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Competitive RT-PCR Analysis of Cytochrome P4501A1 Gene Expression in Atlantic Tomcod Larvae

Nirmal K. Roy [1,3]
Simon Courtenay [2]
Isaac Wirgin [1]

[1] Institute of Environmental Medicine
New York University School of Medicine
57 Old Forge Road
Tuxedo, New York 10987

[2] Canadian Department of Fisheries and Oceans
Moncton, New Brunswick
Canada E1C 9B6

[3] Corresponding Author
Phone- 914-731-3547
Fax-914-351-5472
royn@charlotte.med.nyu.edu

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ABSTRACT

Quantitation of expression of inducible genes such as cytochrome P4501A1 (CYP1A1) in sentinel species is frequentlly used as a biomarker to assess levels of bioavailable chemical contaminants to natural populations. In fish, eggs and larvae are most sensitive to damage from aromatic hydrocarbon pollutants; however, because of their small size quantitative methods are not available to routinely screen for CYP1A1 expression in individual specimens. We developed a quantitative approach to standardize total RNA loading and then used quantitative RT-PCR to compare CYP1A1 mRNA expression levels among individual Atlantic tomcod larvae. Validation of these approach on PCB-exposed and vehicle exposed tomcod larvae showed significant induction of CYP1A1 expression in the chemically exposed group.

INTRODUCTION

One of the ultimate goals of environmental biomonitoring programs is an evaluation of the effects of xenobiotics on ecosystem and human health. Biomarkers in sentinel species are frequently used to assess the relative conditions of impacted terrestrial and aquatic ecosystems (8). Molecular biomarkers can offer the advantage of providing sensitive, dose-responsive, direct links between exposure and biological responses. If a cascade of mechanistic links between responses at lower and immediately higher levels of biological organization are known, molecular changes can be predictive of perturbations at the organismal and sometimes at the population levels (1,12).

Aromatic hydrocarbons are ubiquitous lipophilic contaminants that often concentrate in the sediments and bioaccumulate in the higher trophic levels of polluted aquatic ecosystems. Cytochrome P4501A1 (CYP1A1) induction is widely used as an index of aromatic hydrocarbon contamination of aquatic ecoystems because CYP1A1 expression in fishes is dose-responsive to environmentally relevant concentrations of aromatic hydrocarbons of concern including dioxins, furans, some coplanar PCB congeners, and PAHs (12). CYP1A1-encoded monooxygenase enzymes convert environmental PAHs to reactive electrophiles which can adduct to DNA and proteins. Dioxins and PCBs are refractory or very slowly metabolized, but induction of CYP1A1-encoded activities by these halogenated compounds can still elevate levels of DNA damage through the generation of reactive oxygen species. Populations with high levels of CYP1A1 expression often exhibit elevated levels of DNA damage as detected by ^{32}P postlabelling (13), activation of the K-*ras* oncogene (9), high prevalences of liver tumors (13,6), and early life stage mortality (2). CYP1A1 gene expression can be screened at the mRNA, protein, or enzymatic levels. Evaluation of CYP1A1 expression at the transcriptional level may prove most dose-responsive to bioavailable concentrations of contaminants because of the reduced number of steps in eliciting transcriptional activation compared to induction of translation or enzyme activity.

In many cases, it may be most informative to assess CYP1A1 expression in young life stages of fish from impacted populations. Exposure of young life stages to bioactive xenobiotics is believed to significantly increase teratogencitiy, mortality, decrease recruitment, and subsequent population abundance of adults. Additionally, modulation of CYP1A1 inducibility by hormonal factors is often observed in adults during phases of reproductive activity thus compromising the utility of gene expression in adults as a

measure of contamination (15). Induction of CYP1A1 (10) and bioactivation of PAHs to DNA-reactive metabolites (7) has been demonstrated in fish embryos and larvae.

Atlantic tomcod *Microgadus tomcod* is a bottom-dwelling species whose liver is lipid-rich and serves to bioaccumulate lipophilic xenobiotics such as PCBs and dioxins (5). Tomcod are common in large estuaries along the Atlantic coast of North America from the Hudson River, NY, to Labrador. The Hudson River ecosystem has long been impacted by point and non-point source releases of PCBs, dioxins, and PAHs (16). In the early 1980s, tomcod from the Hudson River exhibited one of the highest prevalences of liver tumors ever observed in a feral population and at the same time the age structure of the Hudson River population was truncated compared to tomcod from elsewhere (6). This suggested that tomcod from the Hudson River were exposed to high levels of bioavailable contaminants and that tomcod were sensitive sentinels of environmental degradation.

We used induction of CYP1A1 mRNA as a biomarker of exposure of adult tomcod to aromatic hydrocarbons in the Hudson River and other, cleaner rivers along the Atlantic coast of North America and as expected levels of gene expression usually corresponded to sediment concentrations of xenobiotic agents that can induce CYP1A1 expression (12). However, when tomcod were collected from the Hudson River, depurated extensively in clean water, and treated in the laboratory under controlled conditions with PCBs and TCDD no induction of CYP1A1 expression was observed. When tomcod from the cleaner Miramichi River, NB, were treated under identical conditions with the same chemicals, high levels of CYP1A1 mRNA induction were observed suggesting that CYP1A1 inducibility with PCBs and TCDD was down-regulated in tomcod from the Hudson River perhaps due to prior exposure to high levels of contaminants (17,5).

We sought to determine if non-inducibility of CYP1A1 in Hudson River tomcod was due to genetic or physiological factors. To address this question, we investigated CYP1A1 mRNA inducibility in hatchery-reared offspring of controlled experimental matings of Hudson River and Miramichi parents. Because of difficulties in husbandry, delays in rearing eggs to adulthood, and the potential for modulation of gene induction in adults by hormonal factors associated with reproductive activity, we analyzed the induciblity of CYP1A1 mRNA in chemically-treated tomcod yolk-sac larvae. Because of their small size and the need in biomarker studies to quantify basal levels of gene expression, it was necessary to develop and validate a quantitative RT-PCR assay for this study.

MATERIALS AND METHODS

Atlantic tomcod broodstock were collected from the Miramichi River in January, 1998, eggs were extruded from 21 females, eggs were fertilized dry with sperm from 21 Miramichi River males, and embryos were divided equally by volume among three McDonald jars. Embyros were reared as described by Peterson (10) until 37-43 d post-fertilization and were then exposed to water-borne PCB congener 77, 3,3',4,4'-tetrachlorobiphenyl (10 ppm) or 0.5% DMSO vehicle for 24 h and then transferred to clean water for an additional 72 hr. Yolk sac larvae were collected and placed in individual 1.5 ml microcentrifuge tubes with 50 µl of H_20, snap frozen in liquid nitrogen, and maintained at -70° C until processing. Larvae were not weighed in this experiment, but the mean wet weight of individual Hudson River tomcod larvae at 7 days posthatch was 0.214 mg and dry weights of larval fish are approximately 10% of wet weight values (C. Chambers, NMFS, pers. comm.).

Five hundred µl of Ultraspec reagent (Biotecx, Houston, TX) was added to each tube containing a single larvae, larvae were homogenized with a micropestle, homogenates were incubated on ice for 10 min, 100 µl of chloroform was added, homogenates were vortexed and incubated on ice for 10 min, and centrifuged at 15,000 g for 15 min at 4° C. The aqueous phase was removed and mixed with an equal volume of cold isopropanol and incubated on ice for 45 min. Total RNA was pelleted by centrifugation at 15,000 g for 15 min at 4° C. RNA pellets were washed twice with cold 70% EtOH, air-dried, resuspended in 50 µl of DEPC-treated water and stored at -80° C.

Using spectrophotometry, we were unable to reliably determine total RNA concentrations of larval samples because absorbance values were too low. Instead, we used slot blots to quantify 18 S and 23 S rRNA hybridization signals in larval RNA samples and compared those to 18 S and 23 S rRNA hybridization signals on the same membranes for adult tomcod liver samples for which total RNA concentrations had been determined previously by spectrophotometry.

For slot blot analysis, larval and adult liver RNAs were brought to 12.5 µl volumes with DEPC-treated H_20, incubated in 1 M glyoxal, 50% (v/v) dimethyl sulphoxide, 10mM sodium phosphate buffer, pH 7.0, at 50° C for 1 hr. Samples were cooled on ice for 30 sec and 10 X SSC stock was added to a final concentration of 6 X SSC. Larval RNAs were then applied with an Schleicher & Schuell (S & S) Slot Blot manifold onto Nytran Nylon Plus (S & S) membranes along with four amounts (0.5 ng, 2.0 ng, 5.0 ng, and 12.5

ng) of adult liver RNAs. Slots were washed 3 times with 6 X SSC and membranes were baked under vacuum for 2 hr. Membranes were prehybridized for 2 hr, hybridized overnight to a ^{32}P radiolabeled rat 18 S rRNA probe (pHRR118) (4) and washed exactly as described in Wirgin et al (12). Hybridization signal for each larval and liver RNA sample was quantified using a Molecular Dynamics PhosphorImager. A standard curve of total adult liver RNA versus hybridization signal was generated for each slot blot and this was used to calculate total RNA concentrations for each larval RNA (Figure 1). This information allowed us to express CYP1A1 levels/ng of total RNA.

For each larval sample, 1 and 10 µl of 1:10 dilutions of RNA were mixed with 0.2 pg of internal control RNA standard (prepared according to Celi et al. (3) in 10 µl volumes. Internal control RNA standard was truncated tomcod CYP1A1 cDNA (11) and designed to include both primer annealing sites. Two µl of random hexamers (150 pmoles each) were added, reactions were incubated at 75°C for 5 min, and chilled on ice for 5 min. Reverse transcriptase mixture was added to each reaction to final concentrations of 1 X M-MLV reverse transcriptase buffer, 0.5 mM dNTPs, 4 U of RNasin ribonuclease inhibitor, 20 U of M-MLV reverse transcriptase (Promega), and reactions were incubated at 42° C for 1 hr. Reaction products were denatured at 98° C for 5 min, chilled on ice for 5 min and an aliquot was subjected to PCR amplification. PCR reactions were in 25 µl volumes containing 1 X reaction buffer (Boehringer Mannheim), 0.2 mM dNTPs, 1 µM of each of the CYP1A1 primers (U2: 5'-CACCAGGAGATCAAGG-3' and D2: 5'-CTGCAGATATAGCAGACAG-3') (18) and 0.5 U of Taq DNA polymerase (Boehringer Mannheim). Reaction conditions were 1 cycle at 95° C for 5 min , 30 cycles for 1 min at 95° C, 1 min at 55° C and 1 min at 72° C, and a final extension at 72° C for 7 min .

Reaction products were electrophoresed in 1.2% agarose gels and stained first with Syber Green I (Molecular Probes) and visualized by scanning with a Molecular Dynamics PhosphorImager and then with ethidium bromide and visualized and photographed on a UV transilluminator (Figure 2). The intensities of the truncated control and native CYP1A1 bands were determined from scans using the IQMac v1.2 (Molecular Dynamics) application.

Calculations of CYP1A1 mRNA in larval samples were made from the relationship (A / B) x C, where A and B are the intensity of the products from total RNA and control RNA respectively and C is the pg amount of the control RNA used in the reverse transcriptase reaction. Consideration was not given to the size of the CYP1A1 mRNA to calculate

picogram amount present.

RESULTS AND DISCUSSION

Our results show the utility of competitive RT-PCR in quantifying CYP1A1 gene expression both in uninduced and PCB-exposed individual tomcod larvae. Basal levels of expression in control samples varied from 16 pg to 107 pg CYP1A1 mRNA/μg total RNA with a mean of 61 pg CYP1A1 mRNA/μg total RNA (SEM= 22). Levels of CYP1A1 mRNA expression in PCB-exposed larvae ranged from 463 pg to 1395 pg CYP1A1 RNA/μg total RNA with a mean of 809 pg CYP1A1 mRNA/μg total RNA (SEM=203). Thus, CYP1A1 expression was significantly induced 14 fold ($p < 0.05$) in PCB-exposed larvae compared to controls exposed to DMSO vehicle. Levels of CYP1A1 mRNA induction in tomcod larvae treated with water-borne TCB (10 ppm) in this experiment were much lower than what we reported in hepatic tissue from adult Miramichi River tomcod i.p injected with an identical dose of this congener (Courtenay et al. In press). In that case, CYP1A1 mRNA induction was approximately 160 fold. Decreased CYP1A1 inducibility in this experiment was almost certainly due to the different routes of exposure, reduced sensitivity of entire larva compared to liver, and perhaps variation in CYP1A1 responsiveness during development.

One of the problems in the use of small tissue samples in quantitative gene expression assays is standardization of the total RNA amounts that are used as template in RT-PCR reactions. The amount of total RNA isolated from individual larval specimens analyzed in this study varied from 765 ng to 1975 ng with a mean of 1267.5 ng± (149.3; SEM.). Since the mean wet weights of 7 day old Hudson River tomcod larvae was 0.214 mg (C. Chamber, pers comm), total yields of RNA from individual larvae ranged between 3.6 ng total RNA/μg larval tissue and 9.2 ng total RNA/μg larval tissue. Thus, we were unable to obtain sufficient yields of total RNA from individual larvae to use spectrophotometry to reliably quantify RNA concentrations. The use of slot blot analyses and hybridization with the rat 18 S rRNA probe to RNAs of known concentrations isolated from adult livers allowed us to circumvent this problem and quantify the concentrations and amounts of total RNAs in individual larval samples as demonstrated in Figure 1. Validity of this approach is based on the assumption that relative concentrations of 18S and 23S rRNAs and total rRNAs remain constant during development and among individual specimens. The use of northern analysis with the 18S rRNA probe also allowed us to evaluate the integrity of RNA. rRNA bands were intact in all the preparations (data not shown).

Our results demonstrate the potential use of competitive RT-PCR as an effective tool to quantify levels of expression of a variety of genes in organisms from impacted and

reference wild populations. Although most laboratories currently employ western blot analyses or enzyme assays as their major tool to assess CYP1A1 gene expression in sentinel species, RT-PCR offers several advantages not afforded by these other techniques. Firstly, the development of reagents such as RNA*later* (Ambion) that allow for the isolation of intact RNA from tissue samples that have been stabilized at room temperature relaxes demands on sample preservation. Secondly, analyses of environmental samples currently focuses on genes such as CYP1A1 or metallothionein and tissues such as liver in which expression of these genes is highly induced in organisms from contaminated sites. However, in the future it may be equally informative to investigate expression of genes such as the aryl hydrocarbon receptor for which exposure to contaminants may reduce levels of expression from constitutive levels or genes such as glutathione-s-transferase for which induction may be minimal, yet still significant. Finally, RT-PCR offers the advantage of assessing gene expression in tissues that are non-destructively collected such as fin clips or blood for which RNA yields may be low or in which levels of gene induction may not be as pronounced as in liver.

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FIGURE LEGENDS

Figure 1. Slot blot analysis of tomcod larval and liver 18 S rRNA. One (columns A and C) and 10 µl (columns B and D) of larval total RNAs were slot blotted along with four different amounts of tomcod adult liver total RNA. Rows 1 and 2 contain RNA from DMSO-treated larvae, rows D and E from TCB-treated larvae, and row C contains 0.5 ng, 2.0 ng, 5.0 ng, and 12.5 ng of adult liver RNA. Tomcod rRNAs were visualized by hybridization to a ^{32}P radiolabelled 18 S rat rRNA probe.

Figure 2. Competitive RT-PCR analysis of CYP1A1 mRNA expression in TCB and DMSO-treated tomcod larvae. One and 10 µl of 1:10 dilutions of total tomcod larval RNAs and 0.2 pg of truncated competitive tomcod CYP1A1 standard were used in RT-PCR analyses. The molecular size of amplicons from larval samples was 566 bp and 469 bp for the truncated CYP1A1 standard. Amplicons were electrophoresed in 1.2% agarose gels and visualised by ethidium bromide staining. Amplicons from DMSO-exposed control larval specimens are in lanes 1-8 and TCB exposed larval samples are in lanes 10-17. Lane 9 contains a 1 kb DNA ladder (Gibco-BRL).

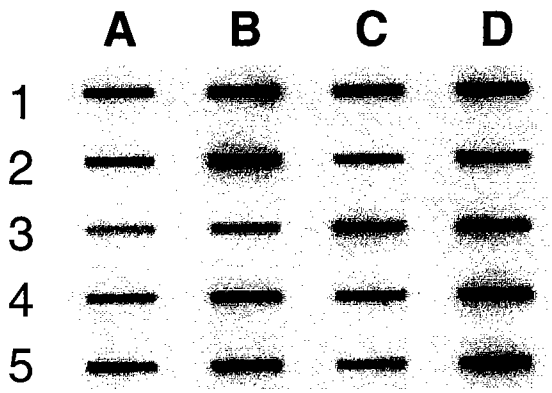

Fig. 1

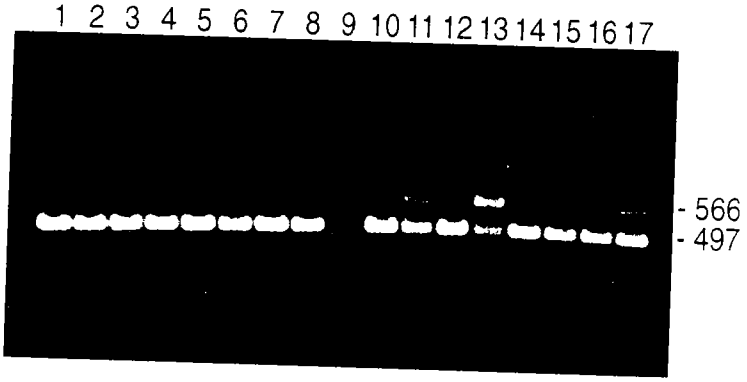

Fig. 2