Biochemical and Biophysical Research Communications **271,** 130–137 (2000)
doi:10.1006/bbrc.2000.2543, available online at http://www.idealibrary.com on IDEAL®

In Situ RT-PCR Detection of CYP1A mRNA in Pharyngeal Epithelium and Chondroid Cells from Chemically Untreated Fish: Involvement in Vertebrate Craniofacial Skeletal Development?

Hisato Iwata*,†,[1] and John J. Stegeman*,[2]

**Biology Department, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543; and †Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University, N18 W9 North Ward, Sapporo, Japan*

Received March 1, 2000

Knowledge about the expression sites of cytochrome P450 1A (CYP1A) mRNA is crucial for a better understanding of the physiological function of CYP1A. We investigated the cellular localization of CYP1A mRNA in chemically untreated fish by use of an *in situ* reverse transcription-polymerase chain reaction (IS RT-PCR) technique. The fathead minnow (*Pimephales promelas*) was formalin-fixed, and paraffin-embedded. Sections (5 μm) were treated with trypsinogen. Following reverse transcription of CYP1A mRNA, the cDNA was amplified *in situ* by PCR with specific primers. Detection of the amplicons was accomplished by a second PCR performed with digoxigenin-labeled dUTP. CYP1A mRNA expression was detected in cytoplasm of chondroid cells surrounding hyaline cartilage in gill arches. This result was consistent with that of immunohistochemical analysis with a CYP1A1-specific monoclonal antibody. CYP1A mRNA also was found in stratified squamous epithelium of the pharynx and gill arches, but no staining was detected in those cells by immunohistochemical analysis. The results indicate that IS RT-PCR is an effective/sensitive technique for localizing low level CYP1A expression. In addition, the sites where we identified expression of CYP1A are targets of retinoic acid, *sonic hedgehog* and *Hox* genes, suggesting that functional CYP1A in vertebrates could participate in craniofacial skeletal development through involvement in the retinoic acid signaling cascade.

***Key Words: in situ* RT-PCR; CYP1A; pharyngeal epithelium; chondroid cell; fathead minnow; retinoic acid; craniofacial skeletal development.**

The cytochrome P450s (CYPs) are a superfamily of monooxygenase enzymes that play an important role in the biotransformation of endogenous and exogenous compounds. Among the CYP isozymes, the members of CYP1A subfamily are known to be inducible by polynuclear aromatic hydrocarbons (PAHs) and planar halogenated aromatic hydrocarbons such as polychlorinated dibenzo-*p*-dioxins, dibenzofurans, and coplaner polychlorinated biphenyls (1).

The toxicological functions of CYP1A enzymes involve the activation of numerous PAHs, initiating cell and genetic damage, and may involve the release of superoxide and hydrogen peroxide (2). On the other hand, due to the high evolutionary conservation of CYP1A over 400 million years (1, 3), and the expression during early mammalian embryogenesis (3), it was speculated that CYP1A may play an important role in development, possibly associated with metabolism of some endogenous regulatory molecule(s) by CYP1A. Although some endogenous substrates specifically metabolized by CYP1A have been recently reported (4, 5), the physiological function of CYP1A is not yet known.

One approach to a better understanding of possible physiological role(s) of CYP1A is to clarify the *in-situ* occurrence of CYP1A protein or mRNA in different types of cells and organs in chemically untreated animals, in which CYP1A may be constitutively expressed at low levels. Previous reports have documented a mild immunohistochemical staining of CYP1A protein in untreated teleost fish but in few sites, such as hepatocyte and gill pillar cells where CYP1A is readily induced by xenobiotics (6, 7). CYP1A mRNA expression was not detectable in any tissues of untreated mouse by *in situ* hybridization (8). Further identifying the cellular localization of putatively constitutive CYP1A, particularly in physiologically relevant sites, would

[1] Present address: Center for Marine Environmental Studies, Ehime University, 3-5-7 Tarumi, Matsuyama 790-8566, Japan.
[2] To whom correspondence should be addressed. Fax: +1-508-457-2169. E-mail: jstegeman@whoi.edu.

0006-291X/00 $35.00

help to understand not only the native function of CYP1A but also the potential targets of toxicity associated with CYP1A expression.

Fathead minnow (*Pimephales promelas*) appears to be moderately sensitive to PAH carcinogenesis compared to some other fish species such as medaka (9). Such low sensitivity to PAH procarcinogens may be due partly to a lesser induction of CYP1A that activates those compounds in this species (10). The low response of CYP1A to xenobiotic inducers suggests that this species may be suitable to investigate endogenous function of CYP1A that is different from xenobiotic metabolism. The present study therefore attempted to detect constitutive CYP1A expression in untreated fathead minnow. Previous immunohistochemical studies using monoclonal antibodies to CYP 1A have revealed that in untreated fathead minnow no staining was detected in sites where CYP 1A commonly is induced, while in fish treated with the non-*ortho* coplanar PCB-77, frequent and strong staining was identified in those cells, for example in endothelium of several organs (10). Application of more sensitive analytical methods may enable the detection of CYP1A expression.

In recent years, a number of papers have reported the possibility of detecting nucleic acids in tissues, taking advantage of the amplifying potential of the polymerase chain reaction (PCR). Particularly, *in situ* reverse transcription-polymerase chain reaction (IS RT-PCR) techniques have been successful in detecting low copy number viral and human RNAs in formalin fixed and paraffin embedded archival material, and in evaluating the cellular localization (11–13). Therefore, this study was designed to develop the IS RT-PCR method for CYP1A, to determine the cellular localization of CYP 1A in untreated animals, and to consider the physiological function(s) associated with expression site(s). To our knowledge, this is the first report on the use of IS RT-PCR to detect CYP 1A in biological tissues.

MATERIALS AND METHODS

Primer design and preliminary experiments. Prior to the *in situ* RT-PCR, sense and antisense primers for CYP1A were designed for fathead minnow. Using the designed primers, PCR conditions and temperatures within the thermal cycles were established by standard PCR in solution phase in order to obtain a neat and single band of the anticipated number of base pair.

Fresh liver samples were taken from two adult male minnows, immediately frozen in liquid nitrogen, and stored at −80°C until use. Total RNAs were extracted from the pooled liver by the AGPC method (14) using RNA STAT-60 (TEL-TEST, Friendswood, TX).

Primers were designed based on the highly conserved regions of 12 teleost CYP1A sequences including killifish (Accession No. AF026800), oyster toadfish (U14161), gilthead sea bream (AF011223), scup (U14162), four-eye butterfly fish (U19855), European sea bass (U78316), plaice (X73631), Atlantic tomcod (L41886), and rainbow trout (1A1V1; S69278, 1A1V2; U62796, 1A1V3; U62797, and 1A3; S69277) which are retrievable from GenBank. The sense primer was (5′-ACA AGG ACA ACA TCC GTG AC-3′) and the antisense primer was (5′-TCA TGG TTG ATC TGC CAC TG-3′), which were positioned upstream from the cysteine-containing heme-binding region. The amplicon of mRNA after RT-PCR is derived from exons 2, 3, 4 and 5. The expected amplicon length from mRNA is 430 bp.

Nine hundred ng of total RNA was dissolved in 20 μl of solution mixture containing 1× PCR buffer II, 5 mM $MgCl_2$, 1 mM dNTP mixture, 2.5 μM Oligo $d(T)_{16}$, 20 units RNase inhibitor and 50 units MuLV reverse transcriptase (Perkin-Elmer, Foster City, CA), and the RNA was reverse-transcribed to cDNA on PCR Thermal Cycler (GeneAmp PCR System 9700, Perkin-Elmer). Temperature conditions were: 10 min for extension of Oligo $d(T)_{16}$ by reverse transcriptase at 30°C, 15 min for further reaction at 42°C, 5 min for transcriptase deactivation at 99°C, and finally 5 min at 5°C. From the reaction mixture, 0.5 μl of the solution was used for the following PCR.

The cDNA was amplified with the 0.5 μM primers in 50 μl PCR mixture containing 1× PCR buffer II, 1.5 mM $MgCl_2$, 0.2 mM dNTP mixture and 1.25 units AmpliTaq Gold DNA polymerase (Perkin-Elmer) on PCR Thermal Cycler. The reaction was started with a single 12 min cycle at 95°C followed by 35 cycles of 30 sec denaturation at 95°C, 1 min annealing at 45°C, 1 min extension at 72°C, and was stopped after 12 min hold at 72°C. PCR products were run on a 2.0% (w/v) agarose gel and a single band of the expected base pair (430 bp) was detected.

The fresh PCR products were purified by the Geneclean III kit (BIO 101, Carlsbad, CA), and immediately cloned into the pGEM-T Easy Vector (Promega, Madison, WI), followed by the transforming of JM109 competent cells with that plasmid DNA. The competent cells were cultured for 1.5 h at 37°C. The cells from the selected colonies were cultured overnight in liquid LB (Luria-Bertani's) broth. The plasmids were purified using QIAprep Spin Miniprep Kit (QIAGEN, Stanford, CA), and were sequenced for the confirmation of minnow CYP 1A mRNA amplification.

Section preparation and IS RT-PCR. Head regions from immature female minnows, split lengthwise were conventionally processed according to the method previously reported (10). Sections (5 μm thick) were mounted on silane-coated slides and dried at room temperature. The slides were then rinsed three times for 10 min in clearant, followed by dehydration twice for 10 min each time in 100% ethanol. The sections were mildly digested by trypsinogen (2 mg/ml in 0.1 N HCl, Sigma Chemical, St. Louis, MO) for 12 min at room temperature. The digestion was stopped by several washes in TBS buffer.

Ingredients in 25 μl reverse transcriptase solution were the same as those for the soluble phase RT. The solution was applied to each section in Frame-Seal Incubation Chambers (MJ Research, Watertown, MA) on slides. The sealed slides were placed on a heat block of the PCR Thermal Cycler, and incubated according to a temperature program as follows: 30°C for 30 min; 42°C for one hour followed by 5°C for 10 min. After incubation, the solution in the incubation chambers was carefully removed by pipetting and the section was briefly washed in PBS buffer.

The PCR mixture (25 μl) identical to the standard PCR solution was applied to each tissue sample on a slide, sealed in incubation chambers, and placed in the PCR Thermal Cycler. The thermal cycling program consisted of 95°C for 12 min, followed by 35 cycles of 95°C for 30 sec, 45°C for 60 sec and 72°C for 60 sec, and finally 72°C for 12 min. The primary amplicon was labeled during a second PCR by including a single cycle of denaturation, annealing, and extension (95°C for 1 min, 45°C for 90 sec, and 72°C for 90 sec) in 25 μl of the PCR mixture containing 0.2 mM of dATP, dCTP and dGTP, 0.13 mM of dTTP, and 0.07 mM of digoxigenin (DIG)-11-dUTP (Roche Molecular Biochemicals, Indianapolis, IN).

Digoxigenin detection. After labeling the PCR products, the slides were washed twice in Tris-NaCl (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl) for 10 min and immersed in blocking buffer (Roche Molec-

ular Biochemicals) for 15 min. The detection of the incorporated DIG-labeled dUTP was performed with a 100 μl of highly specific anti-DIG antibody conjugated with alkaline phosphatase (Roche Molecular Biochemicals) solution, which was diluted 1:500 in blocking buffer and then incubated for 30 min at room temperature. For visualization of the product, the slides were incubated in 15 ml of freshly prepared nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (Roche Molecular Biochemicals) for 15–30 min. The stained sections were mounted with crystal mount.

Controls. In order to account for the possible detection of false signals, three types of controls were used (11). As a positive control to demonstrate whether cDNA is amplified *in situ* using the newly designed primers, and under the examined conditions for PCR mixture and temperatures, we examined a fixed section from heart of scup (*Stenotomus chrysops*) treated with coplanar PCB 77, which previously was shown to have a high level of CYP 1A expression (6). Another control was a minnow sample which was incubated without Mg^{2+} at the RT step. In this section, amplification only of genomic DNA, which should give a nuclear signal, was expected due to the lack of reverse transcription of mRNA to cDNA. An additional control involved incubation of minnow sections with no primers at the DIG-dUTP labeling step. The control was to test for non-specific binding of DIG-dUTP independent of primers. The two controls, omission of Mg^{2+} or primers, were simultaneously processed with the matching serial sections incubated with complete conditions, expected to show specific mRNA signals.

Immunohistochemistry. Immunohistochemical analysis with the CYP1A1-specific monoclonal antibody (Mab) 1-12-3 that recognizes a distinct fish CYP1A epitope was also performed using the serial sections. The analysis was processed according to the protocol provided by Lindstrom-Seppa *et al.* (10).

RESULTS

Cloning of Fathead Minnow CYP1A

Prior to IS RT-PCR for CYP1A, we evaluated primers by cloning a minnow CYP1A fragment. The PCR products (430 base pairs including both 5′- and 3′-end PCR primer regions) obtained from the PCR reaction were cloned and sequenced. The nucleotide and the deduced amino acid sequences of the cDNA fragment between the two primer sites are shown in Fig. 1. The nucleotide sequence has been deposited in the GenBank database under accession number AF232749. The 390 bp nucleotide and 130 amino acid sequences share 72.8–78.2% and 80.8–90.8% identities with corresponding regions of other teleost CYP1A sequences, respectively (Fig. 2), while the percentage identities of the minnow CYP1A nucleotide and amino acid sequences with human CYP1A1 were 66.2 and 64.6%, respectively. The minnow gene has sequences 1-ITDSLIDHCEDRKLDEN-17 and 23-SDEKIVGIVNDLFGAGFDT-41 that are highly conserved among teleost CYP1As, as previously reported (15). Some amino acids of the minnow CYP1A were characteristically identical to the rainbow trout CYP1As (which shared higher identities to minnow CYP1A than any other teleost CYP1A) at residues 22 (V), 49 (A), 51 (V), 67 (L), 73 (M), 78 (R), and 87 (L). Based on the analysis of the PCR fragment, we could confirm that the cDNA product was derived from CYP1A mRNA of fathead minnow.

```
        10        20        30        40        50        60
ATCACCGACTCGCTCATCGACCACTGCGAGGACCGCAAGCTGGACGAGAACTCCAACGTT
 I  T  D  S  L  I  D  H  C  E  D  R  K  L  D  E  N  S  N  V

        70        80        90       100       110       120
CAAGTGTCCGATGAGAAGATCGTCGGAATCGTCAATGACCTCTTCGGGGCCGGCTTCGAC
 Q  V  S  D  E  K  I  V  G  I  V  N  D  L  F  G  A  G  F  D

       130       140       150       160       170       180
ACCATCAGCACGGCTCTGTCCTGGGCTGTCGTCTATCTGGTGGCCTATCCCGAGATCCAG
 T  I  S  T  A  L  S  W  A  V  V  Y  L  V  A  Y  P  E  I  Q

       190       200       210       220       230       240
GAGCGCCTGCAGGGAGAACTGAGAGAGAAGATCGGAATGGATCGTATGCCGCGCTTGTCG
 E  R  L  Q  G  E  L  R  E  K  I  G  M  D  R  M  P  R  L  S

       250       260       270       280       290       300
GACCGAACGGACCTGCCGCTTCTCGAGGCCTTTATCCTGGAGATCTTCCGGCACTCCTCC
 D  R  T  D  L  P  L  L  E  A  F  I  L  E  I  F  R  H  S  S

       310       320       330       340       350       360
TTCCTCCCGTTCACCATCCCTCACTGTACGTCGAAAGACACGTCGCTCAACGGATACTTC
 F  L  P  F  T  I  P  H  C  T  S  K  D  T  S  L  N  G  Y  F

       370       380       390
ATTCCCAGAGACACCTGTGTGTTTATAAAC
 I  P  R  D  T  C  V  F  I  N
```

FIG. 1. Nucleotide and deduced amino acid sequence of the CYP1A cDNA fragment from fathead minnow.

IS RT-PCR Using Scup Sections as a Positive Control

Microscopic examination of heart sections of scup treated with coplanar PCB 77 revealed that CYP1A mRNA expression was found in the endothelial cells of the endocardium in tested sections (Fig. 3A). The staining was present in the cytoplasm, as the expected site of CYP 1A synthesis.

Figure 3B shows a control section in which the RT step was omitted. An intense nuclear staining, derived from the signal of amplified genomic DNA, was observed in most of the cells. In contrast, less staining was apparent in another control sample in which the set of specific primers were omitted at the DIG-dUTP labeling step (Fig. 3C). Thus, the specificity of the cardiac staining site was ascertained by an array of controls, and a similarity of the staining sites between this IS RT-PCR and reported immunohistochemistry (6).

IS RT-PCR Using Fathead Minnow Sections

Microscopic examination of minnow sections (Fig. 4A) revealed that CYP 1A mRNA was found in some so-called chondroid cells (or hyaline-cell cartilage; 16) in gill arches. The cytoplasmic signals in the cells were clearly distinguishable from those in the accompanying two controls, in which strong nuclear (Fig. 4B) and negligible cytoplasmic signals (Fig. 4C) were detected.

Another site where staining derived from CYP1A mRNA was found was in stratified squamous epithelium of the pharynx (Fig. 5A) and gill arches (Fig. 6A). The cytoplasmic signals in these epithelial cells also were distinguishable from those in a series of controls in which Mg^{2+} (Figs. 5B and 6B) or primer (Figs. 5C and 6C) were omitted. No other staining sites were recognized in these head sections.

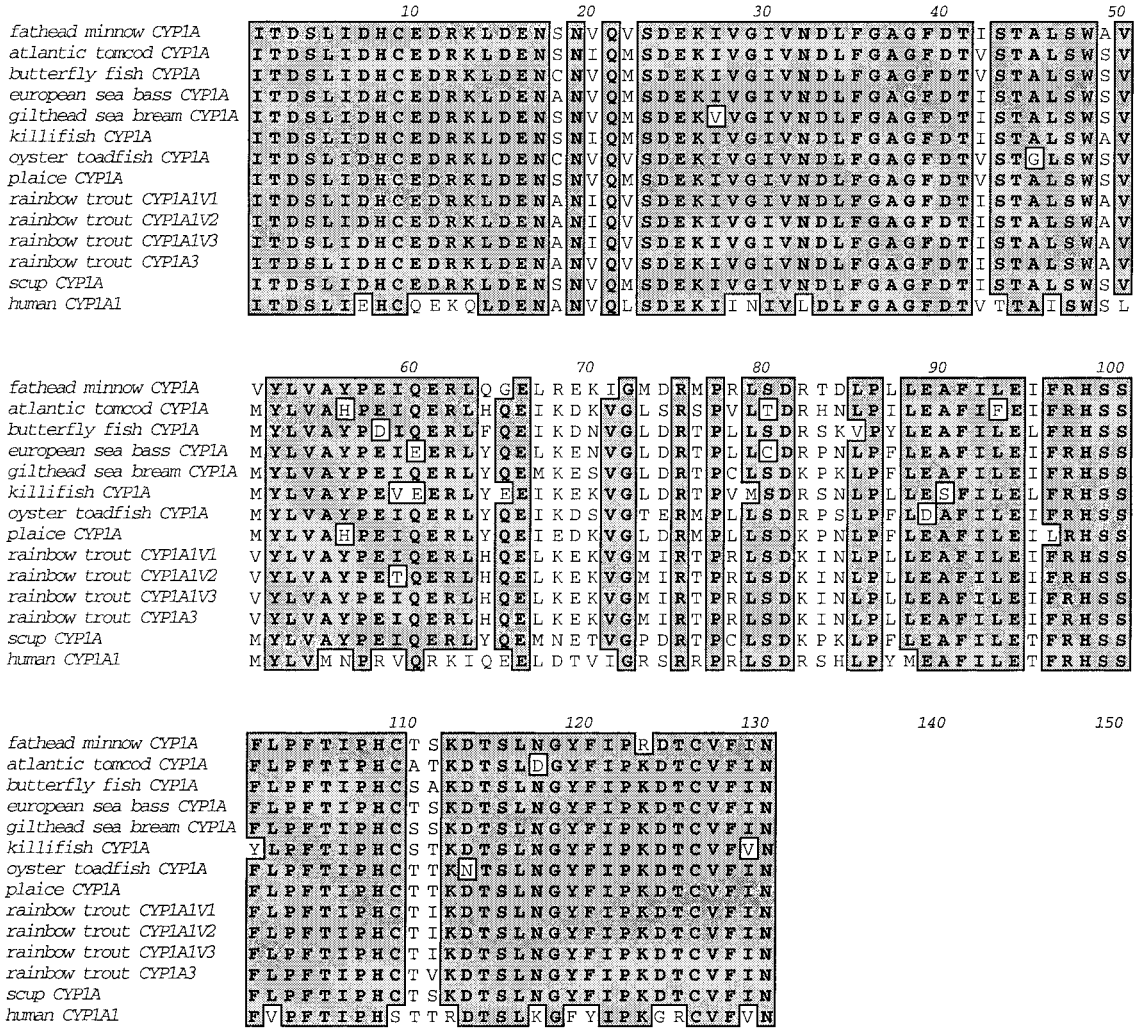


FIG. 2. Alignment of the fathead minnow CYP1A sequence and other teleost and human CYP1A sequences using the MacVector 6.5 program. This coding region corresponds to the 281–410 amino acids in human CYP1A1 sequence. Boxes represent amino acid where more than 75% of the amino acid are identical in all the CYP1A sequences analyzed.

Immunohistochemistry

Staining for CYP 1A protein using sections prepared from the same paraffin-embedded samples as those used for IS RT-PCR was also performed by immunohistochemistry with Mab 1-12-3. The result demonstrated a strong staining in some chondroid cells in which CYP1A mRNA was amplified (Fig. 4D). The finding was compatible with the cellular distribution of the CYP 1A mRNA observed in sections processed with IS RT-PCR. In contrast to this site, no protein staining was detectable in epithelium of pharynx and gill arches by immunohistochemical analyses (Figs. 5D and 6D).

DISCUSSION

IS RT-PCR

This study demonstrates the utility of IS RT-PCR in detecting low copy CYP1A mRNA expression. Previous studies have pointed out that a key parameter in IS RT-PCR is protease digestion, which depends on the type of tissue, the fixative used, and the extent of the fixation (11–13). In this study, we used trypsinogen instead of protease K, which has been commonly used for IS RT-PCR, since initial attempts using protease K led to an over-digestion of minnow sections. The optimal digestion time, which is defined by preserved cell feature and intense nuclear staining in a control section without the RT step, was empirically determined by testing a variety of protease digestion times.

By first testing IS RT-PCR with sections of heart from scup in which CYP1A had been induced by treatment with PCB77, we were able to establish the procedure. CYP1A mRNA was localized in the endothelial cells of the endocardium, where CYP1A proteins had previously been detected by immunohistochemistry (6). This positive control additionally confirmed that

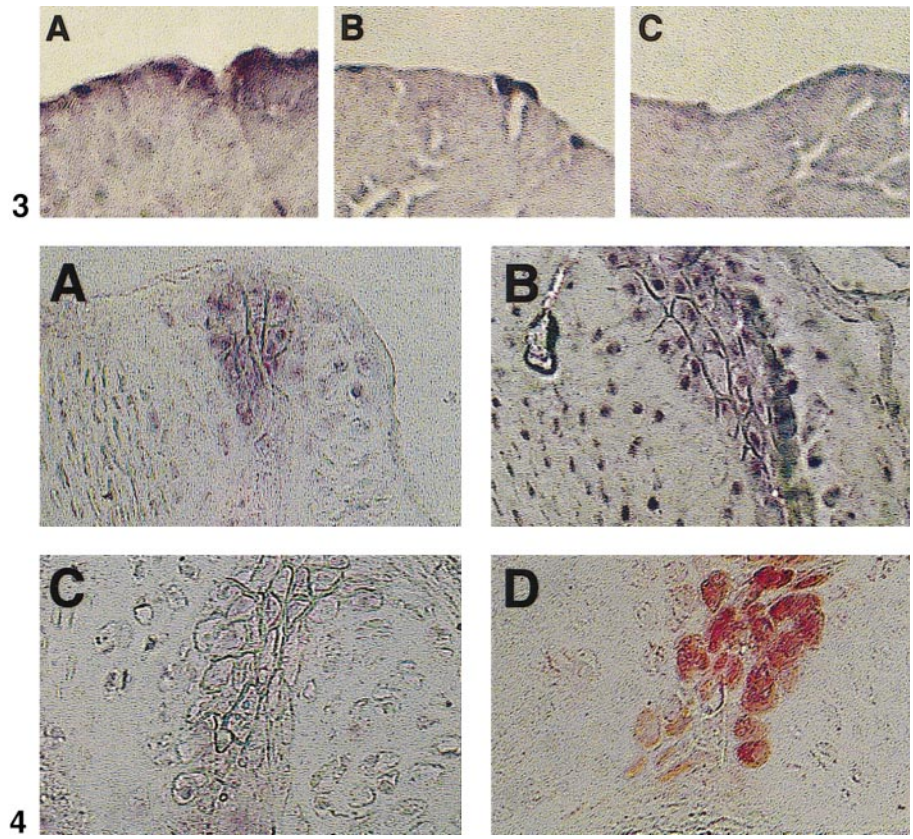


FIG. 3. IS RT-PCR analysis of CYP1A mRNA expression in heart sections from coplanar PCB-77 treated scup. (A) Section showing staining of CYP1A mRNA in cytoplasm of the endocardial endothelium (920×). (B) Control section with no Mg^{2+} for reverse transcription (920×). Nuclear staining for genomic DNA amplification was observed in endocardial cells. (C) Control section with no primers for DIG-dUTP labeled PCR (920×). Less staining was present in endocardial cells.

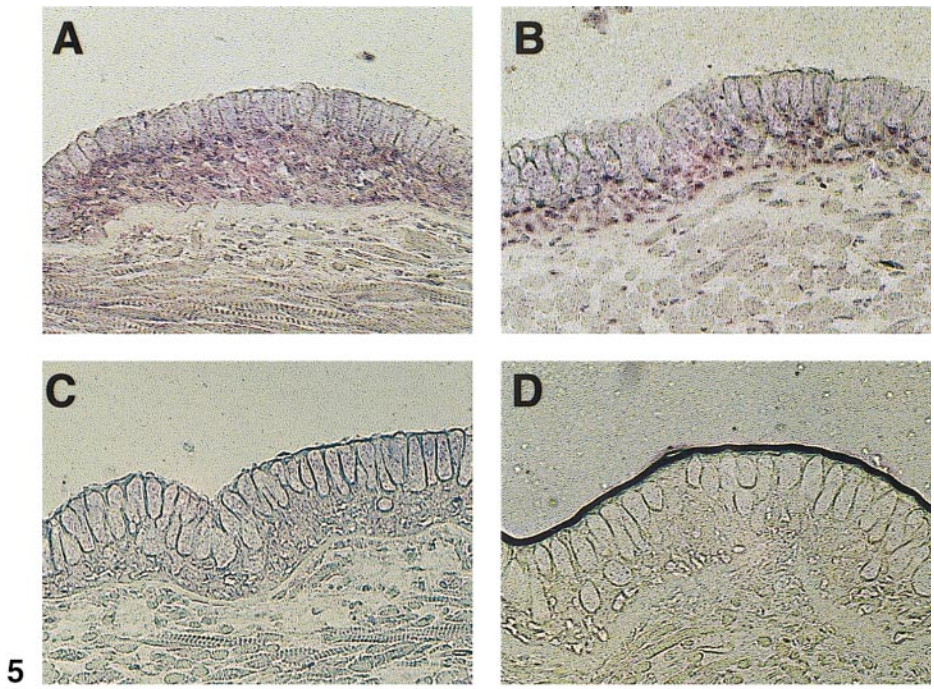
FIG. 4. IS RT-PCR (A, B, and C) and immunohistochemical analyses (D) of CYP1A expression in chondroid cells from chemically untreated fathead minnow. (A) Section showing staining of CYP1A mRNA in cytoplasm of chondroid cells (hyaline-cell cartilage) in gill arches (460×). (B) Control section with no Mg^{2+} for reverse transcription (460×). Nuclear staining for genomic DNA amplification was observed in chondroid cells. (C) Control section with no primers for DIG-dUTP labeled PCR (460×). Less staining was present in chondroid cells. (D) Section showing staining of CYP 1A protein in chondroid cells (460×).

CYP1A induction in teleost endothelial cells, which has been studied extensively, is actually a transcriptional event.

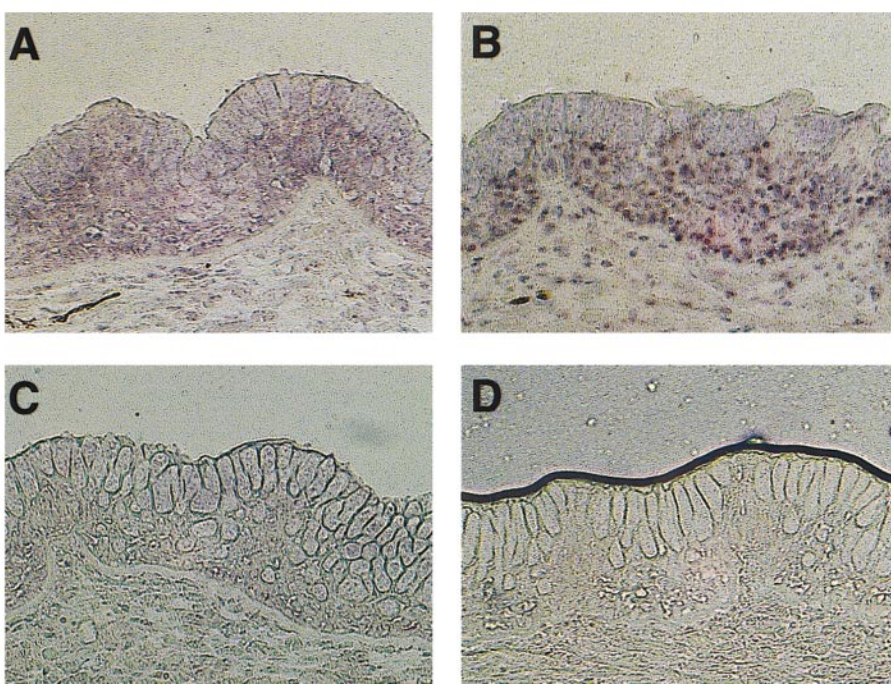
Following the analysis of scup heart sections, the IS RT-PCR technique was applied to tissue sections from chemically untreated fathead minnow. The specificity of the primers determined first by cloning a fragment of minnow CYP1A, enabled us to detect *in-situ* CYP 1A mRNA that seemed to be expressed at low levels. Due to the well-distinguishable spatial resolution of signals, we were able to define the site of these signals to be within the cytoplasm of individual cells. In the fathead minnow samples we examined, CYP1A mRNA was found to be expressed in chondroid cells of gill arches, and also in epithelium of the pharyngeal area.

The success of this method critically depended on an array of controls. The strong dotted staining seen when Mg^{2+} was omitted indicates amplification of genomic DNA, giving a specific signal within nuclei. The clear nuclear signals showed that the diffusion of the amplified products in the nucleus was minimal. In another control where one single PCR cycle was performed without the primer pair but in the presence of DIG-labeled UTP following the normal PCR step, the signal was less, or nondetectable. This indicates that nonspecific binding of DIG-dUTP independent of primers was negligible compared with the primer-dependent specific binding that was seen in the sections simultaneously tested with complete reaction mixture.

Comparison of immunohistochemistry and IS RT-PCR analyses using serial sections of minnow head provided evidence for the sensitivity of IS RT-PCR. In chondroid cells, both techniques gave specific signals, indicating both CYP1A mRNA and CYP1A protein expression. Notably, the immunohistochemical detection of CYP1A protein in pharyngeal chondroid cells observed here was relatively strong, like the results in this cell type in other locations (17). In contrast to the chondroid cells, we were able to detect CYP 1A mRNA signals in epithelium of the pharyngeal area by IS



5



6

FIG. 5. IS RT-PCR (A, B, and C) and immunohistochemical analyses (D) of CYP1A expression in epithelial cells of pharynx from chemically untreated fathead minnow. (A) Section showing staining of CYP1A mRNA in cytoplasm of epithelial cells in pharynx (460×). (B) Control section with no Mg^{2+} for reverse transcription (460×). Nuclear staining for genomic DNA amplification was observed in epithelial cells. (C) Control section with no primers for DIG-dUTP labeled PCR (460×). Less staining was present in epithelial cells. (D) Section showing no staining of CYP 1A protein in epithelial cells (460×).

FIG. 6. IS RT-PCR (A, B, and C) and immunohistochemical analyses (D) of CYP1A expression in epithelial cells of gill arches from chemically untreated fathead minnow. (A) Section showing staining of CYP1A mRNA in cytoplasm of epithelial cells in gill arches (460×). (B) Control section with no Mg^{2+} for reverse transcription (460×). Nuclear staining for genomic DNA amplification was observed in epithelial cells. (C) Control section with no primers for DIG-dUTP labeled PCR (460×). Less staining was present in epithelial cells. (D) Section showing no staining of CYP 1A protein in epithelial cells (460×).

RT-PCR, while no signal was obtained by immunohistochemistry. This indicates that the IS RT-PCR method is a more sensitive technique for localizing CYP1A at lower expression levels than immunohistochemistry, supporting results from studies which employed both methods to examine other genes (12).

The lack of CYP1A protein detection in pharyngeal epithelium suggests low levels of expression. Another possible interpretation of the lack of detection of CYP1A protein is that post-transcriptional suppression of CYP1A may have occurred in this site. It has been shown that some CYP1A inducers that are slowly metabolized CYP1A substrates suppress CYP1A protein levels in vertebrates (18–20). A recent study proposed that reactive oxygen species released from uncoupling of CYP1A may account for the suppression (2). CYP1A suppression by xenobiotics is pronounced only at high doses of planar halogenated aromatic hydrocarbons, which are very slowly metabolized (2). By comparison, that would suggest that some endogenous inducer/substrate capable of uncoupling CYP1A would have to be present at high levels in pharyngeal epithelium. We do not consider it likely that such a process is involved here.

CYP1A Expression Sites and Its Physiological Function

The observation of CYP1A expression in epithelial and chondroid cells in the pharyngeal area of chemically untreated fish does suggest the presence of some endogenous CYP1A inducer/substrate. From a phylogenetic viewpoint these sites are known to be in an area of importance during development. The epithelium of the pharynx contributes to the formation of gills in chordates (21), and the cartilage and surrounding connective tissue are present in the majority of vertebrates, but not in invertebrates. It is thus interesting to consider the evolutionary emergence of CYP1A in association with possible physiological function. CYP1A protein and/or CYP1A genes have been found in early-diverging chordates such as elasmobranch and teleost fish, and all "higher" vertebrates groups (15). The circumstance in agnathan fish is less clear. In hagfish, the earliest diverging agnathan fish, CYP1A protein was not detected by Mab 1-12-3, but polyclonal antibody to cod CYP1A did detect a band, and ethoxyresorufin-*O*-deethylase (EROD) activity was measurable, but neither were inducible. In lamprey, no protein bands were detected using Mab 1-12-3 or anti-cod CYP1A, and no EROD activity was detectable, in untreated or in aryl hydrocarbon receptor (AhR) agonist-treated animals (22). Considering our findings of CYP1A expression sites in minnow and the evidence suggesting a fundamental change in the AhR-CYP1A pathway occurring early in vertebrate emergence, it is possible that both AhR and CYP1A may play crucial roles in developmental mechanisms underlying a key transition in early stages of the vertebrate evolution.

Apart from the information on the CYP1A expression sites and evolutionary emergence, association of CYP1A function with vertebrate development is indirectly suggested by the following knowledge: [1] CYP1A1 in rat and rabbit liver was shown to be responsible for the catalytic reaction from retinal to retinoic acid (4, 5), [2] cartilaginous/chondrogenic cells are a retinoic acid target during skeletal development in chick and mouse embryos (23, 24), [3] excess retinoic acid causes skeletal malformations in vertebrate embryos, [4] retinoic acid treatment depressed *sonic hedgehog* and *Hoxd-4* expression in the pharyngeal area of flounder embryos and led to size reduction of pharyngeal cartilages (25), [5] *sonic hedgehog* in epithelium participates in craniofacial morphogenesis and is down-regulated by excess retinoic acid (26), [6] growth retardation in cartilage of gill arches was observed in TCDD treated zebrafish embryo (27). Our present report in addition to these earlier findings suggest that functional CYP1A in vertebrates could participate in craniofacial skeletal development through involvement in the retinoic acid signaling cascade. Examining further the cellular and developmental expression of CYP1A and related genes (AhR, etc.) in untreated fish may help to clarify this situation.

A recent study demonstrated that a Cyp1a1(−/−) knockout mouse is viable and has no obvious effect on phenotype (28). However, even under chemically untreated conditions, the knockout mouse showed strong expression of CYP1A2, which could have compensated for the absence of CYP1A1 (28). Many early-diverging vertebrate species appear to have only one CYP1A (15), and may provide an interesting counter-point to the Cyp1a1(−/−) knockout mouse study in evaluating a physiological function of CYP1A.

ACKNOWLEDGMENTS

The authors thank Dr. Mark E. Hahn for the critical reading of the manuscript. This study was partly supported by Postdoctoral Fellowships for Research Abroad from the Japan Society for the Promotion of Science, and Grants-in-aid for the Scientific Research from the Ministry of Education, Science and Culture of Japan (Nos. 09306021 and 09876084) awarded to Hisato Iwata. Financial support was also done by NIH grant P42-ES07381 and EPA grant R827102010. Contribution Number 10127 of Biology Department, Woods Hole Oceanographic Institution.

REFERENCES

1. Stegeman, J. J., and Hahn, M. E. (1994) *in* Biochemical, Cellular and Molecular Biological Perspectives in Aquatic Toxicology (Ostrander, D., and Malins, D., Eds.), pp. 87–206 Lewis Press.
2. Schlezinger, J. J., White, R. D., and Stegeman, J. J. (1999) *Mol. Pharmacol.* **56,** 588–597.

3. Dey, A., and Nebert, D. W. (1998) *Biochem. Biophys. Res. Commun.* **251,** 657–661.
4. Tomita, S., Okuyama, E., Ohnishi. T., and Ichikawa, Y. (1996) *Biochim. Biophys. Acta* **1290,** 273–81.
5. Raner, G. M., Vaz, A. D., and Coon, M. J. (1996) *Mol. Pharmacol.* **9,** 515–522.
6. Smolowitz, R. M., Hahn, M., and Stegeman, J. J. (1991) *Drug Metab. Dispos.* **19,** 113–123.
7. Husoy, A. M., Myers, M. S., Willis, M. L., Collier, T. K., Celander, M., and Goksoyr, A. (1994) *Toxicol. Appl. Pharmacol.* **129,** 294–308.
8. Dey, A., Jones, J. E., and Nebert, D. W. (1999) *Biochem. Pharmacol.* **58,** 525–537.
9. Hawkins, W. E., Overstreet, R. M., and Walker, W. W. (1988) *Aquat. Toxicol.* **11,** 113–128.
10. Lindstrom-Seppa, P., Korytko, P. J., Hahn, M., and Stegeman, J. J. (1994) *Aquat. Toxicol.* **28,** 147–167.
11. Martinez, A., Miller, M. J., Quinn, K., Unsworth, E. J., Ebina, M., and Cuttitta, F. J. (1995) *Histochem. Cytochem.* **43,** 739–47.
12. Bates, P. J., Sanderson, G., Holgate, S. T., and Johnston, S. L. (1997) *J. Virol. Methods* **67,** 153–160.
13. Wolfahrt, S., Kleine, B., and Rossmanith, W. G. (1998) *Mol. Hum. Reprod.* **4,** 999–1006.
14. Chomczynski, P., and Sacchi, N. (1987) *Ann. Biochem.* **162,** 156–159.
15. Morrison, H. G., Weil, E. J., Karchner, S. I., Sogin, M. L., and Stegeman, J. J. (1998) *Comp. Biochem. Physiol. C* **121,** 231–240.
16. Benjamin, M. (1989) *J. Anat.* **169,** 153–172.
17. Stegeman, J. J., Lindstrom-Seppa, P., Knipe, T., Suter, S., and Smolowitz, R. M. (1994) *in* 10th International Symposium on Microsomes & Drug Oxidations, p. 339. Toronto, Canada.
18. Gooch, J. W., Elskus, A. A., Kloepper-Sams, P. J., Hahn, M. E., and Stegeman, J. J. (1989) *Toxicol. Appl. Pharmacol.* **98,** 422–433.
19. Stegeman, J. J., Hahn, M. E., Weisbrod, R., Woodin, B. R., Joy, J. S., Najibi, S., and Cohen, R. A. (1995) *Mol. Pharmacol.* **47,** 296–306.
20. White, R. D., Shea, D., Solow, A. R., and Stegeman, J. J. (1997) *Biochem. Pharmacol.* **53,** 1029–40.
21. Ogasawara, M., Wada, H., Peters, H., and Satoh, N. (1999) *Development* **126,** 2539–2550.
22. Hahn, M. E., Woodin, B. R., Stegeman, J. J., and Tillit, D. E. (1998) *Comp. Biochem. Physiol. C* **120,** 67–75.
23. Yamaguchi, M., Nakamoto, M., Honda, H., Nakagawa, T., Fujita, H., Nakamura, T., Hirai, H., Narumiya, S., and Kakizuka, A. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 7491–7496.
24. Koyama, E. Golden, E. B., Kirsch. T., Adams, S. L., Chandraratna, R. A., Michaille, J. J., and Pacifici, M. (1999) *Dev. Biol.* **208,** 375–391.
25. Suzuki, T., Oohara, T., and Kurokawa, T. (1999) *Dev. Growth Differ.* **41,** 143–152.
26. Helms, J. A., Kim, C. H., Hu, D., Minkoff, R., Thaller, C., and Eichele, G. (1997) *Dev. Biol.* **187,** 25–35.
27. Henry, T. R., Spitsbergen, J. M., Hornung, M. W., Abnet, C. C., and Peterson, R. E. (1997) *Toxicol. Appl. Pharmacol.* **142,** 56–68.
28. Dalton, T. P, Dieter, M. Z., Matlib, R. S., Childs, N. L., Shertzer, H. G., Genter, M. B., and Nebert, D. W. (2000) *Biochem. Biophys. Res. Commun.* **267,** 184–189.