

Identification, Functional Characterization, and Regulation of a New Cytochrome P450 Subfamily, the CYP2Ns*

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The screening of liver and heart cDNA libraries from the teleost *Fundulus heteroclitus* with degenerate oligonucleotide probes to conserved α -helical regions in mammalian P450s resulted in the identification of two cDNAs that together represent a novel P450 subfamily, the CYP2Ns. Northern analysis demonstrated that CYP2N1 transcripts are most abundant in liver and intestine, whereas CYP2N2 mRNAs are most abundant in heart and brain. CYP2N1 and CYP2N2 proteins were co-expressed with NADPH-cytochrome P450 oxidoreductase in *Sf9* insect cells, and their ability to metabolize arachidonic acid and xenobiotic substrates was examined. Both CYP2N1 and CYP2N2 metabolize arachidonic acid to epoxyeicosatrienoic acids. Epoxidation is highly regio- and enantioselective with preferential formation of (8*R*,9*S*)-epoxyeicosatrienoic acid (optical purities are 91 and 90% for CYP2N1 and CYP2N2, respectively) and (11*R*,12*S*)-epoxyeicosatrienoic acid (optical purities are 92 and 70% for CYP2N1 and CYP2N2, respectively). CYP2N1 and CYP2N2 also catalyze the formation of a variety of hydroxyeicosatetraenoic acids. Both P450s have benzphetamine *N*-demethylase activities but show minimal alkoxyresorufin *O*-dealkylase activities. To investigate factors affecting CYP2N expression *in vivo*, CYP2N transcripts were examined following starvation and/or treatment with 12-*O*-tetradecanoyl phorbol-13-acetate. Intestinal CYP2N1 mRNAs decrease in starved and/or phorbol ester-treated fish, whereas intestinal CYP2N2 transcripts decrease only following phorbol ester treatment. Interestingly, cardiac CYP2N2 expression decreases following phorbol ester treatment but increases following starvation. These results demonstrate that members of this novel P450 subfamily encode early vertebrate forms of arachidonic acid catalysts that are widely expressed and are regulated by

environmental factors. Given the wealth of information on the functional role of P450-derived arachidonate metabolites in mammals, we postulate that CYP2N1 and CYP2N2 products have similar biological functions in early vertebrates. The identity of the mammalian ortholog(s) of the CYP2Ns remains unknown.

The cytochromes P450¹ (EC 1.14.14.1) comprise a large gene superfamily that encodes over 500 distinct heme-thiolate proteins that act as the terminal oxidases in the mixed function oxidase system (1, 2). Ubiquitous in living organisms, these proteins have been identified in bacteria, yeast, plants, and animals (1). The P450 enzymes catalyze the metabolism of a wide variety of xenobiotics and are responsible for the bioactivation of numerous endogenous compounds including steroids, bile acids, and fatty acids (1, 2). The 14 known mammalian P450 gene families are divided into 29 subfamilies, the largest of which is the CYP2 family that contains eight mammalian subfamilies (1). All vertebrate P450s require NADPH and NADPH-P450 oxidoreductase, which transports electrons from NADPH to the heme-thiolate protein.

Since it was suggested in 1961 (3) that ω -oxidation of fatty acids might occur via the mixed function oxidase system, growing attention has focused on the role of P450s in the metabolism of fatty acids. The observation that P450 inhibitors block arachidonic acid-induced platelet aggregation and the formation of aggregation factors from arachidonic acid by platelet microsomal enzymes (4) first suggested that P450-derived eicosanoids may have important biological functions. Subsequent studies have described the diversity of arachidonate metabolites formed by P450s (5–7) and the potential physiological importance of some of these metabolites (8–10). Products of P450-mediated metabolism of arachidonic acid include four regioisomeric epoxyeicosatrienoic acids (14,15-, 11,12-, 8,9-, and 5,6-EETs), six mid-chain *cis-trans*-conjugated dienols (5-, 8-, 9-, 11-, 12-, and 15-HETEs), and five ω -terminal hydroxyeicosatetraenoic acids (16-, 17-, 18-, 19- and 20-HETEs) (10). The EETs can be hydrated to their corresponding vicinal diols (DHETs) by epoxide hydrolases (10). In particular, the P450 epoxygenase reactions have been extensively investigated because the EETs are endogenous constituents of and have potent biological effects in numerous mammalian tissues (8–10).

Most studies that have identified P450s involved in fatty acid

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¹ The abbreviations used are: P450, cytochrome P450; CYPOR, NADPH-P450 oxidoreductase; DHET, dihydroxyeicosatrienoic acid; EET, epoxyeicosatrienoic acid; GC/MS, gas chromatography/mass spectrometry; HETE, hydroxyeicosatetraenoic acid; HPLC, high performance liquid chromatography; NADPH, nicotinamide adenine dinucleotide phosphate; PFB, pentafluorobenzyl; *Sf9*, *Spodoptera frugiperda*; TMS, trimethylsilyl; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; kb, kilobase pair.

metabolism have focused on mammalian systems, although some have identified arachidonic acid catalysts in birds (11). Importantly, few studies have addressed P450-dependent arachidonic acid metabolism or the identity of the specific P450 arachidonate catalysts in other vertebrate groups. Knowledge of P450 functions in early vertebrates may be useful in unraveling the endogenous roles of these genes in mammals since critical functions are likely to be maintained throughout evolution. In particular, comparisons between early vertebrate and mammalian systems are necessary to address questions of functional conservation and divergence. Herein, we report the cDNA cloning of two novel P450s from the teleost *Fundulus heteroclitus*.² A comparison of the deduced amino acid sequences encoded by these cDNAs with those of other P450s shows that the extent of similarity is limited; hence, these two enzymes represent a new P450 subfamily, the CYP2Ns. We also demonstrate that the recombinant CYP2N proteins are active arachidonic acid epoxygenases and hydroxylases, that CYP2N transcripts are abundantly expressed in hepatic and extrahepatic tissues including the heart, brain, and intestine, and that expression is regulated by environmental factors.

EXPERIMENTAL PROCEDURES

Materials—[1-¹⁴C]Arachidonic acid, [γ -³²P]ATP, and [α -³²P]dCTP were purchased from NEN Life Science Products. Triphenylphosphine, α -bromo-2,3,4,5,6-pentafluorotoluene, *N,N*-diisopropylethylamine, and diazald were purchased from Aldrich. All other chemicals and reagents were purchased from Sigma unless otherwise specified.

Library Screening—Oligo(dT)-primed Uni-ZAP cDNA libraries were constructed from *F. heteroclitus* liver and heart poly(A)⁺ mRNA using a Lambda ZAP-cDNA synthesis kit obtained from Stratagene (La Jolla, CA) according to the manufacturer's instructions. A *Fundulus* λ gt10 liver cDNA library was obtained from Dr. D. Crawford (University of Missouri, Kansas City, MO). Approximately 5×10^5 phage from the λ gt10 liver cDNA library were screened with two degenerate oligonucleotide probes end-labeled with [γ -³²P]ATP as described (12). Multiple CYP2 proteins from several mammalian species (including rat CYP2A1, CYP2B1, CYP2B2, CYP2D1, CYP2E1 and CYP2G1, mouse CYP2A5, human CYP2B6 and CYP2F1, rabbit CYP2B4, CYP2C3, and CYP2C4, dog CYP2C21, chicken CYP2H1 and CYP2H2), and also CYP101A1 from *Pseudomonas putida* (13–15) were aligned and used to design these probes. Regions corresponding to the locations of α -helices in CYP101A1, determined by x-ray crystallography (13), were chosen as the sites for the two degenerate oligonucleotides under the assumption that they encode portions of P450 proteins that tend to be more conserved than regions corresponding to the putative substrate recognition sites. The probe sequences were 5'-CTGCAGCTCGAGGAGCGVATY-CAGGASGARGC-3' and 5'-GGATCCTCTAGATSACBGCVTCNGTG-TAKGGCA-3', with restriction enzyme sites at the 5' ends. They correspond to amino acid residues 144–160 and 349–355 of rat CYP2A1, respectively. Where appropriate, codon usage tables for teleosts were used to eliminate degeneracies (16). Hybridizations were done in 6 \times SSPE, 0.05 \times Blotto, and 20% formamide at 42 °C overnight. Approximately 90 positive clones were identified, 35 of which were rescued into pBluescript SK(+) and the inserts cycle sequenced by the dideoxynucleotide chain termination method (17) using DNA polymerase (Epicentre Technologies) and infrared labeled primers (LI-COR, Inc.). Approximately 6×10^5 phage from each of the Uni-ZAP liver and heart cDNA libraries were screened with cDNA fragments isolated from the λ gt10 library. These fragments were random primer-labeled with [α -³²P]dCTP using Rediprime (Amersham Pharmacia Biotech). Hybridizations were done in 6 \times SSPE, 0.05 \times Blotto, and 50% (liver) or 20% (heart) formamide at 42 °C overnight. Positive clones were isolated from each library, rescued into pBluescript SK(+), and sequenced as above. Seven clones isolated from the liver Uni-ZAP library contained identical sequences that were most similar to CYP2 family P450s. One of these clones (clone LMO-64, 2.2 kb) was completely sequenced using oligonucleotide primers that spanned the entire length of the sense and antisense cDNA strands. Similarly, four clones isolated from the heart Uni-ZAP library contained identical sequences that shared some ho-

mology with CYP2 family P450s. One of these clones (clone HMO-11, 2.4 kb) was completely sequenced as described above.

Phylogenetic Analyses—Phylogenetic comparisons were done with a representative member of each CYP2 subfamily using the majority of the sequence (>94%) or using only the six putative substrate recognition sites. The topologies of the trees were constructed using the minimum evolution criterion or Neighbor-joining Algorithm (18). Bootstrap analysis was performed using the method of Felsenstein (19) to assess relative confidence in the topologies. Clustal alignments were done using GCG software (Genetics Computer Group, Inc., Madison, WI). Conserved sequences were analyzed by searching GenBankTM, EMBL, and SwissProt data bases.

Heterologous Expression of Recombinant CYP2N1 and CYP2N2—The proteins encoded by the LMO-64 (CYP2N1) and HMO-11 (CYP2N2) cDNAs were co-expressed with human CYPOR in *Sf9* insect cells using the pAcUW51-CYPOR shuttle vector (kindly provided by Dr. Cosette Serabjit-Singh, Glaxo Wellcome) (20) and the BaculoGold Baculovirus Expression System (PharMingen). The respective cDNAs were subcloned into the *Bam*HI site in the polylinker region of pAcUW51-CYPOR, and the identity and orientation of the resulting expression vectors (pAcUW51-CYPOR-CYP2N1 and pAcUW51-CYPOR-CYP2N2) were confirmed by sequence analysis and restriction enzyme digestion. In both constructs, the p10 promoter controlled expression of CYPOR and the polyhedrin promoter independently controlled expression of the CYP2Ns. Cultured *Sf9* insect cells were then co-transfected with each of the expression vectors and linearized wild-type BaculoGold viral DNA in a CaCl₂ solution as described (20, 21). Recombinant viruses were purified, and the presence of the CYP2N1 and CYP2N2 cDNAs was corroborated by polymerase chain reaction analysis. Cultured *Sf9* cells, grown in spinner flasks at a density of $1.5\text{--}2.0 \times 10^6$ cells/ml, were infected with high titer viral stocks in the presence of 5 μ M hemin or 100 μ M δ -aminolevulinic acid hydrochloride. Cells co-expressing recombinant CYP2N1 and CYPOR or recombinant CYP2N2 and CYPOR were harvested 72 h after infection, washed twice with phosphate-buffered saline, and used to prepare microsomal fractions by differential centrifugation at 4 °C as described previously (22). The P450 content of the microsomes was determined spectrally according to the method of Omura and Sato (23) with a Shimadzu UV-3000 dual wavelength/double-beam spectrophotometer (Shimadzu Scientific, Columbia, MD).

Arachidonic Acid Metabolism and Product Characterization—Microsomes were resuspended to a final reaction volume (0.2–0.5 ml) in 0.05 M Tris-Cl buffer (pH 7.5) containing 0.15 M KCl, 0.01 M MgCl₂, 8 mM sodium isocitrate, and 0.5 IU of isocitrate dehydrogenase/ml. Reactions were equilibrated at 37 °C with constant mixing for 2 min before the addition of [1-¹⁴C]arachidonic acid (25–55 μ Ci/ μ mol, 50–100 μ M final concentration). Reactions were initiated by the addition of NADPH (1 mM final concentration) and continued at 37 °C with constant mixing. After 30–60 min, lipid-soluble products were extracted into ethyl ether, dried under a nitrogen stream, resolved by reverse-phase HPLC, and quantified by on-line liquid scintillation using a Radiomatic Flo-One β -detector (Radiomatic Instruments, Tampa, FL) as described (24). Products were identified by comparing their reverse- and normal-phase HPLC properties with those of authentic standards and by GC/MS (24–26). For rate determinations, the reactions were terminated after only 5–10 min to ensure that the quantitative assessment of the rates of product formation accurately reflect initial rates. For chiral analysis, the EETs were collected batchwise from the HPLC eluent, derivatized to the corresponding EET-PFB or EET-methyl esters, purified by normal-phase HPLC, resolved into the corresponding antipodes by chiral-phase HPLC, and quantified by liquid scintillation as described previously (24, 27). Uninfected *Sf9* insect cell microsomes, insect cell microsomes expressing CYPOR but not P450, and reactions without the addition of NADPH were used as negative controls.

Metabolism of Xenobiotics—Benzphetamine *N*-demethylation activities of the recombinant P450s were assessed using the same microsomal preparations used for arachidonic acid assays under identical reaction conditions but employing benzphetamine (2 mM, final concentration) as the substrate. The reaction product (formaldehyde) was quantified according to the method of Nash (28). Similarly, the activities of recombinant CYP2N1 and CYP2N2 toward ethoxyresorufin, benzyl-oxyresorufin, methoxyresorufin, and pentoxyresorufin were assessed by quantifying the reaction product, resorufin, using a cytofluor as described previously (29). For the benzphetamine assays, incubations containing microsomes but without NADPH gave background formaldehyde production rates that were always less than 5% of formaldehyde production rates during incubations with NADPH. The low background values were subtracted from the total to obtain the reported rates. Other negative controls included incubations with uninfected *Sf9* cell

² The new sequences reported in this paper have been submitted to the Committee on Standardized Cytochrome P450 Nomenclature and have been designated CYP2N1 and CYP2N2.

microsomes (*i.e.* microsomes that did not contain P450) and incubations with boiled microsomes in which the P450 was rendered inactive. In both cases, formaldehyde production rates were low to undetectable.

RNA Analyses—Total RNA was prepared from *Fundulus* testes, ovaries, eye, heart, gill, kidney, muscle, brain, spleen, intestine (mid-gut), and liver using RNA Stat-60 (Tel-Test B, Inc.) according to the manufacturer's directions. All tissues except liver were pooled from up to six fish. 10 μ g of total RNA was denatured and electrophoresed on 1.0% agarose gels containing 2.2 M formaldehyde and transferred to nylon membranes by downward alkaline capillary as described (30). The blots were hybridized with either the CYP2N1 or the CYP2N2 cDNAs labeled with [α - 32 P]dCTP by nick translation. Hybridizations were at 42 °C in 50% formamide, 6 \times SSPE, 1.0% (w/v) SDS, and 100 μ g/ml heat-denatured calf thymus DNA. Loading and transfer of RNA were monitored by ethidium bromide staining of the gels and filters. Correlation between the stain on the filters and the amount of RNA was ascertained by probing the filters with labeled ribosomal RNA.

Experimental Treatments Including Starvation and Phorbol Ester—The dose and timing of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was based on published work in mice (31, 32) and by extrapolation from *in vitro* studies on effects of TPA on P450 gene expression in cultured fish hepatocytes (33). We are unaware of prior *in vivo* studies involving TPA administration to fish. This dose is well below the predicted LD₅₀ for TPA (~400–500 ng/g based on extrapolation from studies in mammals) and was not toxic to the fish. The fasting regimen was also selected as one that would likely elicit changes in P450 gene expression. There are marked differences between mammals and fish in normal metabolic responses to fasting (34). Fasting for periods of 2–10 weeks or longer has often been employed in other studies of carbohydrate and lipid metabolism in fish (35, 36). Moreover, at lower temperatures, the effects of fasting generally take longer to appear.

Forty-eight female fish that had been held in clean water for greater than six months were acclimated from 20 to 14 °C for 1 week, as fish maintained at lower temperatures tolerate fasting well. Twenty-four of the fish were fed excess TetraMin (Tetra Sales, Blacksburg, VA) throughout the experiment; the rest were held without feeding. After ten days of feeding or fasting, six fish from each group were sacrificed, and intestinal (mid-gut), heart, and brain tissues were frozen in liquid nitrogen for RNA analyses. After 17 days, six fish from each group were dosed intraperitoneally with 0.02 μ g TPA/g in 80% normal saline, 19.8% acetone, 0.2% ethanol (vehicle), vehicle alone, or nothing. Fish were sacrificed 3 days later, and tissues were frozen in liquid nitrogen for RNA analyses. All tissues were analyzed as pooled specimens. The protocol for these studies was approved by the WHOI Institutional Animal Care and Use Committee.

Synthetic Procedures—The [1- 14 C]EET internal standards were synthesized from [1- 14 C]arachidonic acid (55–57 μ Ci/ μ mol) by nonselective epoxidation as described (37). Racemic and enantiomerically pure EETs were prepared by total chemical synthesis according to published procedures (38–41). Methylations were performed using an ethereal solution of diazomethane (42). PFB esters were formed by reaction with α -bromo-2,3,4,5,6-pentafluorotoluene as described (43). TMS ethers were prepared using 25% (v/v) bis(trimethylsilyl)trifluoroacetamide in anhydrous pyridine (44). HETE standards were purchased from Cayman Chemical Co. ω -Terminal alcohols of arachidonic acid were synthesized as described (45).

RESULTS

Molecular Cloning of CYP2N1 and CYP2N2 cDNAs—Screening of *Fundulus* liver and heart cDNA libraries with degenerate oligonucleotide probes to conserved α -helical regions in mammalian CYP2 family P450s resulted in the identification of two cDNAs that are 69% identical and represent a novel P450 subfamily. Clone LMO-64 is 2151 nucleotides long, contains an open reading frame between nucleotides 70 and 1563 flanked by initiation (ATG) and termination (TGA) codons, and contains a 585-nucleotide 3'-untranslated region with a polyadenylation tail (Fig. 1). The cDNA encodes a 497-amino acid protein that has a derived molecular mass of 56,429 Da and contains the putative heme-binding peptide (FSAGKRVCLGEGLA) with the underlined conserved residues and the invariant cysteine at position 444 (Fig. 2). Clone HMO-11 is 2375 nucleotides long, contains an open reading frame between nucleotides 140 and 1633 flanked by initiation (ATG) and termination (TGA) codons, and contains a 739-

nucleotide 3'-untranslated region with a polyadenylation tail (Fig. 1). The protein encoded by HMO-11 contains 497 amino acids, has a derived molecular mass of 56,663 Da, and contains the putative heme-binding peptide (FSAGKRVCLGEGLA) (Fig. 2). Clone HMO-11 also contains a second in-frame initiation codon at position 68 that would be predicted to give rise to a 521-amino acid, 59,131-Da protein; however, this start site was deemed less favorable due to the absence of a 3' purine nucleotide.

A comparison of the nucleotide sequences of clones LMO-64 and HMO-11 with those of other P450s indicates that the extent of similarity is limited (*i.e.* less than 45% nucleic acid identity with members of the CYP1, CYP3, and CYP4 families and 45–55% identity with various members of the CYP2 family). Furthermore, the differences are randomly distributed along the entire length of the cDNAs. Comparison of the deduced amino acid sequences encoded by LMO-64 and HMO-11 with those of other P450s demonstrates the following: (a) 19–30% sequence identity with CYP1, CYP3, and CYP4 family P450s; (b) 37–43% sequence identity with CYP2A, CYP2B, CYP2C, CYP2D, CYP2E, CYP2F, CYP2G, and CYP2H subfamily P450s; and (c) 46–49% identity with members of the CYP2J subfamily. These two new P450s have been designated CYP2N1 (clone LMO-64) and CYP2N2 (clone HMO-11) by the P450 Nomenclature Committee. The two CYP2N amino acid sequences are 76% identical and the primary amino acid sequence alignment shown in Fig. 2 demonstrates that most of the differences represent conservative changes (*i.e.* replacement with residues with overall similar chemical properties). In addition to the putative heme-binding peptide, both CYP2N proteins contain structural features associated with other CYP2 family P450s including an N-terminal hydrophobic peptide and a proline cluster between residues 40 and 51. Comparative analyses of the amino acid sequences of other CYP2 family proteins with three bacterial P450s whose crystal structures have been determined has allowed the tentative identification of the locations of six putative substrate recognition sites (SRSs) within the CYP2 family sequences (13, 46). We have aligned the CYP2N sequences with other CYP2 family members to identify tentatively these components (Fig. 2). Gotoh (13) predicted that amino acid sequences of P450 SRSs should be more variable than the rest of the molecule as a consequence of adaptive evolution. Analysis of the putative CYP2N SRSs reveals that SRS-2 and SRS-3 contain sequences that are more variable (38–63% identical), whereas SRS-4 and SRS-6 contain sequences that are more conserved (79–88% identical) among the CYP2N subfamily members.

Phylogenetic comparisons were done with a representative member of each CYP2 subfamily using the majority of the sequence or using only the six putative substrate recognition sites. The topologies of the trees constructed using the minimum evolution criterion (18) showed that the CYP2N proteins are related most closely to the mammalian CYP2Js. Bootstrap analysis (19) to assess relative confidence in the topologies showed that the CYP2N/CYP2J branch formed >94% of the time using either the majority of the sequences or using only the putative substrate recognition sites. Clustal alignment of seven known CYP2Js with CYP2N1 and CYP2N2 identified a number of sequences that are highly conserved in both subfamilies; however, none of these sequences appear to be CYP2J/CYP2N signature sequences in that they are also present, to varying degrees, in other CYP2 family enzymes.

Heterologous Expression of Recombinant CYP2N1 and CYP2N2 in Insect Cells—The recombinant CYP2N1 and CYP2N2 proteins were co-expressed with recombinant CYPOR in *Sf9* insect cells using the baculovirus expression system.



FIG. 1. Nucleic acid sequence alignment of the CYP2N1 and CYP2N2 cDNAs. The initiation and termination codons are shown in bold.

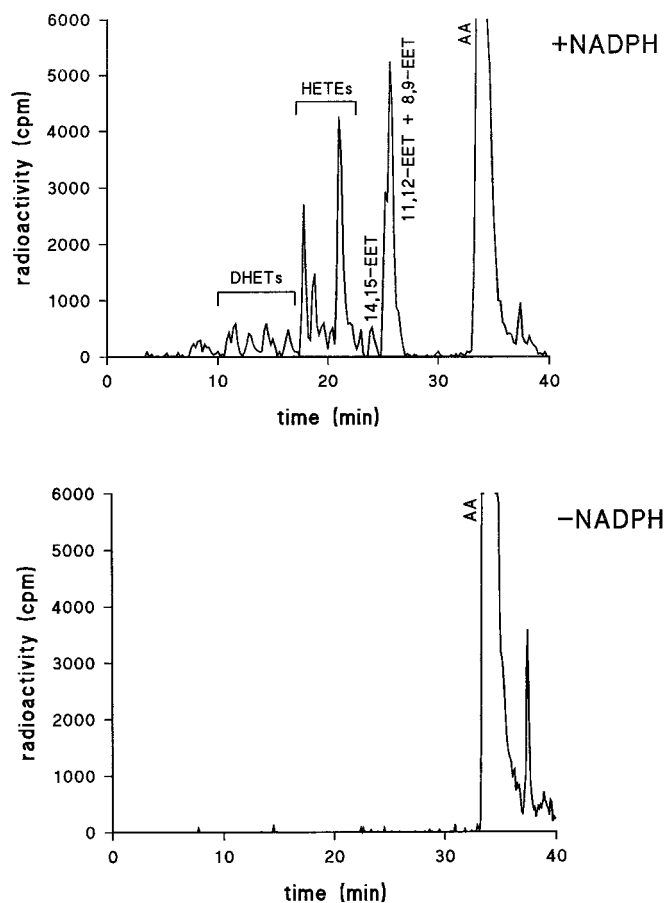


FIG. 3. Reverse-phase HPLC of the organic soluble metabolites generated during incubation of recombinant CYP2N1 with arachidonic acid. Microsomal fractions prepared from CYP2N1/CYPOR-transfected *S/9* insect cells were incubated at 37 °C with [1-¹⁴C] arachidonic acid (50–100 μ M, final concentration) with/without NADPH (1 mM, final concentration) and an NADPH-regenerating system. After 30 min, the reaction products were extracted and resolved by reverse-phase HPLC as described. Peak identifications were made by comparisons of the HPLC properties of individual peaks with those of authentic standards using both reverse-phase and normal-phase HPLC and by GC/MS. Ordinate, radioactivity in cpm; abscissa, time in min. Top panel, incubation with NADPH; bottom panel, incubation without NADPH.

recombinant CYP2Ns suggesting that these P450s are among the predominant enzymes responsible for arachidonic acid metabolism in the liver. Regiochemical analysis of the HETEs reveals a preference for hydroxylation at the 16- and 19-positions (46% and 8% of the total HETEs, respectively) (Table III).

Benzphetamine *N*-Demethylase and Alkoxyresorufin *O*-Dealkylase Activities—Because most CYP2 subfamily enzymes metabolize both endogenous and xenobiotic substrates, we assessed the metabolism of selected aromatic amines and resorufins by the CYP2Ns and by *Fundulus* liver microsomes. The baculovirus-expressed CYP2N1 and CYP2N2 have moderate benzphetamine *N*-demethylase activities (catalytic turnovers: 1.00 and 0.84 nmol of product/nmol of P450/min at 30 °C, respectively) (Table IV). *Fundulus* liver microsomes also catalyze the *N*-demethylation of benzphetamine (catalytic turnover: 0.47 nmol of product/nmol of P450/min at 30 °C) (Table IV). Compared with *Fundulus* liver microsomes, recombinant CYPN1 and CYP2N2 show minimal ethoxy- and methoxy-*O*-dealkylase activities (Table IV). The rates of benzyloxy- and pentoxyresorufin-*O*-dealkylase activities catalyzed by the recombinant CYP2Ns and by liver microsomes are low (Table IV).

Tissue Distribution of CYP2N mRNAs—To determine the tissue distribution of the CYP2N transcripts, total RNA iso-

TABLE I

Regio- and stereochemical composition of CYP2N1-derived EETs and HETEs

The activity of recombinant CYP2N1 was measured in the presence of NADPH and an NADPH-regenerating system as described under "Experimental Procedures." The EET products were extracted, resolved into individual regioisomers by reverse- and normal-phase HPLC, derivatized to corresponding EET-PFB or EET-methyl esters, purified by normal-phase HPLC, and resolved into the corresponding antipodes by chiral-phase HPLC. The HETE products were extracted and resolved into individual regioisomers by reverse- and normal-phase HPLC. Values shown are averages of at least three different experiments with S.E. <5%. ND, not detected.

Regioisomer	Distribution	Enantioselectivity	
		<i>R,S</i>	<i>S,R</i>
% total EETs			
14,15-EET	12	49	51
11,12-EET	29	92	8
8,9-EET	59	91	9
5,6-EET	ND		
Regioisomer	Distribution		
% total HETEs			
5-HETE	37		
16-HETE	23		
12-HETE	16		
9-HETE	6		
11-HETE	4		
Other HETEs	14		

lated from various tissues was blot-hybridized under high stringency conditions with the full-length CYP2N1 and CYP2N2 cDNAs. As shown in Figs. 6 and 7, the CYP2N1 probe hybridizes with three distinct bands (3.0, 2.4, and 1.8 kb) in *Fundulus* liver and intestine (mid-gut) but with a single 2.4-kb band in heart and brain RNA. The identity of these transcripts remains unknown, but they may represent alternate splice variants of CYP2N1 or mRNAs of other genes that share nucleic acid homology with CYP2N1. CYP2N1 transcripts are undetectable in other tissues including testes, ovary, eye, gill, kidney, muscle, and spleen. There are no apparent gender differences in the abundance of CYP2N1 transcripts; however, there is considerable inter-animal variability in CYP2N1 expression that is most pronounced in the liver (Fig. 6). In contrast, the CYP2N2 probe detects a single 2.4-kb transcript, most abundantly in *Fundulus* heart and brain, at lower levels in liver and intestine, and at barely detectable levels in eye, gill, and kidney RNA (Figs. 6 and 7). There is little inter-animal variability in the expression of CYP2N2 mRNAs which are present at comparable levels in male and female fish (Fig. 6).

Regulation of CYP2N1 and CYP2N2 by Starvation and Treatment with Phorbol Ester—To investigate possible factors affecting CYP2N expression *in vivo*, CYP2N1 and CYP2N2 transcript levels were examined in extrahepatic tissues following starvation and/or treatment with TPA, a diacylglycerol mimic that has been shown to increase intracellular free arachidonic acid levels (47, 48). Intestinal CYP2N1 mRNAs are decreased in starved, TPA-treated, and starved/TPA-treated fish (Fig. 7). The effects of starvation are manifest only after 20 days (*i.e.* 10 days of starvation results in the same or slightly higher CYP2N1 mRNA levels as compared with fed fish). Intestinal CYP2N2 transcripts are reduced only following phorbol ester treatment (*i.e.* starvation itself has no consistent or significant effects on CYP2N2 mRNA levels) (Fig. 7). Interestingly, cardiac CYP2N2 levels are decreased following TPA treatment but are increased significantly following starvation (Fig. 7). In contrast, brain CYP2N2 expression remains relatively constant following these manipulations (Fig. 7). The changes in CYP2N1 and CYP2N2 mRNAs are not due to dif-

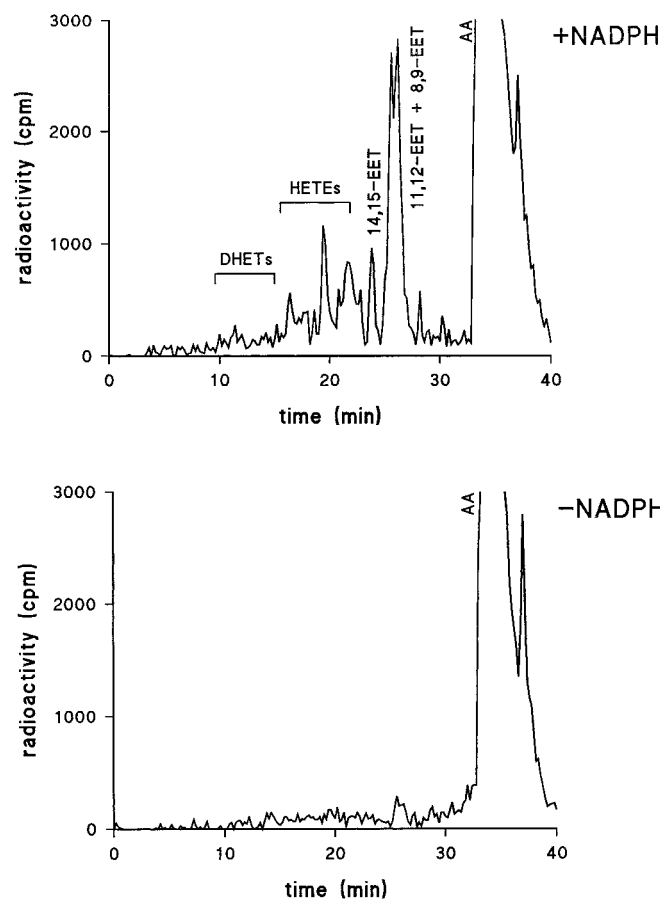


FIG. 4. Reverse-phase HPLC chromatogram of the organic soluble metabolites generated during incubation of recombinant CYP2N2 with arachidonic acid. Microsomal fractions prepared from CYP2N2/CYPOR-transfected *S/9* insect cells were incubated at 37 °C with [1-¹⁴C]arachidonic acid as described in Fig. 1. After 30 min, the reaction products were extracted, resolved by HPLC, and identified as described in Fig. 1. Ordinate, radioactivity in cpm; abscissa, time in min. Top panel, incubation with NADPH; bottom panel, incubation without NADPH.

TABLE II
Regio- and stereochemical composition of CYP2N2-derived EETs and HETEs

The activity of recombinant CYP2N2 was measured in the presence of NADPH and an NADPH-regenerating system as described under "Experimental Procedures." The EET and HETE products were resolved into individual regio- and stereoisomers as described in Table I. Values shown are averages of at least three different experiments with S.E. <5%. ND, not detected.

Regioisomer	Distribution	Enantioselectivity	
		<i>R,S</i>	<i>S,R</i>
% total EETs			
14,15-EET	15	32	68
11,12-EET	19	70	30
8,9-EET	67	90	10
5,6-EET	ND		
Regioisomer	Distribution		
% total HETEs			
9-HETE	30		
20-HETE	12		
Other HETEs	58		

ferences in the amount of RNA applied to each lane as assessed by ethidium bromide staining of gels and membranes. Taken together, these data demonstrate that regulation of CYP2N expression occurs in an isoform- and tissue-specific fashion.

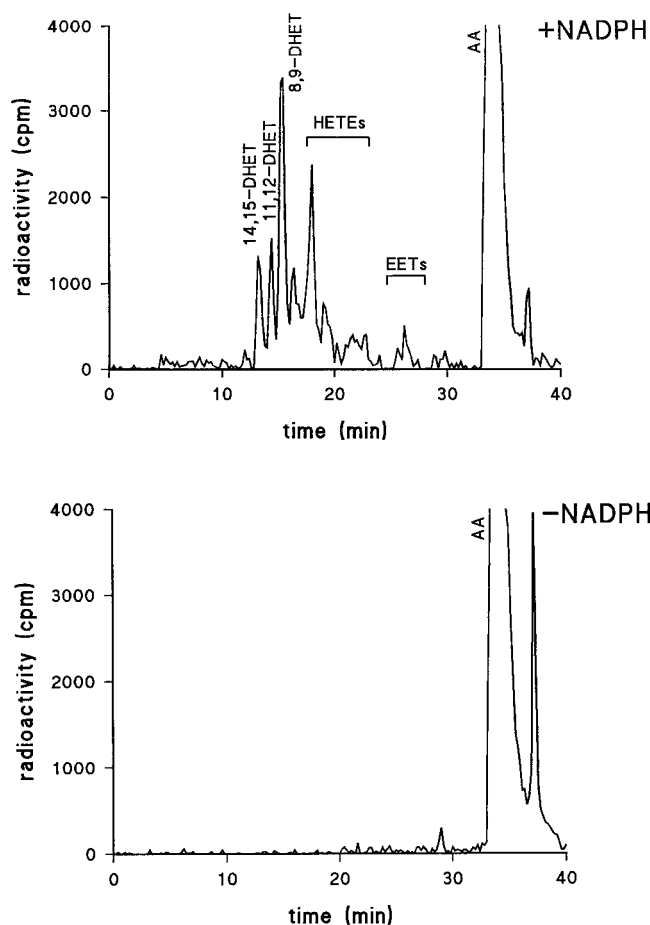


FIG. 5. Reverse-phase HPLC of the organic soluble metabolites generated during incubation of killifish liver microsomes with arachidonic acid. Killifish liver microsomal fractions were incubated at 37 °C with [1-¹⁴C]arachidonic acid as described in Fig. 1. After 30 min, the reaction products were extracted, resolved by HPLC, and identified as described in Fig. 1. Ordinate, radioactivity in cpm; abscissa, time in min. Top panel, incubation with NADPH; bottom panel, incubation without NADPH.

TABLE III
Regiochemical composition of DHETs and HETEs produced by killifish microsomes

The activity of killifish liver microsomes was measured in the presence of NADPH and an NADPH-regenerating system as described under "Experimental Procedures." The DHET and HETE products were extracted and resolved into individual regioisomers by reverse- and normal-phase HPLC. Values shown are averages of at least three different experiments with S.E. <5%. ND, not detected.

Regioisomer	Distribution
% total DHETs	
14,15-DHET	15
11,12-DHET	24
8,9-DHET	61
5,6-DHET	ND
Regioisomer	Distribution
% total HETEs	
16-HETE	46
19-HETE	8
Other HETEs	46

DISCUSSION

Historically, there has been an intense interest in the role of P450 enzymes in metabolic oxidation, peroxidation, and reduction of xenobiotics including drugs, industrial chemicals, environmental pollutants, and carcinogens (1, 2). More recently,

there has been a growing appreciation for the role of these ubiquitous heme-thiolate proteins in the oxidation of lipophilic endobiotics such as arachidonic acid and in the biological significance of the resulting eicosanoid products (8–10). The last decade has given rise to an explosion in the number of P450 sequences that have been reported in bacteria, yeast, plants, and animals (1); however, most of the animal studies have focused on mammalian systems, and few investigations have addressed the identity and/or function of specific P450 catalysts in other vertebrate groups. The information gleaned from such studies might be very useful in unraveling the true biological roles of these enzymes since critical endogenous functions are likely to be maintained throughout evolution. Herein we report the cDNA cloning, heterologous expression, enzymatic characterization, tissue distribution, and regulatory

characteristics of two new P450s from the teleost *F. heteroclitus*. Together, these two P450s represent a novel P450 subfamily, the CYP2Ns.

A fundamental question that remains is whether these new hemoproteins are unique to early vertebrates, and if not, what is the identity of their mammalian counterparts? In some P450 gene families, such as CYP26 which is involved in retinoic acid metabolism (49, 50), diversity is low and orthologous genes in early vertebrates and mammals appear to have similar endogenous functions. The P450s of the CYP2 family present a vastly more complex picture, in part because of their multiplicity and broad substrate specificity. Based upon the deduced amino acid sequences, CYP2N1 and CYP2N2 appear to be most closely related to the mammalian CYP2Js (21, 51). Interestingly, like the CYP2Ns, the CYP2J proteins are abundant in both hepatic and extrahepatic tissues including the heart, intestine, and brain and are active in the metabolism of arachidonic acid to EETs and HETEs (21, 51). Further work will be necessary to determine if the CYP2Js are the mammalian orthologues of the CYP2Ns or whether other, as yet unrecognized, P450s serve this function in mammals.

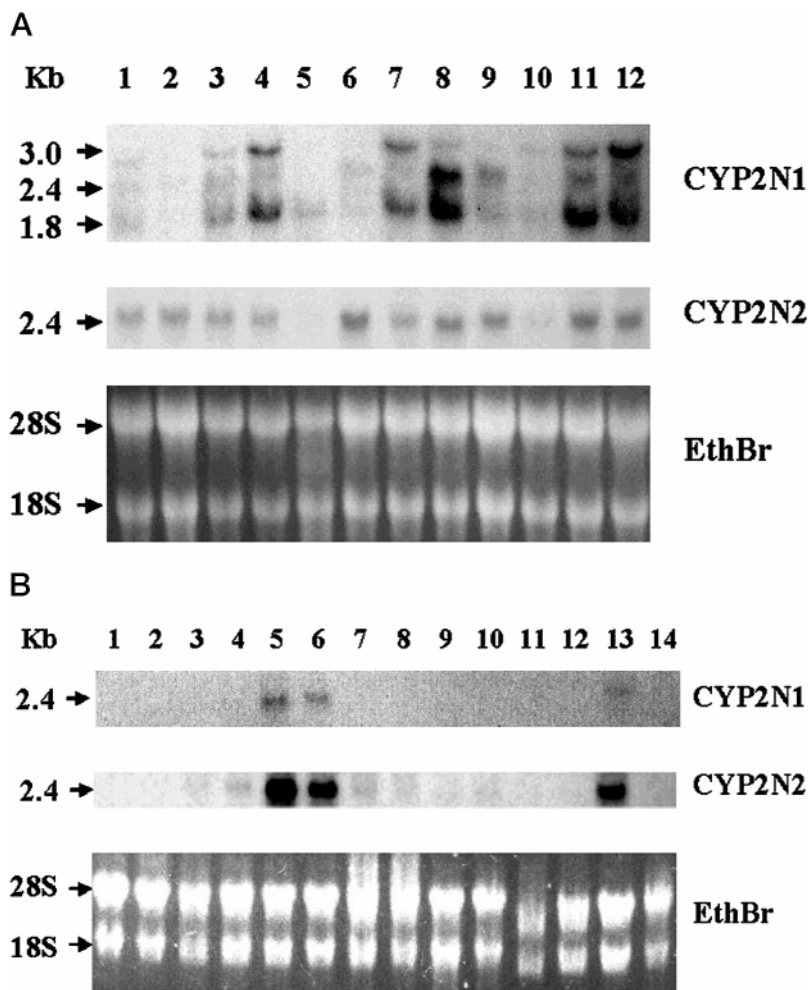
The studies reported herein and those of Oleksiak *et al.*³ provide the first evidence for the existence of multiple, catalytically distinct arachidonic acid epoxygenases and hydroxylases in early vertebrates. Our results show that although CYP2N1 and CYP2N2 exhibit catalytic similarities, they are also enzymologically distinct heme-thiolate proteins. Thus, both

TABLE IV
Catalytic turnover of Recombinant CYP2N1, CYP2N2, and killifish liver microsomes with various substrates

The activities of recombinant CYP2N1, CYP2N2, and killifish liver microsomes were measured in the presence of NADPH and an NADPH-regenerating system as described under "Experimental Procedures." Values shown are averages of at least three different experiments with S.E. <15%. AA, arachidonic acid; BENZ, benzphetamine; ER, ethoxyresorufin; MR, methoxyresorufin; BR, benzyloxyresorufin; PR, pentoxyresorufin; ND, not detected.

	AA	BENZ	ER	MR	BR	PR
	<i>pmol product/nmol P450/min</i>					
CYP2N1	1100	1000	3	2	8	2
CYP2N2	210	840	1	1	4	ND
Liver microsomes	210	470	1463	538	24	9

FIG. 6. Tissue distribution of CYP2N transcripts by nucleic acid blot hybridization analysis. A, total RNA (10 μ g) prepared from male (lanes 1–6) and female (lanes 7–12) *Fundulus* livers was denatured, electrophoresed, transferred to nylon membranes, and hybridized with the radiolabeled CYP2N1 or CYP2N2 cDNA probes as described under "Experimental Procedures." Transcript levels were determined by autoradiography after a 72-h exposure time. Arrows show sizes of bands in kilobases. Ethidium bromide (*EthBr*) staining of the membrane was used to assess quality and relative loading of RNA. B, total RNA (10 μ g) prepared from *Fundulus* male and female extrahepatic tissues was analyzed as described above for hepatic tissues. Lane 1, testes; lane 2, ovaries; lane 3, male eye; lane 4, female eye; lane 5, male heart; lane 6, female heart; lane 7, male gill; lane 8, female gill; lane 9, male kidney; lane 10, female kidney; lane 11, male muscle; lane 12, female muscle; lane 13, female brain; lane 14, female spleen.



³ M. F. Oleksiak, S. Wu, C. Parker, D. C. Zeldin, and J. J. Stegeman, submitted for publication.

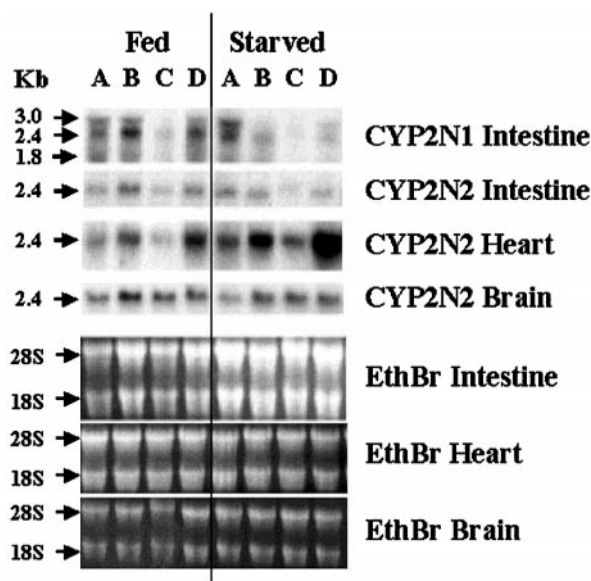


FIG. 7. Effect of TPA and/or starvation on CYP2N expression *in vivo*. Total RNA (10 μ g) from *Fundulus* intestine (mid-gut), heart, and brain was denatured, transferred to nylon membranes, and hybridized with the radiolabeled CYP2N1 or CYP2N2 cDNA probes as described under "Experimental Procedures." Top panels, autoradiograph of blots after a 72-h exposure time. Bottom panels, ethidium bromide-stained filters following transfer. Arrows show sizes of bands in kilobases. A, fish were either fed or starved for 10 days. B, fish were either fed or starved for 20 days. C, fish were either fed or starved for 20 days and treated with TPA. D, fish were either fed or starved for 20 days and treated with vehicle.

CYP2N1 and CYP2N2 are arachidonic acid epoxygenases and favor epoxidation at the 8,9- and 11,12-olefins. Furthermore, both enzymes favor epoxidation at the *re,si* face of these olefins, preferentially producing (8*R*,9*S*)- and (11*R*,12*S*)-EET. In contrast, CYP2N1 produces 14,15-EET as a nearly racemic mixture, whereas CYP2N2 forms (14*S*,15*R*)-EET with 68% optical purity. CYP2N1 and CYP2N2 also differ in the regiochemistry of HETE formation. Importantly, the regio- and stereochemical composition of CYP2N1 and CYP2N2 products are different from that of other P450 enzymes known to metabolize arachidonic acid including members of the CYP1A, CYP2B, CYP2C, CYP2E, CYP2J, and CYP4A subfamilies (21, 22, 52–54).

CYP2N1 and CYP2N2 metabolized arachidonic acid with catalytic turnovers of 1.1 and 0.21 nmol of product/nmol of P450/min, respectively. Although these rates are somewhat lower than those of mammalian P450s involved in metabolism of xenobiotic substrates, they are comparable to those reported for mammalian P450s involved in arachidonic acid metabolism including CYP1A2, CYP2B1, CYP2B4, CYP2B6, CYP2C8, CYP2C9, CYP2E1, CYP2G1, CYP2J2, CYP2J3, and CYP4A1 (21, 22, 51, 53, 55–57). Importantly, the rates of arachidonic acid metabolism obtained in the current study for the recombinant CYP2Ns are comparable to that obtained for *Fundulus* liver microsomes and slightly higher than that obtained for recombinant CYP2P3, a recently described *Fundulus* P450 expressed in liver and intestine.³ The rates for benzphetamine *N*-demethylation by the recombinant CYP2Ns are lower than those reported for many mammalian enzymes that metabolize this compound at significant rates above other enzymes but comparable to those reported for CYP2J3 and CYP2P3 (58).³

The regiochemistry of CYP2N1- and CYP2N2-derived EETs is similar to that of *Fundulus* liver microsomes. These similarities in EET regiochemical distribution, in light of the abundance of CYP2N transcripts in *Fundulus* liver, suggest that the CYP2N proteins may contribute significantly to arachidonic

acid metabolism in this tissue. However, other P450 isoforms are likely involved in arachidonate metabolism in teleost liver. For example, Zacharaewki *et al.* (59) has shown that microsomes prepared from livers of β -naphthoflavone-treated *Raja erinacea* (skate) metabolize arachidonic acid. Our recent studies with *Stenotomus chrysops* (scup) liver microsomes showed that these teleosts biosynthesize EETs and HETEs, and antibody inhibition experiments demonstrated that CYP1A- and CYP2B-like proteins were responsible for up to 70% of total microsomal arachidonic acid metabolism in that species (60). Presumably, homologues of both CYP1A- and CYP2B-like proteins metabolize arachidonic acid in *Fundulus*; however, the relative contribution of these and/or other P450s to hepatic arachidonic acid metabolism is not clear at the present time.

CYP2N1 mRNAs are expressed predominantly in liver and intestine, whereas CYP2N2 transcripts are particularly abundant in heart and brain. The abundance of CYP2Ns in these tissues together with their documented ability to catalyze arachidonic acid metabolism suggest that these P450s may have specialized endogenous functions, in addition to their potential involvement in metabolism of xenobiotic substrates. A number of studies indicate that P450-derived eicosanoids are endogenous constituents of mammalian hepatic and extrahepatic tissues (21, 22, 42, 43, 60) and possess potent biological activities in those tissues (8–10, 21, 61–70). For example, in the liver, the EETs activate phosphorylase α , affect Ca^{2+} flux, and may be involved in vasopressin-stimulated glycogenolysis (61, 62). In the heart, the EETs regulate coronary artery vascular tone, modulate Ca^{2+} transport, and affect heart contractile function following global cardiac ischemia (21, 63, 64). In the intestine, the EETs have been shown to influence microvascular tone (65). In the brain, the EETs affect cerebral vascular tone, activate Ca^{2+} -activated K^{+} channels, and modulate the secretion of neuropeptides (66–68). The EETs also have been shown to affect general physiological processes such as cellular proliferation and tyrosine kinase activity (69, 70). To our knowledge, no studies have evaluated the physiological functions of P450-derived eicosanoids in early vertebrates. However, we postulate that CYP2N eicosanoid products are involved in mediating these and other critical endogenous functions in early vertebrates as they are in mammals.

Fundulus are mainly carnivorous but encounter natural plant products (such as terpenes, phenols and phorbol esters) in their diet (71, 72). In order to examine the role of environmental factors in the regulation of CYP2N expression *in vivo*, we examined CYP2N1 and CYP2N2 mRNA levels in fed, starved, and/or TPA-treated fish. TPA alters the activity of protein kinase C and phospholipase A_2 , thus affecting the availability of free arachidonic acid for metabolism by P450 and other oxygenases (47, 48). We found that intestinal CYP2N1 mRNAs decreased in starved, TPA-treated, and starved/TPA-treated fish, whereas intestinal CYP2N2 transcripts only decreased following TPA treatment. In contrast, cardiac CYP2N2 levels decreased following TPA treatment but increased markedly following starvation, whereas brain CYP2N2 mRNAs remained relatively constant regardless of the treatment. Thus, regulation of CYP2N expression occurred in an isoform- and a tissue-specific fashion. Phorbol esters have been shown to affect function of a variety of cell surface receptors including those for insulin, epidermal growth factor, transferrin, somatomedin C, and catecholamines and to affect multiple intracellular signaling pathways (73). Similarly, starvation can induce a variety of metabolic perturbations including changes in insulin, glucagon, and thyroid hormone levels and cause alterations in protein kinase activity (74–76).

In summary, we have identified two new heme-thiolate pro-

teins that, together, represent a novel subfamily of P450s, the CYP2Ns. Both CYP2N1 and CYP2N2 are catalytically distinct enzymes that are active in the metabolism of arachidonic acid and xenochemicals. Their transcripts are abundant in liver, intestine, heart, and brain and are regulated by starvation and phorbol ester treatments in an isoform- and tissue-specific fashion. We conclude that members of this novel P450 subfamily encode early vertebrate forms of arachidonic acid catalysts that are widely expressed in hepatic and extrahepatic tissues and regulated by environmental factors. Given the wealth of information on the functional role of P450-derived arachidonate metabolites in mammals, we postulate that CYP2N1 and CYP2N2 products have similar biological functions in early vertebrates. Furthermore, studies of P450 functions in early vertebrates may be useful in unraveling the endogenous functions of the mammalian orthologous genes.

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