AN ABSTRACT OF THE THESIS OF

Lijing You for the degree of Master of Science in Biochemistry and Biophysics presented on May 20, 1997. Title: Cloning, Sequencing and Aflatoxin B_1 Metabolism by Multiple Rainbow Trout CYP1A cDNAs Expressed in Yeast.

Abstract approved: Redacted for Privacy

George Bailey

Previous reports indicate the presence of multiple CYP1A sequences in rainbow trout, but their functional differences are unknown. This report describes the cloning and partial characterization of four trout CYP1A cDNAs, which are given the tentative designations CYP1A1v2, v3, v4, and v5. Comparison among these four and three previously reported trout CYP1A sequences reveals that all of the nucleotide and translated amino acid sequences all are closely related (96.9-99.4% cDNA identity; 95.2-99.4% amino acid sequence identity) but none are identical. Six of these sequences encode proteins of 522 amino acids, and one encodes a protein of 536 amino acids. Expression vectors containing the cDNAs for CYP1A1v2, v3, and v4 were transformed into yeast, yielding microsomal hemoprotein CYP contents (63, 156, 96 pmol/mg) comparable to those reported for human CYP1A1 (68-156 pmol/mg) expressed in this system (Eugster et al., 1990, Biochem. Biophys. Res. Commun. 737-744). Kinetic analysis of CYP1A1v2 and v3 proteins indicated similar but not identical Michaelis constants (20±3 vs 13±2 µM) and molar activities (508±47 vs 218±19 pmol/min/nmol P450) for

oxidation of aflatoxin B_1 (AFB_1) to aflatoxin M_1, a reaction characteristic of human CYP1A2. Trout CYP1A1v2 and v3 exhibited lower activity for production of AFB_1-8,9-*exo*-epoxide, also a human CYP1A2 activity. Kinetic data for ethoxyresorufin *O*-deethylation, a prototypical mammalian CYP1A1 activity, also revealed modest but distinct differences in which CYP1A1v3 was more active for this substrate (Km=0.07 ± 0.01 µM, Vm=1398 ± 95 pmol/min/nmol P450) than was CYP1A1v2 (Km=0.15 ± 0.03 µM, Vm=684 ± 83 pmol/min/nmol p450). Interestingly, CYP1A1v4 showed no catalytic activity towards AFB_1, ethoxyresorufin, or 7,12-dimethylbenzanthracene despite formation of a hemoprotein. These results together with previous studies demonstrate the presence in various rainbow trout populations of at least seven *CYP1A* cDNAs representing gene duplication or allelic variation. Present results show that one of three such cDNA sequences encodes a CYP1A hemoprotein with no apparent catalytic activity, that two of the encoded proteins possess certain catalytic properties common to both human CYP1A1 and CYP1A2, and that the sequence differences, though small, are reflected in enzymic properties that can be distinguished.

Cloning, Sequencing and Aflatoxin B$_1$ Metabolism by Multiple Rainbow Trout *CYP1A* cDNAs Expressed in Yeast

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Dean of Graduate School

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Lijing You, Author

ACKNOWLEDGMENT

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DEDICATION

This thesis is dedicated to my wonderful parents for their love, understanding and support.

Cloning, Sequencing and Aflatoxin B$_1$ Metabolism by Multiple Rainbow Trout *CYP1A* cDNAs Expressed in Yeast

INTRODUCTION

The cytochrome P450s (CYP450) consist of a large and expanding superfamily of heme proteins that catalyze oxygenase reactions (1-3). CYP enzymes have been linked to the metabolism of a vast array of substrates, including endogenous compounds such as steroids and fatty acids, as well as xenobiotics such as drugs and chemical carcinogens (2). At least 36 CYP gene families have been identified, many with multiple subfamilies (3).

In mammals there are two known members of the CYP1A subfamily, CYP1A1 and CYP1A2 (4-9). The expression of CYP1A1 and CYP1A2 is induced by polyaromatic hydrocarbons (PAHs) such as 3-methylcholanthrene (3-MC) and chlorinated hydrocarbons including 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (10). The rodent P4501A1 enzyme is known to metabolize benzo[a]pyrene and other polycyclic hydrocarbons, whereas the rodent P4501A2 enzyme is known to metabolize arylamines such as 2-aminofluorene and 4-aminobiphenyl (10). Divergence of the mammalian 1A1 and 1A2 genes is suggested to have occurred subsequent to divergence of fishes and mammals. If so, then fish would not be expected to have diverged CYP1A genes that would be orthologous to mammalian 1A1 and 1A2 (10). The report in 1988 by Heilmann et.al that only one P450 1A cDNA was isolated from 3-MC treated rainbow trout

supported this hypothesis (11). But in 1994 Berntson and Chen cloned two closely related CYP1A genes from rainbow trout liver, which were termed *CYP1A1* and *CYP1A2*, and demonstrated the presence of both *CYP1A1* and *CYP1A2* transcripts in the liver of 3-MC treated rainbow trout (12). Neither of these reported sequences was 100% identical to the trout *CYP1A1* reported by Heilmann et al. Therefore, the number of 1A genes that occur in trout and other fish remain to be defined.

Heterologous expression of single CYP450 genes provides an approach to understanding the catalytic specificity of individual proteins. Similar studies have been carried out with several mammalian P450s in heterologous systems, such as bacteria, yeast, or mammalian cells, but there is no report on in vitro expression of trout CYP1A or catalytic function of the expressed protein. The purpose of this study is to investigate the catalytic activities of individual trout CYP 1A enzymes following expression of cloned P450 cDNAs in yeast, especially their metabolism of aflatoxin B_1 (AFB_1), a potent hepatocarcinogen in several animal models (13), which has been implicated as a hepatocarcinogen in humans (14,15).

LITERATURE REVIEW

Background

The cytochrome P450s comprise a large and expanding superfamily of heme

proteins that catalyze oxygenase reactions (1-3). Omura and Sato first identified the

unusual CO-binding pigment previously found in rat liver (16, 17) as a heme protein, a b-

type cytochrome (18). They were termed P450 after the characteristic CO-bound, reduced

absorption peak at 450 nm (hence Pigment 450). In 1963 it was first demonstrated that

such a CO-binding pigment was catalytically active since it was involved in steroid

hydroxylation in adrenal cortex microsomes (19). The reduced P450-CO complex was

found to be photodissociable, which allowed it to be the participant in many chemical

reactions such as steroid and drug hydroxylation (19). Cytochrome P450 enzymes have

since been linked to the metabolism of a wide range of substrates, with more described

regularly. P450s occur in prokaryotes and eukaryotes, where they are soluble and

membrane bound, respectively (1).

P450 gene superfamily

The current P450 classification system (3) is based on proposed evolutionary

relationships between genes, as inferred from the degree of amino acid sequence identity

between pairs of enzymes (20). For example, P450 proteins showing >40% identical are

defined as being within a single family, and >55% identical are defined as in the same

subfamily. The classification is still evolving. The deficiency of this system is its failure

to consider the evolutionary distance between two species when comparing the sequences of their potentially orthologous CYP forms. So a two-factor system (% identity + species distance) is needed to improve the current one-factor (% identity) system.

As of late 1992 there were 36 CYP gene families reported in the superfamily, many with multiple subfamilies. CYP are now known by their systematic and common names. At present, the assigned name includes the designation of CYP, followed by symbols denoting gene family (Arabic numeral), subfamily (capital letter), and specific gene (Arabic numeral) (20). The number of P450 genes occurring in any one species is not known. However, there is direct evidence for at least 20 and presumptive evidence for 40 distinct P450 genes in rat, and it is estimated that there are likely to be more than 100 distinct P450 genes in a given mammalian species.

Diversity of functions

Cytochrome P450s are very versatile enzymes. They can catalyze a very wide range of different types of reactions (21). These include a variety of reaction types (22), such as oxidative deamination, desaturation of steroids, various heteroatom dealkylations, oxidations, hydroxylations and a number of reductive reactions, including reductive dehalogenation, azo reductive cleavage, and nitro group reduction, as well as ester group oxidative cleavage. Based on the source of substrates, the P450 reactions can be divided into two categories: (i) the synthesis and degradation of endogenous substrates and (ii) the metabolism of foreign chemical (xenobiotic) substrates. In eukaryotes the endogenous compounds include steroids and steroid-derived compounds (cholesterol, androgens,

estrogens, corticosteroids, ecdysteroids, bile acids, vitamin D), fatty acids and fatty acid

derivatives (arachidonic acid, lauric acid), and plant secondary metabolites (cinnamic

acid, tannins, flavonoids). Xenobiotic substrates of P450 in animals also include drugs,

industrial chemicals, pesticides, synthetic intermediates and byproducts, chemical

carcinogens, fungal (mycotoxins) and plant-derived compounds (phytoallexins), and other

natural products.

P450 catalytic cycle

The P450 catalytic cycle is illustrated in Figure 1 (23). The first step of the cycle

is binding of substrate, which usually results in conversion to high spin iron and a shift in

the redox potential that favors reduction of the iron. Subsequently, the iron is reduced by

electron transfer from the flavoprotein NADPH-cytochrome P450 reductase, or in

prokaryotic and mitochondrial P450 reactions with involvement of iron-sulfur proteins

(redoxins). Following iron reduction, O_2 is bound, a critical point at which catalysis may

proceed or be interrupted, resulting in release of active oxygen (superoxide). The next

steps involve addition of a second electron, in some cases via cytochrome b_5, and

formation of a peroxide, followed by cleavage of the O-O bond, the formation of a

substrate radical, and the hydroxylation of that radical and release of the product.
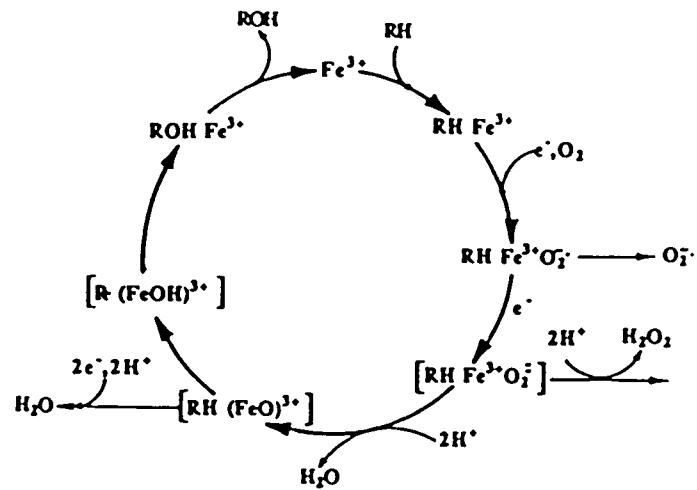
FIG. 1: The P450 catalytic cycle. (From Poulos, T. L., and Ragg, R., *FASEB J.*, 6, 674, 1992)

Cytochrome P450 gene regulation

CYP450 induction was first recognized because induction alters pharmacologic responses (24, 25). One of these, 'barbiturate sleep-time' in rats, has served as an index to the extent of induction (26). CYP induction is not limited to mammals. Several non-mammalian CYP induction systems have been found, including those in birds (27), fish (28), invertebrates (29), bacteria (30), fungi (31) and plants (32, 33).

The general mechanism of P450 induction involves *de novo* protein synthesis (24) and increased transcription (10, 34). Rates of synthesis and degradation (turnover) of both heme and apoprotein will determine the amount of active enzyme. The aryl hydrocarbon (Ah) receptor-mediated induction system is most extensively studied (35, 36). It is understood that the role of Ah receptor is to recognize and bind inducers such as TCDD, and the inducer-receptor complex interacts with transcriptional enhancers on the gene, which stimulates transcription of that gene.

Many compounds can induce mammalian P450s. Inducer categories were created based on the type of specific P450 they induced. Five inducer categories have been established: i.e. phenobarbital (25), 3-methylcholanthrene (24), pregnenolone-16a-carbonitrile (PCN) / glucocorticoids (37), ethanol (38), and peroxisome proliferators such as clofibrate (39). In some cases, there is overlap in these inducer categories. The inducer categories are updated as new inducers are found.

Variables affecting P450 expression

A number of variables have long been known to influence the monooxygenase system in mammals. These include the same variables of health, condition, nutritional status, and reproductive and developmental status that influence many metabolic systems (40). These variables may also influence the expression or function of P450 forms in aquatic species to a certain extent. The mechanisms and degree of effect in different species are less certain. There are strong sex differences in P450 content in mammals and fish, but the forms involved and mechanisms by which these differences are achieved may be different in these groups.

Three approaches to studying P450 structure and functions

Several approaches have been used, separately and in concert, to characterize P450s. The approaches can be grouped into three types:

1. Protein purification and enzymic characterization, amino acid sequencing

2. Immunological probes to study structure and function

3. Gene cloning and nucleic acid probes to study gene regulation

Purification and reconstitution of a P450 form can permit an assessment of some of its intrinsic functional capability and provide immunogens for generating specific antibodies. Purification alone does not enable one to determine how expression of the gene is regulated. On the other hand, cloning of a P450 cDNA or gene will provide nucleic acid and deduced amino acid sequences, often revealing evolutionary relationships, but will not necessarily provide information regarding function.

Functional characterization following expression of cloned P450 cDNA sequences in heterologous systems, such as bacteria, yeast, or mammalian cells, has been carried out with several mammalian P450s (41), but similar studies have been conducted for only three fish P450s, trout CYP11A (P450scc) (42), CYP19 (aromatase) (43) and CYP17 (17α-hydroxylase) (44). A comprehensive characterization- purification, generation of antibodies, determination of catalytic function, and cloning- has been carried out with two fish P450s, trout CYP1A1 and CYP2K1 (11, 45, 46).

Cytochrome P450 in fish

It was originally believed that CYP450s were not present in fish (47). However, later reports by Baker et al (48) and by Potter & O'Brien (49) showed that fish tissues can catalyze oxidation reactions. Since then, research on aquatic species monooxygenase systems has grown rapidly. The failure to detect CYP450 in fish in early studies was probably due to lower temperature optimum in fish than in mammals.

Like mammals, fish are highly responsive to polycyclic aromatic hydrocarbons. It is believed that the Ah receptor- dependent mechanism may also exist in fish based on the knowledge of CYP1A regulation in fish, although direct evidence has not been reported yet. Unlike mammals, the fish MFO systems are not inducible by phenobarbital-like compounds.

There is little direct information on the turnover of P450 in aquatic species. However, there are some studies on fish CYP1A induction. The induction of monooxygenase (AHH) activity in fish liver was first suggested as an indicator of

environmental exposure to petroleum in the early 1970s (50). There is great and growing interest in using CYP1A induction as a biomarker to indicate the exposure of organisms in the wild to inducing compounds, to evaluate the degree and possible risk of environmental contamination (51).

Since fish and other aquatic species have a great variety of habitats, life histories, and ecological relationships, P450s in aquatic species are likely to be as diverse as known mammalian forms, if not more so.

Two known members of the CYP1A subfamily, CYP1A1 and CYP1A2

In mammals there are two known members of the CYP1A subfamily, CYP1A1 and CYP1A2. Divergence of the mammalian 1A1 and 1A2 genes is suggested to have occurred subsequent to divergence of the teleost and mammalian lines (52). If so, then fish would not have diverged CYP1A genes that would be orthologous to both mammalian 1A1 and 1A2. Some regions of the inferred trout 1A1 sequence are identical to all mammalian 1A1 but not 1A2, and others are identical to all 1A2 but not 1A1 (11, 53). This "hybrid" condition could be expected if fish have but single 1A genes, evolved from a single type ancestral to both 1A1 and 1A2 in mammals. Catalytic activities with 7-ethoxyresorufin (7-ER) and benzo[a]pyrene (BaP) indicate a closer similarity to 1A1 than to 1A2, but the hydroxylation of acetanilide, a mammalian 1A2 substrate, by scup CYP1A1 and by trout CYP1A1 (54) is consistent with a 1A1/1A2 hybrid character. Whether the structural relationship of teleost CYP1A to mammalian 1A1 and 1A2 proteins is reflected also in catalytic and other properties needs further examination.

Berntson and Chen recently cloned two closely related CYP1A genes from rainbow trout

liver (12). These may have diverged quite recently. The greater than expected degree of

sequence similarity in the N-terminus of CYP1A from distant teleost families supports

the idea that the rate of evolution could be slower in the P450 genes in fish than in

mammals (53, 55). Sequencing is necessary to establish the extent of similarity and

identity and to address the evolutionary relationship.

Tetraploidy

Studies have concluded that salmonids evolved from a primitive diploid ancestor

by tetraploidization (56-58). Ohno *et al* reported that the diploid chromosome number of

the rainbow trout (*Salmo irideus*) ranges from 58 to 64, but the number of chromosome

arms remains constant at 104. The DNA value of this species is 80 % that of mammals. It

appears that salmonids evolved by tetraploidization from a diploid ancestor which had 48

acrocentrics and a DNA value 40 % that of mammals. Evidence of the persistent

presence of several multivalents in meiotic nuclei of rainbow trout further supported this

conclusion. However, not all salmonid fish in the salmonid superfamily are tetraploid.

For example, the smelt (*Hypomesus pretiosus*) of the family Osmeridae has a diploid

complement and its DNA value is only 21 % that of mammals (58).

Gene duplication played an important role in vertebrate evolution through the

creation of new genes. Products of these several genes which arose by duplication from

an ancestral gene perform the same function but in slightly different ways. It has been

observed that multiple lactate dehydrogenase (LDH) isoforms are present in trout (56,

59). Considering the role P450s play in the metabolism of endogenous as well as exogenous compounds, it is expected that multiple P450s occur in trout.

Aflatoxin B_1

Aflatoxins are secondary metabolites of fungi such as *Aspergillus flavus* and *Aspergillus parasiticus* (60). The aflatoxin family of compounds is among the most carcinogenic of naturally occurring substances. Aflatoxin B_1 is the most toxic and carcinogenic compound among aflatoxin B_1, B_2, G_1, G_2 which produce the most severe pre- and postharvest contamination of foods and feeds.

Evidence shows that humans and many other animal species are sensitive to aflatoxin toxicity. Humans exposed to aflatoxins may demonstrate the following symptoms: vomiting, abdominal pain, pulmonary edema, fat infiltration and necrosis of the liver, even death (61, 62). The International Agency for Research on Cancer (IARC) now classifies AFB_1 aflatoxin as a human carcinogen considering the data for human liver cancer after exposure to aflatoxin (63).

Chicken, duckling and turkey exhibited acute hepatic necrosis, marked bile duct hyperplasia, acute loss of appetite, wing weakness, and lethargy after aflatoxin poisoning (64). Symptoms such as gross subcutaneous edema, ascites, and hepatic injury were also observed in guinea pigs and rats (65, 66) after feeding MRC #18 diet that contained peanut meal (67). Morever, rats ultimately developed liver cancer. The rainbow trout has proved to be the most sensitive animal to the carcinogenicity of AFB_1; diets containing

only few parts per billion of AFB_1, fed for as little as 1 day, will cause liver neoplasms in trout 9-12 months later (68).

The basis for the strain and species difference to the toxicity of AFB_1 is not fully understood. Two factors may play important roles: bioactivation of AFB_1 by phase I enzymes and conjugation of metabolites by phase II enzymes. The activation of AFB_1 to the reactive intermediate 8,9-epoxide is carried out by cytochrome P450. Binding of AFB_1-epoxide to the various cellular macromolecules is believed to be responsible for hepatocellular injury and death.

MATERIALS AND METHODS

RT-PCR to isolate trout *CYP1A* cDNAs

Rainbow trout were treated by intraperitoneal injection of 30 mg/kg of 3-MC dissolved in 100% corn oil as reported for *CYP1A* induction (12). Fish were sacrificed after 24 hr, livers were removed, snap frozen in liquid nitrogen, and stored at -80°C until use. Total RNA was isolated from two fish livers using Tri Reagent (Molecular Research Center, Inc. Cincinnati, OH) and mRNA was purified from total RNA by BioMag mRNA Purification Kit (PerSeptive Diagnostics, Cambridge, MA).

Upstream primer 5'-GCTCATAACGGAAGATTGTGC-3' and downstream primer 5'-CAAAGCCACCATAATAAGTCA-3' were taken from the 5' and 3' untranslated sequence published for *CYP1A3* (12), and found to produce *CYP1A*-related cDNAs of 2.4 kb. Ten to 50 ng of mRNA was used in the first strand cDNA synthesis, following the protocol of Superscript Preamplification System (GIBCO, BRL, Gaitherburg, Maryland). RT-PCR was performed in a volume of 25 µl containing 160 nM each of forward and reverse primers, 200 µM of each dNTP (dATP, dCTP, dGTP, dTTP) and 2U of Taq DNA polymerase. The PCR program was as follows: first denaturing templates at 99°C for 8 sec; then performing 35 cycles of 99°C, 28 sec; 61°C, 45 sec; 68°C, 2 min.

Upstream primer 5'-AATATAACCTCACCTGCAAAC-3' and downstream primer 5'-GAGAATCATAAATGTGACCAT-3' were taken from the 5' and 3' untranslated sequences published for *CYP1A1v1* (12), and generated cDNAs of 1.7 kb.

The RT-PCR reaction conditions were the same as for *CYP1A3* and the PCR program was the same as for *CYP1A3* except that the annealing temperature was 56°C instead of 61°C.

Cloning & sequencing

DNA fragments of 2.4 kb or 1.7 kb were generated from RT-PCR and purified from agarose gel by Geneclean kit (Bio 101 Inc. La Jolla, CA). The purified cDNAs were cloned into a TA cloning vector (Invitrogen, Inc. San Diego, CA), and recombinant pCRII plasmid DNAs were isolated, purified and used for sequencing. Sequencing was carried out using an automated DNA sequencer (Applied Biosystem Inc., Foster City, CA) in the Center for Gene Research and Biotechnology at Oregon State University. (DNA templates were sequenced from both direction. SP6 and T7 promoter in the TA cloning vector were chosen as first set of sequencing primer. The subsequent primers were designed based on the sequence already known). The sequence analysis and comparison were performed using the GCG package (Madison, Wisconsin). Each *CYP1A* cDNA insert was excised from the TA vector by EcoRI digestion and subcloned into the EcoRI site of shuttle vector pHE46 (Fig. 2). Both vector pHE46 and the final expression vector pNW144 (69) were kindly provided by Dr.Christian Sengstag (Institute of Toxicology; Swiss Federal Institute of Technology and University of Zuerich; Schorenstr. 16, CH-8603 Schwerzenbach, Zuerich, Switzerland). This construction placed the cDNA behind the GAPFL promoter and ahead of the PHO5 terminator.

The expression cassette of the putative *CYP1A3* cDNA was isolated as a fragment of NotI digestion/ClaI partial digestion of vector pHE46 plus insert cDNA, and subcloned into the NotI/ClaI site in the dLEU2 region of the final expression vector pNW144 (Fig. 2). The second ClaI site in vector pNW144 is methylated during plasmid replication in E.Coli host, so no cleavage occurred at this position when the vector was digested by NotI/ClaI before ligation with expression cassette. The same procedure was used for expression of the putative *CYP1A1v1* cDNA, except that there is no need of NotI digestion/ClaI partial digestion since these cDNAs lack the ClaI site.
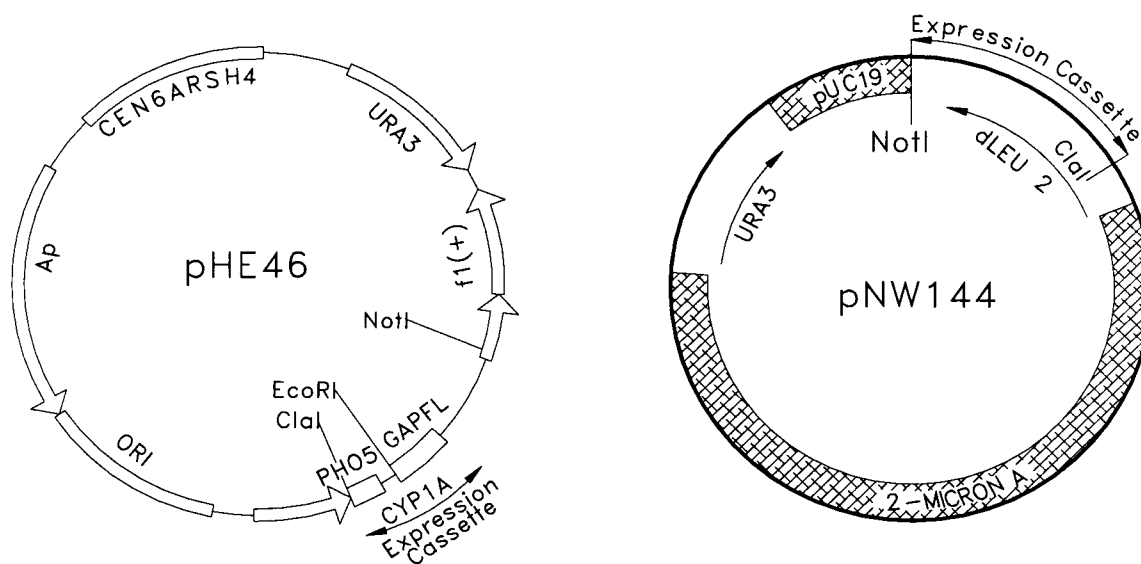
FIG. 2:
Map of expression vectors used in this study. Vector pHE46 provided GAPFL promotor and PHO5 terminator for the expression cassette. CYP1A cDNA was cloned into EcoRI site of pHE46. The CYP1A expression cassette was isolated from the vector pHE46 by NotI /ClaI digestion, and then subcloned into pNW144. Ura 3 in pNW144 served as a selection marker to identify positive yeast transformants.

Yeast transformation

Yeast strain 3090-9d (a; Leu2-3,112; Ura3-52), a generous gift of Dr. Alan

Bakalinsky (Dept.of Food Science and Technology, Oregon State University, Corvallis,

OR 97331), was transformed using the lithium acetate method (70). Sonicated calf

thymus DNA was used as carrier. Yeast transformants were grown on synthetic complete

medium lacking uracil (SC-ura) (71). Yeast microsomes were isolated by the method of

Guengerich et al. (72). Protein concentration in the microsomes was determined by

Lowry assay (73). Microsome P450 content was estimated by reduced CO difference

spectra (18).

Western blotting analysis

Yeast microsomes were resolved on polyacrylamide gels and blotted onto

nitrocellulose membrane. A polyclonal rabbit anti-trout CYP1A antibody (kindly

provided by Dr. D.R. Buhler, Oregon State University, Corvallis, OR 97331) was used as

primary antibody. The blots were subsequently probed with a mouse anti-rabbit secondary

antibody conjugated to horseradish peroxidase (Bio-Rad Laboratories, Richmond, CA).

P450 protein was then detected indirectly by chemiluminescence using an ECL Western

blotting detection kit (Amersham Corp., Arlington Heights, IL).

Enzyme assays

Ethoxyresorufin O-deethylase (EROD) was assayed using the method of Burke et

al. (74). AFB_1 metabolism was examined essentially as described by Stresser et al (75).

We used mouse cytosol plus glutathione (GSH) as an AFB_1-8,9-epoxide trapping system (76) in our primary experiment. Epoxide production proved relatively minor, and the trapping system was not added in the enzyme kinetics studies for AFB_1 metabolism to aflatoxin M_1 (AFM_1). The final reaction mixture included 1 mg/ml microsome proteins; 5, 6.67, 10, or 20 μM AFB_1; 2 mM NADPH in a buffer containing 200 mM sucrose, 64 mM Tris, 20 mM KCl, 4 mM $MgCl_2$ (pH=7.4). The reaction mixture was incubated at 25°C for 30 min (30 min is within the linear range of product formation vs time, data not shown). After 30 min, the reaction was terminated by the addition of 5 μl methanol containing the internal standard aflatoxin G_1 (AFG_1) and 25 μl 2 N acetic acid, and the mixture was frozen overnight. Next day, the frozen mixture was allowed to thaw during centrifugation at 14,000 g for 5 min to remove the precipitated proteins. Metabolites were resolved by HPLC on a 150 x 4.6 mm C_{18} 5 μM Spherisorb ODS-2 column (Phenomenex, CA) and detected by UV absorption at 362 nm. The mobile phase consisted of a combination of 0.1% ammonium phosphate, pH 3.5 (solvent A) and 50:45:5 methanol: acetonitrile: tetrahydrofuran (solvent B).The gradient program for solvent B was: 0-1 min, 10-20%; 1-15 min, 20-40%; 15-17.5 min, 40-60%; 17.5-20 min, constant 60%; 20-21 min, 60-10%; 21-32.5 min, equilibration with 10% solvent B. The flow rate was set at 1.0 ml/min. Metabolites were identified by their respective retention time and quantified using an AFM_1 standard curve and AFG_1 as an internal standard.

RESULTS

RT-PCR and construction of expression cassettes

There were no clones of trout *CYP1A* cDNAs available at the outset of this work. However, Berndtson and Chen (12) published the complete nucleotide sequences of two trout *CYP1A* genes now tentatively termed *CYP1A1v1* and *CYP1A3*. Based on their sequences, we designed RT-PCR primers to selectively amplify these two cDNAs from our strain of trout. In this study four Shasta strain trout were treated with 3-MC to induce *CYP1A* expression, and the livers from two fish were pooled for subsequent RNA isolation. RT-PCR results showed that *CYP1A* mRNAs were indeed expressed in these 3-MC treated Shasta trout, but not in livers of fish without 3-MC treatment (Fig. 3). The two primer sets were found to generate *CYP1A* cDNAs of 2.4 kb and 1.7 kb. TA cloning® was then used to isolate specific RT-PCR products. Two subclonings were performed to construct pNW144 plasmids carrying inserts from the TA-cloned RT-PCR products, which allowed the yeast transformants to grow on synthetic complete medium lacking uracil (SC-Ura).

Sequencing of trout *CYP1A* cDNA inserts

Sequencing of insert DNAs from TA clones revealed four trout *CYP1A*-related insert sequences in this study. *CYP1A1v2* (Genbank accession number U62796) and *CYP1A1v5* were generated from *CYP1A3* primer set, while *CYP1A1v3* (Genbank accession number U62797) and *CYP1A1v4* (Genbank accession number ------) were
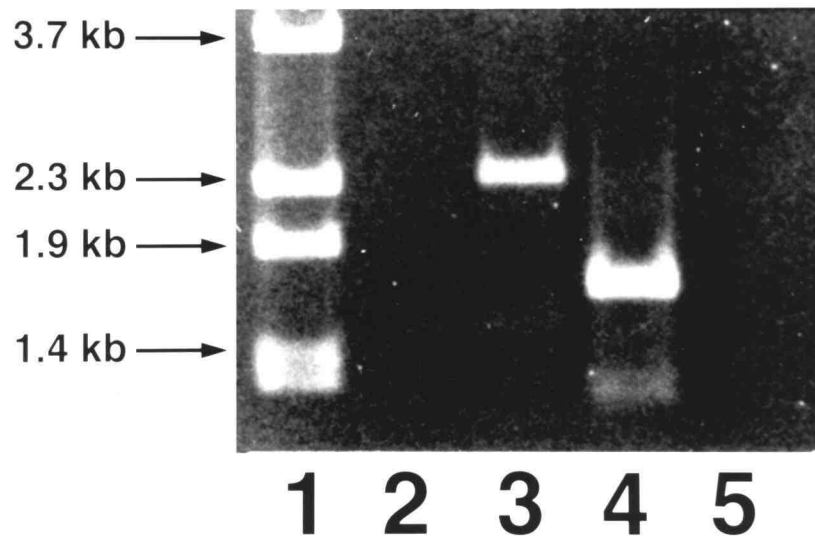
FIG. 3: RT-PCR results. 10 μl of RT-PCR products were subjected to 0.8 % agarose gel electrophoresis.
Lane 1: molecular weight marker: 396 ng of λ DNA digested with BstE II.
Lane 2: control fish without 3-MC treatment amplified with *CYP1A3* primers.
Lane 3: 3-MC treated fish amplified with *CYP1A3* primers.
Lane 4: 3-MC treated fish amplified with *CYP1A1v1* primers.
Lane 5: control fish without 3-MC treatment amplified with *CYP1A1v1* primers.

produced from the *CYP1A1v1* primer set. Figure 4 shows the complete nucleotide

sequence and deduced amino acid sequence of the trout cDNA we term *CYP1A1v2*

cDNA. The deduced amino acid sequences for the two *CYP1A1v1*-related inserts termed

CYP1A1v3 and *v4* are summarized in Figure 5. Figure 5 also gives the amino acid

sequence encoded by *CYP1A1v5* for comparison, though this sequence is regarded as

tentative until more complete bi-directional sequencing is accomplished. Comparisons

among the three previously published trout *CYP1As* and the four cDNA sequences

isolated from our 3-MC induced Shasta trout are shown in Table 1 and 2. None of the

reported nucleotide or amino acid sequences show 100% identity. All sequences are,

however, closely related (96.9-99.4% cDNA identity; 95.2-99.4% amino acid sequence

identity). Though one expects at least one *CYP1A* gene duplication in this ancient

tetraploid species, orthologies among these seven sequences are not readily apparent. At

the cDNA level, the sequences tentatively assigned the designations *CYP1A1v1*,

CYP1A1v3 and *CYP1A1v4* are 99.4-99.6% identical and equally related to *CYP1A1*, and

thus may reasonably represent alleles at a single *CYP1A1* locus. *CYP1A1v2* is less

closely related but there is no genetic basis to exclude it from this series. *CYP1A3* has

already been tentatively assigned as a separate locus. We note that *CYP1A1v5* and

CYP1A3 are actually closely related (99.1%) and approximately equally distant to all

other sequences (96.4-97.7% identity). It is curious, however, that *v5* was isolated using

primers intended to produce *CYP1A3* sequences and that the product was the same length

as *CYP1A1v1*.

FIG. 4: The nucleotide and the deduced amino acid sequences of trout CYP1A1 v2 cDNA. The complete coding region and partial non-coding region are included. The number of nucleotides is shown on both sides.

```
  1 GCTCATAACGGAAGATTGTGCAGAAGCCACAAATAAAACATACAAATGGTTCTCATGATA    60
                                                  M   V   L   M   I
 61 CTACCCATTATCGGCTCAGTCTCTGCGTCTGAGGGGCTGGTGGCCATGGTAACACTATGC   120
     L   P   I   I   G   S   V   S   A   S   E   G   L   V   A   M   V   T   L   C
121 CTGGTGTACATGATCATGAAGTACATGCACACAGAGATCCCTGAGGGACTGAAACGGCTC   180
     L   V   Y   M   I   M   K   Y   M   H   T   E   I   P   E   G   L   K   R   L
181 CCAGGACCAAAGCCCCTTCCCATCATCGGGAATATGCTGGAGGTGCACAATAACCCTCAC   240
     P   G   P   K   P   L   P   I   I   G   N   M   L   E   V   H   N   N   P   H
241 CTCAGCCTGACTGCCATGAGCGAGCGCTACGGCTCAGTCTTCCAGATCCAAATAGGGATG   300
     L   S   L   T   A   M   S   E   R   Y   G   S   V   F   Q   I   Q   I   G   M
301 CGGCCTGTGGTTGTTCTGAGTGGCAATGAGACAGTCCGCCAGGCTCTTATCAAGCAAGGG   360
     R   P   V   V   V   L   S   G   N   E   T   V   R   Q   A   L   I   K   Q   G
361 GAAGACTTCGCCCGGGAGGTCCGATCTATACAGCTTCAAATTCATCAACGACGGCAAGAGC   420
     E   D   F   A   G   R   S   D   L   Y   S   F   K   F   I   N   D   G   K   S
421 TTGGCCTTCAGCACCGACAAGGCTGGGGTGTGGCGCCCGCCGCCGCAAGCTAGCTATGAGT   480
     L   A   F   S   T   D   K   A   G   V   W   R   A   R   R   K   L   A   M   S
481 GCCCTCCGCTCTTTCGCCACACTGGAGGGAACGACCCCAGAGTACTCCTGTGCCCTGGAG   540
     A   L   R   S   F   A   T   L   E   G   T   T   P   E   Y   S   C   A   L   E
541 GAGCACGTCCTCAAGGAGGGAGAATACTTGGTAAAACAGCTGACCTCCGTCATGGATGTC   600
     E   H   V   L   K   E   G   E   Y   L   V   K   Q   L   T   S   V   M   D   V
601 AGTGGCAGCTTTGACCCCTTCCGCCATATTGTCGTATCGGTGGCCAACGTCATCTGTGGA   660
     S   G   S   F   D   P   F   R   H   I   V   V   S   V   A   N   V   I   C   G
661 ATGTGCTTCGGCCGGCGCTACAGCCATGATGACCAGGAGCTGTTGGGCTTGGTGAACATG   720
     M   C   F   G   R   R   Y   S   H   D   D   Q   E   L   L   G   L   V   N   M
721 AGTGATGAGTTTGGGCAGGTGGTGGGCAGCGGCAACCCTGCAGACTTCATTCCCATCCTT   780
     S   D   E   F   G   Q   V   V   G   S   G   N   P   A   D   F   I   P   I   L
781 CGTTACCTGCCCAACCGCACCATGAAGAGGTTTATGGATATCAATGACCGTTTCAACAAC   840
     R   Y   L   P   N   R   T   M   K   R   F   M   D   I   N   D   R   F   N   N
841 TTTGTGCAGAAGATTGTCAGTGAGCACTATGAAAGCTATGACAAGGACAACATCCGTGAC   900
     F   V   Q   K   I   V   S   E   H   Y   E   S   Y   D   K   D   N   I   R   D
901 ATCACTGACTCCCTCATTGACCACTGTGAGGACAGGAAACTAGATGAGAACGCCAACATC   960
     I   T   D   S   L   I   D   H   C   E   D   R   K   L   D   E   N   A   N   I
961 CAGGTTTCTGATGAGAAGATTGTGGGCATTGTCAATGATCTGTTTGGTGCAGGTTTTGAC  1020
     Q   V   S   D   E   K   I   V   G   I   V   N   D   L   F   G   A   G   F   D
1021 ACCATCAGCACAGCTCTGTCATGGGCTGTTGTGTACCTTGTGGCTTATCCAGAGACCCAG  1080
     T   I   S   T   A   L   S   W   A   V   V   Y   L   V   A   Y   P   E   T   Q
1081 GAAAGACTGCATCAGGAACTGAAGGAAAAGGTGGGAATGATTCGCACTCCCCGTCTCTCA  1140
     E   R   L   H   Q   E   L   K   E   K   V   G   M   I   R   T   P   R   L   S
1141 GACAAAATCAACTTACCTCTGCTGGAAGCCTTTATCCTGGAGATCTTCCGGCACTCTTCC  1200
     D   K   I   N   L   P   L   L   E   A   F   I   L   E   I   F   R   H   S   S
1201 TTCCTGCCGTTCACCATCCCACACTGCACGATCAAGGACACATCCCTCAATGGCTACTTC  1260
     F   L   P   F   T   I   P   H   C   T   I   K   D   T   S   L   N   G   Y   F
1261 ATTCCCAAGGACACCTGTGTCTTCATCAACCAGTGGCAGGTCAACCATGACCCGGAGCTG  1320
     I   P   K   D   T   C   V   F   I   N   Q   W   Q   V   N   H   D   P   E   L
1321 TGGAAGGAGCCTTCTTCATTCAACCCTGACCGTTTCCTGAGTGCTGATGGCACAGAACTC  1380
     W   K   E   P   S   S   F   N   P   D   R   F   L   S   A   D   G   T   E   L
1381 AACAAGCTGGAGGGGGAGAAAGTGCTCGTATTCGGCATGGGCAAGCGCCGCTGCATCGGT  1440
     N   K   L   E   G   E   K   V   L   V   F   G   M   G   K   R   R   C   I   G
1441 GAGGCCATCGGACGCAACGAGGTCTACCTCTTCTTGGCCATCCTGCTCCAAAGGCTGCGC  1500
     E   A   I   G   R   N   E   V   Y   L   F   L   A   I   L   L   Q   R   L   R
1501 TTCCAGGAGAAACCTGGGCACCCGCTGGACATGACCCCAGAATACGGCCTCACCATGAAG  1560
     F   Q   E   K   P   G   H   P   L   D   M   T   P   E   Y   G   L   T   M   K
1561 CACAAGCGCTGTCAGCTGAAGGCTAGCATGCGGCCATGGGGGCAGGAGGAGTGAGGGCCA  1620
     H   K   R   C   Q   L   K   A   S   M   R   P   W   G   Q   E   E   *
1621 TGGTCACATTTATGATTCTCAACATCACTATAACTGATTTATAGTTAGCCCTACATACTT  1680
1681 GATGGCATGAGATGAGTTCAGATTTAAATGTCCGAGGGTACATTTTCTCTCTAGGAACGA  1740
1741 CTGGGCTTGACACCTCTCTTGATTCATTGGAGAATTAACAGTGAGGGAAAACTGAGTCAA  1800
1801 ATTGTAGACTATTTGAAATCGATAAAATTATAATCTGAAATGGTATGTAGGGGGATTCCA  1860
1861 AACAAGTGTTGTATTGGATAAAGGACCTTCACAAACTCATAGAGTTGATTGGTTTCTAAG  1920
1921 ACCAGCATACTACAGGCCCTACTACAGTTTGTCAGGTGTTTCTTCAGGAGATATCAGGAG  1980
1981 AGACTATAGCTGTTTAGCTGCCTTTTTGTCTACATAATGTGTTTACTTCTCCTCTCGTT  2040
2041 TTGATCACAGCTCATATACTGTATCAGATCACTTTAAAGAGTATGAATCTATAAAACATA  2100
2101 CACATAAACCCATGGTGGGGTGTTAAAGAGCATTGTTTTTAGTTCCATGTTTGTGTAGAA  2160
2161 TTTTTTGCTACATTGCCTGTTCTCGTACTTTTGAGTTATTGTTCAGGTGGTCATAGATCA  2220
2221 CAGGTAGTTGAATGGATGCATCCTCCCATTTCTAAGCTAGATATTTTTTATTCCACTGAA  2280
2281 TAAATCAGTAAAATGTGGACACAACACACTGAAGCTGTGTTTGTATGCCACCAACATGTG  2340
2341 ATCCTTTTTTGAAATGTGCCTAAAATGTGTATATATTGTGGTGACTTATTATGGTGGCTTT  2401
```

Figure 4

```
          1                                                          51                                                100
CYP1A1    HVLHILPIIG  SVSASEGLVA  HVTLCLVYHI  HKYHHTEIPE  GLKRLPGPKP  LPIIGHVLEV  HNNPHLSLTA  HSERYGSVFQ  IQIGHRPVVV  LSGSETVRQA
CYP1A1_v1 ..........  ...V......  I........L  ...K......  ..........  ..........  Y.........  ..........  ..........  ...N......
CYP1A1_v2 ..........  ..........  ..........  ..........  ..........  .......H...  ..........  ..........  ..........  ...N......
CYP1A1_v3 ..........  ...V......  I........L  ...K......  ..........  ..........  Y.........  ..........  ..........  ...N......
CYP1A1_v4 ..........  ...V......  I........L  ...K......  ..........  P.........  Y.........  ..........  ..........  ...N..D...
CYP1A1_v5 ..........  ..........  ..........  ..........  ..V...      ..........  ..........  ..........  ..........  ..........
CYP1A3    ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........

          101                                                        151                                               200
CYP1A1    LIKQGEDFAG  RPDLYSFKFI  NDGKSLAFST  DKAGVWRARR  KLAHSALRSF  ATLEGTTPEY  SCALEEHVCK  EGEYLVKQLT  SVHDVSGSFD  PFRHIVVSVA
CYP1A1_v1 ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
CYP1A1_v2 ..........  .S........  ..........  ..........  ..........  ..........  .......L..  ..........  ..........  ..........
CYP1A1_v3 ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
CYP1A1_v4 ..........  ..........  ...N......  ..........  ..........  ..........  ....S....   ..........  ..........  ...Y......
CYP1A1_v5 ..........  ..........  ..........  ..........  ..........  .....S....  ..........  ..........  .......R.. .Y........
CYP1A3    ..........  ..........  ..........  ..........  ..........  .....S....  ..........  ..........  ..........  ..........

          201                                                        251                                               300
CYP1A1    NVICGHCFGR  RYSHDDQELL  GLVNHSDEFG  QVVGSGNPAD  SIPILRYLPN  RTHKRFHDIN  DRFNTFVQNI  ISEHYESYDK  DNIRDITDSL  IDHCEDRKLD
CYP1A1_v1 ..........  ..........  ..........  ..........  F.........  ..........  ....N...K.  V.........  ..........  ..........
CYP1A1_v2 ..........  ..........  ..........  ..........  F.........  ..........  ....N...K.  V.........  ..........  ..........
CYP1A1_v3 ..........  ..........  ..........  ..........  F.........  ..........  ....N...K.  V.........  ..........  ..........
CYP1A1_v4 ..........  ..........  ..........  ..........  F.........  ..........  ....N...K.  V.........  ..........  ..........
CYP1A1_v5 ..........  ..........  ..........  ..........  F.........  .......Y..  ........K.  V....D....  ..........  ..........
CYP1A3    ..........  ..........  ..........  ..........  F.........  ..........  ........K.  V....D.....  ..........  ..........

          301                                                        351                                               400
CYP1A1    ENANIQVSDE  KIVGIVNDLF  GAGFDTISTA  LSWAVVYLVA  YPEIQERLHQ  ELKEKVGHIR  TPRLSDKINL  PLLEAFILEI  FRHSSFLPFT  IPHCTIKDTS
CYP1A1_v1 ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
CYP1A1_v2 ..........  ..........  ..........  ..........  ...T......  ..........  ..........  ..........  ..........  ..........
CYP1A1_v3 ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
CYP1A1_v4 ......H....  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........I  ..........
CYP1A1_v5 ....V.....  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ......V....
CYP1A3    ....V.....  ..........  ..........  ..........  ....-----   ----------  ----------  ----------  ----------  --...V....

          401                                                        451                                               500
CYP1A1    LNGYFIPKDT  CVFINQHQVN  HDPDLWKEPS  SFNPDRFLSA  DGTELNKLEG  EKVLVFGHDK  RRCIGEAIGR  NEVFLFLAIL  LQRLRFQEKP  GHPLDHTPEY
CYP1A1_v1 ..........  ..........  ...E......  ..........  ..........  ..........  ..........  ...Y......  ..........  ..........
CYP1A1_v2 ..........  ..........  ...E......  ..........  ..........  ........G.  ..........  ...Y......  ..........  ..........
CYP1A1_v3 ..........  ..........  ...E......  ..........  ..........  ........G.  ..........  ...Y......  ..K.......  ..........
CYP1A1_v4 ..........  ..........  ...E......  ..........  ..........  ..........H ...Y......  ..........  ..........
CYP1A1_v5 ..........  ..........  ...E......  ........I.  ..........  ..........  ..........  ...Y......  ....C.....  ..........
CYP1A3    ..........  ..........  ...E......  ..........  ..........  ........G.  ..........  ...Y......  ....C.....  ..........

          501                                  536
CYP1A1    GLTHGHKRCQ  LKASHRPHGQ  EE
CYP1A1_v1 ..........  ..........  ..
CYP1A1_v2 ..........  ..........  ..
CYP1A1_v3 ..........  ..........  ..
CYP1A1_v4 ..........  ..........  ..
CYP1A1_v5 ..........  ....L.L...  ..
CYP1A3    ..........  ....L.HG.R  KSEGHGHIYD  SQHHYN
```

FIG. 5: Multiple alignment of seven trout CYP1A amino acid sequences.
Comparison was done using Multalign by Florence Corpet, described in: Multiple sequence alignment with hierarchical clustering, F.Corpet, 1988, Nucl. Acids Res. 16, 10881-10890.
Web site: http://www.toulouse.inra.fr/multalin.html
Symbol comparison table: blosum62
Gap weight: 12
Gap length weight: 2

TABLE 1. Comparison between different trout *CYP1A* cDNAs

(% identity of nucleotide sequences)

	PREVIOUS REPORTS			THIS STUDY			
	1A3[a]	*1A1*[b]	*1A1 v1*[c]	*1A1 v2*	*1A1 v3*	*1A1 v4*	*1A1 v5*
1A3	100	97.9	97.4	97.7	97.2	96.9	99.1
1A1		100	98.9	98.8	98.5	98.3	97.2
1A1 v1			100	98.7	99.6	99.4	96.9
1A1 v2				100	98.9	98.6	96.9
1A1 v3					100	99.4	96.6
1A1 v4						100	96.4
1A1 v5							100

[a] Originally called *CYP1A1* by Berndtson and Chen (1994), now called *CYP1A3*.

[b] As originally specified by Heilmann et al. (1988).

[c] Originally called *CYP1A2* by Berndtson and Chen (1994), now called allelic variant *v1* of *CYP1A1*.

TABLE 2. Comparison between different trout CYP1A Proteins

(% identity of amino acid sequences)

	PREVIOUS REPORT			THIS STUDY			
	1A3	1A1	1A1 v1	1A1 v2	1A1 v3	1A1 v4	1A1 v5
1A3	100	96.7	96.2	96.7	96.4	95.2	97.9
1A1		100	97.9	97.7	97.3	96.6	96.7
1A1 v1			100	97.9	99.4	98.6	96.2
1A1 v2				100	98.1	96.9	96.4
1A1 v3					100	98.5	96.0
1A1 v4						100	95.2
1A1 v5							100

Relationships among the protein sequences were examined using the PHYLIP version 3.572c protein parsimony algorithm. As may be anticipated, the closeness of the sequences results in several unrooted or rooted solutions, one of which is shown in Figure 6. By this solution, CYP1A1 v1, v3, and v4 are recently diverged alleles within the CYP1A1 locus. CYP1A3 and CYP1A1v5 are approximately equaly distant from all other proteins and from each other, and could be denoted in this solution as separate loci. Since the protein products CYP1A1v5 and CYP1A3 share a less distinctive relationship than do their cDNAs, and in the absence of information on *CYP1A1v5* regulation, we are reluctant to assign this as a *CYP1A3* allelic variant.

Trout CYP1A expression in yeast

Western blot analysis was carried out to establish if the heterologous P450s were expressed as proteins in yeast. Yeast microsomes from different transformants were isolated and the proteins were resolved on polyacrylamide gel. Microsomes from β-naphthoflavone (BNF) treated trout were used as a positive control (data not shown). Yeast transformed with pNW144/*CYP1A1v2, v3,* or *v4* gave positive signals on Western blotting analysis (Fig. 7), whereas yeast transformed with pNW144 vector alone did not show any cross reactions. CYP1A1v2 and v3 proteins exhibited identical mobility on Western blot, while CYP1A1v4 had slightly greater mobility. No heterologous expression studies were carried out with *CYP1A1v5.*
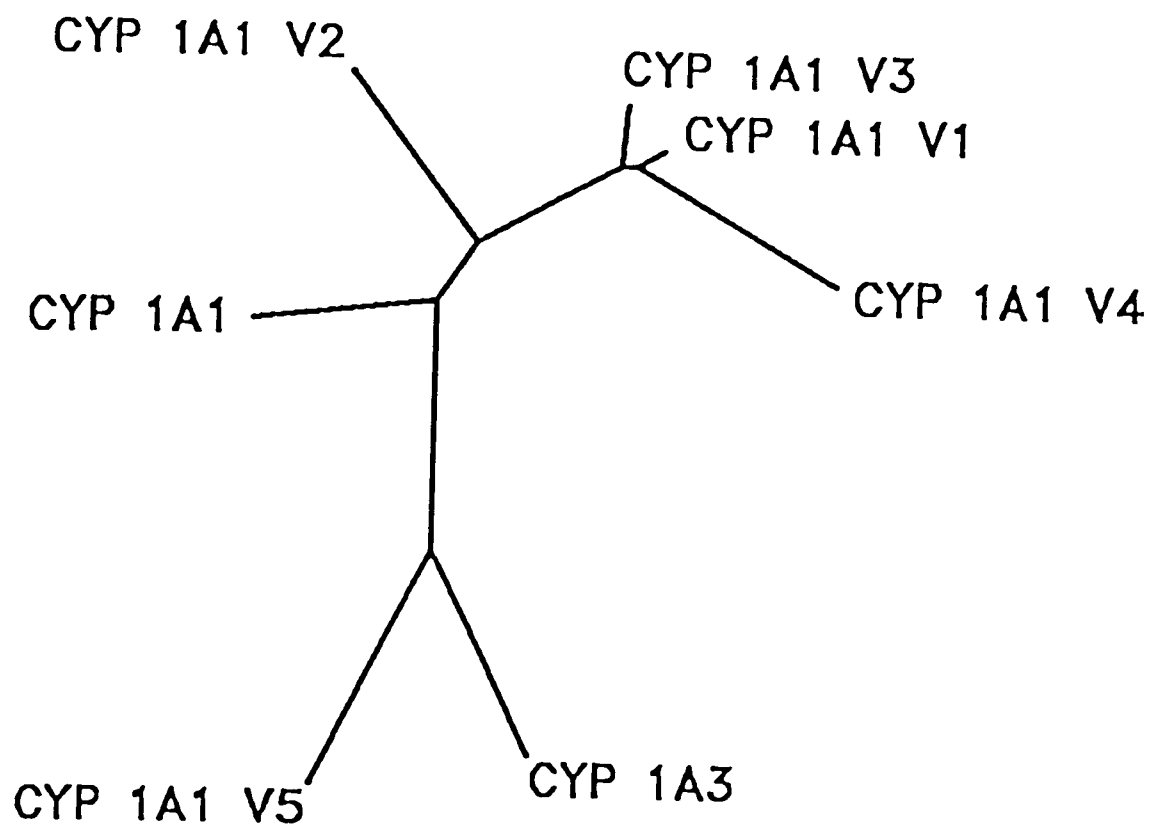
FIG. 6: Phylogenetic tree showing the relationship between seven trout CYP1A proteins.
PHYLIP version 3.572c:
ProtDist : Dayhoff PAM matrix
FITCH : default settings
DRAWTREE: unrooted tree

FIG. 7: Western blot demonstrating the presence of trout CYP1A proteins in yeast microsomes transformed with trout CYP1A cDNA. Yeast microsomes were loaded on SDS-PAGE gel, transferred to nitrocellulose membrane after electrophoresis, then incubated with primary rabbit-anti-trout antibody and subsequently with secondary mouse-anti-rabbit antibody. Secondary antibody was then detected with chemiluminescence.
Lane 1: CYP1A1 v3, 10.2 ug of microsome protein.
Lane 2& 3: two purified CYP1A proteins provided by Dr. Donald Buhler.
Lane 4: vector alone. 1.6 ug of microsome protein.
Lane 5: CYP1A1 v2, 1.6 ug of microsome protein.
Lane 6: CYP1A1 v4, 1.8 ug of microsome protein.

Enzyme activity

Cytochrome P450 contents of the microsomes were determined by reduced CO-difference spectrophotometry. The spectrum of pNW144/CYP1A1v2, v3, and v4 each showed a characteristic peak at 450 nm (data not shown). The pNW144 vector alone failed to show a peak at 450 nm. This indicates there was negligible endogenous yeast CYP content and that the heterologous CYP proteins accounted for all the P450s present in the yeast. The P450 contents for microsomal preparations of pNW144/CYP1A1v2, v3, and v4 used for subsequent catalytic experiments were 63, 156, and 96 pmol/mg respectively.

EROD activity was assayed using these transformed yeast microsomes. There was no detectable EROD activity in control microsomes transformed with vector pNW144 alone. Both CYP1A1v2 and CYP1A1v3 were capable of catalyzing the O-deethylation of ethoxyresorufin. Enzyme kinetics studies show that there are significant differences between these two enzymes (Fig. 8). CYP1A1v3 (Km=0.07 ± 0.01 μM, Vm=1398 ± 95 pmol/min/nmol P450) appears to be more active towards the substrate ethoxyresorufin than does CYP1A1v2 (Km=0.15 ± 0.03 μM, Vm=684 ± 83 pmol/min/nmol P450). Surprisingly, microsomes from yeast transformed with pNW144/CYP1A1v4 showed no EROD activity, even though this CYP1A protein exhibited a reduced CO spectrum and was present in substantial amounts.

Both CYP1A1v2 and v3 were also able to catalyze the formation of AFB$_1$ metabolites (Fig. 9), while microsomes from yeast transformed with vector alone or with CYP1A1v4 failed to metabolize AFB$_1$. Comparison with HPLC standards demonstrated
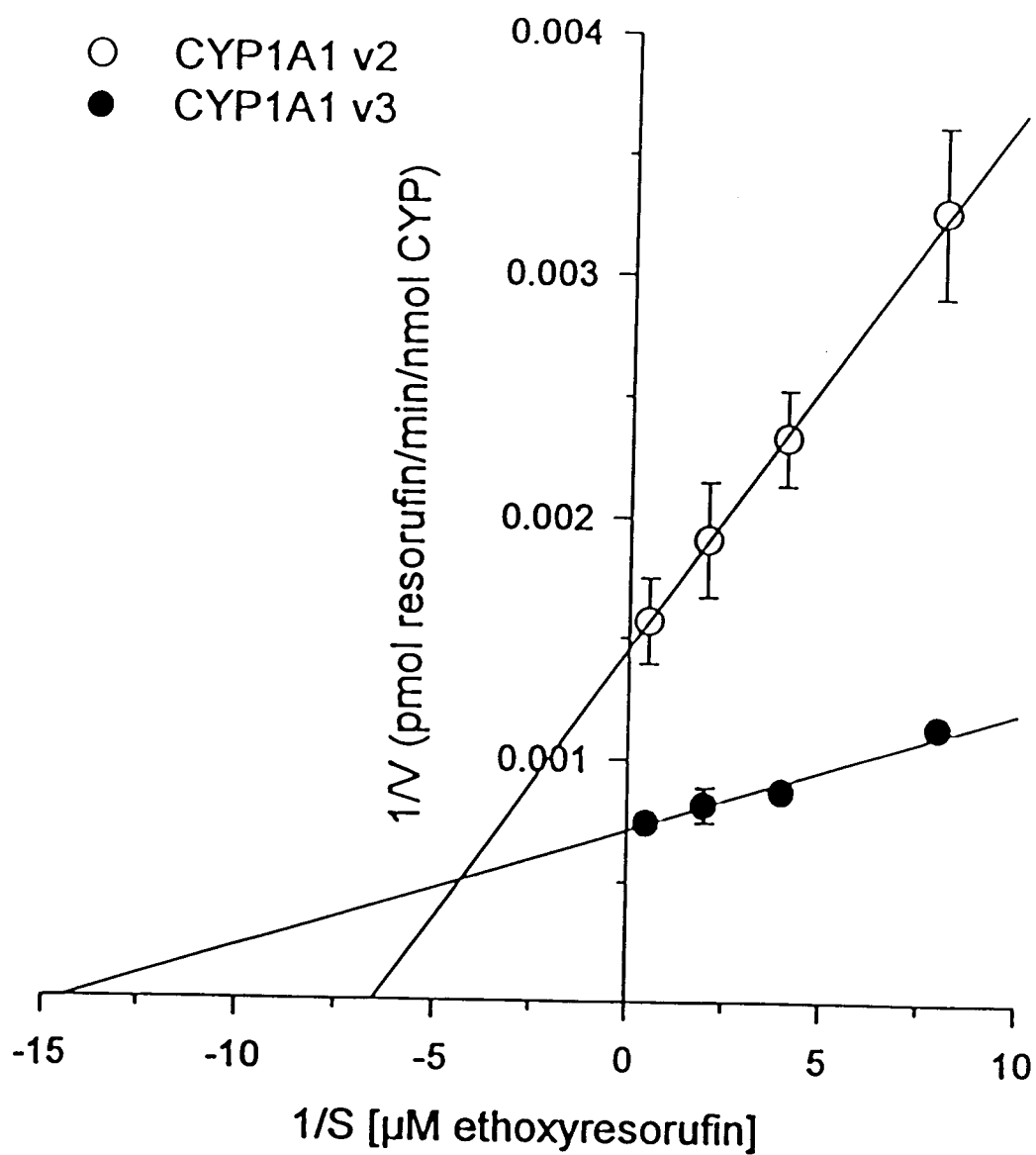
FIG. 8: Lineweaver-Burke plot showing EROD activity of trout CYP1A1v2 and CYP1A1v3. Data are means of triplicate samples ± SE. Curve fitting was done by non-linear regression. EROD was assayed at 25°C.
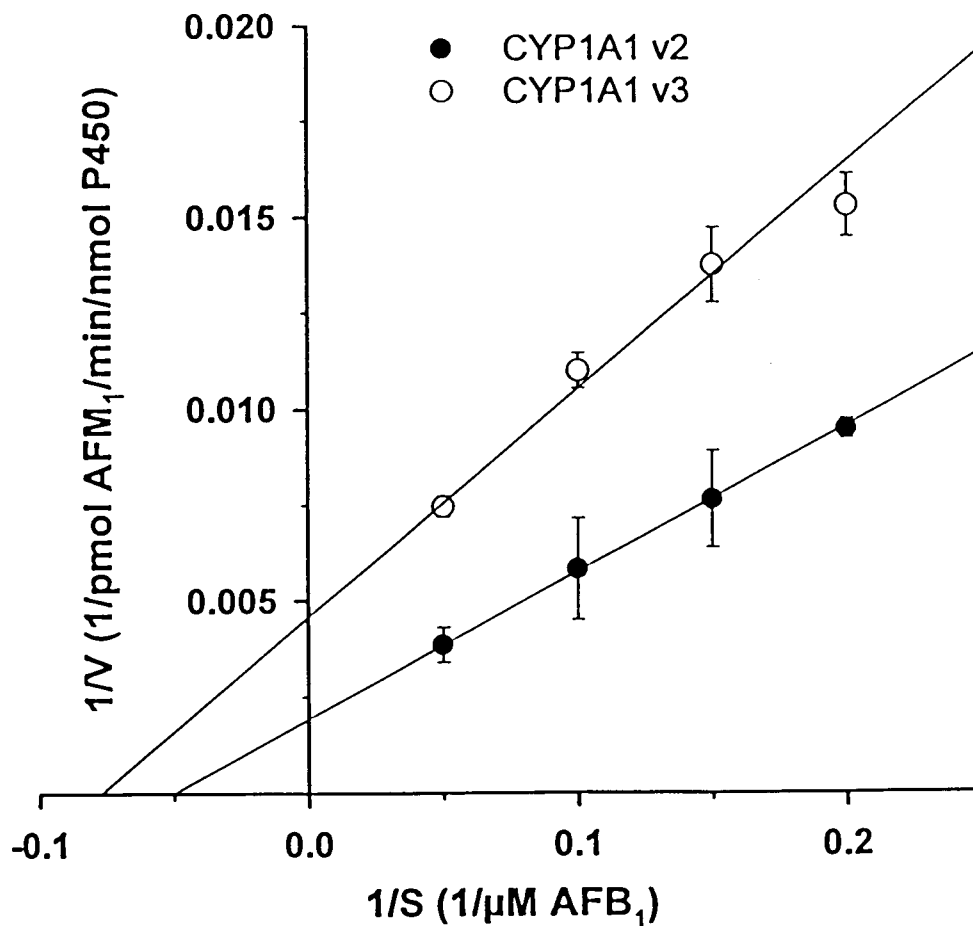
FIG. 9: Lineweaver-Burke plot showing AFB$_1$ metabolism by trout CYP1A1v2 and CYP1A1v3. Data are means of triplicate samples ± SE. Curve fitting was done by non-linear regression. AFB$_1$ metabolism was measured at 25°C and AFB$_1$ concentration of 5, 6.67, 10 or 20 μM.

that AFB_1 was mainly hydroxylated to AFM_1 by these two enzymes. Production of AFB_1-8, 9-epoxide was detectable in the HPLC profile (Fig. 10) only at the highest substrate concentration used in these studies, 20 µM AFB_1. The amount of AFB_1-8,9-epoxide metabolite produced by these two enxzymes at this substrate concentration was about 6-10% that of AFM_1 metabolite formed in the same reaction. Interestingly, CYP1A1v2 had greater molar activity than v3 (508 ± 47 *vs* 218 ± 19 pmol/min/nmol P450) for AFB_1 hydroxylation, though it had lower activity for ethoxyresorufin deethylation (Table 3). By contrast CYP1A1v2 had the greater Michaels constant for both substrates.
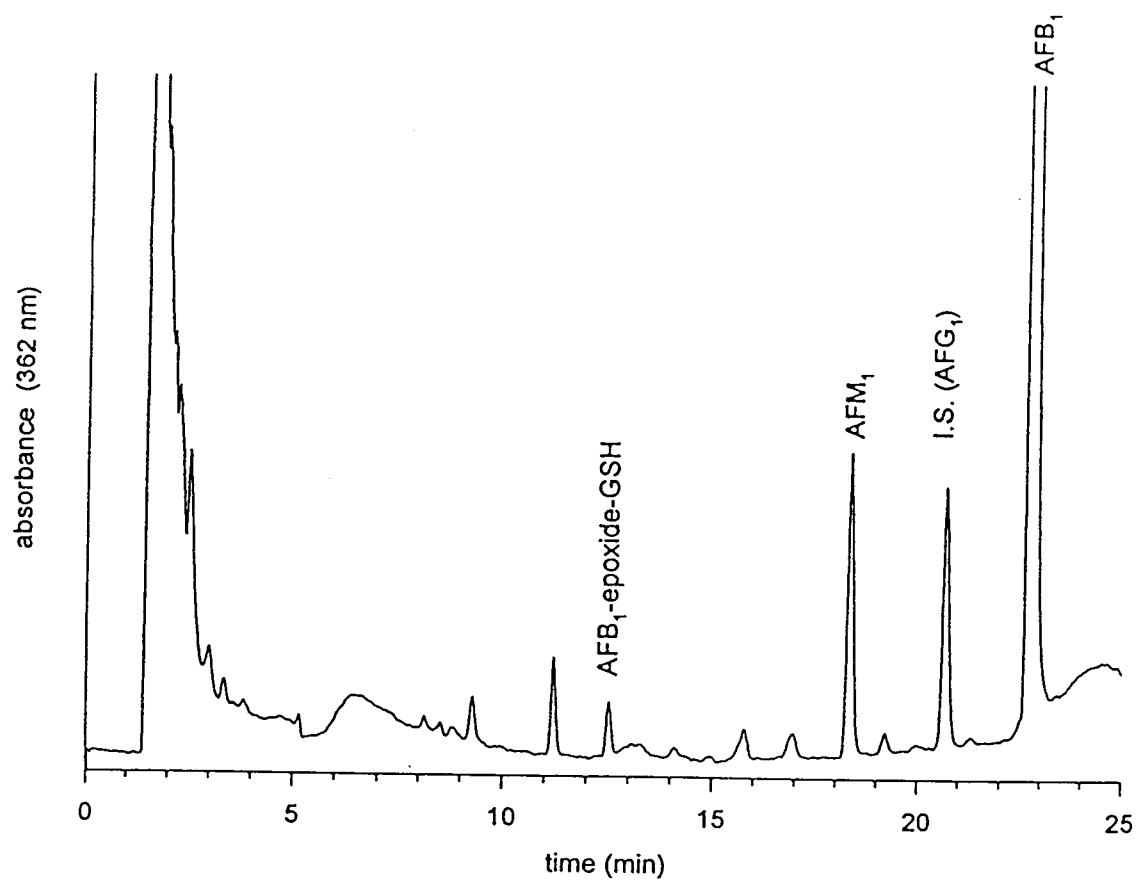
FIG. 10: Representative HPLC chromatogram showing AFB_1 metabolism by CYP1A1v3.

TABLE 3. Catalytic activities of the microsomes from yeast transformed with trout *CYP1A*

	AFM$_1$ metabolite formation		EROD	
	Km	V$_m$	Km	V$_m$
	(uM)	(pmol/min/nmol P450)	(uM)	(pmol/min/nmol P450)
CYP1A1 v2	20 ± 3	508 ± 47	0.15 ± 0.03	684 ± 83
CYP1A1 v3	13 ± 2	218 ± 19	0.07 ± 0.01	1398 ± 95
CYP1A1 v4	nd	nd	nd	nd

nd denotes not detectable

DISCUSSION

Multiple *CYP1A* genes in trout

Berndtson and Chen first described the expression of multiple *CYP1A* genes in liver in response to 3-MC treatment in rainbow trout (12). These researchers isolated two distinct rainbow trout CYP1A genes, now tentatively designated *CYP1A1v1* and *CYP1A3*, from a genomic library probed with a 514-bp PCR fragment coding for the first 514 base pairs of a trout *CYP1A1* cDNA. Though the results demonstrated that two *CYP1A* genes were expressed at the mRNA level in response to 3-MC treatment, the study did not establish if the proteins corresponding to these two genes were either present or catalytically active. We now report the cloning of four additional trout *CYP1A* cDNAs and the functional expression in yeast of three of these. Two 2.4 kb cDNA clones (*CYP1A1v2* and *v5*) showed 96.9 % identity to each other, while two 1.7 kb cDNA clones (*CYP1A1v3* and *CYP1A1v4*) showed 99.4% identity to each other. Even the most closely related sequences, however, encode five differences in amino acid sequence, which we believe are too numerous to be considered as reverse transcriptase or PCR errors. In order to verify the reliability of our sequencing strategy, we carried out partial sequencing of nine additional TA clones, one generated by the *CYP1A3* primer set and eight generated by the *CYP1A1v1* primer set. The 400-500 nucleotide partial sequences examined for each of these nine clones indicated that one clone was *CYP1A1v2* and eight were *v3*. Most importantly, among this secondary data set of over 4000 bases there were no differences from the full-length *v2* and *v3* sequences reported here. This result indicates

that the cloning and sequencing strategy was reliable. We have not, however, conducted totally independent *CYP1A* isolates from Shasta trout nor has this been done for the other reported trout sequences, so the possibility of isolation artifacts within the reported trout *CYP1A* sequences cannot be completely discounted.

Western blot and spectral analyses indicated that trout CYP1A1v2, v3, and v4 hemoproteins were expressed in transformed yeast microsomes. The apparently inactive v4 protein showed slightly greater electrophoretic mobility than the active v2 and v3 proteins. Our results clearly demonstrate that there are multiple *CYP1A* cDNAs present and expressed in rainbow trout. Since two trout livers were pooled for RNA preparation in our studies, it is formally possible that all four *CYP1A* isoforms represent allelic variants at a single locus, and this is how we have tentatively classified them. The degree of sequence and regulatory divergence suggest that at least two trout genes, *CYP1A1* and *1A3*, have arisen through duplication and subsequent functional divergence. Evidence for divergence in catalytic properties, however, is restricted to sequences *CYP1A1v2* and *v3* in the present study. CYP1A3 protein has not been expressed and the one sequence from our study that could possibly be considered a *CYP1A3* allele, *CYP1A1v5*, has not been expressed.

The sequence of CYP 1A1v3 and the inactive variant v4 showed eight amino acid differences. Several of the changes occurred in sequence motifs that are conserved in P450 families (77). The change at position 459 from glycine to aspartic acid was located in the heme binding site, which is one of the five sequence motifs characteristic of a P450 protein (78) and conserved in all P450 proteins. A search of the PRINTS database, P450

superfamily section (79, 80), containing 354 P450 sequence entries, was conducted to

determine the frequency of amino acid changes in the heme binding site. The results

showed that only two entries, CP11_oncmy and S69278 showed a change from glycine to

aspartic acid at a position equivalent to amino acid 459 in the inactive variant v4.

Interestingly, both sequences, currently named CYP1A1 (CP11_oncmy) and CYP1A1v1

(S69278) are the previously published rainbow trout liver DNA sequences (11, 12). For

comparison, changes of a second glycine (position 457) in the heme binding site are much

more common (181 glycine, 155 serine, 17 asparagine and 2 histidine), emphasizing the

highly conserved nature of glycine 459. The appearance of the glycine - aspartic acid

change at an otherwise highly conserved site and its concurrent occurrence in an inactive

protein suggest the possibility that sequences with aspartic acid code for non functional

proteins, although the inactivity of variant v4 might have been caused by some of the

other detected amino acid changes.The successful verification of the proposed effect of

aspartic acid on CYP 1A enzyme activity will help in elucidating the significance of

multiple, closely related CYP1A sequences in rainbow trout. More exhaustive repetitive

cloning will be necessary before the origin of the differences in reported trout *CYP1A*

sequences from the three laboratories are clearly understood.

AFB$_1$ metabolism by trout CYP1A proteins

AFB$_1$ is metabolized by the CYP-dependent mixed function oxidase system in

various species to a variety of more polar metabolites, including AFM$_1$, aflatoxin Q$_1$

(AFQ$_1$), and the AFB$_1$-8,9-epoxide. The formation of AFM$_1$ and AFQ$_1$ represents

production of less toxic metabolites, while the *exo*-isomer of AFB_1-8,9-epoxide is a potent electrophile that binds covalently to protein, RNA, and DNA (81). The rainbow trout is very sensitive to AFB_1, and has long been used as an animal model for AFB_1-induced hepatocarcinogenesis (81). In trout, CYP2K1 has high activity for metabolizing AFB_1 to AFB_1-8,9-epoxide (81). It has been shown that the potent induction of CYP1A enzyme(s) by β-naphthoflavone in both trout and rodents is accompanied by increased conversion of AFB_1 to the less toxic metabolite AFM_1 in vivo (82) and in vitro (83-88). In trout, AFB_1 is also reduced by cytosolic enzymes to aflatoxicol (AFL) (89, 90), which is further metabolized to aflatoxicol M_1 ($AFLM_1$), possibly by CYP1A enzymes (91). However, the enzymes actually responsible for AFM_1 production in trout have not previously been directly identified.

The data presented herein show that trout CYP1A1v2 and v3 both have the capability of catalyzing AFM_1 formation, and that they do so with similar but not identical Km and significantly different Vm values. The enzyme kinetic data for EROD activity also indicate the existence of distinct though relatively modest differences between these two enzymes. Among mammals the human, mouse and rat CYP1A2 isozymes appear principally responsible for the formation of AFM_1 (92-96). Formation of the 8,9-epoxide can also be catalyzed by rat (95) and human (96) CYP1A2, as well as human CYP1A1 (97) and CYP3A4 (96). It is interesting that both CYP1A1v2 and v3 can produce AFB_1-8,9-*exo*-epoxide. While human CYP1A2 has been argued to be primarily responsible for formation of AFB_1-8,9-epoxide in liver due to its low Km and relatively high expression level, this appears to be a relatively minor reaction for trout

CYP1A1v3 (Fig. 9). It is important to note that the epoxide trapping assay with mouse cytosol is highly selective for the *exo* epoxide. We do not know the extent, if any, to which these two trout enzymes support formation of the relatively non-genotoxic *endo* AFB_1-8,9-epoxide isomer. In summary, with respect to AFB_1 metabolism, the predominant reaction supported by trout CYP1A1v2 and v3, AFM_1 production, most closely resembles that of the human CYP1A2 enzyme.

Catalytic differences of trout CYP1A proteins

None of the proteins for the three previously reported trout CYP1A cDNAs were characterized. This report examined differences in three trout CYP1A proteins using heterologous expression in yeast. One of these, CYP1A1v4, showed no catalytic activity toward ethoxyresorufin, AFB_1 or 7, 12-dimethylbenz[a]anthracene (data not shown) even though it generated a strong reduced CO-difference spectrum indicative of heme incorporation. At present we have identified no xenobiotic substrate for this protein and tentatively conclude it to be an expressed but inactive gene product.

The functional significance of the relatively modest catalytic differences so far determined for CYP1A1v2 and v3 is not understood, but could be important. In support of this idea, Curtis et al. have reported that two trout CYP1A proteins are regulated differently in vivo by 2,4,5,2',4',5'-hexachlorobiphenyl (98). The relationship between their trout "CYP1A1" and "CYP1A2", and the CYP1A proteins we have identified is presently not known.

Although trout lack an ortholog to mammalian *CYP1A2*, it is possible that the multiple trout CYP1A proteins collectively accommodate at least some of the catalytic functions that distinguish mammalian CYP1A1 and CYP1A2. Even at the allele level, we note that CYP1A1v2 and v3 display the EROD activity characteristic of mammalian CYP1A1, and the AFM_1 production (as well as some 8, 9-epoxide production) characteristic of mammalian CYP1A2. More detailed studies of arylamine and PAH metabolism by these enzymes and CYP1A3 should help clarify the extent to which trout CYP1As may have diverged with respect to CYP1A1-like and CYP1A2-like catalytic properties.

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