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Cloning of CYP4F7, a Kidney-Specific P450 in the Sea Bass *Dicentrarchus labrax*

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A cDNA sequence coding for a cytochrome P450 of the CYP4F subfamily was isolated from total RNA of sea bass kidney by rapid amplification of cDNA ends. The full length sequence coded for a protein of 526 amino acids. The amino acid sequence shared 39% to 56% residue identities with the mammalian CYP4F sequences, and thus was named CYP4F7 (accession number AF123541). RNA blot analysis using CYP4F7 cDNA as a probe indicated that the corresponding mRNA was only detected in kidney. Expression in the kidney was constitutive, and no induction of this mRNA was detected in this or other tissues, with any of the inducers tested, including peroxisome proliferators.

Key Words: **CYP4 family; cytochrome P450; *Dicentrarchus labrax;* kidney; molecular cloning.**

Members of the cytochrome P450 family CYP4 are found in a wide variety of organisms and tissues. The role of mammalian CYP4 enzymes in ω- and (ω-1)-hydroxylation of fatty acids and prostaglandins is well established (1-2). Most of them are expressed preferentially in liver and kidney. Their expression is regulated both by endogenous substances, such as hormones (3, 4), and by some xenobiotics, the peroxisome proliferators in particular (5). The peroxisome proliferators include compounds with no obvious strutural similarity: hypolipidaemic drugs (such as clofibrate), industrial plasticizers (such as di-2-ethylhexylphthalate), herbicides (such as chlorophenoxyacetic acid), insecticides (such as dimethrin), antifungal agents (such as bifonazole), wood preservatives (such as chlorophenolates), and several other industrial chemicals (6).

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Abbreviations used: 2,4-D, 2,4-dichlorophenoxy acetic acid; CYP, cytochrome P450; DEHP, di(2-ethylhexyl) phthalate; DIG, digoxigenin; LTB4, leukotriene B4; RACE-PCR, rapid amplification of cDNA ends-polymerase chain reaction.

To our knowledge, members of the CYP4F subfamily have been identified only in rats and humans. Purification or expression of CYP4F2 (7) and CYP4F3 (8) in heterologous systems have demonstrated that LTB4 ω-hydroxylation was specifically catalysed by these enzymes. The metabolic role of the other CYP4F enzymes is still unknown. LTB4, which is derived from arachidonic acid, acts as a mediator of inflammation through the activation of polymorphonuclear leukocytes (9). The catabolism of LTB4 and thus its inactivation leads to stop the inflammation process. The ω-hydroxylation catalysed by CYP4F isoforms is the first step in the degradation of LTB4, which is further catabolized by β-oxidation in peroxisomes (10). As LTB4 degradation occurs primarily in neutrophils or in liver, LTB4 ω-hydroxylases have been isolated from these tissues. Thus, CYP4F2 is highly expressed in human liver (11), and CYP4F3 in neutrophils (8).

Several P450 enzymes have been described in fish (12, 13). P450 from families CYP1, 2, 3, 11, 17, and 19 were isolated. Most of them have been purified or cloned from the rainbow trout, *Oncorhynchus mykiss* (13). As CYP1A enzyme activities or mRNA concentrations are used as a biomarker to estimate xenobiotic exposure in aquatic environments, CYP1A isoforms have been cloned in numerous freshwater and marine fishes (7 various species). Cytochromes P450 from the family 4 were isolated from rainbow trout and sea bass (14, 15). Their physiological role is still unknown. The relative tissue distribution and regulation of the CYP4T2 isoform cloned from the sea bass, *Dicentrarchus labrax*, present original features as compared to mammals. This gene is predominantly expressed in kidney and small intestine, and is induced by DEHP and 2,4-D but not by clofibrate (15).

We report here the molecular cloning of an another CYP4 cDNA sequence from kidney of sea bass. Surprisingly the deduced amino acid sequence shared from 39% to 56% identity with the mammalian CYP4F sequences, and thus this gene was named CYP4F7. We

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have investigated the tissue localization of this gene and its induction features by various xenobiotics, including peroxisome proliferators.

MATERIAL AND METHODS

Chemicals. Clofibrate, DEHP, 2,4-D, benzo[a]pyrene, prochloraz, and all other chemicals were purchased from Sigma-Aldrich Chimie (France). Restriction enzymes were from Roche Diagnostics (Germany).

Animals. Sexually immature sea bass (*Dicentrarchus labrax*, 100-200 g) were obtained from the fish farm "Cannes Aquaculture" (France) and placed in aquaria containing 120 l of artificial sea water (28‰ salinity) at 18°C. Fish were acclimated for at least 2 weeks to reduce possible stress-related responses. Each group of 5 fishes was treated daily for 3 days by intraperitoneal injection of clofibrate (100 mg/kg body weight/day), DEHP (1.5 g/kg body weight/day), 2,4-D (20 mg/kg body weight/day), prochloraz (20 mg/kg body weight/day), benzo[a]pyrene (10 mg/kg body weight/day), or NaCl 9‰ (control).

Poly $(A)^+$ mRNA isolation. Total RNAs from DEHP-treated sea bass kidney were extracted by the guanidine thiocyanate method as described by Chomczynski and Sacchi (16). Poly $(A)^+$ mRNAs were isolated by affinity chromatography on an oligo(dT)-cellulose column as described in Sambrook *et al.* (17).

Molecular cloning of CYP4F7. Isolation of 5′ and 3′ ends of the gene was performed using the Marathon cDNA Amplification Kit (Clontech, USA). A library of adaptor-ligated double stranded cDNA was constructed from kidney poly $(A)^+$ mRNAs according to the manufacturer's instructions. The 3′-end fragment (857 bp) was obtained by RACE-PCR using a primer (3′-EGAD) designed from the "laurate box" of the CYP4T2 gene (15). The sequence of the primers used to clone the 3′-end were "3′-EGAD" (sense, 5′-GGGCCATGACACCACAGCCAGTGCG-3′) and "AP1" (antisense, 5′-GCCCTATAGTGAGTCGTATTAGGATGG-3′). A CYP4F7 gene specific primer (5′-NWEDL) was designed from the 3′ cDNA fragment previously cloned to generate the 5′ fragment. The sequence of the primers used to clone the 5′-end were "AP1" (sense, 5′-CCATCCTAATACGACTCACTATAGGGC-3′) and "5′-NWEDL" (antisense, 5′-GGGAAGGTTAGACAGATCCTCCCAC-3′). 3′ and 5′ RACE-PCR reactions were performed using the Advantage klenTaq Polymerase Mix (Clontech, USA) on a ThermojeT thermal cycler (Eurogentec, Belgium) with the following program: 94°C for 1 min, 30 cycles of 94°C, 30 sec, and 68°C, 4 min, followed by a final 10 min extension at 68°C. Fragments of expected size were purified on a agarose gel then subcloned by inserting into the EcoRI site of the plasmid vector pCR 2.1 (Invitrogen, The Netherlands). Plasmid DNA from the clones of interest was purified using the QIAprep Spin Miniprep Kit (Qiagen Inc., USA) before sequencing.

DNA sequencing. cDNA sequencing was performed by Genome Express (Grenoble, France). cDNA sequencing was done by Dye Deoxy Terminator Cycle Sequencing (Applied Biosystems, Weiterstadt, Germany) according to the manufacturer's instructions. The reactions were performed on a GeneAmp 9600 thermal cycler (Perkin Elmer/Cetus), and were primed with universal primers (M13 reverse primer and M13 forward (−20) primer). Sequences were analysed on the Applied Biosystems DNA Sequencing System (model 373A).

DNA sequence analysis. The nucleotide sequence obtained was compared to the EMBL and GenBank databases using the BLAST 2.0 program (18). This cDNA sequence was then aligned with the human CYP4F2 (U02388) and CYP4F3 (Q08477), and the rat CYP4F1 (M94548), CYP4F4 (U39206), CYP4F5 (U39207), and CYP4F6 (U39208) sequences using the CLUSTAL X program (version 1.64b, 02/1998). The CYP4F7 sequence was also compared to all other full length CYP4 sequences available in GenBank. Sequences were retrieved using GenBank accession numbers from the D. Nelson web site (19). A phylogenetic tree was constructed with the distance-matrix method using the Clustal X program.

Northern blot analysis. Total RNAs from sea bass tissues were separated by electrophoresis on a 1.2% denaturating agarose gel, transferred to nylon membranes (Roche Diagnostics), and UV cross-linked. Digoxigenin (DIG)-labeled CYP4F7 probes (3′-, 5′-cDNA fragments, or full length sequence) was prepared by PCR using the specific required primers, and the DIG DNA Labeling Mix (Roche Diagnostics). A cDNA encoding for the sea bass ribosomal protein rpl 17A (Sabourault, unpublished results; GenBank AF 074720) was used as an internal standard for normalizing the quantity of mRNA on the gel (15). A DIG-labeled rpl 17 cDNA probe was prepared using the same procedure. Prehybridization and hybridization (14-16 h at 50°C), washes (at 65°C), and chemiluminescent detection were performed according to the manufacturer's instructions of the DIG system (20, 21).

The resulting films were scanned and analysed using the NIH Image 1.62 software (available by anonymous FTP from zippy.nimh.nih.gov). The integrated values were normalized with rpl 17 mRNA. Various exposure times were needed to avoid saturation of the signal due to rpl 17 mRNA, and therefore aberrant results in densitometry.

RESULTS AND DISCUSSION

Several clones were obtained during isolation of the CYP4T2 cDNA by 3′ RACE-PCR reactions (15). Among these clones was the CYP4T2 3′-fragment, and a new CYP4 sequence, which has been named CYP4F7 (accession number AF123541) by Dr. D. Nelson (P450 Gene Superfamily Nomenclature Committee). The sequence of this new cDNA revealed that the 3′-primer (positions 1062-1086) covered a very homologous region between the 2 genes (15). The other part of the CYP4F7 sequence was obtained by RACE-PCR using a 5′-gene specific primer (positions 1191-1215). The full length sequence (1917 bp) of this new CYP4 gene contained an open reading frame (positions 82-1659) followed by a TGA termination codon (positions 1660-1663) and a 3′-untranslated sequence of 255 bp (Fig. 1). This open reading frame encoded a protein of 526 amino acids with an deduced molecular weight of 60,326.

This sequence was compared to other CYP4F sequences. The sea bass CYP4F7 predicted protein sequence shared 54% amino acid identity with the human CYP4F2. The alignment of all CYP4F amino acid sequences showed several domains that are conserved among human, rat, and sea bass sequences (Fig. 2). The 5 regions of highest conservation are found at positions 150-162, 199-208, 319-334, 368-389, and 456-473. The region [456-473] contains the residues surrounding the heme-binding cysteine (position 467) conserved in all P450s. The region [319-339] may correspond to the "laurate box" initially described by Bradfield *et al.* (22), which is found exclusively in CYP4 sequences. There are however 3 differences in this 13-amino acid peptide. Residue 325 is Ala in all CYP4F sequences, but is Val in all other CYP4. Residue 326 is Asn in sea bass CYP4F7 but is Asp in all other CYP4,

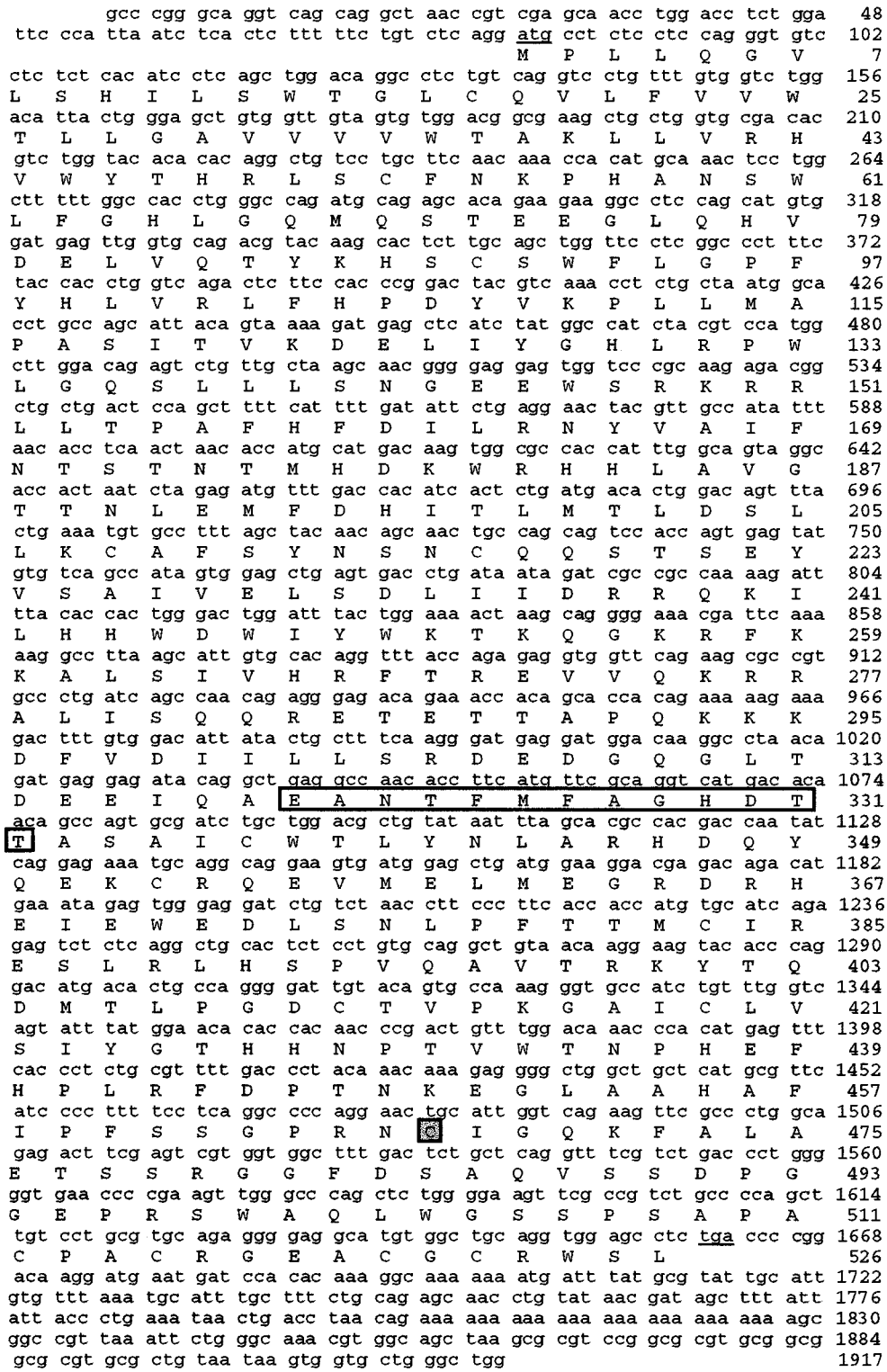

FIG. 1. Nucleotide and deduced amino acid sequences of the CYP4F7 transcript. Putative translation start codon (ATG), and stop codon (TGA) are underlined. The 13-amino acid peptide, which is characteristic of the CYP4 family, is boxed. The putative heme-binding cysteine residue is shaded.

including the CYP4F sequences. Residue 331, which is Glu in most CYP4 sequences (except for the *Drosophila* CYP4D1) is variable in the CYP4F sequences (Glu, Gly, or Ala for CYP4F7). This 13-amino acid peptide was described to be closely related to the substrate recognition domain (22), despite the diversity of sub-

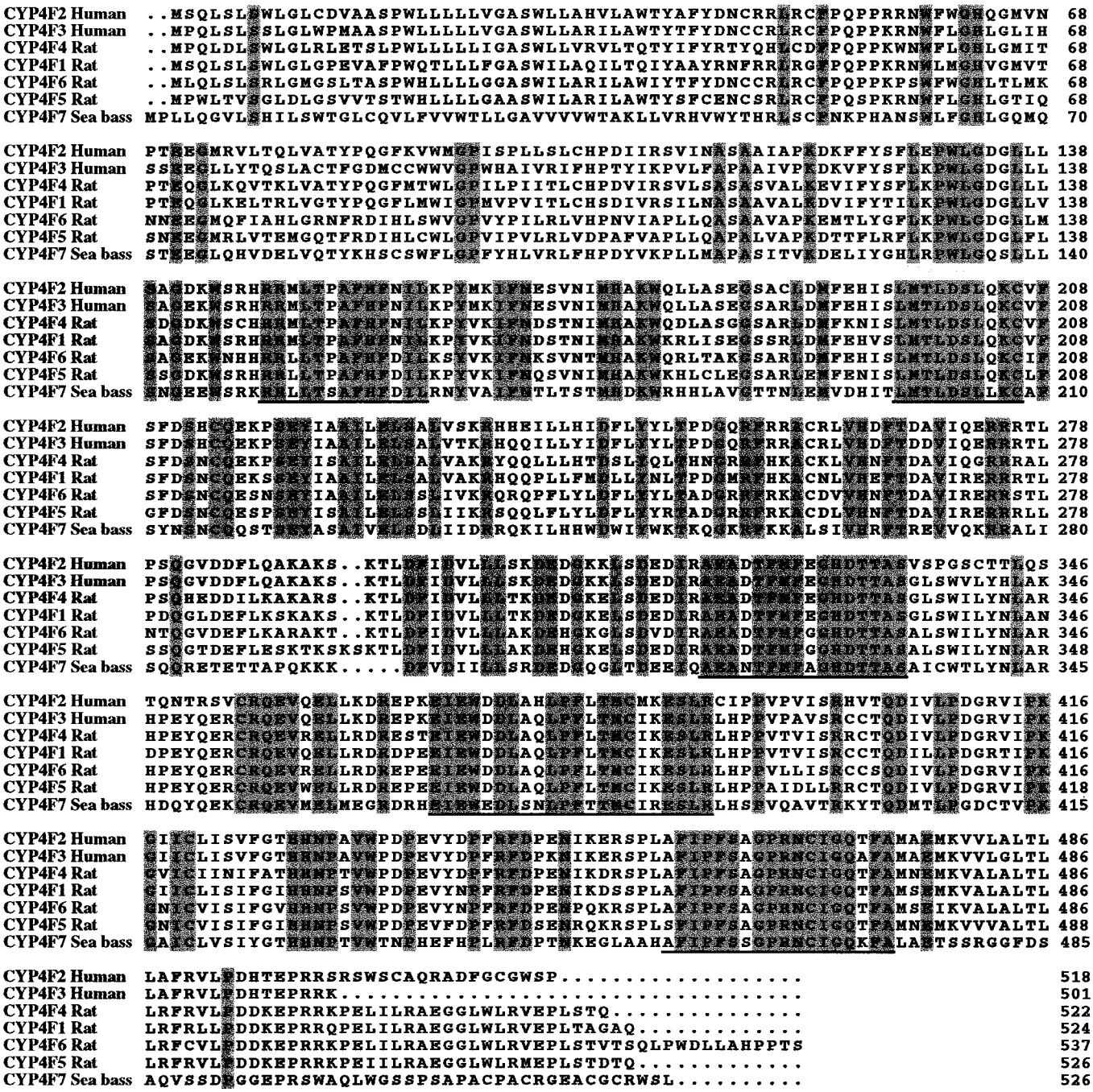

FIG. 2. Alignment of the deduced amino acid sequences of sea bass CYP4F7 with human CYP4F2 and CYP4F3, and rat CYP4F1, CYP4F4, CYP4F5, and CYP4F6. Residues conserved in all sequences are shaded.

strates (fatty acids, prostaglandins, leukotrienes) and the catalytic variability among CYP4s. The CYP4F7 sequence was compared to all other full CYP4 sequences available in GenBank to infer the phylogenetic relationships between the mammalian, insect, and fish CYP4 genes. These CYP4 sequences were also compared to representative mammalian and fish members of gene families CYP1, 2, 11, and 17. A phylogenetic tree was generated using the distance-matrix method (Fig. 3). All vertebrate CYP4 sequences clustered together but the CYP4Fs formed a group quite distinct from the others (CYP4As, CYP4Bs, and CYP4T2). These data may be correlated with the fact that CYP4Fs have specific metabolic functions, quite different from those of the other CYP4s. Within the CYP4Fs subgroup, the fish sequence formed a separate branch.

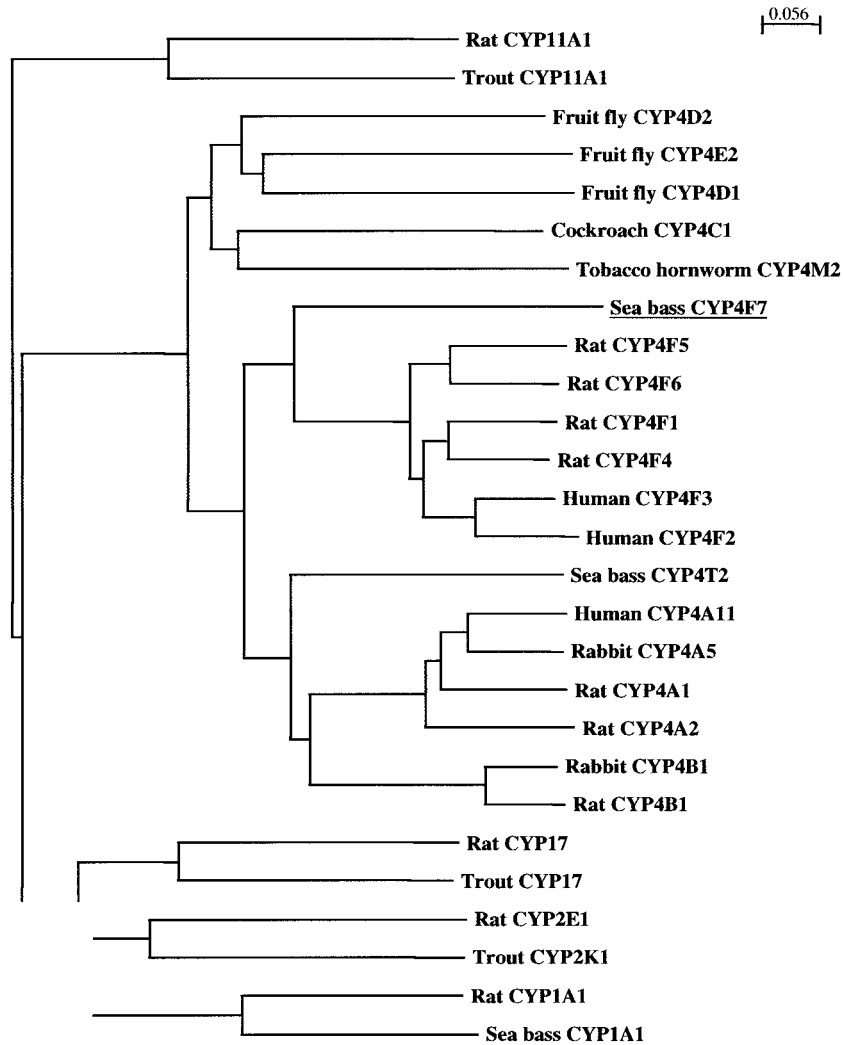

FIG. 3. Phylogenic tree of the full length CYP4 sequences inferred by the distance-matrix method using the Clustal X program. Distances between genes are represented by the sum of their horizontal separation. The scale bar indicates the distance corresponding to 10 differences in 100 positions.

Most of CYP4s are suspected to be involved primarily in endogenous metabolic pathways. Mammalian CYP4F catalyse ω-hydroxylation, and thus inactivation of the potent proinflammatory agent LTB4 (7, 8). They play an important role in regulating the biological activity of LTB4 and their concentration in the organism (10). Thus, these enzymes might serve as a negative feedback mechanism involved in the modulation of some inflammatory responses. To date, CYP4F enzymes were isolated only in human and rat (8, 11, 23, 24). These enzymes are predominantly expressed in liver (7, 11, 23, 24), and polymorphonuclear leukocytes (8, 11), but also in kidney (11, 24), and brain (24).

In order to investigate expression of this gene in sea bass tissues, Northern blot analysis were performed with full length, 5′-end, or 3′end cDNAs of CYP4F7 as digoxigenin-labeled probes. The same results were obtained with the various probes (not shown). As presented in Fig. 4, CYP4F7 transcripts are exclusively present in the kidney of untreated sea bass. The CYP4F7 cDNA hybridized to a unique band of 2.1 kb, which is the expected size for the CYP4F7 transcript. While the major site of expression for most CYPs in mammals is the liver, it appears that the kidney is a key organ for the expression of various P450s in fish (13, 15). CYP1A and CYP2K4 isoforms are strongly expressed in trunk kidney of male rainbow trout, and expression of CYP3A27 increases with age in this tissue (Sabourault, unpublished results, 13).

Sea bass were also treated with various xenobiotics, such as peroxisome proliferators, and some “classical inducers” of other CYP families (benzo[a]pyrene, prochloraz, ethanol). For each fish, RNAs were isolated from 7 organs and analysed. No CYP4F7 transcript

was detected in tissues other than kidney, even in treated sea bass (data not shown). No induction of CYP4F7 was seen in kidney of xenobiotic-treated animals. While the amounts of renal mRNAs in clofibrate- and 2,4-D-treated sea bass was slightly affected, a treatment of fish by prochloraz or acetone resulted in a 40% decrease of the transcripts (Fig. 5). The lowest amount of CYP4F7 mRNA was found after a treatment by DEHP, benzo[a]pyrene, or ethanol (40% of the control). It is noteworthy that ethanol inhibits the LTB4 metabolism in rat hepatocytes (25), and that benzo-[a]pyrene induce an inflammatory response in mouse skin (26). Thus it appeared that this isoform is not inducible by xenobiotics, unlike the sea bass CYP4T2, which was strongly induced by DEHP or 2,4D (15).

CYPs belonging to 7 families (CYP1, CYP2, CYP3, CYP4, CYP11, CYP17, and CYP19) have been isolated from fish, especially from rainbow trout (13). Most of them (except for the CYP1A) are involved in endogenous metabolism, such as fatty acids hydroxylation or steroid biosynthesis. In view of the biological importance of CYP4F genes in mammals, it is not so surprising to find a member of this CYP4 subfamily in fish. However, the tissue-specific expression of CYP4F7 in kidney is quite unexpected as compared to mammals. Little is known about LTB4 generation and its biological activities in fish. Their potential pro-inflammatory ability has not been tested. Leukotrienes B4 are produced by the macrophages and by the thrombocytes (platelet equivalent cells) in rainbow trout (27, 28). The presence of putative receptors for LTB4 on trout macrophages was reported (29), suggesting a biological mechanism similar to that in mammals.

The tissue-specific and constitutive expression of the CYP4F7 gene in sea bass kidney, and the great homology with mammalian CYP4F isoforms suggest an important physiological role that remains to be established. Heterologous expression of the gene and the screening of potential substrates will be required to identify the physiological substrates and roles of this new CYP4.

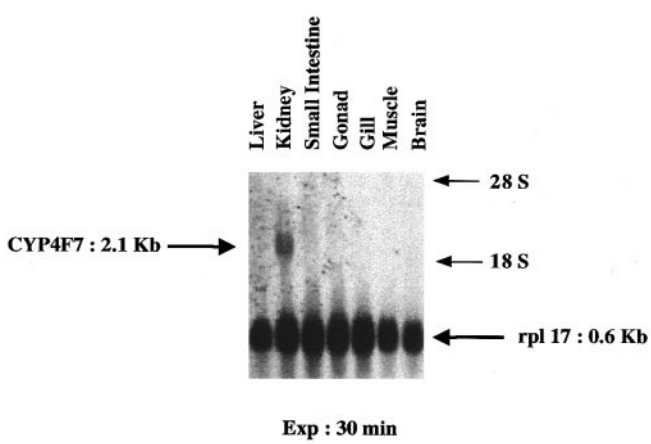

FIG. 4. Blot analysis of total RNA from various sea bass tissues. Total RNA (20 μg per lane) were separated on a 1.2% denaturing agarose gel, transferred to a nylon membrane, and hybridized with a digoxigenin-labeled CYP4F7-specific probe (5′end). As a loading control, the membrane was cohybridized with a rpl 17 probe, which is constitutively expressed.

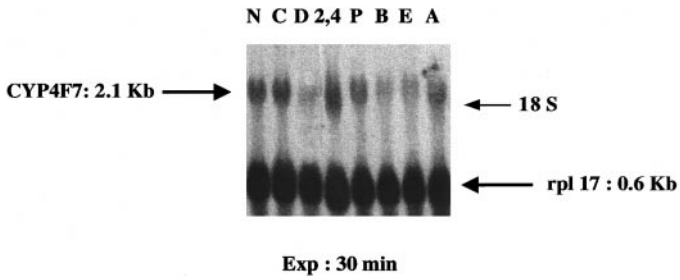

FIG. 5. Blot analysis of total RNA from kidney of sea bass treated with various xenobiotics, including some peroxisome proliferators. N, control; C, Clofibrate; D, DEHP; 2,4, 2,4-D; P, Prochloraz; B, Benzo[a]pyrene; E, Ethanol; A, Acetone.

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