed in *Spodoptera frugiperla* (Sf9) insect cells. After extraction with sodium cholate, the heterologously expressed CYP2K1 (BV-CYP2K1) catalyzed the regiospecific hydroxylation of lauric acid (LA) and the epoxidation of aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) in the presence of rat NADPH-cytochrome P450 reductase, with a turnover number of 10.01 (for LA) and 2.02 for (AFB<sub>1</sub>) nmole/min/nmole P450. The formation of AFB<sub>1</sub>-8,9-epoxide was indicated by the appearance of an AFB<sub>1</sub>-GSH conjugate in the incubation mixtures containing mouse liver cytosol or rat liver glutathione transferases and glutathione in phosphate buffer. HPLC analysis of AFB<sub>1</sub>-GSH produced by BV-CYP2K1 and purified P450 LMC2 showed

retention times identical to that produced by human CYP3A4, an enzyme known to form exclusively the AFB<sub>1</sub> exo-epoxide. This result indicated that CYP2K1 is one of the P450s responsible for the exo-epoxidation of AFB<sub>1</sub> in rainbow trout. Expression of CYP2K3 protein in the recombinant virus infected Sf9 cells, however, yielded a protein that did not display the typical CO-difference spectrum and exhibited no enzymatic activity toward lauric acid or AFB<sub>1</sub>.

## Introduction

Rainbow trout (*Oncorhynchus mykiss*) possess a complex cytochrome P450 monooxygenase system which is involved in the metabolism of endogenous and exogenous chemicals (Buhler, 1995). At least five constitutive P450s (LMC1 to LMC5) (Miranda et al., 1989) and two  $\beta$ -naphthoflavone (BNF)-inducible isoforms (a CYP1A1 ortholog and CYP1A3) have been purified (Williams and Buhler, 1983; Gooneratne et al., 1997) and cloned (Heilmann et al., 1988; Berndtson and Chen 1994) from liver of rainbow trout. Three of the constitutive hepatic P450s, LMC1, LMC2 and LMC5, also have been cloned, sequenced (Yang et al., 1997a; Buhler et al., 1994; Lee et al., 1997) and designated as CYP2M1, CYP2K1 and CYP3A27 by the P450 Nomenclature Committee, respectively (Nelson et al., 1996).

CYP2K1 is the first cytochrome P450 that has been cloned from the liver of untreated rainbow trout (Buhler et al., 1994). Based on its nucleotide sequence, this fish P450 was designated as the first member of a new P450 subfamily, CYP2K (Nelson et al., 1996). CYP2K1 cDNA is 1,859 bp long containing an open reading frame encoding a protein of 504 amino acids with a deduced molecular weight of 56,795 Da.

It has been indicated that the protein encoded by CYP2K1 cDNA is identical to P450 LM2 (Williams and Buhler 1983) and P450 LMC2 (Miranda et al., 1989), previously purified from the liver of rainbow trout. The CYP2K1 cDNA was obtained from a trout cDNA expression library screened with both polyclonal and monoclonal antibodies prepared

against trout LMC2. The deduced N-terminal sequence of CYP2K1 matched 12 of the 15 amino acids derived from the purified P450 LMC2 by Edman degradation. The molecular weight of the deduced CYP2K1 protein (56,795 Da) is only 100 daltons different from that of LMC2 (56,695 Da) as determined by MALDI-TOF mass spectrometry. It thus appeared that the cloned CYP2K1 likely expressed the same enzyme as the purified LMC2. The P450 LMC2 form has been shown to exhibit lauric acid ( $\omega$ -1)- and ( $\omega$ -2)-hydroxylase activity (Buhler et al., 1997) and convert AFB<sub>1</sub> to AFB<sub>1</sub>-8,9-epoxide (Williams and Buhler, 1983). Human and rat microsomes bioactivate AFB<sub>1</sub> to produce a mixture of *endo*- and *exo*-epoxides, which can be trapped with glutathione (GSH) and the resulting conjugates separated by HPLC (Raney et al., 1992). The *exo*-epoxide is much more mutagenic than the *endo*-epoxide because the *exo*-form is more efficient in forming a DNA-adduct (Eaton and Gallagher 1994). However, the identity of the epoxide produced by trout P450 LMC2 has not been determined.

In a previous report, Northern, Western and Southern blot analyses indicated the presence of other P450 genes that were closely related to CYP2K1. In the present study, we have isolated a cDNA (CYP2K3) using 5' and 3' RACE-PCR (Rapid Amplification of cDNA Ends-Polymerase Chain Reaction). The catalytic activities of the recombinant CYP2K1 and CYP2K3 proteins toward the diagnostic substrates of purified LMC2, lauric acid and AFB<sub>1</sub>, then were determined. The identity of the AFB<sub>1</sub>-epoxides produced by the P450 LMC2 and the baculovirus expressed CYP2K1 (BV-CYP2K1) also was investigated.

## Materials and Methods

### Experimental animals

Sexually mature 3-year old post-spawning male rainbow trout (*Oncorhynchus mykiss*) of the Mt. Shasta strain were obtained from the Marine/Freshwater Biomedical Sciences Center of Oregon State University.

### Materials

Restriction enzymes (BamHI, HindIII, EcoRI) were from Stratagene (La Jolla, CA) or New England Biolab (Beverly, MA). Marathon cDNA Amplification Kit and TaqStart antibody were purchased from CLONTECH Laboratory Inc. (Palo Alto, CA). TRI REAGENT was purchased from Molecular Research Center (Cincinnati, OH). T4 DNA ligase, fetal bovine serum (FBS), *Trichoplusia ni* Medium-Formulation Hink (TNM-FH) and SF-900 II serum free medium were from GIBCO BRL (Grand Island, NY). pBlueBacIII and Sf9 cells were the kind gifts from Dr. George F. Rohrmann, Department of Agricultural Chemistry, Oregon State University. Linear AcMNPV (*Autographa californica* nuclear polyhedrosis virus) transfection kit and TA cloning kit were purchased from Invitrogen (San Diego, CA). Hybond-N<sup>+</sup> nylon membrane, [ $\gamma$ -<sup>32</sup>P]-dATP, and [1-<sup>14</sup>C]-lauric acid were from Amersham (Amersham, UK). [<sup>3</sup>H(G)]-aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) was purchased from Moravek Biochemicals (Brea, CA). Taq/Pwo DNA

polymerase was obtained from Boehringer Mannheim (Indianapolis, IN). Coomassie plus Protein Assay Reagent was from Pierce Chemical Co. (Rockford, IL). Wizard Miniprep DNA purification system was from Promega (Madison, WI). Acetonitrile (HPLC grade) was from J.T. Baker Chemical (Phillipsburg, NJ). Lauric acid (sodium salt), AFB<sub>1</sub>, purified rat glutathione transferases (GSTs), bovine serum albumin (BSA), hemin and NADPH, were purchased from Sigma Chemical (St. Louis, MO).

#### **Total RNA preparation**

Total RNA from liver of sexually mature male rainbow trout was prepared using TRI Reagent as described previously (Buhler et al., 1994).

#### **cDNA library construction for 5'- and 3'-RACE polymerase chain reaction**

The cDNA library for PCR reactions was made by using Marathon cDNA Amplification kit. For the first strand cDNA synthesis, one  $\mu\text{g}$  of total RNA was primed with a modified lock-docking oligo(dT) primer which contains two degenerate nucleotides at the 3' end. These two degenerate nucleotides position the oligo(dT) primer at the start of the poly-A tail, therefore eliminating the 3' heterogeneity associated with the conventional oligo(dT) priming method. RNaseH<sup>-</sup> Moloney murine leukemia virus (MMLV) reverse transcriptase was used to synthesize the

first strand cDNA; *E.coli* DNA polymerase, RNaseH and *E.coli* DNA ligase then were used to complete the second strand cDNA synthesis. The double stranded cDNA was made blunt ended by T<sub>4</sub> DNA polymerase and used for adaptor ligation. The adaptor ligated cDNA library was diluted 50 times and used for PCR reactions.

The 5'-RACE reactions were performed as follows: in a final volume of 50  $\mu$ l, 5  $\mu$ l of diluted cDNA library was added to 1x PCR buffer (1.75 mM MgCl<sub>2</sub>), 200  $\mu$ M dNTP, 10 pmole specific primer, corresponding to the antisense strand of CYP2K1 cDNA sequences, (5'-CTCAGACCCGGCTCACAGCAC-3'); 10 pmole of adaptor primer 1 (AP1) (5'-CCATCCTAATACGACTCACTATAGGGC-3') and 2.5 unit of Taq/Pwo DNA polymerases. TaqStart antibody was also included to hot start the PCR reaction. The mixtures were incubated at 94°C for 5 min in a DNA Thermal Cycler (Perkin-Elmer Cetus) to start the reaction. The amplification was done for 35 cycles using the following conditions: 94°C for 30 sec; 63°C for 30 sec; 68°C for 3 min; and a final extension at 68°C for 10 min.

The 3'-RACE reactions were performed in a final volume of 50  $\mu$ l containing 5  $\mu$ l diluted adaptor-ligated cDNA library, 1x PCR buffer (1.75 mM MgCl), 200  $\mu$ M dNTP, 10 pmole specific primer corresponding to the sense strand of CYP2K3 cDNA (5'-GGGATATTTTCATCAAAAAGGATG-3'), 10 pmole of adaptor primer 1 (AP1), 0.25-0.5 unit of Perfect Match polymerase enhancer, TaqStart antibody and 2.5 unit of Taq/Pwo DNA polymerases. The PCR reactions were performed as follows: 94°C for 5 min to start the reaction; 35 cycles

of 94°C for 30 sec; 55°C for 30 sec; 68°C for 3 min; and a final extension at 68°C for 10 min.

Aliquots of PCR reactions were separated on 1% agarose gel, stained with ethidium bromide, and then visualized under UV light. The resulting PCR products were excised from the agarose gel and purified using a Prep-A-Gene DNA purification system. Purified PCR products were then ligated to the pCR II vector with a TA cloning kit. Plasmid DNA from positive clones was purified by Wizard miniprep DNA purification system. The presence of correct insert DNA was confirmed by EcoRI restriction digestion followed by agarose gel electrophoresis. Positive clones were selected for sequencing using fluorescence labeled oligonucleotide primers on automated sequencers (ABI Model 373A or 377) serviced by the Center for Gene Research and Biotechnology at Oregon State University. Each base was determined at least once from each strand. Sequence data were analyzed using the MacVector program (International Biotechnologies, Inc., New Haven, CT).

#### **cDNA-directed expression of CYP2K1 and CYP2K3 in Sf9 insect cells**

The CYP2K1 cDNA (Buhler et al., 1994) was excised from pSPORT1 vector by BamHI and HindIII digestion and ligated to the compatible ends of baculovirus transfer vector pBlueBacIII. To remove CYP2K3 cDNA from the pCR II vector, EcoRI was used to digest the insert-containing plasmid and then the insert cDNA was gel purified as

described above. In order to obtain the appropriate restriction sites (5'-BamHI and 3'-HindIII) for directional cloning into pBlueBacIII, the purified CYP2K3 cDNA fragment was first subcloned to EcoRI digested pBK-RSV plasmid. The clones with correct insert orientation (as determined by diagnostic restriction digestion) were digested with BamHI and HindIII to excise the cDNA insert. Each recombinant baculovirus was generated by cotransfection of linear AcMNPV DNA and cDNA containing pBlueBacIII using the AcMNPV linear transfection kit. Insect cells (Sf9) were maintained in complete TNM-HF/10% FBS at 27°C.

The recombinant virus, a homologous recombination product of the pBlueBacIII and the wild type baculovirus AcMNPV DNA, placed CYP2K1 and CYP2K3 expression under the control of virus polyhedrin promoter and  $\beta$ -galactosidase under the virus early-to-late promoter. The recombinant virus was isolated using plaque assay and X-gal for color selection (O'Reilly, 1992). Identification of recombinant virus was first done by visualization of blue and occlusion body negative plaques. The purity of the plaques and the presence of cDNA insert were then confirmed by PCR using recombinant baculovirus PCR forward (5'-TTTACTGTTTTTCGTAACAGTTTTG-3') and reverse (5'-CAACAACGCACAGAATCTAGC-3') primers. The PCR products were further analyzed by diagnostic restriction digestion. Due to the differences in expression level between each recombinant virus, at least three different recombinant viruses were selected from each transfection to examine the protein yield by Western blotting (Laemmli, 1970)

probed with rabbit polyclonal antibodies raised against P450 LMC2 (Miranda et al., 1989). The most productive virus was amplified to prepare high titer stock for expression. The presence of cDNA and the absence of contaminating virus in the high titer stock were confirmed again using PCR.

To express CYP2K1 and CYP2K3, Sf9 cells were grown in SF-900 serum-free medium to a density of  $1.8-2 \times 10^6$  cells/ml in spinner flasks (Bellco Glass) at room temperature (23-24°C). Cells were infected at MOI (multiplicity of infection) of 1 to 5. An equal molar mixture of BSA and hemin stock solution, prepared as described (Gragan et al., 1995) was added at the time of infection to a final concentration of 1.0 µg/ml of hemin to compensate for the low endogenous levels of hemin in the insect cells. Production of P450 during the infection was monitored by the CO-reduced difference spectral measurement as described by Omura and Sato (1964). Cells were harvested 84-90 hr after infection, washed once with phosphate buffer saline (PBS), and resuspended in 100 mM potassium phosphate pH 7.4, 1 mM EDTA, 1 mM dithiothreitol (DTT), 20% glycerol and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). Cell lysates were prepared by sonication in an ice bath at a power setting of 2 (SONICATOR, model W-10, Heat Systems-Ultrasonics, Plainview, NY) until more than 80% of cells were broken as determined microscopically. The total P450 content was measured using reduced CO-difference spectrometry (Omura and Sato 1964). Since it has been reported that a P450-containing insect cell homogenate treated with sodium cholate

exhibited higher catalytic activity than the crude cell lysate (Sigle et al., 1994; Yang et al., 1997a), aliquots of cell lysate were exposed to 1.4% sodium cholate overnight at 4 °C, then centrifuged at 16,000 x g for 20 min. The cholate-treated, P450-containing supernatant and crude cell lysate were used for enzyme assays. The protein concentration was determined by using Coomassie Plus Protein Assay Reagent.

### **Western blot analysis**

Total cell lysate proteins from recombinant baculovirus-infected Sf9 cells were separated by electrophoresis in SDS-7.5% polyacrylamide gel. The separated proteins were electro-blotted to a nitrocellulose membrane. The protein containing membrane was blocked with 5% nonfat dry milk (Lucerne) in TBS/T buffer (20 mM Tris HCl pH 7.5, 0.5M NaCl, 0.05% Tween 20). After blocking, the membrane was incubated for 30 min with rabbit anti-trout LMC2 polyclonal antibodies (Miranda et al., 1989) or mouse anti-trout LMC2 monoclonal antibodies (3M1 and 3M3) (Wang et al., in preparation). Unbound antibody was removed by washing in TBS/T buffer. Detection was carried out using [<sup>125</sup>I]-protein A for polyclonal antibodies and autoradiography with X-ray film (XAR5), or goat anti-mouse antibodies conjugated to horseradish peroxidase and ECL Western blotting detection reagents for monoclonal antibodies.

## Enzyme assays

Purified LMC2 (0.05 nmole) or 0.1 nmole of expressed BV-CYP2K1 (cholate-treated or crude lysate) or 2 mg of BV-CYP2K3 (cholate-treated or crude lysate) were preincubated with 0.4 nmole of purified rat liver NADPH-cytochrome P450 reductase, 0.2 nmole of rabbit cytochrome b5 and 10  $\mu$ g of dilaurylphosphatidylcholine (DLPC) at room temperature for 10 min. Buffer (50 mM Tris-HCl, pH 7.4) and substrate (200  $\mu$ M [1-<sup>14</sup>C]-lauric acid or 100  $\mu$ M [<sup>3</sup>H]-AFB<sub>1</sub>, final concentration) were then added to the mixture followed by addition of NADPH to 1 mM in a total volume of 0.5 ml. After a 1-hr incubation at 27°C, the reaction was stopped by the addition of 0.1 ml of 10% sulfuric acid (for lauric acid hydroxylation) or 0.5 ml methanol (for AFB<sub>1</sub> oxidation). Lauric acid metabolites were extracted and analyzed by HPLC as described by Buhler et al. (1997). Because the standards for lauric acid metabolites are not available for HPLC, GC/MS was used to further analyze the metabolites as described previously (Buhler et al., 1997). Metabolites of AFB<sub>1</sub> in the supernatant obtained after a 20-min centrifugation of the reaction mixture was analyzed by HPLC. Separation of AFB<sub>1</sub> metabolites (as Tris-diols or dihydrodiols) by HPLC was performed on a 5  $\mu$ m Prodigy ODS-2 column (250 x 4.6 mm, Phenomenex) at a flow rate of 1 ml/min with detection at 362 nm and by radioactivity monitoring (FLO-ONE  $\beta$ eta, Packard). Mobile phases were methanol and 0.1% ammonium phosphate, pH 3.0. Solvent flow was 28% methanol for 15 min followed by a linear gradient to 80% methanol in 5 min. At 25 min, the methanol was returned to

28% in 2 min and the column equilibrated for an additional 13 min to return to baseline before the next injection. Based on the conversion efficiency of AFB<sub>1</sub> to AFB<sub>1</sub>-dihydrodiol or AFB<sub>1</sub> Tris-dihydrodiol conjugate, turnover numbers (nmole/min/nmole P450) were calculated.

Glutathione S-transferase (GST)-catalyzed conjugation of AFB<sub>1</sub> bioactivated by BV-CYP2K1, BV-CYP2K3 and purified LMC2 was performed under the following conditions. The incubation mixtures consisted of 0.05 nmole purified LMC2, 2 mg of CYP2K3-containing Sf9 cell homogenate, or 0.1 nmole [(BV-CYP2K1, or baculovirus expressed human CYP3A4 and CYP1A2 (GENTEST Corporation) as positive controls] of P450, 0.4 nmole of rat NADPH-cytochrome P450 reductase, 0.2 nmole of cytochrome b5, 10 µg of dilaurylphosphatidylcholine (DLPC), 1 mM glutathione (GSH), 0.3 mg of purified rat GST (or 0.5 mg of mouse liver cytosol), 1 mM NADPH and 100 µM [<sup>3</sup>H]-AFB<sub>1</sub> in a total volume of 0.5 ml. After a 1-hr incubation at 27°C (for BV-CYP2K1, BV-CYP2K3 and purified LMC2) or 37°C (for CYP3A4 and CYP1A2), the reaction was stopped by the addition of 100 µl of 2M acetic acid. When juvenile male trout liver microsomes were used, the NADPH-cytochrome P450 reductase, cytochrome b5 and DLPC were omitted in the incubation mixture. After incubation the samples were frozen for 2 hr and then thawed and centrifuged at 16,000 x g for 20 min. The supernatant was analyzed by HPLC using a Pirkle-concept chiral column packed with D-phenylalanine covalently bound to aminopropyl silica (4.6 x 250 mm) as described by Stresser et al. (1994).

## Results

### **CYP2K3 cDNA cloning and sequence analysis**

To isolate CYP2K1-related cDNA from rainbow trout liver, a PCR primer at the 3' end coding region of CYP2K1 cDNA was used along with AP1 in the 5'-RACE reaction. This primer contained the stop codon and its flanking region, which is highly conserved among the CYP2K cDNAs examined (unpublished observation). The resulting PCR product (about 1.5 Kb) was subjected to TA cloning. Five clones were selected and partially sequenced. One clone with the complete open reading frame was found to contain a 39-bp deletion close to the heme-binding domain. Surprisingly, the deletion did not disrupt the open reading frame but resulted in the loss of 13 amino acids. The amino acid sequences next to the 5' and 3' ends of the deletion remain unchanged. To further analyze and confirm the presence of this cDNA, a specific primer, 5'-GGGATATTTTCATCAAAAAG≈GATG-3', where the "≈" marks the deletion site, flanking the deletion region was used in the 3'-RACE reactions. Two identical cDNA fragments were identified in two separate PCR reactions. Sequence analysis of the 5'-and 3'-RACE products revealed a full-length cDNA that was 1,914 bp in length and contained an open reading frame coding for a protein of 491 amino acids with a calculated molecular weight of 55,284 Da, a 77-bp 5'-untranslated region and a 364-bp 3'-untranslated region with two putative polyadenylation signals and a poly(A) tail (Fig. 3.1). On the basis of high similarity to

CYP2K1, this P450 was designated CYP2K3 by the P450 Nomenclature Committee. Sequence comparison between CYP2K1 and CYP2K3 exhibited 96.5% identity in amino acid sequence (Fig. 3.2) and 97.6% identity in nucleotide sequence of the coding region.

### **CYP2K1 and CYP2K3 expression in Sf9 cells**

The coding regions of CYP2K1 and CYP2K3 cDNA were ligated to the pBlueBacIII baculovirus transfer vector under the control of polyhedrin promoter. The resulting recombinant baculovirus were plaque-purified, amplified and used in infecting Sf9 cells for overexpression. A typical P450 CO-difference spectrum with maximum at 450 nm was detected from the homogenate of CYP2K1 producing Sf9 cells (Fig. 3.3). No P450-type CO-difference spectrum was formed in the homogenate from control cells and cells infected with CYP2K3 recombinant and wild type viruses. The specific content of spectrally active CYP2K1 was estimated at 30-70 nmole/L culture. Lysates from CYP2K1 and CYP2K3 recombinant baculovirus infected cells contained proteins that cross-reacted with polyclonal anti-LMC2 antibodies. Similar electrophoretic mobilities were exhibited by P450 LMC2, CYP2K1 and CYP2K3 upon Western blotting analysis (Fig. 3.4A). However, CYP2K3 was not recognized by monoclonal antibodies (3M1 and 3M3) raised against LMC2 as shown in Fig. 3.4B and 3.4C. Cell homogenate from Sf9 cells or Sf9 cells infected with wild type virus did not show any cross-reactivity with either antibody (data not shown).

### Catalytic properties of cDNA-expressed P450s and purified P450 LMC2

P450 LMC2 was the major P450 catalyzing the ( $\omega$ -1)-hydroxylation of lauric acid (Buhler et al., 1997) and bioactivation of AFB<sub>1</sub> (Williams and Buhler 1983) in liver of rainbow trout. Therefore, lauric acid and AFB<sub>1</sub> were used as substrates to determine the catalytic activities of baculovirus-expressed CYP2K1 (BV-CYP2K1) and CYP2K3 (BV-CYP2K3). Crude lysate from BV-CYP2K1 containing insect cells showed lauric acid hydroxylase activity with turnover number of 1.33 nmole/min/nmole P450. Cholate treatment increased the activity of BV-CYP2K1 to 10.01 nmole/min/nmole P450 (Table 3.1). Purified P450 LMC2 gave a turnover number of 5.07 nmole/min/nmole P450 (Table 3.1), with ( $\omega$ -1)-hydroxylauric acid the major metabolite. A small amount of ( $\omega$ -2)-hydroxylation product also was produced by both BV-CYP2K1 and purified LMC2 (Fig. 3.5). The identity of lauric acid hydroxylation products was further confirmed by GC/MS analysis (data not shown).

As seen in the lauric acid hydroxylation, cholate-extracted cell lysate gave higher catalytic activity toward AFB<sub>1</sub> than the crude lysate. Both purified P450 LMC2 and BV-CYP2K1 converted AFB<sub>1</sub> to dihydrodiols with catalytic activities of 6.88, 0.39 (crude lysate) and 2.02 (cholate-treated) nmole/min/nmole P450, respectively (Table 3.1).

It was not known if BV-CYP2K1, or purified LMC2 are capable of forming the *exo*- and *endo*-epoxides of AFB<sub>1</sub>. To answer this question, incubations were carried out using the trout P450s to generate the AFB<sub>1</sub>-epoxides which were then

assayed as glutathione (GSH) conjugates when incubations were performed in the presence of purified rat liver glutathione transferases (GSTs) or mouse liver cytosol. Glutathione transferases from rat were partially purified and contained alpha- and mu-class GSTs that have been shown to conjugate both endo- and exo-epoxides of AFB<sub>1</sub>. The mouse liver cytosol (mainly alpha-class) conjugates the exo-epoxide almost exclusively (Raney et al., 1992). As a positive control, insect cell (BTI-TN-5B1-4) microsomes containing cDNA-expressed human CYP1A2 and CYP3A4 (GENTEST Corporation) were used as catalysts for the formation of the two forms of AFB<sub>1</sub>-epoxides. Purified P450 LMC2, BV-CYP2K1 and human CYP3A4 produced a single AFB<sub>1</sub>-GSH peak in the presence of mouse liver cytosol and GSH (Fig. 3.6A, 3.6B, and 3.6C) with retention time at 15 min. A single AFB<sub>1</sub>-GSH peak (retention time = 15 min) was also produced when rat GSTs was used in place of mouse liver cytosol in incubations containing BV-CYP2K1, purified LMC2 or human CYP3A4 (Fig. 3.7A, 3.7B, and 3.7C). Microsomes from liver of juvenile rainbow trout incubated with either rat GSTs or mouse liver cytosol produced a single AFB<sub>1</sub>-GSH peak with identical retention time observed in BV-CYP2K1 and purified LMC2 (data not shown). Human CYP3A4 and CYP1A2, however, each gave a single AFB<sub>1</sub>-GSH peak (Fig. 3.7C, 3.7D) with different retention times (16.2 min for CYP1A2 and 15.0 min for CYP3A4) in the presence of rat GST. When mouse liver cytosol was used instead of rat GSTs, an AFB<sub>1</sub>-GSH peak was formed by CYP3A4 but not by CYP1A2. In addition to the AFB<sub>1</sub>-GSH conjugates, aflatoxin M<sub>1</sub> (AFM<sub>1</sub>) and aflatoxin Q<sub>1</sub>

(AFQ<sub>1</sub>) were also formed by CYP1A2 and CYP3A4, respectively (Fig. 3.6C, 3.6D, 3.7C, 3.7D).

Under the identical reaction condition, no lauric acid hydroxylation or AFB<sub>1</sub> metabolites were detected in cells infected with CYP2K3-containing recombinant baculovirus, Sf9 cells only, and Sf9 cells infected with wild type virus.

### *Discussion*

A CYP2K1-related P450, designated as CYP2K3, was cloned from the liver of sexually mature male rainbow trout using 5'- and 3'-RACE. The fidelity of the cDNA sequences was ascertained by comparing the sequence of two PCR products from two separate PCR reactions. CYP2K1 and CYP2K3 shared high homology in their coding regions (96.5 % in amino acid, 97.6% in nucleotide sequences, respectively); however, they showed only 47% identity in the 3'-untranslated region. Two putative polyadenylation signals were located 11 bp and 31 bp upstream the poly(A) tail. Multiple polyadenylation signals were also found in the cDNA of CYP11A from southern stingray (Nunez and Trant 1997), porcine P450arom (Choi et al., 1996), and human CYP2E1 (Seree et al., 1995). Most amino acid changes in CYP2K3 were conserved alterations as compared to that of CYP2K1 (Fig. 3.2). Comparison of the CYP2K3 amino acid sequence by alignment with some eukaryotic P450s and prokaryotic P450s of known structure (Peterson and Graham-Lorence, 1995; Wachenfeldt and Johnson, 1995) revealed that the 13-amino acid deletion found in the CYP2K3 corresponds to the P450 conserved core, where the secondary structure arranged in  $\beta$ 1 sheet and  $\alpha$ K' helix. The deletion may disrupt the normal structural organization of the P450. A glycine residue, (G396), a highly conserved residue in family 2 P450s, is in the deletion region of CYP2K3. Western blot analysis of BV-CYP2K1 and BV-CYP2K3 probed with anti-LMC2 polyclonal antibodies showed that cDNA-directed expression of P450 did occur in insect cells. The lack of cross-reactivity between the anti-LMC2 monoclonal antibodies

(3M1 and 3M3) and CYP2K3 suggested that the epitopes recognized by these two antibodies may lie within the deletion region of the CYP2K3 or where other amino acids are changed. These results imply that CYP2K3 protein was expressed in Sf9 cells but was either unstable or could not form the correct conformation for heme insertion because of the deletion and/or other amino acid alterations. Nonfunctional transcripts caused by point mutation or deletion also have observed in CYP2A (Yamano et al., 1990), CYP2B (Yamano et al., 1989), and CYP17 (Fardella, et al., 1993)

As observed in other reports (Sigle et al., 1994; Yang et al., 1997a), cell lysate treated with cholate increased the catalytic activities. The lauric acid hydroxylation activity of BV-CYP2K1 increased 7.5-fold after treated with cholate (1.33 vs 10.01 nmole/min/nmole P450). The cholate-treated BV-CYP2K1 exhibited 2 times higher activity toward lauric acid than reconstituted P450 LMC2 (5.07 nmole/min/nmole P450) (Table 3.1). The BV-CYP2K1 treated with cholate was more active than the reconstituted P450 LMC2 which suggests that under the condition used, the insect cells can better support CYP2K1 lauric acid hydroxylase activity. Cholate has been shown to facilitate the incorporation of P450 or other proteins into functional complexes, and protein-protein interaction is increased by such incorporation into liposomes (Ingelman-Sundberg 1977; Ingelman-Sundberg and Glaumann, 1980). The stimulation of enzyme activity by cholate also has been demonstrated as the result of enhanced interaction of P450 and NADPH-cytochrome P450 reductase in the reconstitution systems of several

P450s in CYP3A subfamily (Imaoka et al., 1992) and rabbit CYP1A2 (Yun, et al., 1997).

The catalytic activity of BV-CYP2K1 toward AFB<sub>1</sub> was also improved 5-fold by cholate extraction with turnover number of 0.39 vs 2.02 nmole/min/nmole P450. However, the maximum activity of BV-CYP2K1 attained was only 30% that of reconstituted P450 LMC2 (6.88 nmole/min/nmole P450) (Table 3.1). This observation indicated that BV-CYP2K1-mediated AFB<sub>1</sub> metabolism might not be fully active under the conditions used, or some of the AFB<sub>1</sub>-epoxide was bound to the DNA or proteins in the cell homogenate before being trapped by Tris or converted to dihydrodiol, resulting in less metabolite in the HPLC profile. Further purification of BV-CYP2K1 and the optimization of reaction conditions are necessary to improve the activity of expressed CYP2K1.

Although AFB<sub>1</sub>-epoxides have not been isolated from biological systems, their presence can be detected indirectly by trapping agents such as DNA (Daniel et al., 1990), GSH/GST (Degen and Neumann, 1978; Hayes et al., 1991; Raney et al., 1992), and Tris (Neals and Colley, 1979). BV-CYP2K1, purified P450 LMC2 and trout liver microsomes catalyzed the metabolic activation of AFB<sub>1</sub> to epoxides that bind to GSH in the presence of mouse liver cytosol or purified rat liver GSTs.

Human CYP1A2 has been shown to catalyze the conversion of AFB<sub>1</sub> to *exo*- and *endo*-epoxides whereas CYP3A4 catalyzes only the formation of the *exo*-epoxide (Ueng et al., 1995). The *exo*-epoxide is the form primarily responsible for AFB<sub>1</sub>-induced carcinogenesis. These two epoxides are conjugated with reduced glutathione (GSH) in the presence of GSTs. In

humans and rats, the mu-class GSTs have the highest catalytic activity for AFB<sub>1</sub> *exo*- and *endo*-epoxides. By contrast, the mouse liver cytosol, which contains mainly alpha-class GST, conjugates the *exo*-epoxide almost exclusively (Raney et al., 1992; Eaton and Gallagher, 1994). Trout liver cytosol (containing GSTs), however, has little or no conjugating activity toward AFB<sub>1</sub> epoxides (Valsta et al., 1988).

To verify the identity of AFB<sub>1</sub>-epoxide produced by BV-CYP2K1 or purified P450 LMC2, mouse liver cytosol was added to the incubation mixtures. Upon separation by chiral HPLC, only one AFB<sub>1</sub>-GSH conjugate peak was found with BV-CYP2K1, purified P450 LMC2 and trout liver microsomes with retention times identical to the *exo*-epoxide GSH conjugate produced by human CYP3A4. These results suggest that the AFB<sub>1</sub>-epoxide produced by trout P450 was mainly in the *exo*-form. To detect the presence of *endo*-epoxide, purified rat hepatic GSTs were added to the incubation mixture containing phosphate buffer. Human CYP1A2, which is known to form both the AFB<sub>1</sub> *exo*- and *endo*-epoxide (Ueng et al., 1995), was used as a positive control. Unexpectedly, only one AFB<sub>1</sub>-GSH peak, instead of two peaks, was formed during incubation with rat liver GSTs (Fig. 3.7D). The retention time of this peak (16.2 min) was longer than the AFB<sub>1</sub>-GSH peak (retention time = 15 min) formed by CYP3A4 (Fig. 3.7C), a P450 known to produce only the *exo*-epoxide. The AFB<sub>1</sub>-GSH peak produced by CYP1A2 may represent the *endo*-epoxide, which is known to appear later than the *exo*-form during HPLC separation using the chiral column (Raney et al., 1992). Contrary to the previous finding of Ueng et al. (1995), the cDNA-expressed

human CYP1A2 did not appear to form the *exo*-epoxide in our incubations (Fig. 3.6 and 3.7). However, in our study, only one peak (retention time = 15 min) was produced by BV-CYP2K1, purified CYP2K1 or trout liver microsomes during incubation with rat GSTs. Based on the retention times, it appeared that the AFB<sub>1</sub>-GSH conjugate formed by cDNA-expressed CYP2K1, purified P450 LMC2 or trout liver microsomes corresponds to the *exo*-epoxide produced by CYP3A4.

In summary, we have cloned a new cytochrome P450 CYP2K3 that is closely related to the previously identified CYP2K1. However, CYP2K3 cDNA-directed expression in insect cells did not produce a functional enzyme. A 13-amino acid deletion in CYP2K3 disrupting the conserved core structure of the P450 may account for the inactivity of this protein. The results of immunological and enzymatic assays indicate that cDNA-expressed CYP2K1 and the purified trout hepatic P450 LMC2 are identical proteins. The AFB<sub>1</sub>-epoxide formed by cDNA-expressed CYP2K1 or purified trout liver LMC2 binds to GSH in the presence of a purified rat GST mixture or mouse liver cytosol. Only one single peak was detected with retention time identical to that produced by human CYP3A4 which is known to form exclusively the AFB<sub>1</sub> *exo*-epoxide. This indicates that trout CYP2K1 converted AFB<sub>1</sub> only to the *exo*-epoxide metabolite.

### *Acknowledgement*

The authors would like to thank Ms. Marilyn C. Henderson for HPLC analysis and Mr. Maciej Kedzierski for computer graphing. We also thank Dr. Jerry D. Hendricks, Mr. Ted Will and Mr. Dan Arbogast of the Marine/Freshwater Biomedical Sciences Center, OSU for their help in the care and maintenance of the trout used in this study. The authors would also like to thank Dr. George Rohrmann and his associates of Department of Agricultural Chemistry, Oregon State University for providing the cell culture facility and assistance in insect cell culture and Mr. Larry Hodges for help in baculovirus and insect cell culture. This work was supported by NIEHS grants ES00210, ES03850 and ES04766. This manuscript was issued as Technical Paper No. xxxxx from Oregon Agricultural Experiment Station, Oregon State University, Corvallis, OR.

TABLE 3.1. Comparison of the catalytic activities of BV-CYP2K1, BV-CYP2K3 and purified LMC2

**ω-1 Hydroxylation of lauric acid**

	<u>Turnover number (nmole/min/nmole P450)</u>
BV-CYP2K1	1.33
BV-CYP2K1 (cholate treated)	10.01
BV-CYP2K3	ND*
BV-CYP2K3 (cholate treated)	ND*
Purified LMC2	5.07

**AFB1-diol formation**

	<u>Turnover number (nmole/min/nmole P450)</u>
BV-CYP2K1	0.39
BV-CYP2K1 (cholate treated)	2.02
BV-CYP2K3	ND*
BV-CYP2K3 (cholate treated)	ND*
Purified LMC2	6.88

\* ND, not detectable

**Fig. 3.1** Nucleotide and deduced amino acid sequences of CYP2K3 cDNA. The nucleotides of the translation initiation codon, ATG, are indicated by underline and bold. The stop codon is denoted by (\*). Two putative polyadenylation signals in the 3'-untranslated region are highlighted.

cacacacttggtctattaaggagcattggtgtgtgtgatttttagcccagtataagggtgatgagctcagctcgaagATGTCTCTTTAAGAAGGTCTTCT  
M S L I E G L L  
ACAGACATCAAGCACAGTGACACTGCTGGGTAAGTGTGCTGTTTCTGCTGGTCTCTACCTCCGCTCCTCTGGTTCAGCTCAGAGGGACAGGGGAAGGAG  
Q T S S T V T L L G T V L F L L V L Y L R S S G S S S E G Q G K E  
CCTCCAGGACCGAGGCCGCTGCCCTGCTTGGTAACATGCTCCAGCTGGACCTCAAGAAGCCCTACTGCACTCTCTGCGAGCTCTCCAAGAAATATGGTT  
P P G P R P L P L L G N M L Q L D L K K P Y C T L C E L S K K Y G  
CCATCTTCACGTTTCACTTTGGACCCAAGAAAGTGGTTGTTCTGGCAGGATAACAAGACGGTCAAGCAGGCGCTGGTCAACCAGGCAGAGGACTTTGGGGA  
S I F Y F H F G P K K V V V L A G Y K T V K Q A L V N Q A E D F G D  
CAGGGACATCACTCTGTGTTCTATGATTTCAACCAAGGACATGGGATTCTGTTTGGCAATGGAGACTCATGGAAAAGAGATGAGGCGCTTTGCCCTGACA  
R D I T P V F Y D F N Q G H G I L F A N G D S W K E M R R F A L T  
AACCTTAGAGACTTTGGGATGGGCAAGAAAGGGAGTGAGGAGAAAATCTTGGAGGAAAATCCCCTACCTGATTGAAGTGTGAAAACATGAGGGTAAGG  
N L R D F G M G K K G S E E K I L E E I P Y L I E V F E K H E G K  
CATTGACACCACCCAGTCTGTGCTTTATGCCGTCTCCAACATAATCTCAGCCATTGTCTATGGCAGCAGGTTTGAATACACTGACCCACTATTACAGG  
A F D T T Q S V L Y A V S N I I S A I V Y G S R F E Y T D P L F T G  
CATGGCGGACAGGGCCAAGGAGATTTACACCTTACAGGTTCTGCATCAATTCAGATGTACAACATGTTTCCCTGGTTGGGTCCGTGGATAAACCAACCTG  
M A D R A K E S I H L T G S A S I Q M Y N M F P W L G P W I N N L  
ACACGTCTGAAAAAGAATATTGCTGATATGAAGATGGAGGTGACAGAGCTGGTGAGGGGCCTGAAGGAGACTCTGAACCCTCACATGTGTAGAGGCTTTG  
T R L K K N I A D M K M E V T E L V R G L K E T L N P H M C R G F  
TGGACTCGTTCCCTCGTCCGAAAGCAAACCCTGGAGGAATCTGGCCATATGGATTCTTCTACCATGACGACAACCTGGTATTTAGTGTTGGTAACCTGTT  
V D S F L V R K Q T L E E S G H M D S F Y H D D N L V F S V G N L F  
TTCTGCTGGTACCGACACCACCGGGACAACCCTGCGCTGGGGTCTGCTGCTAATGACCAAGTACCCCATATACAGGATCAGGTCCAGGAGGAGATCAGC  
S A G T D T T G T T L R W G L L L M T K Y P H I Q D Q V Q E E I S  
GGGGTCATAGGAAGTCGTCAAACCTTGGTAGAGGACAGGAAGAACCTGCCCTACACGGATGCAGTGATCCATGAGACCCAGAGACTGGCCAATATTGCAC  
G V I G S R Q T L V E D R K N L P Y T D A V I H E T Q R L A N I A  
CCATGTCCATCCCTCACACCACCGAGATGTGACCTTCCAGGGATATTTTCATCAAAAAGGATGATAGCGAATGGGAGAGCCCTCACACCTTAACCCC  
P M S I P H T T S R D V T F Q G Y F I K K D D S E W E S P H T L T P  
CTCCCCTTTCTGGATGAGAAGGGAGGATTTGTCAAGAGGGACGCCTTTCATGGCCTTCTCTGCAGGTTCGTAGGGTGTGTCTGGGGGAGGGTCTGGCCAGG  
S H F L D E K G G F V K R D A F M A F S A G R R V C L G E G L A R  
ATGGAGCTCTTCTTCTTTCACCTCCCTCCTGCAGCACTTTCGTTTCTCTCCTCCTGGAGTAACAGAGGATGATCTGGACCTGACACCATCCGTGG  
M E L F L F F T S L L Q H F R F S P P G V T E D D L D L T P S V  
AGTTCACCCACAATCCGTCACCTACCAGCTATGTGCTGTGAGCCGGTCTGAgattagaacatgaaaatattggtggggcaaacatcaaaaaagtg  
E F T H N P S P H Q L C A V S R V \*  
aataatcccagaattagtttccgccaataacacaaacattattatatttagtttatgaacggttcatagggcacctccaataccttttacatttaattac  
Actgaagactacggtagtggaattatatttatagagtgaagtctattttacttcattttcagcaatgggtctgagctattgctaattgctaagattgct  
Aagagtattgggacgcagctccaacacggttctgagggtagttgcatccgtctgtctgttaataaaataatcctgttaagaaataaagcaacaaaaaaaaa  
aaaaaaaaaaaaaaaa

```

2K1  MSLIEDILQT SSTVTLLGTV LFLLVLYLRS SGSSSEEQ GK EPPGPRPLPL LGNMLQLDLK KPYCTLCELS 70
2K3  -----GL----- -G-----

2K1  KKYGSIFYVH FGPKKVVVLA GYKTVKQALV NQAEDFGDRD ITPVFYDFNQ GHGILFANGD SWKEMRRFAL 140
2K3  -----F-----

2K1  TNLRDFGMGK KGSEEKILEE IPYLIEVF EK HEGKAFD TTQ SVLYAVSNII SAIVYGSRFE YTDPLFTGMA 210
2K3  -----

2K1  DRAKESIHLT GSASIQMYNM FPWLG PWINN LTRLKKN IAD MKMEVIELVR GLKETLNPHM CRGFVDSFLV 280
2K3  -----T-----

                * * *
2K1  RKQTL EESGH MDSFYHDDNL VFSVGNLFSA GTDTTGT TLR WGLLLMTKYP HIQDQVQEEI SRVIGSRQTL 350
2K3  -----G-----

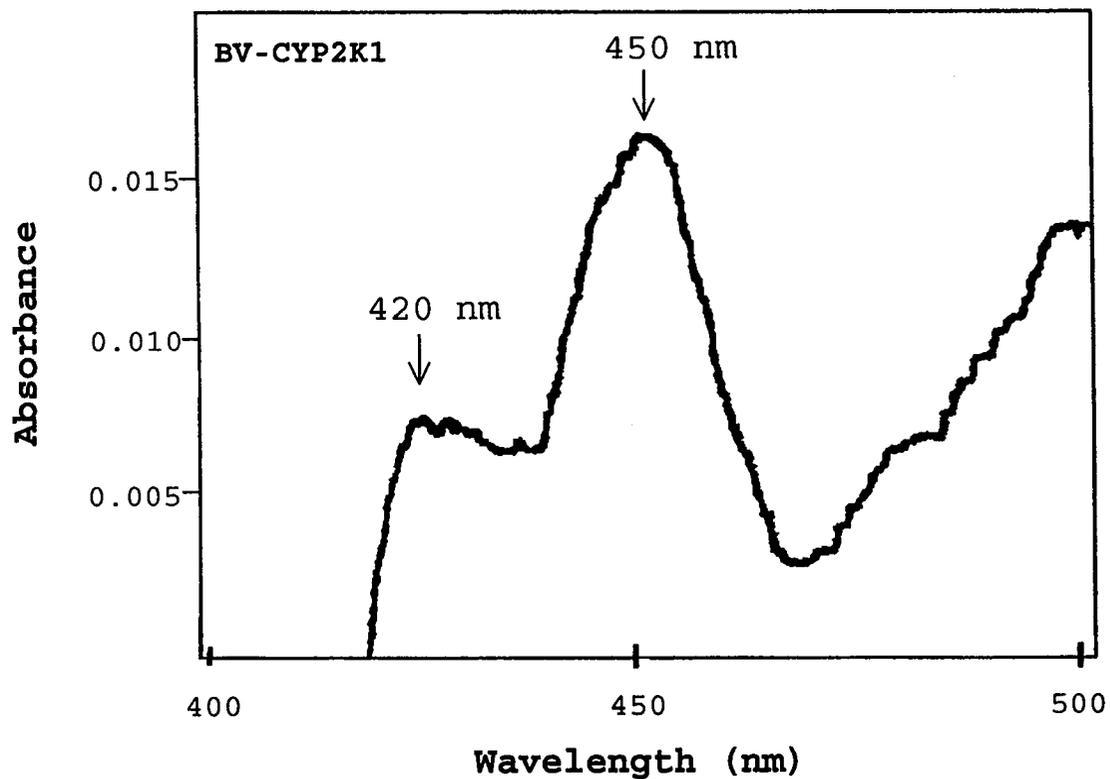
                * *                **                *
2K1  VEDRKNLPYT DAVIHETQRL ANIVPMSVPH TTSRDVTFQG YFIKKGTSVI PLLTSVLODD SEWESPNTFN 420
2K3  -----A--I----- [ ] -----H-LT

                * * * *
2K1  PSHFLDEQGG FVKRDAFMA F SAGRRVCLGE GLARMELFLF FTSL LQRFRF SPPPGVTEDD LDLTPLLGFT 490
2K3  -----K-----H-----SVE--

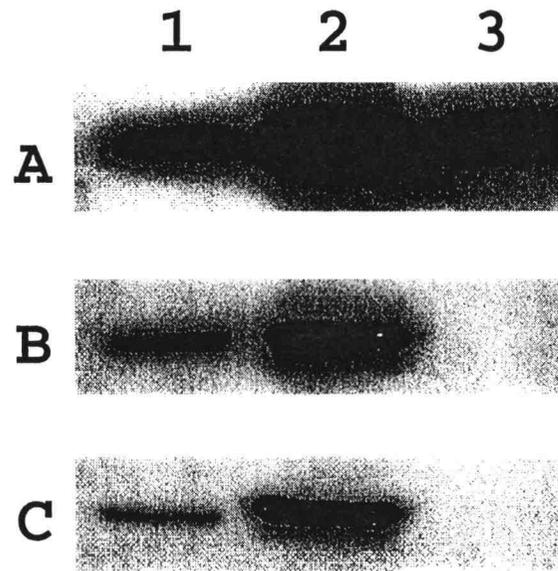
2K1  LNPSPHQLCA VSRV
2K3  H-----

```

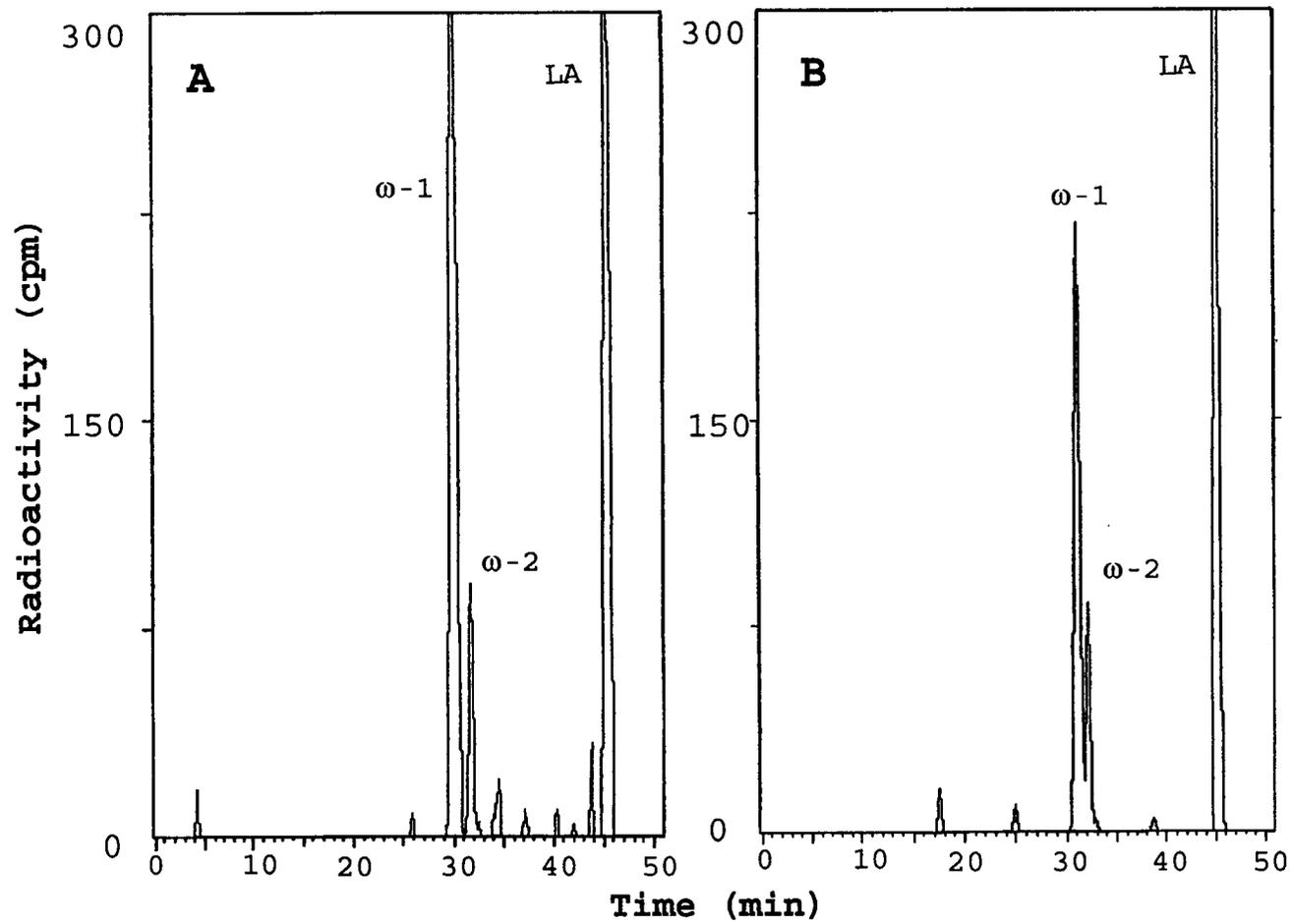
**Fig. 3.2** Amino acid sequence alignment between CYP2K1 and CYP2K3. Sequence alignment was performed using the MacVector program. Dash line indicates the identical amino acid residues. Open box presents the site of deletion found in CYP2K3. An asterisk above the aligned sequences indicates the highly conserved residue found in family 2 of cytochromes P450. The conserved heme-binding domain is shown in the box.



**Fig. 3.3** CO-reduced difference spectrum of BV-CYP2K1. The spectrum of the cDNA-expressed CYP2K1 was measured at 450 nm in 0.1 M potassium phosphate buffer pH 7.4, 1 mM EDTA, 20% glycerol, and 1 mM DTT using cell homogenate from CYP2K1 recombinant virus infected Sf9 cells.

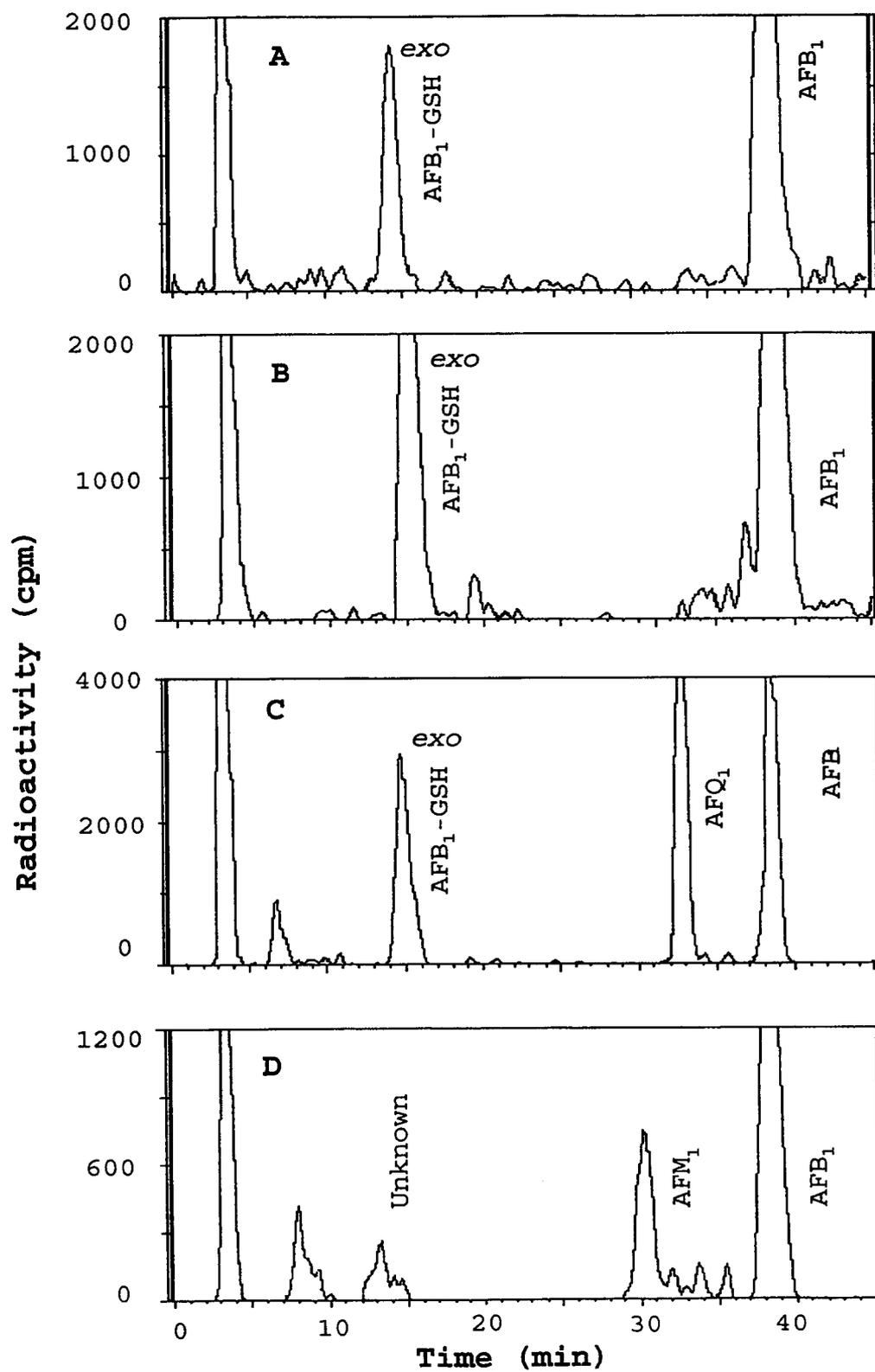


**Fig. 3.4** Western blotting of baculovirus-expressed CYP2K1 and CYP2K3. Blots were probed with polyclonal anti-LMC2 antibodies (A) and monoclonal anti-LMC2 antibodies - 3M1 (B) and 3M3 (C). Lane 1: 0.25 pmole of purified LMC2; lane 2: BV-CYP2K1 in 2.5  $\mu$ g of Sf9 cell homogenate; lane 3: BV-CYP2K3 in 2.5  $\mu$ g of Sf9 cell homogenate.

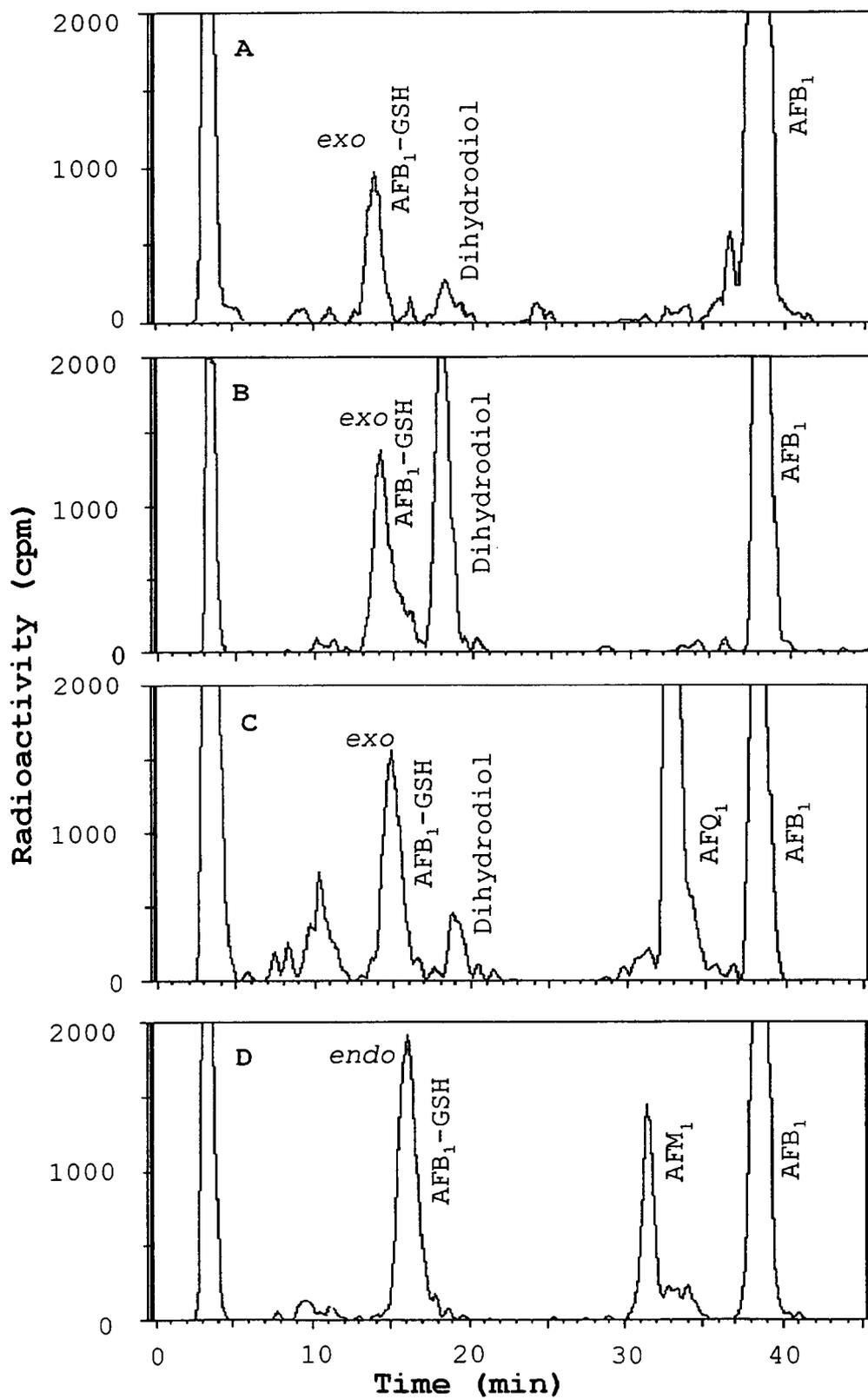


**Fig. 3.5** HPLC chromatograms of [ $^{14}\text{C}$ ]-lauric acid hydroxylation metabolites produced by BV-CYP2K1 (A) and purified LMC2 (B).

**Fig. 3.6** HPLC chromatograms of AFB<sub>1</sub>-GSH conjugates produced by BV-CYP2K1 (A), purified LMC2 (B), human CYP3A4 (C) or human CYP1A2 (D) in the presence of mouse liver cytosol and GSH.



**Fig. 3.7** HPLC chromatograms of AFB<sub>1</sub>-GSH conjugates produced by BV-CYP2K1 (A), purified LMC2 (B), human CYP3A4 (C) or human CYP1A2 (D) in the presence of purified rat GSTs and GSH.



## CHAPTER 4

**CYP2K4: a New Cytochrome P450 Isoform from Male Trunk  
Kidney of Post-Spawning Rainbow Trout**

Yea-Huey Yang<sup>1,2</sup>, Tommy. B. Andersson<sup>5</sup>, Byung-Woo Ryu<sup>4</sup>,  
Jun-Lan Wang<sup>2</sup>, Donald R. Buhler<sup>1,2,3,4</sup>

<sup>1</sup>Genetics Program, <sup>2</sup>Department of Agricultural Chemistry,  
<sup>3</sup>Marine/Freshwater Biomedical Sciences Center, and  
<sup>4</sup>Toxicology Program, Oregon State University, Corvallis, OR,  
97331

<sup>5</sup>University of Göteborg, Sweden

Correspondence to

Dr. Donald R. Buhler  
Department of Agricultural Chemistry  
Oregon State University  
ALS 1007  
Corvallis OR 97331-7301

Formatted for submission

### Abstract

To investigate the nature of the 1.9 and 2.8 Kb bands previously found to hybridize with a 440 bp 3'-terminal cDNA probe (2K1,7c) for CYP2K1, a  $\lambda$ -gt11 cDNA library from the trunk kidney of post-spawning (3-yr) male rainbow trout was screened using a CYP2K1 full-length cDNA as a probe. By combination of the nucleotide sequences from the resulting kid8 clone together with 5'- and 3'- RACE products, a full-length cDNA was assembled. This cDNA was 2,601 bp in length, contained an open reading frame encoding for a protein of 504 amino acids with a calculated MW of 56,745 Da. It contained an extra long 1 Kb 3'-untranslated region (2K4-3'u) which was not found in CYP2K1. On the basis of P450 sequence comparisons, this trout P450 was assigned as CYP2K4 by the P450 Nomenclature Committee. The deduced N-terminal amino acid sequence of CYP2K4 matched 12 out of 16 identified N-terminal amino acids of KM2, a male-specific P450 previously purified from the trunk kidney of mature male rainbow trout. Northern blot analysis was performed using the 1,055 bp 3'-RACE PCR product that contained the 3'-untranslated region plus a small segment of the open reading frame as a probe for the liver and kidney of 3-yr trout. A single 2.8-Kb CYP2K4 cross-hybridized mRNA band was detected in the trunk kidney and liver of 3-yr old male trout but not in females of the same age, or juvenile liver and trunk

kidney of both sexes. The expression of this 2.8 Kb transcript in the trunk kidney, therefore, was male specific, with mRNA concentrations much higher in the trunk kidney than in the liver.

## Introduction

The cytochrome P450-dependent monooxygenase system constitutes a superfamily of heme-thiolate enzymes that are active in the oxidative metabolism of various structurally different endogenous and xenobiotic compounds. Cytochromes P450 are expressed primarily in the liver, but also are present in other tissues.

As seen in mammals, multiple forms of P450 occur in fishes (Stegeman and Hahn, 1994). At least five constitutive P450 forms (P450 LMC1 to LMC5) and two  $\beta$ -naphthoflavone (BNF)-inducible forms (a CYP1A1 ortholog and CYP1A3) were isolated and characterized from the liver of juvenile rainbow trout (*Oncorhynchus mykiss*) (Miranda et al., 1989; Williams and Buhler, 1983; 1984; Gooneratne et al., 1997). Three of the constitutive cytochrome P450s [CYP2K1 (LMC2) (Buhler et al., 1994) and CYP2M1 (LMC1) (Yang et al., 1997a) CYP3A27 (LMC5) (Lee et al., 1997)] have been cloned and sequenced. The steroidogenic cytochrome P450s cDNA were also cloned from the ovary of rainbow trout and their catalytic activities characterized (Sakai et al., 1992; Tanaka et al., 1992; Takahashi et al., 1993).

In general, the liver is considered to be the major organ expressing cytochromes P450, however, trunk kidney of rainbow trout produces certain P450s with levels comparable or higher than liver depending on the developmental stage examined (Williams et al., 1986; Andersson, 1990). The kidneys of most fishes are slender, elongate, dark red organs extending along the dorsal

aspect of the body. The kidney of teleost fish such as rainbow trout is divided into two distinct regions with differing structure and function. The head kidney (anterior portion) contains lymphoid, hemopoietic, interrenal and chromaffin tissues. The interrenal and chromaffin tissues correspond to the adrenal cortex and medulla of higher vertebrates, respectively (Bond 1996). The trunk kidney (posterior portion) has the normal mammalian renal functions. The P450-dependent monooxygenase activity was 3- to 14- fold higher in the trunk kidney of rainbow trout than in the head kidney (Pesonen et al., 1990). Sexually mature rainbow trout displayed a pronounced sex difference in the levels and activities of P450-mediated reactions. This sexual dimorphism was much pronounced in trunk kidney (~20-fold) than in liver (~2-fold). The high level of P450 expressed in sexually mature male trout trunk kidney corresponded to previously purified LM2 (Williams and Buhler, 1984) [also identified as LMC2 or CYP2K1 (Miranda et al., 1989; Buhler et al., 1994)] immunologically and enzymatically (Williams et al., 1986; Yang et al., 1997b). An earlier report (Andersson 1992) described the purification of a P450 isoform (KM2) with an apparent MW of 52,000 Da from the trunk kidney of sexually mature male trout. This major cytochrome P450 isoform constituted 66% of the total P450 present. Western blot analysis exhibited extensive sexual dimorphism in the expression of P450 KM2. Levels of the KM2 P450 were 120-fold lower in the kidney of sexually mature female rainbow trout than in sexually mature male

trout. This P450 KM2 also strongly cross-reacted with polyclonal anti-LMC2 antibodies.

In our previous study, we observed similar sexual dimorphism in the expression of CYP2K1 in liver and kidney of rainbow trout using both Western and Northern blotting (Buhler et al., 1994). Trunk kidney from mature male trout expressed two CYP2K1 cDNA hybridizable mRNAs, in the 1.9 Kb and 2.8 Kb regions. A 1.9 Kb mRNA was found at similar levels in the juvenile trunk kidney of both sexes. As rainbow trout reached the late reproduction stage, however, the 2.8 Kb mRNA was expressed very strongly in the trunk kidney of male rainbow trout but in the trunk kidney of mature females, CYP2K1 cDNA hybridizable mRNAs were very weak or nondetectable.

In the present study, we have cloned and sequenced a full-length cDNA from sexually mature trout kidney by cDNA library screening and RACE-PCR (Rapid Amplification of cDNA Ends-Polymerase Chain Reaction). To further understand the nature of this new P450 and its relationship to the 2.8 Kb CYP2K1 hybridizable mRNA transcript, Northern blot analysis with a CYP2K4 specific 3'-RACE PCR product as a probe was used to examine the transcriptional expression of this cytochrome P450.

## *Material and Methods*

### **Experimental animals**

Three-year-old post-spawning untreated sexually mature male and female rainbow trout (*Oncorhynchus mykiss*) of Mt. Shasta strain were obtained from Marine/Freshwater Biomedical Sciences Center of Oregon State University were used in this study.

### **Total and poly(A)<sup>+</sup> RNA isolation**

Total RNA from the liver and trunk kidney of rainbow trout was prepared using TRI Reagent as described by Buhler et al. (1994). Poly(A)<sup>+</sup> RNA from trunk kidney of a single but representative sexually mature male rainbow trout was isolated by Quick Prep micro mRNA purification kit from Pharmacia (Uppsala, Sweden).

### **Construction and screening of $\lambda$ -gt11 cDNA library**

Poly(A)<sup>+</sup> mRNA from trunk kidney of a sexually mature male rainbow trout was used for cDNA synthesis. A cDNA library was prepared in the  $\lambda$ -gt11 vector using BRL SuperScript Choice System and Gigapack (Stratagene, La Jolla, CA) *in vitro* lambda packaging system according to

the manufacturers' instructions. The library was amplified by growing in *E.coli* Y1088.

Approximately  $3 \times 10^5$  pfu were screened with [ $^{32}\text{P}$ ]-labeled full-length CYP2K1 cDNA probe using Prime-it II random primer labeling kit (Stratagene). Hybridization was performed in Quikhyb solution (Stratagene) and washed as described previously (Buhler et al., 1994). The positive clones were selected and plaque-purified by repeated screening. The cDNA inserts were excised from the  $\lambda$ -gt11 vector by NotI restriction enzyme and then subcloned to pSPORT1 vector for sequencing.

#### **Rapid amplification of cDNA ends by polymerase chain reaction (5'- and 3'-RACE)**

RACE-PCR was used to obtain the 5'- and 3'-ends of cDNA isolated from a  $\lambda$ -gt11 cDNA library from trunk kidney of sexually mature male rainbow trout. Briefly, poly(A)<sup>+</sup> mRNA was used to make the cDNA library for PCR reactions. The cDNA was synthesized by using a Marathon cDNA amplification kit (CLONTECH Laboratory, Palo Alto, CA) according to manufacturer's instructions. For the first strand cDNA synthesis, one  $\mu\text{g}$  of mRNA was primed with a modified lock-docking oligo(dT) primer which contained two degenerate nucleotides at the 3' end. These two degenerate nucleotides positioned the oligo(dT) primer at the start of the poly-A tail, therefore eliminating the 3'-heterogeneity associated with the conventional oligo(dT) priming method. The double stranded cDNA was

made blunt ended by T<sub>4</sub> DNA polymerase and then used for adaptor ligation.

The 5'-RACE reactions were performed as follows: in a final volume of 50  $\mu$ l, 5  $\mu$ l of diluted cDNA library was added to 1x PCR buffer (1.75 mM MgCl<sub>2</sub>), 200  $\mu$ M dNTP, 10 pmole of specific primer (GSP1) corresponding to the antisense strand of CYP2K1 cDNA sequences (5'-CTCAGACCCGGCTCACAGCAC-3'), 10 pmole of adaptor primer 1 (AP1) (5'-CCATCCTAATAC GACTCACTATAGGGC-3'), and 2.5 unit of Taq/Pwo DNA polymerases (Boehringer Mannheim, Indianapolis, IN). TaqStart antibody (CLONTECH Laboratory) was also included to hot start the PCR reaction. The mixtures were incubated at 94°C for 5 min to start the reaction in a DNA Thermal Cycler (Perkin-Elmer Cetus), then the amplification was done for 35 cycles under the following conditions: 94°C for 30 sec, 63°C for 30 sec, 68°C for 3 min, and a final extension at 68°C for 10 min. The design of GSP1 was based on the cDNA sequence of cloned P450 obtained from the screening of kidney cDNA library. The resulting PCR product should contain the complete open reading frame.

The 3'-RACE reactions were performed in final volume of 50  $\mu$ l containing 5  $\mu$ l diluted cDNA library, 1x PCR buffer (1.75 mM MgCl<sub>2</sub>), 200  $\mu$ M dNTP, 10 pmole of specific primer (GSP2) corresponding to the sense strand of P450 cDNA isolated from kidney cDNA library, 10 pmole of adaptor primer 1 (AP1), 0.25 - 0.5 unit of Perfect Match polymerase enhancer (Stratagene, La Jolla, CA), TaqStart antibody and 2.5 unit of Taq/Pwo DNA polymerases. The mixtures were incubated at 94°C for 5 min to start the

reaction in a DNA Thermal Cycler (Perkin-Elmer Cetus), then the amplification was done for 35 cycles under the following conditions: 94°C for 30 sec, 55°C for 30 sec, 68°C for 3 min, and a final extension at 68°C for 10 min. The resulting PCR product should contain the 3'-untranslated region and 38 bp of 3'-end of the open reading frame.

The resulting PCR products were gel purified and then ligated to the pCR II vector with a TA cloning kit (Invitrogen, San Diego, CA).

#### **cDNA sequencing**

Positive clones from cDNA library screening and TA cloning of PCR products were selected for sequencing using fluorescence labeled oligonucleotide primers on automated sequencers (ABI Model 373A or 377) serviced by the Center for Gene Research and Biotechnology. At least two separate clones from two independent PCR reactions were sequenced. Each base was determined at least once from each direction. Sequence data were analyzed using the MacVector program (International Biotechnologies, Inc., New Haven, CT).

#### **Northern blot analysis**

Total RNA was extracted from liver and trunk kidney using TRI Reagent (Molecular Research center, Cincinnati,

OH). Northern blots were performed exactly as described previously (Buhler et al., 1994). Briefly, total RNA was glyoxylated, separated by 0.8% agarose gel electrophoresis and then transferred to Hybond-N<sup>+</sup> (Amershan) nylon membrane. Hybridization with [<sup>32</sup>P]-labeled 3'-RACE product (2K4-3'u) or CYP2K1,7c was carried out following the same procedure as described in the cDNA library screening. The RNA-containing membrane was also stripped and reprobbed with an 18S ribosomal oligonucleotide (5'-ACGGGCGGTGTGTRC-3') as an internal standard.

## Results

### Cloning and sequencing analysis

A full-length CYP2K1 cDNA was used as a probe to screen a  $\lambda$ -gt11 kidney cDNA library. Several clones were isolated but none of them was full-length. Therefore, the RACE-PCR approach was used to clone the 5'- and 3'-ends of the cDNA. Because of the designed of the primers, the 5'-RACE product should contain the complete coding region of the gene and the 3' RACE-product should contain the entire 3'-untranslated region plus 38 bp at the 3'-terminal of the reading frame. As expected, a major 1.6 Kb PCR product was found in the 5'-RACE reaction, and a single 1 Kb fragment was seen in 3'-RACE (Fig. 4.1). A full-length cDNA was assembled from the positive clone of the  $\lambda$ -gt11 cDNA library (kid8) and the RACE-PCR products (Fig. 4.2). The resulting cDNA was 2,601 bp containing an open reading frame of 1,512 bp encoding a protein of 504 amino acids, a 5'-untranslated region of 72 bp, and a 3'-untranslated region of 1,017 bp. The putative polyadenylation signal is located 14 bp upstream of the poly A tail (Fig. 4.3). On the basis of deduced amino acid sequence, this new P450 was designated as CYP2K4 by the P450 Nomenclature Committee. The sequence of the N-terminal end of CYP2K4 matches closely that of KM2 obtained by Edman degradation since twelve amino acid residues out of 16 identified are identical (Fig. 4.4).

### **Northern blot analysis**

To investigate the expression of CYP2K4 at the transcriptional level, total RNA from liver and trunk kidney of sexually mature rainbow trout were used in Northern blot analysis. Because CYP2K1 and CYP2K4 share a very high homology (91.2%) in nucleotide sequence of the coding region, the CYP2K4-specific CYP2K4-3'UTR cDNA was used as a probe. Only a single 2.8 Kb mRNA band was detected in the liver and trunk kidney of 3-yr male trout, with a higher level of expression in the trunk kidney than in liver. This transcript was not observed in the liver and trunk kidney of juvenile (data not shown) and sexually mature female trout (Fig. 4.5A). The expression of CYP2K4 was male specific based on the Northern blot analysis. A separate hybridization with the CYP2K1,7c probe showed two bands at 1.9 and 2.8 Kb (Fig. 4.5B) as observed in the previous report (Buhler et al., 1994).

### *Discussion*

In our previous report, we have shown two CYP2K1,7c cDNA-hybridizable mRNA bands from liver and trunk kidney of sexually mature male trout in Northern blot analysis (Buhler et al., 1994). Based on the size of the 3'-RACE product, the longer transcript (2.8 Kb) has a longer 3'-untranslated region. Using this PCR product (CYP2K4-3'u) as a probe in the Northern blot hybridization, only the 2.8 Kb band was detected. When CYP2K1,7c was used as a probe, however, male liver and kidney clearly showed the presence of both 1.9 and 2.8 Kb bands (Fig. 4.5B). Both transcripts were not observed in the liver and trunk kidney of 3-yr sexually mature female trout. Separate experiments also failed to show a 2.8 Kb mRNA species in the liver and trunk kidney of juvenile trout (data not shown). These results further indicated that the size difference observed in the Northern blot was caused by the different length of the 3'-untranslated region and the expression of CYP2K4 was male specific.

Western blot analysis of microsomal protein from trunk kidney of male trout cross-hybridized strongly with monoclonal antibodies raised against P450 LMC2 (CYP2K1) (Buhler et al., 1994) and polyclonal antibodies raised against P450 KM2 (Andersson et al., 1992). Enzymatic analysis using microsomes from trunk kidney of sexually mature male rainbow trout exhibited high activity toward lauric acid and aflatoxin B<sub>1</sub> (Williams et al., 1986) that were also metabolized by CYP2K1 (Yang et al., 1997b). These results suggest the presence of CYP2K-related

proteins in the trunk kidney of sexually mature male trout.

Comparison of N-terminal amino acid sequence of CYP2K4 and KM2 indicates similarity (12 amino acids are identical) but not identity as 4 of the first 21 amino acid residues are different. Definitive information on the similarity of the two sequences is not possible as the identity of 5 of the amino acids of KM2 was uncertain from Edman degradation.

Sequence comparison between CYP2K4 and CYP2K1 showed remarkable homology in the 5'-flanking and the coding regions. Only two nucleotide differences were found in the first 58 bp of the 5'-untranslated region of CYP2K1. 94.2% identity in amino acid sequences and 91.2% identity in nucleotide sequences were observed in the coding region. However, CYP2K4 had a much longer 3'-untranslated region of 1,017 bp while CYP2K1 had a shorter 289 bp 3'-untranslated region which shared about 57% of homology to the corresponding sequences in CYP2K4. CYP2K4 also exhibited high homology with CYP2K3 isolated from liver of sexually mature male rainbow trout (Yang et al., 1997b), 92.9% identity in the deduced amino acid sequences and 96% identity in the nucleotide sequences of the coding region. The amino acid sequences of the conserved heme-binding domain were 100% identical in CYP2K1, CYP2K3 and CYP2K4.

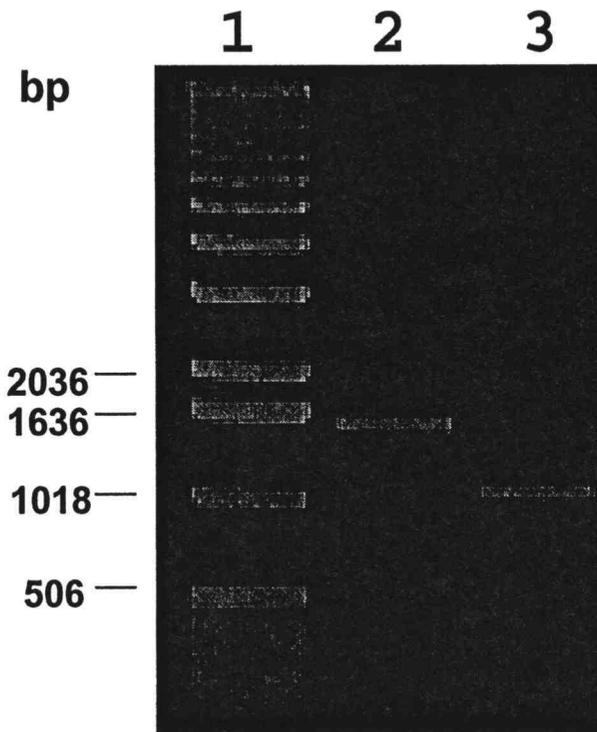
Accumulation of mutation, gene conversion, P450 gene duplication and deletion caused by the unequal crossover, and the natural selection are the major driving mechanisms for the P450 evolution observed in mammalian P450 superfamily (Kimura et al., 1989; Heim and Meyer, 1992).

The complexity of CYP2K genes observed in rainbow trout also could be the result of gene duplication and continuing evolution of CYP2K genes.

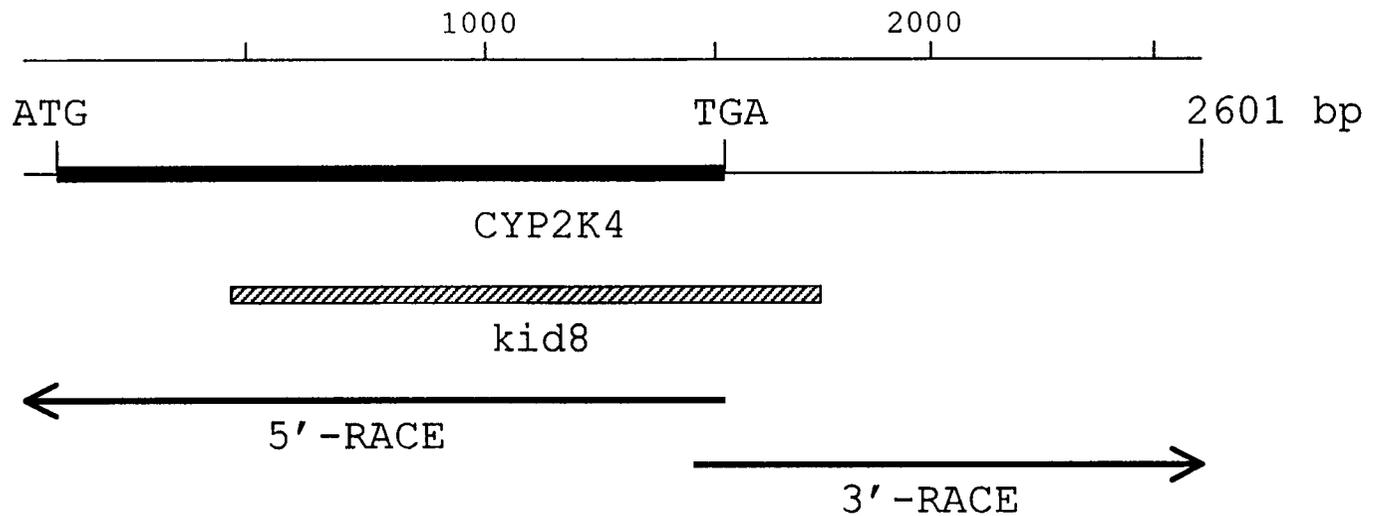
In summary, we have cloned a new cytochrome P450 designated as CYP2K4 from the trunk kidney of sexually mature male rainbow trout. The deduced amino acid sequence of CYP2K4 is 92.9% and 94.0% identical to trout CYP2K3 and CYP2K1, respectively. CYP2K4 also shared high homology to the N-terminal amino acid residues of 450 KM2. CYP2K4 expression is age- and sex-dependent as also observed in the expression of CYP2K1 and CYP2M1. The levels of 2.8 Kb mRNA were high in the trunk kidney of sexually mature male trout but no signal was detected in the liver and trunk kidney of juvenile fish from both sexes (data not shown) and sexually mature female rainbow trout (Fig. 4.5A). CYP2K4 was expressed also in the liver of mature male trout but to a lesser extent. Further study of the substrate specificity and catalytic activity of CYP2K4 using cDNA-directed expression in the baculovirus expression system will contribute the understanding of the physiological function of this P450 and its role in xenobiotic metabolism.

*Acknowledgement*

This work was supported by NIEH grants ES00210, ES03850 and ES047661.



**Fig. 4.1** RACE-PCR products. The PCR products were separated by 1% agarose gel electrophoresis. Lane 1: 1 Kb DNA ladder; lane 2: 10  $\mu$ l of 5'-RACE product; lane 3: 10  $\mu$ l of 3'-RACE product.

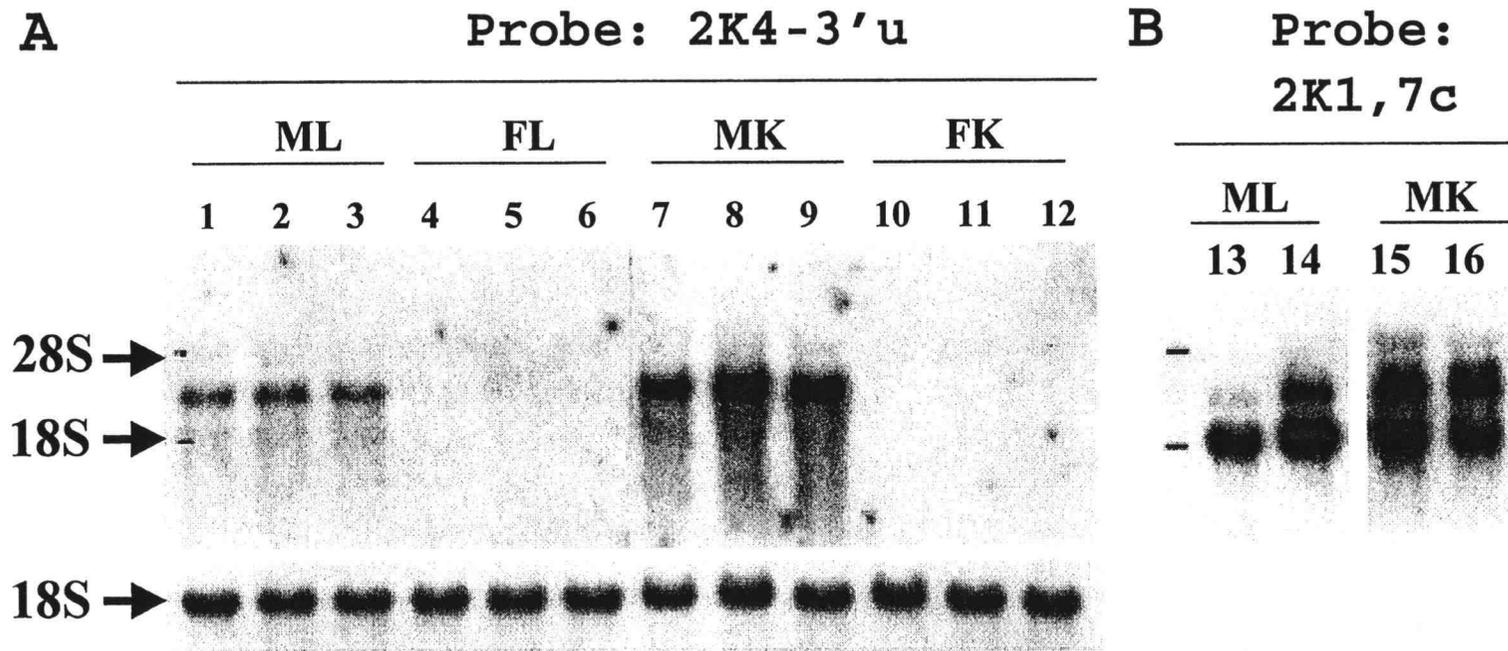


**Fig. 4.2** Assembly strategy for the cloned CYP2K4 cDNA. The open reading frame of CYP2K4 is indicated by a closed box. The hatched box (kid8) indicates the sequence obtained from the screening of cDNA library using CYP2K1 cDNA as a probe. The direction and extent of 5'- and 3'-RACE are indicated by horizontal arrows.



2K4	MSLIEGLLQT	SSTVTL LGTV	IFLLVPYLRS	SGSSSEEQ GK	EPPGPRPL PL	LG NMLQLDLK	KPYCTLCELS	70
KM2	xx---D---	xx---GxLF						
2K1	---DI---		---L---					
2K3				---G---				
2K4	KKYGSIFTVH	FGSKKVVVLA	GYKTVKQALV	NQAEDFGERD	ITPAFYDFNQ	GHGILFTNGD	SWKEMRRFAL	140
2K1		---P---		---D---	---V---	---A---		
2K3		---F---						
2K4	TNLRDFGMGK	KGSEEKILEE	IPYLIEVLEK	HEGKAFTTQ	SVHHA VS NII	SAIVYGSRFE	YTDPLFTGMV	210
2K1			---F---		---LY---		---A---	
2K3								
2K4	DRV NENVHLI	GSASIQMYNM	FPWLG PWINN	LTRLKKNVAD	LKMEVIELVR	GLKETLNPHM	CRGFVDSFLV	280
2K1	--AK-SI--T			---I---	M---			
2K3					---T---			
2K4	RKQTL EESGK	MDSFYHDDNL	LFSIGNLFGA	GTDTTGTTLR	WGLLLMAKYP	HIQDQVQEEI	SRVIGSRQTL	350
2K1	-----H		V--V--S-		---T---			
2K3							---G---	
2K4	VEDRK NLPYT	DAVIHETQRL	ANISPM AVPH	TTSRDVTFQG	YFIKKGTSVI	PLLMSVLQDD	NEWESPNTFN	420
2K1			---V--S---			---T---	S-----	
2K3			---A--I---					
2K4	PSHFLDEQGG	FVKRDAFMAF	SAGRRVCLGE	GLARMELFLF	FTSLLQRF RF	SPPPGVTEDD	LDLTPLLGF T	490
2K1								
2K3	-----K--				---H---		---SVE--	
2K4	LHPSPHQLCA	VSRV						
2K1	-N-----							
2K3	H-----							

**Fig. 4.4** Amino acid sequence alignment between CYP2K4, CYP2K1, CYP2K3 and the N-terminal region of P450 KM2. The heme-binding region is shown in a solid box. "x" indicates the unidentified amino acid residue. The open box shows the deletion region in CYP2K3.



**Fig.4.5** Northern blot analysis of CYP2K4. A. The blot was probed with CYP2K4-3'u (a 1,055 bp 3'-untranslated region plus 38 bp of open reading frame). Lane 1-3: 3-yr male liver (ML); lane 4-6: 3-yr female liver (FL); lane 7-9: 3-yr male trunk kidney (MK); lane 10-12: 3-yr female trunk kidney (FK). The bottom lanes showed the 18S rRNA as internal standard which was probed as described in methods. B. Selected lanes of a Northern blot of liver (ML, lane 13 and 14) and trunk kidney (MK, lane 15 and 16) from 3-yr male trout probed with CYP2K1,7C.

## CHAPTER 5

### Conclusion

This study, in addition to the further characterization of the previously sequenced rainbow trout (*Oncorhynchus mykiss*) cytochrome P450 2K1, has resulted in the cloning, sequencing and characterization of three additional constitutive trout cytochrome P450s. A cDNA encoding hepatic P450 LMC1 purified from rainbow trout has been cloned, sequenced and assigned by the P450 Nomenclature Committee to a new P450 subfamily as CYP2M1. Northern blot analysis revealed marked sexual dimorphic expression of CYP2M1 mRNA in liver and trunk kidney. Expression of CYP2M1 was highest in the liver of sexually mature male trout; however, the CYP2M1 mRNA was barely detectable in the trunk kidney from both male and female fish of the same age. A marked decrease in CYP2M1 expression also was observed in the trunk kidney of mature female trout, while liver maintained a similar level of expression both in juvenile and mature trout. Heterologous expression of CYP2M1 in COS-7 and baculovirus/insect cells produced a protein hydroxylating lauric acid at the ( $\omega$ -6)-position. This unique regiospecific hydroxylation also was catalyzed by the purified P450 LMC1 but was not observed as a product of other trout or mammalian P450s. The physiological function of this P450 or the ( $\omega$ -6)-hydroxylation reaction is unknown at this time. However, with ample amounts of

functional CYP2M1 expressed in insect cells, various endogenous and xenobiotic compounds can be used in the future to obtain the information on substrate specificity of this P450, thus leading to a better understanding of the biological function of this P450 isoform.

CYP2K1, CYP2K3 and CYP2K4 represent the closely-related members in a multigene P450 family in rainbow trout resulting from gene duplication and divergent evolution. CYP2K1 and CYP2K3 were expressed in a baculovirus/insect cell expression system. A functional CYP2K1 was produced in this system and its catalytic activity characterized. The regioselectivity of lauric acid hydroxylation and the bioactivation of AFB<sub>1</sub> to the ultimate carcinogen AFB<sub>1</sub>-exo-epoxide by CYP2K1 were demonstrated. The presence of AFB<sub>1</sub>-exo-epoxide was detected by glutathione conjugation and resolved by HPLC on a chiral column. CYP2K1 is the major cytochrome P450 present in the liver of rainbow trout. The high efficiency of AFB<sub>1</sub> activation by CYP2K1 and lack of a constitutive or inducible glutathione S-transferase with appreciable activity toward AFB<sub>1</sub>-epoxide may help explain the high incidence of tumor formation induced by AFB<sub>1</sub> in rainbow trout.

CYP2K3 shared 96.5% homology with CYP2K1 but exhibited a 39-bp deletion close to the conserved heme-binding domain. This deletion likely disrupted the conserved core structure within the CYP2K3 protein and might be responsible for the lack of enzymatic activity of this protein following its expression in the baculovirus system.

CYP2K4, a male-specific P450, was cloned from the trunk kidney of sexually mature rainbow trout. This 2.6 Kb cDNA has a long 3'-untranslated region (1,017 bp) compared with that of CYP2K1 (289 bp). CYP2K4 shared 94.2% homology with CYP2K1 in its amino acid sequence. The sequence similarity in the open reading frame and size difference in the 3'-untranslated region of CYP2K4 and CYP2K1 explains, to a large part, the multiple banding pattern observed previously in the Northern blot analysis using 2K1,7c as a probe (Buhler et al., 1994). While there is considerable similarity between CYP2K4 and a purified trout kidney P450 form, KM2, the relationship is not certain at this time. The two isoforms are similar but not identical based on the N-terminal amino acid sequence comparison. Further studies are needed to elucidate the catalytic activity and substrate specificity of these two isoforms.

The sexual dimorphism observed in the expression of cytochrome P450 in sexually mature trout has also been reported in mammals. The regulation of P450 expression in liver (Waxman and Chang, 1995) and kidney (Henderson et al., 1990) has been linked to the hormone status of the animals. Rat P450 enzymes CYP2C11 and CYP2C12 are the prototypic examples of sex-specific P450s in liver. The neonatal androgen imprinting of the hypothalamic-pituitary axis shortly after birth determines the growth hormone (GH) release pattern later in the life of the animals. Pituitary GH secretory patterns regulate the sex-specific expression of liver P450 through a transcriptional mechanism. Gonadal hormones also modulate the expression of these P450s, but

the effects are largely indirect and are mediated by the hypothalamic-pituitary axis and its control of plasma GH levels (Waxman and Chang, 1995). Treatment of juvenile rainbow trout with androgens, estrogens and growth hormone has shown that estradiol is a suppressor of P450 expression at the level of transcription (Miranda et al., unpublished observation). Studies with hypophysectomized rainbow trout indicate that the actions of estradiol are not mediated by pituitary hormones (Hansson and Gustafsson, 1981), thus the mechanism by which estradiol exerts this down-regulation of trout P450s is unknown. Cloning and characterization of the promoter regions of P450 genes will likely provide some answers to these questions.

Knowledge of the catalytic activity, tissue distribution and P450 expression during different developmental stages facilitates the understanding of the physiological function of P450s and especially their role in the metabolism of xenobiotics in rainbow trout.

## Bibliography

- Andersson, T. (1990) Sex differences in cytochrome P-450 dependent xenobiotic and steroid metabolism in the mature rainbow trout kidney. *J. Endocrinol.* 126: 9-16.
- Andersson, T. (1992) Purification, characterization and regulation of a male-specific cytochrome P450 in the rainbow trout kidney. *Mar. Environ. Res.* 34: 109-112.
- Ayoma, T., Hardwick, J.P., Imaoka, S., Funae, Y., Gelboin H.V., Gonzalez, F.J. (1990) Clofibrate-inducible rat hepatic P450s IVA1 and IVA3 catalyze the omega- and (omega-1)-hydroxylation of fatty acids and the omega-hydroxylation of prostaglandins E1 and F2 alpha. *J. Lipid Res.* 31: 1477-1482.
- Bailey, G.S., Williams, D.E., Wilcox, J.S., Loveland, P.M. Coulombe, R.A., and Hendricks, J.D. (1988) Aflatoxin B<sub>1</sub> carcinogenesis and its relation to DNA adduct formation and adduct persistence in sensitive and resistant salmonid fish. *Carcinogenesis* 9: 1919-1926.
- Bailey, G.S., Williams, D.E., and Hendricks, J.D. (1996) Fish models for environmental carcinogenesis: the rainbow trout. *Environ. Health Perspect.* 104: 5-21.
- Barnes, H.J., Arlotto, M.P., Waterman, M.R. (1991) Expression and enzymatic activity of recombinant cytochrome P450 17-hydroxylase in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 88: 5597-5601.
- Battula, N. (1989) Transduction of cytochrome P3-450 by retroviruses: constitutive expression of enzymatically active microsomal hemoprotein in animal cells. *J. Biol. Chem.* 264: 2991-2996.
- Bell, M.V., Henderson, R.J., and Sargent J.R. (1986) The role of polyunsaturated fatty acids in fish. *Comp. Biochem. Physiol.* 83B: 711-719.

- Berndtson, A.K. and Chen, T.T. (1994) Two unique CYP1 genes are expressed in response to 3-methylcholanthrene treatment in rainbow trout. *Arch. Biochem. Biophys.* 310: 187-195.
- Bond, C.E. (1996) Excretion and osmoregulation. In: *Biology of fishes*. 2<sup>nd</sup> Ed. pp. 395-416 Saunders college publishing, Florida.
- Brodie, B.B. and Maickel, R.P. (1962) Comparative biochemistry of drug metabolism. In: Brodie B.B., Erdos, F.G. (eds) *Proc 1<sup>st</sup> Int Pharmacology Meeting*, Macmillan, New York, pp. 299-324.
- Buhler D.R. (1966) Hepatic drug metabolism in fishes. *Federation Proc.* 25: 343.
- Buhler, D.R. and Rasmusson, M.E. (1968) The oxidation of drugs by fishes. *Comp. Biochem. Physiol.* 25: 223-239.
- Buhler, D.R. and Williams, D.E. (1990) Enzymes involved in metabolism of PAHs by fishes and other aquatic animals. In: *Metabolism of polynuclear aromatic hydrocarbons in the aquatic environment*. Varanasi, U. (ed), pp. 151-184, CRC press, New York.
- Buhler, D.R. (1995) Cytochrome P450 expression in Rainbow trout: an overview. In *Molecular aspects of oxidative drug metabolizing enzymes: their significance in environmental toxicology, chemical carcinogenesis and health*. Arinc, E., Schenkman, J.B., and Hodgson, E. (eds) pp. 159-180, Springer-Verlag, Berlin Heidelberg, Germany.
- Buhler, D.R., Miranda, C.L., Deinzer, M.L., Griffin, D.A., and Henderson, M.C. (1997) The regiospecific hydroxylation of lauric acid by rainbow trout (*Oncorhynchus mykiss*) Cytochrome P450s. *Drug Metab. Dispos.* 25: 1176-1183.
- Buters, J.T., Shou, M., Hardwick, J.P., Korzekwa, K.R., and Gonzalez, F.J. (1995) cDNA-directed expression of human cytochrome P450 CYP1A1 using baculovirus. Purification,

- dependency on NADPH-P450 oxireductase, and reconstitution of catalytic properties without purification. *Drug Metab. Dispos.* 23: 696-701.
- CaJacob, C.A., Chan, W.K., Shephard, E., and Ortiz De Montellano, P.R. (1988) The catalytic site of rat hepatic lauric acid omega-hydroxylase. Protein versus prosthetic heme alkylation in the omega-hydroxylation of acetylenic fatty acids. *J. Biol. Chem.* 263: 18640-18649.
- Capdevila, J.H., Falck, J.R., and Estabrook, R.W. (1992) Cytochrome P450 and arachidonate cascade. *FASEB J.* 6: 731-736.
- Castle, P.J., Merdink, J.L., Okita, J.R., Wrighton, S.A., and Okita, R.T. (1995) Human liver lauric acid hydroxylase activities. *Drug Metab. Dispos.* 23: 1037-43.
- Celander, M., Ronis, M., and Fölin, L. (1989) Initial characterization of a constitutive cytochrome P-450 isozyme in rainbow trout liver. *Mar. Environ. Res.* 28: 9-14.
- Celander, M., Buhler, D.R., Förlin, L., Goksøyr, A., Miranda, C.L., Woodin B.R., and Stegmann, J.J. (1996) Immunochemical relationships of cytochrome P4503A-like proteins in teleost fish. *Fish Physiol. Biochem.* 15: 323-332
- Choi, I., Simmen, R.C., and Simmen, F.A. (1996) Molecular cloning of cytochrome P450 aromatase complementary deoxyribonucleic acid from periimplantation porcine and equine blastocysts identifies multiple novel 5'-untranslated exons expressed in embryos, endometrium, and placenta. *Endocrinology* 137: 1457-1467.
- Claude, A. (1946) Fractionation of mammalian liver cells by differential centrifugation. *J. Exp. Med.* 84: 51-89.
- Crespi, C.L., Gonzalez, F.J., Steimel, D.T., Turner, T.R., Gelboin H.V., Penman, B.W. and Langenback, R. (1991) A metabolically component cell line expressing five cDNAs

encoding procarcinogen-activating enzymes: application to mutagenicity testing. *Chem. Res. Toxicol.*, 4: 566-572.

- Croy, R.G., Nixon, J.E., Sinnhuber, R.O., and Wogan, G.N. (1980) Investigation of covalent aflatoxin B<sub>1</sub>-DNA adducts formed *in vivo* in rainbow trout (*Salmo gairdneri*) embryos and liver. *Carcinogenesis* 1: 903-909.
- Dai, Y., Rashba-Step, J., and Cederbaum, A.I. (1993) Stable expression of human cytochrome P450E1 in HepG2 cells: characterization of catalytic activities and production of reactive oxygen intermediates. *Biochemistry* 32: 6928-6937.
- Daniels, J.M., Liu, L., Atewart, R.K., and Massey, T.E. (1990) Biotransformation of aflatoxin B<sub>1</sub> in rabbit lung and liver. *Carcinogenesis*. 11: 823-827.
- Degen, G.H., and Neumann, H.G. (1978) The major metabolite of aflatoxin B<sub>1</sub> in the rat is a glutathione conjugate. *Chem.-Biol. Interact.* 22: 239-255.
- Dewaide, J.H. and Henderson, P.T. (1968) Hepatic N-demethylation of aminopyrine in rat and trout. *Biochem. Pharmacol.* 17: 1901-1907.
- Doehmer, J. (1993) V79 Chinese hamster cells genetically engineered for cytochrome P450 and their use in mutagenicity and metabolism studies. *Toxicology* 82: 105-118.
- Doehmer, J. and Oesch, F. (1991) V79 Chinese hamster cells genetically engineered for stable expression of cytochromes P450. *Methods Enzymol.* 206: 117-123.
- Eaton, D.L., and Gallagher, E.P. (1994) Mechanisms of aflatoxin carcinogenesis. *Annu. Rev. Pharmacol. Toxicol.* 34: 135-172.
- Estabrook, R.W., Cooper, D.Y., and Rosenthal, O. (1963) The light reversible carbon monoxide inhibition of the steroid C21 hydroxylation system in the adrenal cortex. *Biochem. Z.* 338: 741-755.

- Falck, J.R., Lumin, S., Blair, I., Dishman, E., Martin, M.V., Waxman, D.J., Guengerich, F.P., and Capdevila, J.H. (1990) Cytochrome P-450-dependent oxidation of arachidonic acid to 16-, 17-, and 18-hydroxyleicosatetraenoic acid. *J. Biol. Chem.* 265: 10244-10249.
- Falckh, P.H.J., Wu, Q.K., and Ahokas, J.P. (1997) CYP4T1-a cytochrome P450 expressed in rainbow trout (*Oncorhynchus mykiss*) liver. *Biochem. Biophys. Res. Commun.* 236: 302-305.
- Fardella, D.E., Zhang, L.H., Mahachoklertwattana, P., Lin, D., and Miller, W.L. (1993) Deletion of amino acid Asp487-Ser488-Phe489 in human cytochrome P450c17 causes severe 17-alpha-hydroxylase deficiency. *J. Clin. Endocrinol. Metab.* 77: 489-493.
- Fisher, C.W., Caudle, D.L., Martin-Wixtrom, C., Quattrochi, L.C., and Tukey, R.H. (1992) High-level expression of functional human cytochrome P450 1A2 in *Escherichia coli*. *FASEB. J.* 6: 759-764.
- Fukuda, T., Imai, Y., Komori, M., Nakamura, M., Kusunose, E., Satouchi, K., and Kusunose M. (1994) Different mechanisms of regioselection of fatty acid hydroxylation by laurate ( $\omega$ -1)-hydroxylating P450s, P450 2C2 and P450 2E1. *J. Biochem.* 115: 338-344.
- Garfinkel, D. (1958) Studies on pig liver microsomes. I. Enzymic and pigment composition of different microsomal fractions. *Arch. Biochem. Biophys.* 77: 493-509.
- Goksøyr, A. (1985) Purification of hepatic microsomal cytochromes P-450 from  $\beta$ -naphthoflavone-treated Atlantic cod (*Gadus morhua*), a marine teleost fish. *Biochem. Biophys. Acta.* 840: 409-417.
- Goksøyr, A. and Förlin, L. (1992) The cytochrome P-450 system in fish, aquatic toxicology and environmental monitoring. *Aquatic Toxicol.* 22:287-312.

- Gonzalez, F.J. (1990) Molecular genetics of the P-450 superfamily. *Pharmacol. Ther.* 45: 1-38.
- Gonzalez, F.J. and Gelboin, H.V. (1993) Roles of cytochromes P-450s in risk assessment and susceptibility to environmentally based disease. *J. Toxicol. Environ. Health* 40: 289-308..
- Gonzalez, F.J. and Gelboin, H.V. (1994) Role of human cytochromes P450 in the metabolic activation of chemical carcinogens and toxins *Drug Metab. Rev.* 26: 165-183.
- Gonzalez, F.J. and Korzekwa, K.R. (1995) Cytochromes P450 expression systems. *Annu. Rev. Pharmacol. Toxicol.* 35: 369-390.
- Gonzalez, F.J., Aoyama, T., and Gelboin, H.V. (1991) Expression of mammalian cytochrome P450 using vaccinia virus. *Methods Enzymol.* 206: 85-9.2
- Gonzalez, F.J., Crespi, C.L., and Gelboin, H.V. (1991) DNA-expressed cytochrome P450s: a new age of molecular toxicology and human risk assessment. *Mutat. Res.* 247: 113-127.
- Gooneratne, R., Miranda, C.L., Henderson, M.C., and Buhler, D.R. (1997)  $\beta$ -Naphthoflavone induced CYP1A1 and CYP1A3 proteins in the liver of rainbow trout (*Oncorhynchus mykiss*). *Xenobiotica* 27: 175-187.
- Gragan, J., Shou, M., Andrusiak, E.A., Tamura, S., Buters, J.T., Gonzalez, F.J., Korzekwa, K.R. (1995) Cytochrome P450 2A1, 2E1, and 2C9 cDNA-expression by insect cells and partial purification using hydrophobic chromatography. *Biochemical Pharmacology.* 50: 1509-1515.
- Guengerich, F.P. (1988) Roles of cytochrome P450 enzymes in chemical carcinogenesis and cancer chemotherapy. *Cancer Res.* 48: 2946-2954.
- Guengerich, F.P. (1991) Reactions and significance of cytochrome P-450 enzymes. *J. Biol. Chem.* 266: 10019-10022.

- Guengerich, F.P. (1992) Metabolic activation of carcinogens. *Pharmacol Ther.* 54:17-61.
- Guengerich, F.P. (1993) The 1992 Bernard B. Brodie Award Lecture. Bioactivation and detoxication of toxic and carcinogenic chemicals. *Drug Metab. Dispos.* 21: 1-6.
- Guengerich, F.P. and MacDonald, T.L. (1990) Mechanisms of Cytochrome P450 catalysis. *FASEB J.* 4: 2453-2459.
- Hansson, T. and Gustafsson, J.-A. (1981) In vitro metabolism of 4-androstene-3,17-dione by hepatic microsomes from the rainbow trout (*Salmo gairdneri*): effects of hypophysectomy and estradiol-17B. *J. Endocrinol.* 90: 103-112.
- Hayes, J.D., Judah, D.J., McLellan, L.I., and Neals, G.E. (1991). Contribution of the glutathione S-transferases to the mechanisms of resistance to aflatoxin B<sub>1</sub> *Pharmacol. Ther.* 50: 443-472.
- Heilmann, L.J., Sheen, Y.-Y., Bigelow S.W., and Nebert, D.W. (1988) Trout P450 IA1: cDNA and deduced protein sequence, expression in liver and evolutionary significance. *DNA* 7: 379-387.
- Heim, M.H. and Meyer, U.A. (1992) Evolution of a highly Polymorphic human cytochrome P450 gene cluster: CYP2D6. *Genomics* 14: 49-58.
- Henderson, C.J., Scott, A.R., Yang, C.S., and Wolf C.R. (1990) Testosterone-mediated regulation of mouse renal cytochrome P-450 isoenzymes. *Biochem. J.* 266: 675-681.
- Imai, Y. (1981) The roles of cytochrome b5 in reconstituted monooxygenase systems containing various forms hepatic microsomal cytochrome P-450. *J. Biochem. (Tokyo)* 89: 351-362.
- Imai, Y., Uno, T., and Nakamura, M. (1990) Characterization of rabbit liver P450IIE1 synthesized in transformed yeast cells. *J. Biochem.* 108: 522-524.

- Imaoka, S., Imai, Y., Shimada, T., and Funae, Y. (1992) Role of phospholipids in reconstituted cytochrome P450 3A form and mechanism of their activation of catalytic activity. *Biochem.* 31: 6063-6069.
- Imaoka, T., Yamada, T., Hiroi, K., Hayashi, T., Sasaki, T., Yabusaki, Y., and Funae, Y. Multiple forms of human P450 expressed in *Saccharomyces cerevisiae*. (1996) Systematic characterization and comparison with those of the rat. *Biochem. Pharmacol.* 51: 1041-1050.
- Ingelman-Sundberg, M. (1977) Phospholipid and detergents as effectors in the liver microsomal hydroxylase systems. *Biochem. Biophys. Acta.* 488: 225-234.
- Ingelman-Sundberg, M. and Gaumann, H., (1980) Incorporation of purified components of the rabbit liver microsomal hydroxylase system into phospholipid vesicles. *Biochem. Biophys. Acta.* 599: 417-435.
- Jansson, I., Tamburini, P.P., Favreau, L.V., and Schenkman, J.B. (1985) The interaction of cytochrome b5 with four cytochrome P-450 enzymes from untreated rat. *Drug Metab. Dispos.* 13: 453-458.
- Jefcoate, C.R. (1986) Cytochrome P-450 enzymes in sterol biosynthesis and metabolism. In: *Cytochrome P-450. Structure, mechanism, and biochemistry.* Ortiz de Montellano P.R. (ed), pp.387-428, Plenum Press, New York.
- Kimura, S., Umeno, M., Skoda, R., Meyer, U.A., and Gonzalez, F.J. (1989) The human debrisoquine 4-hydroxylase (CYP2D) locus: sequence and identification of the polymorphic CYP2D6 gene, a related gene, and a pseudogene. *Am. J. Hum. Genet.* 45: 889-904.
- Kitts, P.A. and Possee, R.D. (1993) A methods for producing recombinant baculovirus expression vectors at high frequency. *Biotechnology* 14: 810-817.
- Klingenberg, M. (1958) Pigments of rat liver microsomes. *Arch Biochem. Biophys.* 75: 376-386.

- Klotz, A.V., Stegeman, J.J., and Walsh, C. (1991) An aryl hydrocarbon hydroxylating hepatic cytochrome P-450 from the marine fish *Stenotomus chrysops*. Arch. Biochem. Biophys. 226: 578-592.
- Kochs, G. and Grisebach, H. (1989) Phytoalexin synthesis in soy bean: purification and reconstitution of cytochrome P450 3,9-dihydroxypterocarpan 6 $\alpha$ -hydroxylase and separation from cytochrome P450 cinnamate 4-hydroxylase. Arch. Biochem. Biophys. 273: 543-553.
- Kochs, G., Werck-Reichhart, D., and Grisebach, H. (1992) Further characterization of cytochrome P450 involved in phytoalexin synthesis in soybean: cytochrome P450 cinnamate 4-hydroxylase and 3,9-dihydroxypterocarpan 6 $\alpha$ -hydroxylase. Arch. Biochem. Biophys. 293: 187-194.
- Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.
- Lane, D.J., Pace, B., Olsen, G.J., Stahl, D.A., Sogin, M.L. and Pace, N.R. (1985) Rapid determination of 16S ribosomal RNA sequences for phylogenetic analysis. Proc. Natl. Acad. Sci. USA 82: 6955-6959.
- Larson J.R., Coon, M.J., Porter, T.D. (1991) Purification and properties of a short ended form of cytochrome P-450 2E1: deletion of the NH<sub>2</sub>-terminal membrane insertion signal peptide does not alter the catalytic activities. Proc. Natl. Acad. Sci. USA. 266: 19186-19191.
- Leaver, M.J., Pirrit, L., and George, S.G. (1993) Cytochrome P450 1A1 cDNA from plaice (*Pleuronectes platessa*). Mol. Marine Biol. Biotechnol. 2: 338-345.
- Lee, C.A., Kadwell, S.H., Kost, T.A., and Serabjit-Singh, C. (1995) CYP3A4 expressed by insect cells infected with a recombinant baculovirus containing both CYP3A4 and human NADPH-cytochrome P450 reductase is catalytically similar to human liver microsomal CYP3A4. Arch. Biochem. Biophys. 319: 157-167.

- Lee, C.A., Kost, T.A., and Serabjit-Singh, C. (1996) Recombinant baculovirus strategy for coexpression of functional human cytochrome P450 and P450 reductase. *Methods Enzymol.* 272: 86-95.
- Lee, S.J., Yang, Y.-H., Wang, J.-L., Miranda, C.L., Cok, I., Lech, J., and Buhler, D.R. (1997) Cloning, sequencing and tissue expression of the first CYP3A form from fish (CYP3A27) in rainbow trout. *FASEB J.* 11: A284.
- Lemaire, P., Lafaurie, M. Weissbart, D., Durst, F., Pflieger, P., Mioskowski, C., and Salaun, J.-P. (1992) Subterminal hydroxylation of lauric acid by microsomes from a marine fish. *Lipids* 27: 197-191.
- Lewis, S., Korsmeyer, K.K., and Correia, M.A. (1993) Matrix-assisted laser desorption mass spectrometry of cytochromes P450. *Rapid Commun. Mass Spectro.* 7: 16-19.
- Lewis, D.F. (1996) Evolution of the P450 superfamily. In *Cytochromes P450 structure, function and mechanism.* pp. 55-77, Taylor & Francis, Bristol, PA.
- Li, Y.C., and Chaing, J.Y. (1991) The expression of catalytically active cholesterol 7 $\alpha$ -hydroxylase in *Escherichia coli*. *J. Biol. Chem.* 266: 19186-19191.
- Lu, A.Y.H. and Coon, M.J. (1968) Role of hemoprotein P-450 in fatty acid  $\omega$ -hydroxylation in a soluble enzyme system from liver microsomes. *J. Biol. Chem.* 243: 1331-1332.
- Luckow, V.A., Lee, S.C., Barry, G.F. and Olins, P.O. (1993) Efficient generation of infectious recombinant baculoviruses by site specific transposon-mediated insertion fo foreign genes into a baculovirus genome propagated in *Escherichia coli*. *J. Virol.* 67: 4566-4579.
- Mansuy, D and Renaud, J.-P. (1995) Heme-thiolate proteins different from cytochromes P450 catalyzing monooxygenations. In *Cytochrome P450: Structure, mechanism and Biochemistry*, 2<sup>nd</sup> Edition (Paul R. Ortiz de Montellano Ed.) pp. 537-574, Plenum Press, New York.

- March, B.E., (1993) Essential fatty acid in fish physiology. *Can. J. Physiol. Pharmacol.* 71: 684-689.
- Mesnil, M. and Testa, B. (1984) Xenobiotic metabolism by brain monooxygenases and other enzymes. In: *Advances in drug research*. Vol. 13 (B. Testa Ed) pp. 95-207, Academic Press, London.
- Miranda, C.L., Wang, J.-L., Henderson, M.C., and Buhler, D.R. (1989) Purification and characterization of hepatic steroid hydroxylases from untreated rainbow trout. *Arch. Biochem. Biophys.* 268: 227-238.
- Miranda, C.L., Wang, J.-L., Henderson, M.C., and Buhler, D.R. (1990a) Immunological characterization of constitutive isozymes of cytochrome P-450 from rainbow trout. Evidence for homology with phenobarbital-induced rat P-450s. *Biochem. Biophys. Acta.* 1037: 155-160.
- Miranda, C.L., Wang, J.-L., Henderson, M.C., Williams, D.E., and Buhler, D.R. (1990b) Regiospecificity in the hydroxylation of lauric acid by rainbow trout hepatic cytochrome P450 isozymes. *Biochem. Biophys. Res. Commun.* 171: 537-542.
- Miranda, C.L., Wang, J.-L., Henderson, M.C., Zhao. X., Guengerich, F.P., and Buhler, D.R. (1991) Comparison of rainbow trout and mammalian cytochrome P450 enzymes. Evidence for structural similarity between trout P450 LMC5 and huamn P450IIIA4. *Biochem. Biophys. Res. Commun.* 176: 558-563.
- Mizukami, M., Okauchi, M., Ariyoshi, T., and Kito, H. (1994) The isolation and sequence of cDNA encoding a 3-methylcholanthrene-inducible cytochrome P450 from wild red sea bream, *Pagrus major*. *Marine Biol.* 120: 343-349.
- Morgan, E.T. and Coon, M.J. (1984) Effects of cytochrome b5 on cytochrome P-450 catalyzed reactions. *Drug Metab. Dispos.* 12: 358-364.
- Morrison, H.G., Oleksiak, M.F., Cornell, N.W., Sogin, M.L., and Stegeman, J.J. (1995) Identification of cytochrome P4501A genes from two teleost fish, toadfish (*Opsanus tau*) and scup (*Stenotomus*

- chrysops*), and phylogenetic analysis of CYP1A genes. *Biochem. J.* 308: 97-104.
- Neals, G.E., and Colley, P.J. (1979) The formation of 2,3-dihydro-2,3-dihydroxy aflatoxin B<sub>1</sub> in vitro by liver microsomes. *FEBS lett.* 101: 382-386.
- Nebert, D.W. and Gonzalez, F.J. (1985) Cytochrome P450 gene expression and regulation. *Trends in Pharmacol. Sci.* 6:160-164.
- Nebert, D.W. and Gonzalez, F.J. (1987) P450 genes: Structure, evolution and regulation. *Anal. Rev. of Biochem.* 56:945-993.
- Nebert, D.W. and Gonzalez, F.J. (1990) The P450 gene superfamily. *Frontiers in Biotransformation.* 2:35-61.
- Nebert, D.W. and Nelson, D.R. (1991) P450 gene nomenclature based on evolution. *Methods in Enzymology.* 206: 3-11.
- Nebert, D.W., Adesnik, M., Coon, M.J., Estabrook, R.W., Gonzalez, F.J., Guengerich, F.P., Gunsalus, I.C., Johnson, E.F., Kemper, B., Levin, W., Phillips, I.R., Sato, R., and Waterman, M.R. (1989) The P450 gene superfamily: recommended nomenclature. *DNA* 6: 1-11
- Nelson, D.R., Kamataki, T., Waxman, D.J., Guengerich, F.P., Estabrook, R.W., Feyereisen, R., Gonzalez, F.J., Coon, M.J., Gunsalus, I.C., Gotoh, O., Okuda, K., and Nebert, D.W. (1993) The P450 superfamily - update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. *DNA Cell Biol.* 12: 1-51.
- Nelson, D.R. and Strobel, M.W. (1987) Evolution of cytochrome P450 Proteins, *Molecular Biology and Evolution.* 4:572-593.
- Nelson, D.R., Koymans, L., Kamataki, T., Stegeman, J.J., Feyereisen, R., Waxman, D.J., Waterman, M.R., Gotoh, O., Coon, M.J., Estabrook, R.W., Gunsalus, I.C., and Nebert, D.W. (1996) P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics* 6: 1-42.

- Nerurkar, P.V., Park, S.S., Thomas, P.E., Nims, R.W., and Lubet, R.A. (1993) Methoxyresorufin and benzyloxyresorufin: substrates preferentially metabolized by cytochromes CYP1A2 and 2B, respectively, in the rat and mouse. *Biochem. Pharmacol.* 46: 933-943.
- Nunez, S., and Trant, J.M. (1997) Isolation of the putative cDNA encoding cholesterol side chain cleavage cytochrome P450 (CYP11A) of the southern stingray (*Dasyatis americana*) *Gene* 187: 123-129.
- Oeda, K., Sakaki, T., and Ohkawa, H. (1985) Expression of rat liver cytochrome P-450MC cDNA in *Saccharomyces cerevisiae*. *DNA Cell Biol.* 4: 167-175.
- Oleksiak, M., Wu, S., Zeldin, D.C., and Stegeman, J.J. (1997) Characterization of members of the novel cytochrome P450 subfamilies CYP2N and CYP2P from the fish *Fundulus heterolitus*. Ninth International symposium on Pollutant Responses in Marine Organisms, Bergen, Norway, April 27-30.
- Omura, T. and Sato, R. (1962) A new cytochrome in liver microsomes. *J. Biol. Chem.* 237: 1375-1376.
- Omura, T. and Sato, R. (1964) The carbon monoxide-binding pigment of liver microsomes. *J. Biol. Chem.* 239: 2370-2378.
- O'Reilly, D.R. (1992) in *Baculovirus Expression Vectors: A Laboratory Manual*, pp. 130-132, Freeman, New York.
- Ortiz de Motellano, P.R. (1989) Cytochrome P-450 catalysis: radical intermediates and dehydrogenation reactions. *Trends Pharmacol. Sci.* 10:354-35.
- Paine, M.J., Gilham, D., Roberts, G.C., and Wolf, C.R. (1996) Functional high level expression of cytochrome P450 CYP2D6 using baculovirus expression system. *Arch. Biochem. Biophys.* 328: 143-150.

- Pesonen, M., Hansson, T., Förlin, L., and Andersson, T. (1990) Regional distribution of microsomal xenobiotic and steroid metabolism in kidney microsomes from rainbow trout. *Fish Physiol. Biochem.* 8: 141-145.
- Peterson, J.A and Graham-Lorence, S.E. Bacteria P450s. in *Cytochrome P450 2<sup>nd</sup> Ed.* (Ortiz de Montellano, P.R. ed.) pp.151-180, Plenum Press, New York.
- Pompon, D. (1988) cDNA cloning and functional expression in yeast *Saccharomyces cerevisiae* of naphthoflavone-induced rabbit liver LM4 and LM6. *Eur. J. Biochem.* 177: 285-293.
- Porter, T.D. and Coon, M.J. (1991) Cytochrome P450: multiplicity of isoforms, substrates and catalytic and regulatory mechanisms. *J. Biol. Chem.* 266: 13469-13472.
- Poulos, T.L. and Raag, R. (1992) Cytochrome P450cam: crystallography, oxygen activation and electron transfer. *FASEB J.* 6: 674-679.
- Raney, K.D., Meyer, D.J., Ketterer, B., Harris, T.M., and Guengerich, F.P. (1992) Glutathione conjugation of aflatoxin B<sub>1</sub> exo and endo-epoxides by rat and human glutathione S- transferases. *Chem. Res. Toxicol.* 5: 470-478.
- Roman, L.J., Palmer, C.N.A., Clark, J.E., Muerhoff, A.S., Griffin, K.J., Johnson, E.F., and Masters, B.S.S. (1993) Expression of rabbit cytochromes P450 A which catalyze the  $\omega$ -hydroxylation of arachidonic acid, fatty acids, and prostaglandins. *Arch. Biochem. Biophys.* 307: 57-65.
- Romano, M.C., Straub, K.M., Yodis, L.A.P., Eckardt, R.D., and Newton, J.F. (1988) Determination of microsomal lauric acid hydroxylase activity by HPLC with flow-through radiochemical quantitation. *Anal. Biochem.* 170: 83-93.

- Roy, N.K., Konkle, B.A., Kreamer, G.-L., Grunwald, C., and Wirgin, I.I. (1995) Characterization and prevalence of a polymorphism in the 3' untranslated region of cytochrome P4501A1 in cancer-prone Atlantic tomcod. *Arch. Biochem. Biophys.* 322: 204-213.
- Sakai, N., Tanaka, M., Adachi, S., Miller, W.L., and Nagahama, Y. (1992) Rainbow trout cytochrome P-450c17 (17 $\alpha$ -hydroxylase/17,20-lyase) cDNA cloning enzymatic properties and temporal pattern of ovarian P-450c17 mRNA expression during oogenesis. *FEBS Lett.* 301: 60-64.
- Sakaki, T., Oeda, K., Miyoshi, M. and Ohkawa, H. (1985) Characterization of rat cytochrome P-450mc synthesized in *Saccharomyces cerevisiae*. *J. Biochem.* 98: 167-175.
- Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989a) *Molecular Cloning: A Laboratory Manual*, 2nd ed, Vol. 1, p. 7.40; Vol.2, p. 9.16, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989b) *Molecular Cloning: A Laboratory Manual*, 2nd ed, Vol.1, 1.42, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Sandhu, P., Baba, T., and Guengerich, F.P. (1993) Expression of modified cytochrome P450 2C10 (2C9) in *Escherichia coli*, purification, and reconstitution of catalytic activity. *Arch. Biochem. Biophys.* 306: 443-450.
- Seree, E.M., Botto F., Pisano, P. Lechevalier, E., Desobry, A., and Barra, Y. (1995) Evidence for the existence of two human CYP2E1 cDNAs using different polyadenylation signals. *Biochem. Biophys. Res. Commun.* 209: 717-722.
- Sigle, R.O., Titus, M.A., Harada, N., and Nelson, S.D. (1994) Baculovirus mediated high level expression of human placental aromatase (CYP19A1) *Biochem. Biophys. Res. Commun.* 201: 694-700.
- Southern, E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Molec. Biol.* 98: 503-517.

- Spoerel, N.A. and Kafatos, F. (1987) Identification of genomic sequences corresponding to cDNA clones. *Methods Enzymol.* 152: 588-597.
- Stegeman, J.J. and Hahn, M.E. (1994) Biochemistry and molecular biology of monooxygenases: current perspectives on forms, functions and regulation of cytochrome P450 in aquatic species. In: *Aquatic Toxicology*, Malins, D.C. and Ostrander, G.K. (eds.), pp.87-206, CRC Press, Boca Raton Florida.
- Stermo, G.D., Schneider, T.D., and Gold, L.M. (1982) Characterization of translational initiation sites in *E. coli*. *Nucleic Acid Res.* 10: 2971-1996.
- Straub, P., Johnson, E.F., and Kemper, B. (1993) Hydrophobic side chain requirements for lauric acid and progesterone hydroxylation at amino acid 113 in cytochrome P450 2C2, a potential determinant of substrate specificity. *Arch. Biochem. Biophys.* 306: 521-527.
- Stresser, D.M., Williams, D.E., McLellan, L., Harris, T.M., and Bailey, G.S. (1994) Indole-3-carbinol induces a rat liver glutathione transferase subunit (Yc2) with high activity toward aflatoxin B<sub>1</sub> exo-epoxide. *Drug Metab. Disp.* 22: 392-399.
- Strobel, H.W., Lu, A.Y.H., Heidema, J., and Coon, M.J. (1970) Phosphatidylcholine requirement in the enzymatic reduction of hemoprotein P-450 and in fatty acid, hydrocarbon, and drug hydroxylation. *J. Biol. Chem.* 245: 4851-4854.
- Takahashi, M., Tanaka, M., Sakai, N., Adachi, S., Miller, W.L., and Nagahama, Y. (1993) Rainbow trout ovarian cholesterol side-chain cleavage cytochrome P450 (P450scc). *FEBS Lett.* 319: 45-48.
- Takahashi, N., Miranda, C.L., Henderson, M.C., Buhler, D.R., Williams, D.E., and Bailey, G.S. (1995) Inhibition of *in vitro* aflatoxin B<sub>1</sub>-DNA binding in rainbow trout by CYP1A inhibitors:  $\alpha$ -naphthoflavone,  $\beta$ -naphthoflavone and trout CYP1A1 peptide antibody. *Comp. Biochem. Physiol.* 110C: 273-280.

- Tanaka, M., Telecky, T.M., Fukada, S., Adachi, S., Chen, S., and Nagahama, Y. (1992) Cloning and sequence analysis of the cDNA encoding P-450 aromatase (P450arom) from a rainbow trout (*Oncorhynchus mykiss*) ovary; relationship between the amount of P450arom mRNA and the production of oestradiol-17 $\beta$  in the ovary. *J. Mol. Endocrinol.* 8: 53-61.
- Ueng, Y.-F., Shimada, T., Yamazaki, H., and Guengerich, F.P. (1995) Oxidation of aflatoxin B<sub>1</sub> by bacterial recombinant human cytochrome P450 enzymes. *Chem. Res. Toxicol.* 8: 218-225.
- Valsta, L.M., Hendricks, J.D., and Bailey, G.S. (1988) The significance of glutathione conjugation for aflatoxin B<sub>1</sub> metabolism in rainbow trout and coho salmon. *Food Chem. Toxicol.* 26: 129-135.
- Vaz, A.D.N., Roberts, E.S., and Coon, M.J. (1990) Reductive  $\beta$ -scission of the hydroperoxides of fatty acids and xenobiotics: role of alcohol-inducible cytochrome P-450. *Proc. Natl. Acad. Sci. USA* 87: 5499-5503.
- Wachenfeldt, C.V. and Johnson E.F. (1995) Structures of eukaryotic cytochrome P450 enzymes. in *Cytochrome P450* 2<sup>nd</sup> Ed. Ortiz de Montellano, P.R. (ed) pp.183-223, Plenum Press, New York.
- Waterman, M.R., John, M.E., and Simpson, E.R. (1986) Regulation of synthesis and activity of cytochrome P-450 enzymes in physiological pathways. In: *Cytochrome P-450. Structure, mechanism, and biochemistry.* Ortiz de Montellano P.R. (ed), pp.345-386, Plenum Press, New York.
- Waxman, D.J. and Chang, T.K.H. (1995) Hormonal regulation of liver cytochrome P450 enzymes. In: *Cytochrome P-450. Structure, mechanism, and biochemistry.* 2<sup>nd</sup> Ed. Ortiz de Montellano P.R. (ed), pp.391-417, Plenum Press, New York.
- Williams, D.E. and Buhler, D.R. (1982) Purification of cytochromes P-448 from  $\beta$ -naphthoflavone-treated rainbow trout. *Biochem. Biophys. Acta.* 717: 398-404.

- Williams, D.E. and Buhler, D.R. (1983a) Purified form of cytochrome P-450 from rainbow trout with high activity toward conversion of aflatoxin B<sub>1</sub> to aflatoxin B<sub>1</sub>-2,3-epoxide. *Cancer Res.* 43: 4752-4756.
- Williams, D.E. and Buhler, D.R. (1983b) Comparative properties of purified cytochrome P-448 from beta-naphthoflavone treated rats and rainbow trout. *Comp. Biochem. Physiol. Pt. C* 75: 25-32.
- Williams, D.E., Becker, R.R., Potter, D.W., Guengerich, F.P., and Buhler, D.R. (1983) Purification and comparative properties of NADPH-cytochrome P-450 reductase from rat and rainbow trout: differences in temperature optima between reconstitution and microsomal trout enzymes. *Arch. Biochem. Biophys.* 225: 55-65.
- Williams, D.E. and Buhler, D.R. (1984) Benzo[a]pyrene hydroxylase catalyzed by purified isozymes of cytochrome P-450 from  $\beta$ -naphthoflavone-fed rainbow trout. *Biochem. Pharmacol.* 33: 3743-3753.
- Williams, D.E., Masters, B.S.S., Lech, J.J., and Buhler, D.R. (1986) Sex differences in cytochrome P-450 isozyme composition and activity in kidney microsomes of mature rainbow trout. *Biochem. Pharmacol.* 35: 2017-2023.
- Xiaodong, S., Gatti, G., Bartoli, A., Cipolla, G., Crema, F., and Perucca, E. (1994) Omeprazole does not enhance the metabolism of phenacetin, a marker of CYP1A2 activity, in healthy volunteers. *Therapeutic Drug Monitoring* 16: 248-250.
- Yamano S., Nhamburo, P.T., Aoyama T., Meyer, U.A., Inaba, T., Kalow, W., Gelboin, H.V. McBride, O.W., and Gonzalez F.J. (1989) cDNA cloning and sequence and cDNA-directed expression of human P450IIB1: identification of a normal and two variant cDNAs derived from the CYP2B locus on chromosome 19 and differential expression of the IIB mRNAs in human liver. *Biochemistry* 28: 7340-7348.

- Yamano S., Tatsuno, J., and Gonzalez F.J. (1990) The CYP2A3 gene product catalyzes coumarin 7-hydroxylation in human liver microsomes. *Biochemistry* 29: 1322-1329.
- Yang, Y.-H., Wang, J.-L., Miranda, C.L., and Buhler, D.R. (1997) CYP2M1: Cloning, sequencing and expression of a new cytochrome P450 from rainbow trout liver with fatty acid ( $\omega$ -6)-hydroxylation activity. *Arch. Biochem. Biophys.* Submitted.
- Yang, Y.-H., Miranda, C.L., Wang, J.-L., and Buhler, D.R. (1997) Cloning, sequencing and heterologous expression of CYP2K1 and CYP2K3 from sexually mature rainbow trout liver, and their roles in aflatoxin B<sub>1</sub> activation. *Arch. Biochem. Biophys.* In preparation.
- You, L., Harttig, U., Buhler, D.R., and Bailey, G. Cloning, sequencing and aflatoxin B<sub>1</sub> metabolism by multiple rainbow trout CYP1A cDNAs expressed in yeast. (1997) Submitted.
- Yun, C.-H., Song, M., and Kim, H. (1997) Conformational change of cytochrome P450 1A2 induced by phospholipids and detergent. *J. Biol. Chem.* 272: 19725-19730.
- Zhang, Y.S., Goksoyr, A., Andersson T, and Forlin, L. (1991) Initial purification and characterization of hepatic microsomal cytochrome P-450 from BNF-treated perch (*Perca fluviatilis*). *Comp. Biochem. Physiol. B* 98B: 97-103.
- Zuber, M.X., Simpson, E.R., and Waterman, M.R. (1986) Expression of bovine 17 $\alpha$ -hydroxylase cytochrome P-450 cDNA in nonsteroidogenic (COS1) cells. *Science* 234: 1258-1261.