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MOLECULAR CLONING OF CYTOCHROME P4501A cDNA OF MEDAKA (*ORYZIAS LATIPES*) AND MESSENGER RIBONUCLEIC ACID REGULATION BY ENVIRONMENTAL POLLUTANTS

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Abstract—The sequence of cytochrome P4501A (CYP1A) cDNA of medaka (*Oryzias latipes*) was determined, and its messenger ribonucleic acid (mRNA) regulation by β-naphthoflavone (βNF) was evaluated. The determined cDNA sequence contained 2,349 base pairs (bp), and the open reading frame contained a total of 1,563 bp encoding 521 predicted amino acids. The induction of CYP1A mRNA in medaka was evaluated using reverse transcription–polymerase chain reaction. The concentration-dependent induction of CYP1A mRNA in the liver was observed after exposure to βNF at nominal concentrations of 20, 100, and 500 μg/L for 2 d. Time-dependent changes of CYP1A mRNA levels were also observed in the liver, gill, gut, and caudal fin tissues of medaka exposed to 100 μg/L of βNF for 7 d. Our results showed that the degree of CYP1A mRNA induction in the gill, gut, and caudal fin after exposure to βNF was relatively higher than that in the liver, possibly because of low basal levels of CYP1A mRNA in the gill, gut, and caudal fin of nonexposed fish. The induction of medaka CYP1A mRNA was also observed after exposure to an environmental sample, landfill leachate. The CYP1A mRNA inductions in the gill, gut, and caudal fin were also higher than that in the liver as shown in the βNF-treated groups. These results show that CYP1A mRNA determination in the gill, gut, and caudal fin, which are in direct contact with the polluted water, may become a useful method for monitoring CYP1A-inducible chemicals.

Keywords—Medaka Cytochrome P4501A Cloning Reverse transcription–polymerase chain reaction Environmental pollutants

INTRODUCTION

The cytochrome P4501A (CYP1A) is a subfamily of the cytochrome P450-dependent monooxygenase enzyme system catalyzing the first step in the biotransformation of many xenobiotics in vertebrates. The CYP1A is induced by some environmental pollutants, such as polycyclic aromatic hydrocarbons (PAHs); halogenated aromatic hydrocarbons (HAHs), including dioxin and furan; and polychlorinated biphenyls (PCBs). The products of biotransformation of these inducers may be detoxified, whereas some of the metabolites are turned into metabolites that are more cytotoxic and/or carcinogenic than the parent compounds [1,2]. Therefore, the CYP1A induction may indicate potential adverse effects resulting from the exposure to such contaminants. Many studies have shown the induction of CYP1A gene expression in teleost fish after exposure to xenobiotics and the potential application of this induction response as an environmental biomarker of the aquatic environment [3–5]. In fact, CYP1A gene expression and its catalytic activity in fish liver have been proposed to serve as a sensitive biomarker for the presence of CYP1A inducers [6]. Because of the relatively high CYP1A-specific activity in fish liver compared to that in other tissues, the liver was proposed to be the major site of CYP1A-catalyzed biotransformation in fish [7]. In addition, the pattern of CYP1A induction in different tissues has been inconsistent, mainly because of the different routes of exposure and different rates of biotransformation in many tissues [8,9].

The medaka (*Oryzias latipes*) has been used as a valuable model system to study general toxicity, developmental and reproductive toxicity, and the effect of endocrine disruption by environmental pollutants [10–15], because the fish are small and require less volume of test water. In addition, they are relatively inexpensive to maintain and easy to manipulate. The CYP1A activity determined by 7-ethoxyresorufin *O*-deethylase (EROD) in medaka has been reported [16,17]. The measurement of CYP1A messenger ribonucleic acid (mRNA) level by reverse transcription–polymerase chain reaction (RT-PCR) can be easier and more sensitive than immunodetection of proteins or enzymatic assays. Therefore, CYP1A mRNA detection could become a more useful tool as an early warning against potential pollution. Despite these advantages in using medaka, to our knowledge the CYP1A cDNA sequence of medaka is unknown, and no study has examined CYP1A induction at the mRNA level in medaka.

The present study aimed to determine medaka CYP1A cDNA sequence and to establish a screening tool for xenobiotics inducing CYP1A in aquatic environment by assessing the level of medaka CYP1A mRNA using RT-PCR. First, we cloned and sequenced CYP1A cDNA from medaka liver, and then we prepared a pair of oligo-primers based on the determined CYP1A cDNA sequence for RT-PCR. Induction of

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CYP1A mRNA was studied in the liver, gill, gut, and caudal fin after exposure to a chemical and landfill leachate to evaluate the utility of CYP1A mRNA measurement as a useful biomarker.

MATERIALS AND METHODS

Fish

The orange-red variety of the medaka (*O. latipes*) used in the present study was maintained at the Environmental Toxicology Laboratory, National Institute of Environmental Research (Incheon, Korea). Fish were raised under constant 16:8-h light:dark cycles and a temperature of 25 ± 1°C, and fed twice a day with nauplii of the brine shrimp (*Artemia salina*). Dechlorinated tap water was used for the maintenance of fish and the dilution of test solution. Adult medaka, ranging in age from three to five months, were used.

Preparation of primers for RT-PCR

To determine the medaka CYP1A cDNA sequence, several primers for initial PCR were designed based on a previously published cDNA sequence from mummichog *Fundulus heteroclitus* [18] as follows: FCyp1A-p1, 5′-tctcggtgtctgagggtttg-3′ (forward); FCyp1A-p2, 5′-ccaaagcacatgccacagat-3′ (reverse); FCyp1A-p3, 5′-atctgtggcatgtgctttgg-3′ (forward); FCyp1A-p4, 5′-gtgaggccatactccgggggtc-3′ (reverse). Three primers for the 5′-/3′-rapid amplification of cDNA ends (5′-/3′-RACE) and six primers for the analysis of internal sequences of CYP1A cDNA were synthesized based on the partial CYP1A cDNA sequence of medaka after sequencing of the initial PCR products as follows: 5Race-p5, 5′-aaggcctccagggatctctt-3′ (reverse); 5Race-p6, 5′-aatgcttgaggagtagatag-3′ (reverse); 3Race-p7, 5′-gcgaggtcatagcacgaaat-3′ (forward); MCyp1A-p8, 5′-gaatactcatgcatgctgga-3′ (forward); MCyp1A-p9, 5′-ttcctgtcttcacagtgatc-3′ (reverse); MCyp1A-p10, 5′-gatcactgtgaagacaggaa-3′ (forward); MCyp1A-p11, 5′-tcaagtccaaaggctctcct-3′ (reverse); MCyp1A-p12, 5′-cacgtgtgtctttatcaacc-3′ (forward); MCyp1A-p13, 5′-ataggctgcagtgctgttaa-3′ (reverse). For the 3′-RACE, Adapter Primer (5′-ggccacgcgtcgactagtacttttttttttttttttt-3′; Life Technologies, Gaithersburg, MD, USA) and Abridged Universal Amplification Primer (AUAP; 5′-ggccacgcgtcgactagtac-3′, reverse; Life Technologies) were also used. Abridged Anchor Primer (AAP; 5′-ggccacgcgtcgactagtacgggiigggiigggiig-3′, forward; Life Technologies) was used for 5′-RACE.

The housekeeping β-actin gene of medaka was used as the internal standard, and the primers were synthesized as follows: β-actin-p14, 5′-ttcaacagccctgccatgta-3′ (forward); β-actin-p15, 5′-ataccgcaggactccataccaa-3′ (reverse).

CYP1A induction and RNA isolation for cloning

To study the CYP1A mRNA induction, medaka were exposed to β-naphthoflavone (βNF), which is known as a classical CYP1A inducer, at a nominal concentration of 100 μg/L for 2 d. A stock solution of 1,000 mg/L of βNF in ethanol was diluted in the dechlorinated tap water.

Total RNA was carefully extracted from the liver tissues of βNF-treated fish using RNAgents Total RNA Isolation System (Promega, Madison, WI, USA) according to the manufacturer's instruction. A pool of four livers was used for each RNA extraction. Purified RNA samples were stored at −80°C until further use, and the concentration of RNA was adjusted to 1 μg/μl for RT-PCR.

Reverse transcription–polymerase chain reaction

The RT-PCR was performed with total RNA extracted from the medaka livers treated with βNF. The RT reaction mixture contained 1 μg of total RNA, 1 μl of 20 μM oligo (dT) primer, 0.5 μl (40 U/μl) of human placenta ribonuclease inhibitor, and diethylpyrocarbonate-treated water. The reaction mixture was heated to 80°C for 10 min and quickly chilled on ice. After cooling, 2 μl of 10× reaction buffer containing 250 mM Tris-HCl (pH 8.3), 500 mM KCl, 20 mM dithiothreitol, 50 mM $MgCl_2$, 4 μl of 25 mM $MgCl_2$, 2 μl of deoxynucleotide triphosphates (dNTPs; 2.5 mM each), and 0.14 μl (35 U/μl) of AMV reverse transcriptase XL (Takara Shuzo, Shiga, Japan) were added to a total volume of 20 μl, and the reaction mixture was incubated for 30 min at 50°C. The reaction mixture was then heated to 99°C for 5 min to stop the RT.

Initial PCR was carried out to analyze CYP1A cDNA sequence of medaka. The PCR reactions contained 3 μl of the RT reaction mixture as a cDNA template, 5 μl of 10× Ex Taq PCR buffer (Takara Shuzo) containing 20 mM Mg^{2+}, 4 μl of dNTPs (2.5 mM each), 0.5 μl (5 U/μl) of Ex Taq polymerase (Takara Shuzo), and 0.4 μM (final concentration in PCR reactions) of both forward and reverse primers. The total volume of the reaction mixture was 50 μl. Primer sets used for initial PCR were FCyp1A-p1 (forward)/FCyp1A-p2 (reverse), FCyp1A-p3 (forward)/FCyp1A-p4 (reverse), and FCyp1A-p1 (forward)/FCyp1A-p4 (reverse). The PCR conditions were as follows: Initial denaturation at 94°C for 2 min; 25 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min; final extension at 72°C for 10 min. The PCR products were analyzed by electrophoresis on 1.5% agarose gels.

Rapid amplification of cDNA ends

To determine the unknown sequences at the 3′- and 5′-ends of CYP1A cDNA of medaka, 3′- and 5′-RACE were performed according to the manufacturer's instructions. Each RACE was performed using the 3′- and 5′-RACE kit (Life Technologies). Primers used for each RACE were prepared based on the defined internal sequences by the initial PCR. The PCR conditions were initial denaturation at 94°C for 2 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min; final extension at 72°C for 7 min. The PCR products were analyzed by electrophoresis on 1.5% agarose gels.

Cloning and sequencing of CYP1A cDNA

The slices of amplified cDNA fragments were cut out from the agarose gels, followed by purification with a Concert Matrix Gel Extraction System (Life Technologies, Grand Island, NY, USA). Amplified cDNA fragments, recovered from agarose gels, were ligated and subcloned into a pGEM-T EasyVector (Promega). Recombinant clones were selected by blue-white screening. White colonies were grown, and plasmids were purified using a plasmid purification kit (NucleoGen, Ansan, Korea). Finally, cDNA sequences were determined using an automatic DNA sequencer (ABI3700; Applied Biosystems, Foster City, CA, USA). The full sequence of CYP1A cDNA of medaka was determined with two or three clones per each fragment in Figure 1. Each clone was sequenced two to four times.

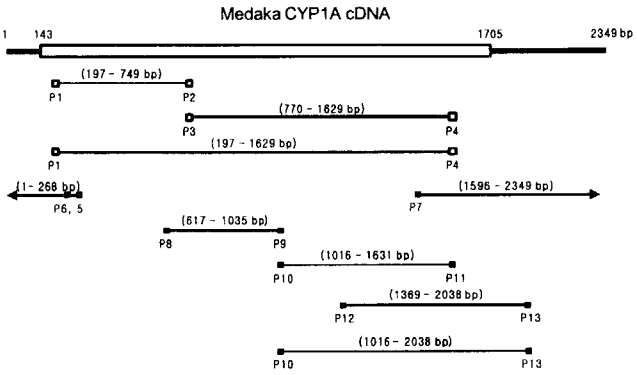

Fig. 1. Positions of the primers used to amplify cytochrome P4501A (CYP1A) cDNA of medaka (*Oryzias latipes*) and polymerase chain reaction products used for cloning. Primers of P1 to P4 and P5 to P13 were prepared based on the CYP1A cDNA sequences of *Fundulus heteroclitus* and *O. latipes*, respectively. The open bar represents the coding region of the medaka CYP1A protein. bp = base pair.

Chemical exposure and quantification of CYP1A mRNA

Induction of medaka CYP1A mRNA was studied with two different exposure schemes. In experiment 1, fish were exposed to βNF at nominal concentrations of 20, 100, and 500 μg/L for 2 d to observe the concentration-dependent induction of CYP1A mRNA in medaka liver. Each of three groups of four fish (two male and two female per group) was exposed to chemical solution of each concentration. Each group was placed in a glass aquarium containing 1 L of water with the chemical. A stock solution of 2,000 mg/L of βNF in ethanol was prepared, and each exposure solution was diluted in the dechlorinated tap water. The control group was exposed to the 0.005% vehicle solution.

In experiment 2, fish were exposed to 100 μg/L of βNF to investigate the pattern of CYP1A mRNA induction in the liver, gill, gut, and caudal fin of medaka. Each of three groups of 16 fish (eight male and eight female per group) was exposed to βNF for 7 d. Each group was placed in a glass aquarium containing 3 L of the chemical solution. On days 0, 1, 4, and 7, the liver, gill, gut, and caudal fin tissues from four fish (two male and two female) were removed from each treatment group. Experimental fish were fed once a day with artemia (*A. salina*), which were consumed within 5 min. The exposure solutions were exchanged every 2 d. The collected liver, gill, gut, and caudal fin tissues were each placed in a 1.5-ml sterile microcentrifuge tube and stored at −70°C before RNA isolation. To quantify the induced CYP1A mRNA, RT-PCR, which is a semiquantitative method, was performed. The RT and PCR after RNA isolation were performed using AccuPower RT Premix and AccuPower PCR Premix (Bioneer, Daejon, Korea), respectively. Total RNA (0.5 μg/reaction) was used in RT. The RT reaction was performed for 60 min at 42°C. The reaction mixture was then heated at 94°C for 5 min to stop the RT. Primers used in PCR were primer MCyp1A-p10 (forward)/MCyp1A-p13 (reverse) for CYP1A and β-actin-p14 (forward)/β-actin-p15 (reverse) for β-actin as a housekeeping gene to normalize mRNA levels. The PCR conditions were initial denaturation at 94°C for 2 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min; final extension at 72°C for 10 min. All PCR products were run on the 1.5% agarose gel, and the band densities of amplified products were calculated by 1D Image Analysis Software (Kodak Digital Science, Rochester, NY, USA).

Application of detection system of CYP1A mRNA induction to landfill leachate

The leachate of a landfill site in Seocheon County (Chungcheongnam-do, Korea), used as an environmental sample, was kindly provided from Waste Research Department, National Institute of Environmental Research. The acute toxicity of the leachate was tested to determine the appropriate dilution ratio (10- to 50-fold) of the leachate after exposure for 24 h. The final ratio was 50-fold dilution of the original leachate, in which all exposed fish were alive after exposure for 24 h. Liver, gill, gut, and caudal fin tissues were removed from each of the fish (n = 4) exposed to the diluted leachate for 24 h and then combined before further procedures. The RNA isolation and RT-PCR were carried out according to the procedures described above.

Statistical analysis

Differences between treatment groups and controls in the concentration study by βNF and the time course study with several tissues were determined using one-way analysis of variance with Dunnett's t test. Statistical significance was considered to be at $p < 0.05$.

RESULTS

In the present study, we determined medaka CYP1A cDNA sequence and observed the induction of CYP1A mRNA in medaka exposed to βNF and landfill leachate by RT-PCR.

cDNA sequence coding for CYP1A protein of medaka

Several primers (FCyp1A-p1, FCyp1A-p2, FCyp1A-p3, and FCyp1A-p4) for initial RT-PCR were prepared based on the cDNA sequence of mummichog (*F. heteroclitus*) [18]. The primer combinations FCyp1A-p1/FCyp1A-p2, FCyp1A-p3/FCyp1A-p4, and FCyp1A-p1/FCyp1A-p4 were designed to produce overlapping PCR products. These primers yielded a 600–base pair (bp) PCR product with FCyp1A-p1/FCyp1A-p2, a 900-bp product with FCyp1A-p3/FCyp1A-p4, and a 1,500-bp product with FCyp1A-p1/FCyp1A-p4 on agarose gels after electrophoresis of initial RT-PCR products. The size of these PCR products was similar to the predicted size of CYP1A cDNA of *F. heteroclitus*. These initial PCR products were subsequently cloned and sequenced. The DNA sequencing showed the combined sequence of 1,433 bp (Fig. 1). Part of the internal cDNA sequence was used to design new specific primers for 3′- and 5′-RACE to get the complete CYP1A cDNA sequence of medaka.

The complete cDNA sequence of medaka CYP1A was obtained by employing 3′- and 5′-RACE. To capture the unknown 3′-end sequence of cDNA, we used the internal primer 3Race-p7 and AUAP. The PCR product of 3Race-p7/AUAP primers was cloned and sequenced. Subsequently, the 754-bp sequence including the poly(A) tail was determined (Fig. 1). To capture the unknown 5′-end cDNA sequence, we used the primer 5Race-p5 or 5Race-p6, specific for two different internal sequences, and AAP. The sequence of 268 bp in the 5′ region was determined after 5′-RACE as assembled in Figure 1. Finally, the full CYP1A cDNA sequence of medaka was obtained through the initial PCR followed by subsequent 3′- and 5′-RACE reactions. To confirm the accuracy of the CYP1A cDNA sequence, several internal primers were prepared and subjected

```
1                                                    AAAGACATCAAGAGTGGTAATT   22
23   CTAACTATCACTCAAGTCAGCAGGCTTACCCTTTTTGTTTAATTTTGGCAAGCTCCTCTG   82
83   AGGTTTTCTTCTTTCCTTTCTTCAACTAAGGTCAATTAGTCGAGCAAAAAAGGCTGTCATC  142
143  ATGGCATTAATGGTACTGCCATTCATCGGTCCTCTGTCAGTACTTGAGGGTTTGATTGCT  202
       M  A  L  M  V  L  P  F  I  G  P  L  S  V  L  E  G  L  I  A
203  CTGACTACAGTGTGTGTGGTCTATCTACTCCTCAAGCATTTTAACAAAGAGATCCCTGGA  262
       L  T  T  V  C  V  V  Y  L  L  L  K  H  F  N  K  E  I  P  G
263  GGCCTTCGTCAGCTGCCGGGCCCCACACCACTCCCCATCATTGGGAATCTACTGGAGCTG  322
       G  L  R  Q  L  P  G  P  T  P  L  P  I  I  G  N  L  L  E  L
323  GGTAGCAAACCCTACCTGAGCCTCACCGAAATGAGCAAGCGATTTGGAGATGTGTTTCAA  382
       G  S  K  P  Y  L  S  L  T  E  M  S  K  R  F  G  D  V  F  Q
383  ATCCAGATCGGCATGCGTCCTGTCGTCGTTTTGAGTGGTAATGAAACGGTGAGACAGGCT  442
       I  Q  I  G  M  R  P  V  V  V  L  S  G  N  E  T  V  R  Q  A
443  CTCATCAAACAAGGAGATGACTTTTCTGGCAGGCCCGACTTGTATAGCTTCCAGTTTATC  502
       L  I  K  Q  G  D  D  F  S  G  R  P  D  L  Y  S  F  Q  F  I
503  AATGACGGCAAGAGTTTGGCTTTCAGCACAGACCAAGCAGGGGTTTGGCGGCTCGCAGA  562
       N  D  G  K  S  L  A  F  S  T  D  Q  A  G  V  W  R  A  R  R
563  AAGTTGGCCTACAGTGCATTGCGCTCTTTCTCAAGCCTAGAGGGCAGCAATGCAGAATAC  622
       K  L  A  Y  S  A  L  R  S  F  S  S  L  E  G  S  N  A  E  Y
623  TCATGCATGCTGGAGGAACACATCTGCAAAGAGACAGAGCACCTGGTCAAAGAGATAGAA  682
       S  C  M  L  E  E  H  I  C  K  E  T  E  H  L  V  K  E  I  E
683  AAAGTAATGAAGACAGAAGGCAAATTTGACCCCTATCGATACATTGTTGTGTCTGTGGCC  742
       K  V  M  K  T  E  G  K  F  D  P  Y  R  Y  I  V  V  S  V  A
743  AATGTTATCTGTGGCATGTGCTTTGGACGACGCTATGACCACCATGACCAGGAGCTGGTA  802
       N  V  I  C  G  M  C  F  G  R  R  Y  D  H  H  D  Q  E  L  V
803  GGCCTGGTAAACCTCAGTGAAGATTTTGTCCAAGTGACGGGCAGCGGCAACCCTGCAGAC  862
       G  L  V  N  L  S  E  D  F  V  Q  V  T  G  S  G  N  P  A  D
863  TTCATCCCTGCCCTGCGGTATCTACCCAGCAAAGCAATGAAAAAGTTGTTGAAATCAAC  922
       F  I  P  A  L  R  Y  L  P  S  K  A  M  K  K  F  V  E  I  N
923  AACCGCTTCCAGTGTTTTGTTCAAAAGATCGTCAATGAGCATTATGCCACGTATGATAAG  982
       N  R  F  Q  C  F  V  Q  K  I  V  N  E  H  Y  A  T  Y  D  K
983  GACAACATCCGTGACATTACAGACTCCCTTATTGATCACTGTGAAGACAGGAAGCTAGAT 1042
       D  N  I  R  D  I  T  D  S  L  I  D  H  C  E  D  R  K  L  D
1043 GAAAATTCCAACATCCAGATGTCGGATGAAAAAGTAGTCGGCATCGTCAATGATCTCTTT 1102
       E  N  S  N  I  Q  M  S  D  E  K  V  V  G  I  V  N  D  L  F
1103 GGAGCAGGTTTTGACACAATCTCTACTGCTCTGTCCTGGGCAGTGGGGTATTTGGTGGCC 1162
       G  A  G  F  D  T  I  S  T  A  L  S  W  A  V  G  Y  L  V  A
1163 TACCCAGACATAGAAAAGAGACTTTTGAAGAAATAAAGGAAAACATCGGCCTGAACCGA 1222
       Y  P  D  I  E  K  R  L  F  E  E  I  K  E  N  I  G  L  N  R
1223 AATCCTACCATATCTGACAGAAGCAACTTACCTTTAACGGAGGCCTTTATCTTGGAGATC 1282
       N  P  T  I  S  D  R  S  N  L  P  L  T  E  A  F  I  L  E  I
1283 TTTCGCCATTCTTCATTTCTCCCTTTCACAATTCCACACTGCACAACAAGGGACACATCT 1342
       F  R  H  S  S  F  L  P  F  T  I  P  H  C  T  T  R  D  T  S
1343 CTGAATGGTTACTATATTCCCAAAGACACGTGTGTCTTTATCAACCAGTGGCAAATAAAC 1402
       L  N  G  Y  Y  I  P  K  D  T  C  V  F  I  N  Q  W  Q  I  N
1403 CATGACCCGAAACTGTGGCAGGATCCATCATCCTTTAACCCAGATCGTTTCCTGAGTGAA 1462
       H  D  P  K  L  W  Q  D  P  S  S  F  N  P  D  R  F  L  S  E
1463 GATGGAAGTGAGGTTAATCGGCTAGACGGAGAGAAAGTGATGGTTTTTGGTCTGGGAAAG 1522
       D  G  S  E  V  N  R  L  D  G  E  K  V  M  V  F  G  L  G  K
1523 CGGCGTTGCATTGGCGAGGTCATAGCACGAAATGAAGTTTTCCTCTTTTTGGCAATCATG 1582
       R  R  C  I  G  E  V  I  A  R  N  E  V  F  L  F  L  A  I  M
1583 ATTCAGAAATTGTGCTTTGAGGAGATGCCAGGAGAGCCTTTGGACTTGACCCCAGAGTAC 1642
       I  Q  K  L  C  F  E  E  M  P  G  E  P  L  D  L  T  P  E  Y
1643 GGGCTCACAATGAAGCACAAGCGCTGCAATGTTAGAGCATCACTGAGGCTAAAGGATGGA 1702
       G  L  T  M  K  H  K  R  C  N  V  R  A  S  L  R  L  K  D  G
1703 TGCTGAAGCTGTTCATAATCCAGCATTTATGACTTTGAAGTCAGCACATGTTGACTGTGA 1762
       C  *
1763 CACTTTAGGAAAAAGCCACCATAATCTGTGTCAGATTCGATGGCATTACAGGCATTGATG 1822
1823 CAAAAAAAGTTGTAAAACATTGCTCAATGAAACTGGTAAGAAGTGTCTAGAAATGTGTCA 1882
1883 ATTCGTGTGGTTTGGGTCAATCAACAGTCTTGGGTCAATCAAATGATGCTTTGAAGGCAC 1942
1943 CCAAAAGGAGAAAAAAGAAACAGAACAACTCACAGCAAAGCTTGTTCAGTTTGGGGACAA 2002
2003 AACATACAAATTGCTATTAACAGCACTGCAGCCTATTGGCTATTTGTCTGTAAAGACTCT 2062
2063 GAAGTAACAGATATTACGAGTAAGCTAGTTTTTATCTTTTATTATGTTGTAGATATCAA 2122
2123 CACTGAAGCTACATTTTTATCCCAAAATGTGATTTTTGTTTTTTGTCTGTCAAGATCACT 2182
2183 ATTTTAAATGACTGGTTTGTGTATGATGTACTTGCTGTATTTTGCTTTTGAATACTTTTC 2242
2243 TATACCAAAACATGATTTACCAGGGCATTTTAAATGGCAGTTGTATGTTTTAAAGGGCTA 2302
2303 TAAAATAAACTTGTCTAACGTGTCACTTAAAAAAAAAAAAAAAAAA              2349
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Fig. 2. The cDNA nucleotide and deduced amino acid sequences of cytochrome P4501A (CYP1A) of medaka (*Oryzias latipes*; GenBank accession no. AY297923). The medaka CYP1A cDNA is 2,349 base pairs in length and encodes a predicted protein of 521 amino acids.

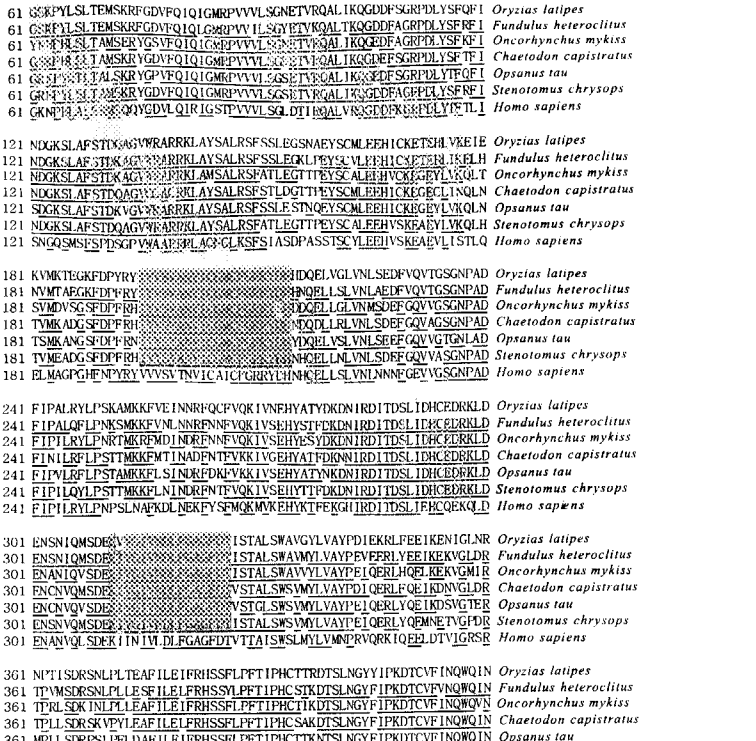

Fig. 3. Sequence alignment of the medaka (*Oryzias latipes*) cytochrome P4501A (CYP1A) protein with those in other teleost species and humans. The identical amino acids with the medaka CYP1A sequence are underlined. Shaded areas represent teleost-specific amino acids. GenBank accession number of each sequence is indicated in parentheses.

to further analysis, including PCR. To produce overlapping products, the primer combinations MCyp1A-p8/MCyp1A-p9, MCyp1A-p10/MCyp1A-p11, MCyp1A-p12/MCyp1A-p13, and MCyp1A-p10/MCyp1A-p13 were used (Fig. 1). The internal sequences of the PCR-amplified CYP1A were also determined by direct sequencing of these products.

Figure 2 shows the cDNA nucleotide and deduced amino acid sequences of the medaka CYP1A. The medaka CYP1A cDNA is 2,349 bp in length, and its open reading frame extends from nucleotide 143 to 1,705 (1,563 bp), encoding for 521 amino acid protein. The deduced amino acid sequence of medaka CYP1A was aligned to the already-known CYP1A protein sequences in other known teleosts and humans (Fig. 3). Comparison of the deduced medaka CYP1A protein with already-known CYP1A proteins was also performed using the basic local alignment search tool (BLAST) through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/) (Table 1). The nucleotide and amino acid sequences of medaka CYP1A showed 74 to 78% nucleotide and 65 to 82% amino acid similarity with other teleost CYP1As.

Induction of CYP1A mRNA in medaka by βNF

Up-regulation of CYP1A mRNA in medaka liver by βNF was studied. The up-regulation of CYP1A mRNA in medaka liver was dependent on βNF concentrations (Fig. 4). The level of CYP1A mRNA was significantly elevated after exposure to 100 or 500 μg/L of βNF for 2 d.

In addition, we studied the pattern of CYP1A mRNA induction in gill, gut, and caudal fin as well as in liver with

Table 1. Comparison of cytochrome P4501A (CYP1A) nucleotide and amino acid sequences of medaka and other species

CYP1A in different species	CYP1A in medaka (*Oryzias latipes*) % Identity of cDNA	% Identity of amino acid
Mummichog (*Fundulus heteroclitus*)	78	82
Rainbow trout (*Oncorhynchus mykiss*)	75	75
Butterflyfish (*Chaetodon capistratus*)	77	77
Tomcod (*Microgadus tomcod*)	74	65
Toadfish (*Opsanus tau*)	74	77
Scup (*Stenotomus chrysops*)	77	76
Zebrafish (*Danio rerio*)	74	70
Human (*Homo sapiens*)	76	54

different exposure duration. Table 2 represents the levels of CYP1A mRNA in each organ on days 1, 4, and 7 compared with those on day 0. Liver, gut, and caudal fin tissue exhibited a similar pattern of time-dependent changes in CYP1A mRNA induction, the levels of which peaked on day 1 and decreased gradually thereafter. The level of hepatic CYP1A mRNA decreased to basal levels on day 7. However, time-dependent change in CYP1A mRNA in the gill was distinct from those in three other tissues, mainly because of its maximal induction observed on day 4.

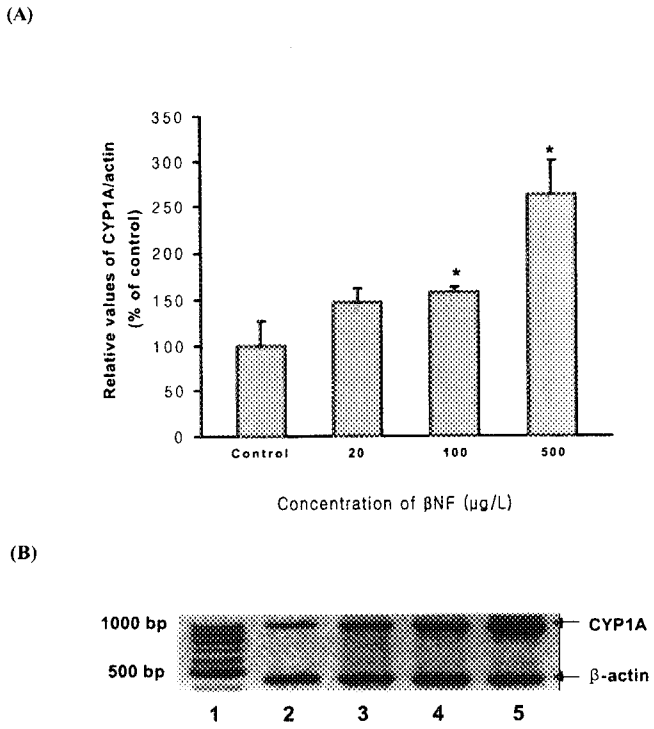

Fig. 4. Concentration-dependent increase in cytochrome P4501A (CYP1A) messenger ribonucleic acid (mRNA) in medaka (*Oryzias latipes*) liver exposed to β-naphthoflavone (βNF). (**A.**) Relative ratio of CYP1A and β-actin mRNA was calculated after densitometric analysis of each band. Values obtained from the vehicle control samples were taken as 100%. Results represent the mean ± standard deviation from three different experiments. Asterisks indicate values significantly different from those in the control group ($p < 0.05$). Each sample is a pool of four medaka livers. (**B.**) Typical result of reverse transcription-polymerase chain reaction. Upper bands show CYP1A mRNA expression, and lower bands show the expression of β-actin mRNA. Lane 1: size markers (100–base pair [bp] ladders); lane 2: vehicle control; lane 3: 20 μg/L of βNF; lane 4: 100 μg/L of βNF; lane 5: 500 μg/L of βNF. The inverse image of the gel is presented.

Induction of CYP1A mRNA in medaka by landfill leachate

One-day exposure of medaka to the 50-fold dilution of leachate led to induction of CYP1A mRNA in the gill, gut, and caudal fin (Fig. 5). As the result of a densitometric analysis of amplified CYP1A band after RT-PCR, the expression values of CYP1A mRNA in the liver, gill, gut, and caudal fin were 97.5, 165.2, 173.9, and 141.4%, respectively, of control group. The CYP1A mRNA levels in the gill, gut, and caudal fin were increased, but the hepatic CYP1A mRNA level was not increased at this exposure concentration of leachate.

DISCUSSION

For the rapid biological screening of CYP1A-inducing pollutants by measuring the level of CYP1A mRNA using RT-PCR, it is necessary to have complete information about the CYP1A cDNA sequence needed for designing specific PCR primers. Three initial RT-PCR products from total RNA of medaka liver were produced using several primers based on the cDNA sequence of mummichog (*F. heteroclitus*) to elucidate the complete CYP1A cDNA sequence of medaka. Because the mummichog belongs to the atherinomorpha under the teleosts (as does medaka), mummichog, out of seven teleost species in Table 1, is the closest to medaka taxonomically. When the DNA sequences of these initial PCR products were analyzed by BLAST, they were homologous to CYP1A cDNAs from other fish. The final CYP1A cDNA sequence was determined by cloning several PCR products using internal primers, which were part of the cDNA sequences of initial PCR products. The amino acid sequence of medaka CYP1A protein includes the teleost-specific amino acid sequences (Fig. 3). In teleost CYP1As, the conserved amino acid sequences (195-IVVSVANVICGMCFGRRYDH-214 and 308-SDEKIVGIVNDIFGAGFDT-326) have been reported [18,19]. These conserved regions were also observed in the CYP1A amino acid sequence of medaka. However, one amino acid in the 308-SDEK*V*VGIVNDIFGAGFDT-326 region of medaka CYP1A is different from the same region (308-SDEK*I*VGIVNDIFGAGFDT-326) of many other teleosts. This different amino acid sequence is also observed in the same regions of gilthead seabream (*Sparus aurata*) and gray mullet (*Liza aurata*) CYP1A proteins [20]. In addition, the amino acid sequence of medaka CYP1A includes the same amino acid, a putative heme-binding cysteine at position 463, as mummichog CYP1A protein does [18].

Some chemicals, such as βNF, 3-methylcholanthrene, and Aroclor 1254 (Hope, RI, USA) have been used as the CYP1A inducers in fish [21–23]. The up-regulation of CYP1A mRNA in medaka liver exposed to 20, 100, and 500 μg/L of βNF for 2 d validated that the cDNA fragment by RT-PCR with primer MCyp1A-p10 and MCyp1A-p13 could be useful as a biomarker for CYP1A induction. The up-regulation of CYP1A mRNA by βNF is consistent with the results of the previous study [16], in which the concentration-dependent induction of EROD activity in the medaka liver exposed to varied concentrations from 1 to 1,000 μg/L of βNF was shown.

Although CYP1A is rapidly and markedly induced in the liver by CYP1A inducers, including βNF, many other studies have shown the induction of CYP1A in extrahepatic tissues, such as gill, heart, gut, kidney, and gonad [9,24–27]. The gill, gut, and caudal fin are in direct contact with various pollutants

Table 2. Time-dependent induction of cytochrome P4501A (CYP1A) messenger ribonucleic acid (mRNA) in the liver, gill, gut, and caudal fin of medaka after exposure to β-naphthoflavone (βNF)

Tissue	Day	CYP1A/actin[a] %	CYP1A/actin[a] Relative values (%)[b]
Liver	0	182.3 ± 23.9	100.0 ± 13.1
	1	272.7 ± 92.5	149.6 ± 50.8
	4	220.5 ± 19.3	121.0 ± 10.6
	7	159.4 ± 11.0	98.4 ± 14.1
Gill	0	82.8 ± 23.2	100.0 ± 28.0
	1	261.2 ± 89.1*	315.5 ± 107.7*
	4	315.2 ± 42.7*	380.6 ± 51.5*
	7	121.8 ± 9.2	147.1 ± 11.1
Gut	0	46.3 ± 7.0	100.0 ± 15.0
	1	317.8 ± 48.0*	686.8 ± 103.8*
	4	191.7 ± 46.7*	414.3 ± 101.0*
	7	105.4 ± 9.3	227.7 ± 20.2
Caudal fin	0	48.1 ± 2.3	100.0 ± 4.9
	1	258.6 ± 14.0*	538.1 ± 29.1*
	4	145.4[c]	302.5[c]
	7	130.7 ± 4.4*	272.0 ± 9.2*

[a] Results represent the mean ± standard deviation from three different experiments. All values were derived from densitometric analysis of amplified CYP1A band relative to actin band on the agarose gel. Each tissue represents a pool of the same tissues from two males and two females.

[b] The average of values obtained from four samples per each tissue on day 0 was taken as 100%.

[c] Only one sample was analyzed because of bad RNA quality in other two samples.

* $p < 0.05$ vs. day 0.

through aqueous and dietary exposure. Route-specific pattern of CYP1A induction was observed in several tissues with immunohistochemical analysis [9]. Aqueous exposure of mummichog to benzo[*a*]pyrene (B*a*P; 10 μg/L) resulted in high levels of immunohistochemical staining of CYP1A in gill pillar cells, heart endothelium, and vascular endothelium, whereas dietary exposure to B*a*P (10 μg/g) only led to mild to moderate staining in these tissues, compared to high-intensity staining in gut mucosal epithelium. In addition, coexposure to both aqueous and dietary B*a*P resulted in a pattern of induction reflecting both routes of exposure. The CYP1A induction in most tissues except gut mucosa seemed to be sensitive to aqueous exposure. In the present study, marked induction of CYP1A mRNA in the gut might have resulted from the ingestion of βNF with food. Van Veld et al. [9] speculated that the low sensitivity of hepatocellular CYP1A to dietary B*a*P exposure could be caused by efficient biotransformation of B*a*P in the gut before reaching the liver.

Acute exposure to CYP1A inducers may lead to higher gill expression of CYP1A mRNA compared to liver expression [9], and low concentrations of certain xenobiotics may be sufficient to induce CYP1A in tissues such as gill pillar cells [28,29]. The gill is an organ in direct contact with xenobiotic-contaminated water. The gill also contains a cytochrome P450 monooxygenase system similar to that in the liver. However, CYP1A activity in the gill usually shows a much lower activity compared to that in the liver [30,31]. Therefore, in the present study, the delayed peak of CYP1A mRNA induction in the gill of βNF-treated fish relative to the hepatic counterpart may result from its lower catalytic rate. A study on the induction of CYP1A in liver and gill of rainbow trout exposed to B*a*P via water has been reported [24]. The hepatic CYP1A mRNA of rainbow trout treated with B*a*P peaked within 24 h of exposure and then decreased to basal levels between 72 and 120 h of exposure. In contrast, the gill CYP1A mRNA rapidly increased after 6 h of exposure and then gradually increased more through 120 h of exposure. In the present study, the hepatic CYP1A mRNA increased after 1 d of exposure to βNF and then decreased to basal levels, whereas CYP1A mRNA levels in the gill peaked after 4 d of exposure. The time with the maximal induction of CYP1A mRNA in the gill coincided with the period during which the maximally induced hepatic CYP1A mRNA returned to the basal level. Other studies suggested that CYP1A-catalyzed, first-pass metabolism of certain CYP1A inducers in the gill reduces the amount of parent inducers, which then reach internal organs, such as the liver [31–33]. Thus, the gill as well as the liver may play an important role in the biotransformation of waterborne xenobiotics. A study with mummichog exposed to 10 μg/L of B*a*P, which may surpass the CYP1A-mediated, first-pass metabolic capacity for B*a*P in the gill, showed that the CYP1A protein level remained elevated in hepatocytes and gill pillar cells over the 12-d exposure period [9]. At higher doses, the dietary inducer can escape the metabolism in the gut and, thus, reach the liver [34]. Waterborne and dietary contaminants enter the body through the gill and intestine, but they can be subjected to initial biotransformation in these tissues. The remaining parent xenobiotics can finally reach the liver, stimulating the prolonged expression of CYP1A mRNA [9,35]. Thus, the exposure route and dose may affect the pattern of CYP1A induction in various tissues. Another study concerning the pattern of CYP1A induction in several tissues of tilapia (*Oreochromis mossambicus*) has been reported [36]. The present result is in contrast with that of this earlier report on the pattern of CYP1A induction in tilapia, in which CYP1A mRNA in the gill did not change after exposure to coastal sediment samples for 3 or 7 d. These differences may also result from the difference of exposure route. Both PAHs and PCBs have very

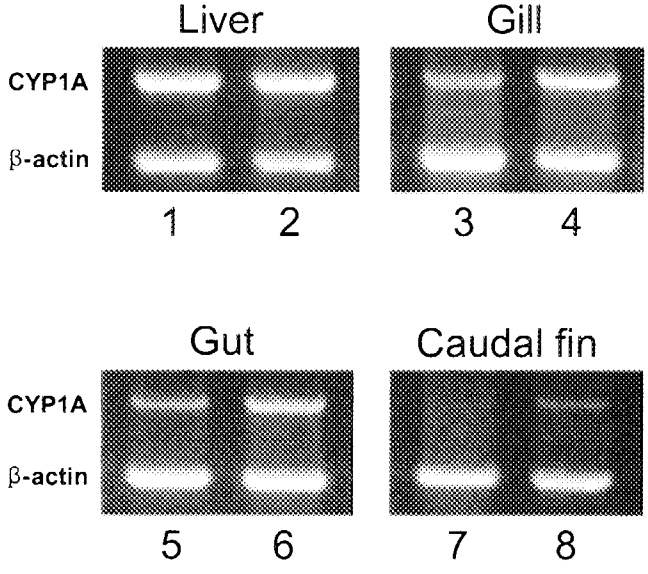

Fig. 5. Reverse transcription-polymerase chain reaction results of cytochrome P4501A (CYP1A) mRNA in liver, gill, gut, and caudal fin of medaka (*Oryzias latipes*) exposed to landfill leachate. Each tissue as indicated represents a pool of the same tissues from four fish of unexposed control (lane 1, 3, 5, and 7) and exposed fish (lane 2, 4, 6, and 8), respectively.

low water solubility. In addition, ingestion of sediments and bottom detritus might be the major route of sediment exposure [36]. Therefore, CYP1A induction by exposure to sediment samples was influenced mainly by oral intake of xenobiotics rather than by aqueous contact. Furthermore, constant oral intake of sediments would result in a continued induction of CYP1A mRNA in intestine. The lower induction of CYP1A mRNA in the liver relative to the gill and gut observed in the current study may result from the first-pass biotransformation of βNF in the gill and gut.

The CYP1A expression in the skin of fish has been hardly examined, although CYP1A induction was observed in the skin of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-exposed larvae of zebrafish [37] and in larvae of gilthead seabream [38]. The present results show relatively greater induction of CYP1A mRNA in the caudal fin of βNF-treated fish compared to that on exposure day 0 in spite of its lower basal levels of CYP1A mRNA. Therefore, measurement of CYP1A mRNA level in the caudal fin may also serve as a good biomarker. Wong et al. [36] showed that induction of the CYP1A gene of fish could become a sensitive early warning biomarker. That study also indicated that the level of CYP1A mRNA in nonhepatic tissues, such as the gill, gut, and caudal fin, might serve as an indirect but useful biomarker for environmental pollution.

To evaluate the utility of CYP1A mRNA measurement in medaka by RT-PCR as a useful biomarker, we studied the expression of CYP1A mRNA in liver, gill, gut, and caudal fin after exposure to landfill leachate. Water percolating through landfills produces leachate, which may contain undesirable or toxic chemicals rich in chloride, heavy metals, and hydrocarbon compounds (e.g., PAHs and HAHs). Some of these chemicals are known to induce the CYP1A gene. Although the chemical composition of the leachate sample used in the present study was not analyzed, CYP1A mRNA was induced in extrahepatic tissues of medaka after exposure to the diluted leachate. The pattern of CYP1A mRNA induction in each tissue of medaka after 1 d of exposure to the diluted leachate was not exactly identical with the time-dependent induction study by βNF exposure, because the leachate as mixture could affect the CYP1A induction differently from a case of exposure to a chemical. At any rate, we could observe the induction of CYP1A mRNA in medaka by exposure to the leachate sample. Therefore, this result indicated that the leachate might contain CYP1A-inducing xenobiotics.

In the present study, the full sequence of CYP1A cDNA of medaka (*Oryzias latipes*) was determined first. A proper pair of primers based on the CYP1A cDNA sequence of medaka were prepared to assess CYP1A mRNA induction by RT-PCR. The results of our experiments using this CYP1A detection system indicated that the gill, gut, and caudal fin were more effective organs than the liver to assess CYP1A mRNA induction in medaka after exposure to βNF and landfill leachate. Therefore, the present study showed a utility for the measurement of CYP1A mRNA levels in the gill, gut, and caudal fin to predict the presence of CYP1A-inducing pollutants in environmental samples.

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