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Characterization of the Aromatic Hydrocarbon Receptor Gene and Its Expression in Atlantic Tomcod[1]

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Cytochrome P4501A1 (CYP1A1) mRNA is not inducible in Atlantic tomcod from the Hudson River that are treated with halogenated aromatic hydrocarbons (HAHs). In contrast, CYP1A1 mRNA is inducible in Hudson River tomcod that are treated with polycyclic aromatic hydrocarbons (PAHs) and in tomcod that are collected from cleaner rivers and treated with HAHs or PAHs. We hypothesize that CYP1A1 transcription is inhibited in Hudson River tomcod because of down-regulation of the aromatic hydrocarbon receptor (AhR) pathway and that separate molecular pathways modulate CYP1A1 transcription in fish treated with HAHs and PAHs. We initially evaluated levels of hepatic nuclear protein binding at enhancer elements (DREs) in the regulatory region of tomcod CYP1A1. No difference in levels of protein binding was observed between tomcod from the Hudson and Miramichi (cleaner) rivers that were untreated or were treated with benzo[a]pyrene. In contrast, levels of protein binding were lower in tomcod from the Hudson River that were treated with TCB than in similarly treated fish from the Miramichi River, suggesting differences between the populations in the structure or expression of AhR pathway molecules. To address this possibility, AhR DNA sequences were characterized from tomcod cDNA and genomic DNA libraries. In tomcod and mammals, AhR is represented by 11 exons, overall peptide sizes are similar, and amino acid sequences at basic, helix–loop–helix, PAAS A, and PAAS B domains are highly conserved. In contrast, little similarity was observed between tomcod and mammals in the sizes or sequences of AhR exons 10 and 11, including the absence in tomcod of glutamine-rich domains. No differences in levels of hepatic AhR mRNA were observed between the two populations or treatment groups when tomcod were untreated or were treated with aromatic hydrocarbons. In contrast, variation in levels of AhR mRNA expression was observed among tomcod tissues; however, no relationship was observed between levels of AhR mRNAs and CYP1A1 mRNAs in tissues from chemically or vehicle control-treated fish. RFLP analysis revealed extensive variation in exons 10 and 11 of AhR cDNA among tomcod from different rivers. Our results suggest that variation between tomcod populations in CYP1A1 mRNA inducibility is reflected by differences in levels of inducible hepatic protein binding to DREs. However, levels of hepatic AhR mRNA are not down-regulated in the Hudson River population, are not affected by AH treatments, and levels of AhR mRNA expression are not responsible for the differential inducibility of CYP1A1 transcription. © 1997 Academic Press

Key Words: **Aromatic hydrocarbon receptor; cytochrome P4501A1; gene expression; genetic resistance; Atlantic tomcod.**

Cytochrome P450 genes encode for enzymes that metabolize both endogenous and exogenous substrates including xenobiotics such as aromatic hyrocarbon compounds (AHs).[3] Cytochrome P4501A1 (CYP1A1)-encoded enzymes oxidize environmental procarcinogenic PAHs to forms which ultimately can either adduct to DNA and proteins or alternatively be eliminated from the body by conjugation with carrier molecules such as glutathione. In fishes, only a CYP1A subfamily with a

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[3] Abbreviations used: AhR, aromatic hydrocarbon receptor; ARNT, aromatic receptor nuclear translocator; CYP1A1, cytochrome P4501A1; PCR, polymerase chain reaction; PAH, polycyclic aromatic hydrocarbon; HAHs, halogenated aromatic hydrocarbons; PCBs, polychlorinated biphenyls; TCDD or dioxin, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCB, 3,3′,4,4′-tetrachlorobiphenyl; B[a]P, benzo[a]pyrene; β-NF, β-naphthoflavone; RFLP, restriction fragment length polymorphism analysis; bp, base pairs; kb, kilobases; HLH, helix–loop–helix; PAAS, *P*er-*A*hR-*A*RNT-*S*im; EMSA, Electrophoretic Mobility Shift Assay.

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single member, CYP1A1, has been definitively identified and it is highly induced by exposure to PAHs, coplanar PCB congeners, dioxins, and furans (1). As a result, levels of CYP1A mRNA, protein, and enzyme activities are frequently evaluated in sentinel fish species as biomarkers to assess contaminant levels in aquatic systems (2, 3).

We have used levels of hepatic CYP1A1 mRNA induction in Atlantic tomcod (*Microgadus tomcod*) to assess levels of bioavailable AHs in estuaries along the North American Atlantic coast. Tomcod collected in the early 1980s from the Hudson River exhibited a prevalence of hepatocellular carcinomas that exceeded 50% in 1-year-old and 90% in 2-year old fish (4); whereas tumors were either absent or in very low prevalence (< 5%) in tomcod from other more pristine rivers (5). Furthermore, the age structure of the Hudson River population was truncated compared to that in other rivers (4). Very recent years (1995–1996) have witnessed a decline in the frequency of tumors in the Hudson River population and the proportion of older fish (>1 year old) in the spawning aggregation has increased (J. Young, Con Edison, personal communication). This reduction in the prevalence of tumors and increase in the longevity of the population could be due to reduced contaminant influxes or to the development of resistance to xenobiotic exposure. While it is true, that both point and nonpoint discharges of contaminants such as PCBs, dioxin, and furans into the Hudson River estuary have decreased (6), these compounds are persistent, particularly in bottom sediments (7, 8). As a result, tomcod from the Hudson River are still exposed to unusually high levels of PCBs which bioaccumulate in liver (9, 10) and hepatic levels of dioxins and furans are high in other sympatric species of finfish from the Hudson River estuary (11).

In our studies, tomcod from the Hudson River exhibited significantly higher levels of bile metabolites of PAHs, CYP1A1 mRNA, and bulky hepatic DNA adducts than tomcod from four other cleaner rivers (3). However, in controlled laboratory dose-and-time response experiments, we observed that CYP1A1 mRNA induction is inhibited in tomcod which are collected from the Hudson River and are then maintained in clean laboratory water (20 to 305 days) and treated with the halogenated aromatic hydrocarbon (HAH) compounds; 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) or the coplanar PCB congener; 3,3′,4, 4′-tetrachlorobiphenyl (TCB). However, CYP1A1 mRNA is inducible in Hudson River tomcod, which are treated with the nonhalogenated compounds: benzo[a]pyrene (B[a]P) or β-naphthoflavone (β-NF) (9, 12). In contrast, both HAHs and PAHs induce CYP1A1 mRNA in tomcod from cleaner rivers (9, 12, 13). These results suggest that noninducibility of CYP1A1 transcription in tomcod is due to chronic exposure to elevated levels of xenobiotics in the Hudson River environment and that separate molecular mechanisms regulate CYP1A1 transcription for halogenated and nonhalogenated AHs in fish. Furthermore, it is possible that reduced CYP1A1 inducibility could result from a multigenerational genetic adaptation or may be due to a single generational acclimation response.

In mammalian models, CYP1A1 transcription is mediated by activation of the aromatic hydrocarbon receptor (AhR) pathway (14). In untreated cells or organisms, the AhR is localized in the cytoplasm and is complexed with hsp90. Exposure to AHs results in cytoplasmic binding of ligand to the AhR and release of AhR from hsp90. The ligand–AhR complex then translocates to the nucleus where a heteromeric complex is formed between the aromatic receptor nuclear translocater (ARNT), liganded-AhR, and perhaps other accessory proteins. The activated AhR complex may then bind to dioxin responsive elements (DREs) 5′ of CYP1A1 and to a host of other genes in the AhR battery (15) and thereby induce their transcription by altering the chromatin structure of the proximal promoter region and allowing access to other transcription factors (16). Polymorphic forms of AhR have been identified among inbred mouse strains (17), murine cell lines (18), and human populations (19) and variant AhRs have exhibited differing functional activities in terms of ligand (18) and DNA binding (16).

AhR protein has been identified in species of cartilagineous and bony fishes, although not in jawless fish (20) and a partial cDNA sequence was reported for the common killifish (*Fundulus heteroclitus*) (21). Additionally, four consensus DRE sequences were identified 5′ to the tomcod CYP1A1 transcriptional start site and inducible nuclear protein binding to these elements was observed with hepatic extracts prepared from tomcod environmentally exposed in the Hudson River and in tomcod treated under controlled laboratory conditions with B[a]P and with protein extracts from DMBA-treated human MOLT 4 cells and rat liver (22). Furthermore, two forms of ARNT were characterized from a rainbow trout gonadal cell line, and both were shown to support CYP1A1 induction in TCDD-treated, ARNT deficient, murine hepatoma cells (23). These preliminary results suggest that mechanisms of CYP1A1 induction are similar in fish and mammals, although the opportunity for variability in the AhR pathway may be greater in fish because of their exposure to unusually high levels of environmental mutagens, frequency of gene duplication events in some fish taxa, relatively short generation times, and reduced capacity for DNA repair (24).

We hypothesize that a population-wide adaptation, inhibition of CYP1A1 transcription by down-regulation of the AhR pathway, has occured in the Hudson River tomcod population because of chronic exposure to high

levels of environmental AHs. As a first step in assessing the mechanistic basis of noninducibility of CYP1A1, we characterized the complete AhR cDNA sequence and partial AhR genomic DNA arrangement in tomcod and compared levels of AhR gene expression in tomcod from the Hudson River and Miramichi River (cleaner) that were untreated or treated with B[a]P, TCB, or TCDD. Additionally, we compared levels of protein binding to DREs in these fish to determine if early AhR pathway-mediated events are responsible for noninducibiilty of CYP1A1 mRNA in the Hudson River population.

METHODS

Tomcod collections and treatments for EMSA and RNA analyses. Tomcod were collected during January 1996 from the Hudson River, NY, with unbaited box traps and with smelt bag nets from the Miramichi River, New Brunswick. Tomcod from both populations were depurated in clean laboratory water (5°C, 5 ppt salinity) for > 60 days prior to treatment. Tomcod from the Miramichi River were then shipped to New York where they were maintained for 14 days until treatment under identical conditions as fish from the Hudson River.

For analysis of inducible protein binding to DREs, size- and sex-matched tomcod from both the Hudson River and Miramichi River populations were i.p. injected with identical doses of B[a]P (10 mg/kg of fish); TCB (100 μg/kg of fish); vehicle controls (emulphor acetone or DMSO, respectively) in 0.1- to 0.3-ml volumes, or were untreated. Fish injected with B[a]P were sacrificed 6 and 24 h after treatment; fish injected with TCB were sacrificed 24 and 120 h after treatment. These concentrations of chemicals and durations of exposure were selected because these conditions elicited at least a 50-fold induction of hepatic CYP1A1 mRNA expression in *in vivo* experiments with tomcod (9, 13, 12). Brain, gill, heart, kidney, liver, and spleen were removed from each fish, snap frozen in liquid nitrogen (except for a subset of liver samples), and stored at −80°C until processing. Additional samples for hepatic RNA analysis were obtained from tomcod that were collected earlier from the Hudson and Miramichi rivers, depurated extensively in clean laboratory water for more than 20 days, and treated with TCDD, TCB, β-NF, or vehicle controls as described (25, 9, 13).

Analysis of inducible protein binding to tomcod DREs. Nuclear protein extracts were prepared as described from fresh tomcod livers (22). Tissues were homogenized in 0.6 ml of buffer A (0.25 M sucrose, 0.14 M NaCl, 15 mM Tris–Cl (pH 7.9), 10 mM KCl, 1 mM DTT, 1 mM EDTA, 0.5 mM EGTA, 0.5 mM spermidine, 0.5 mM PMSF, 2 μg/ml leupeptin, 4 μg/ml pepstatin, 2 μg/ml antipain, and 4 μg/ml aprotinin), and centrifuged at 250g for 1 min at 4°C. The resulting supernatant was transferred to a new tube, Noniodet P-40 was added to a final concentration of 0.5%, homogenized, and centrifuged at 1000g for 10 min at 4°C. The resulting nuclear pellet was washed with 0.4 ml of buffer A, centrifuged at 2500g for 10 min at 4°C, and lysed by incubation for 15 min in 0.15 ml of 0.42 M NaCl, 10% glycerol, 20 mM Hepes (pH 7.9), 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 2 μg/ml leupeptin, 4 μg/ml pepstatin, 2 μg/ml antipain, and 4 μg/ml aprotinin. The lysate was centrifuged at 12,000g for 15 min at 4°C, the supernatant was retained, and aliquots were stored in liquid nitrogen. Protein concentrations of the extracts were determined (26).

Four pairs of complementary oligomers containing core tomcod DRE sequences (Table I) were annealed (27) and ^{32}P radiolabeled with polynucleotide kinase. Three μg of nuclear protein extracts were incubated with 100,000 cpm of labeled double-stranded oligonucleotides in total volumes of 20 μl containing 10 mM Tris–Cl (pH 7.5), 50 mM NaCl, 10% glycerol, 0.05% NP-40, and 2 μg of poly (dI-dC).

TABLE I

Oligonucleotides Used in Electrophoretic Mobility Shift Assays[a]

Primer	Sequence	5′ end begins at tomcod CYP1A1 nt[b]
DREP9	5′-GCTGCCTTCGCGTGACTGAC-3′	−2459
DREP11	5′-GTTTCTCACGCGAACTACAG-3′	−2365
DREP13	5′-CGCTCTCACGCTACTGGTCA-3′	−838
DREP15	5′-GCTGCGGTCGCGTGTGAACT-3′	−762

[a] Complementary oligonucleotides are not shown.
[b] From (22).

Complementary unlabeled oligonucleotides containing consensus core mammalian DRE sequences (5′ GATCTCTTCTCACGCAACTCCGAG 3′ and 5′ GATCCTCGGAGTTGCGTGAGAAGA - 3′) were used as competitors. One-hundred-fold molar excess of unlabeled competitor oligonucleotides were added to the incubation reactions prior to the addition of protein extracts. Reactions were incubated at 23°C for 30 min and terminated by the addition of 2 μl of gel loading dye (50% glycerol, 0.03% each of bromophenol blue, and xylene cyanol). Mobility shifts were analyzed in 4% nondenaturing polyacrylamide gels run at 4°C; gels were vacuum dried on filter paper and autoradiographed.

Development of tomcod genomic and cDNA libraries. A genomic DNA library was developed from *Sau*3A partially digested genomic DNA from a single tomcod collected from the Hudson River. Size-fractionated DNA fragments were ligated to *Bam*HI-digested λ Dash II arms (Stratagene) and ligation products were packaged using Gigapack II extract (Stratagene) and infected into XL1-Blue MRA (P2) host cells (22).

A cDNA library was developed from a single tomcod that was collected from the Hudson River, extensively depurated in clean water (20 days), i.p. injected with β-NF (10 mg/kg fish), and sacrificed 24 h later. Total RNA was prepared from liver (25), poly(A)-enriched RNA was isolated, and a $d(T)_{12-18}$ oligonucleotide was used for first strand synthesis. Size fractionated cDNA fragments larger than 500 bp were ligated to an *Eco*RI linker and inserted in the *Eco*RI site of λ gt-10. Ligation products were packaged using Gigapack II extract and were used to infect C600 *hfl* cells, amplified, and titered (28).

Development of a tomcod-specific AhR genomic DNA probe. Tomcod genomic DNA was PCR amplified with primers derived from PAAS sequences conserved between *F. heteroclitus* (21) and mouse (17) in the 7th exon of AhR (AhRK1 and AhRK2; Table II). A 193-bp tomcod amplicon was obtained and directly sequenced (29) from both ends with these PCR primers. The derived tomcod sequence showed similarity to mammalian and killifish AhR sequences and was used to design tomcod-specific nested primers (AhRT1 and AhRT2; Table II) to amplfy a smaller portion of tomcod genomic DNA. Using this primer pair, a 136-bp amplicon was obtained and this was directly sequenced and found to contain an internal sequence that was identical to that of the original 193-bp tomcod amplicon. This 136-bp amplicon was used to screen the tomcod genomic DNA library in plaque hybridizations.

Approximately 10^6 plaques from the tomcod genomic DNA library were screened. Seven plaques were positive in the primary screening, but only one proved positive in secondary screenings. Its DNA was isolated and the tomcod insert was determined to be approximately 18 kb based on *Eco*RI, *Hin*dIII, and *Bam*HI digests. The 18-kb tomcod insert was digested with a battery of restriction enzymes and Southern blot analysis was used to determine which of the smaller frag-

TABLE II

Primers Used in PCR Amplification of Tomcod AhR Genomic DNA and Sequencing of cDNA

Primer	Sequence	5′ end begins at nt
AHRK1	5′-GAAGTTCCACGGGCGACTAAAG-3′	1089
AHRK2	5′-CAACGCCTGTTGGTGTGAAGTC-3′	1279
AHRT1	5′-CACGGCCAGAGCATGATGA-3′	1117
AHRT2	5′-GATGCTTGGTCTGGAAG-3′	1252
AHRT3	5′-CTTCCAGACCAAGCATC-3′	1236
AHRT9	5′-GGAAGCCAGAGGAGTTG-3′	1081
AHRT10	5′-GCTCGTACACGCTCTGG-3′	859
AHRT11	5′-CAACTCCTCTGGCTTCC-3′	1065
AHRT12	5′-CCATGCCAGCGTGGC-3′	3110
AHRT13	5′-CAAGTGTCCCCGGAGC-3′	2219
AHRT14	5′-TTGAGGGAGGGTGGG-3′	2956
AHRT15	5′-CTTTGTTAAGTAAAC-3′	3842
AHRT16	5′-CAGTGTCTGAACATCC-3′	4392
AHRT17	5′-GTTTGGCTGGTCTGG-3′	1667
AHRT18	5′-GACTGTTTTCCGACTG-3′	1422
AHRT19	5′-CGGTATAAACAACAC-3′	73

ments hybridized to the tomcod AhR 7th exon probe. A restriction map was developed for the largest DNA fragment that hybridized (8 kb), smaller restriction fragments were generated, subcloned, and sequenced using M13 universal forward and reverse primers as well as the tomcod-specific primers listed in Table II. This approach provided tomcod genomic DNA sequence that exhibited significant similarity to mammalian AhR between exons 5 and 9 and based on additional analysis extended into exon 10.

Approximately, 10^6 plaques from the tomcod cDNA library were screened in plaque hybridizations with a 1.6-kb tomcod AhR genomic DNA probe spanning exons 5–7. Twelve plaques were positive upon primary screening and six of these were positive after tertiary screening. DNA was isolated from four of these positive plaques, characterized, and insert size for all four was 4.6 kb. Two of these inserts were subcloned into pUC19, a restriction map was developed, and smaller fragments were subcloned back into pUC19. These were sequenced using M13 universal forward and reverse primers and primers listed in Table II developed from tomcod sequence. Nucleotide sequence for both 4.6-kb inserts was identical.

Genomic and cDNA sequences were assembled into a contiguous sequence using fragment assemby programs (GELSTART, GELENTER, GELOVERLAP, GELASSEMBLE, GELMERGE, GELVIEW) in the GCG computer package (Madison, WI). Homology searches were done with BESTFIT and deduced amino acid sequence was determined with TRANSLATE.

Primer extension analysis. Primer extension analysis was used to determine the start site for transcription of tomcod AhR from total hepatic RNAs isolated from 12 tomcod from the Hudson and Miramichi rivers. Tomcod primer AhRT19 was end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase and 0.5 pmol were used to reverse transcribe 5 μg of RNA from each sample in 10 μl of 10 mM Tris–Cl buffer (pH 7.6) containing 1 mM $MgCl_2$ and 0.5 mM DTT (28). Reactants were denatured at 95°C for 5 min, electrophoresed in denaturing 6% polyacrylamide gels, and gels were dried and autoradiographed. Lengths of the extension products were determined by comparison to a ladder of a sequencing reaction using primer AhRT19 and an upstream tomcod AhR cDNA recombinant.

Northern and slot blot analyses. Total RNA was isolated from tomcod tissues (brain, heart, gill, kidney, liver, and spleen) using Ultraspec reagent (Biotecx, Houston, TX) as recommended by the manufacturer and modified according to Kreamer *et al.* (25). RNA concentrations and purities were determined spectrophotometrically at 260 and 280 nm. Poly(A)+ RNA was purified from 100 μg of total RNA from a subset of liver samples ($n = 8$) using a Quick Prep mRNA Purification kit (Pharmacia) as described by the manufacturer. Total RNA isolated from rat brain and rat heart were included on slot blots to evaluate the specificity of the hybridization reaction with the tomcod AhR probe.

Initally, formaldehyde agarose gels were used to evaluate the integrity of all total RNA samples. Ten micrograms of total RNAs were loaded into 1.0% formaldehyde agarose gels (30) and electrophoresed at 120 V for 2 h and gels were stained in ethidium bromide solution and photographed. Northern blot analysis was then performed with approximately 1 μg of hepatic poly(A)+ RNA from eight fish to visualize tomcod AhR hybridizable transcripts and estimate their molecular size. Slot blots were then used to quantify levels of AhR and CYP1A1 mRNAs for all of the samples. For slot blots, 3 μg of total RNA were denatured (31) and directly applied to Maximum Strength Nytran Plus (0.45 mm) membranes (Schleicher and Schuell) using a Bio-Rad Bio-Dot SF manifold.

Membranes were prehybridized for 3 h and hybridized overnight at 65°C (32, 25). Final wash stringencies were 1.0× SSPE/0.1% SDS and 0.4× SSPE/0.1% SDS at 65°C for 1 h. Probe DNAs were the full-length (4.6 kb) tomcod AhR cDNA (pNR120), a full-length (2.6 kb) tomcod CYP1A1 cDNA (pGLK2.6) isolated from a β-NF treated tomcod (28), and a rat 18S rRNA housekeeping gene (33) and were ^{32}P radiolabeled using random priming (34). Membranes were stripped of AhR and CYP1A1 probes by immersion three times in boiling 0.1× SSC/0.5% SDS and rehybridized. Levels of AhR, CYP1A1 mRNAs, and 18S rRNA were quantified from autoradiographs using the Whole Band Analysis Package in the Millipore BioImage Analysis System.

Southern blot analysis. DNA was isolated from the livers of tomcod from the Hudson ($n = 8$), St. Lawrence ($n = 2$), Margaree ($n = 2$), and Miramichi ($n = 2$) rivers using 0.2% Triton X-100/1 N NH_4OH buffer, standard phenol-chloroform extractions, and alcohol precipitations (35). From each sample, 10 μg of DNA was digested each with *Hae*III, *Hin*dIII, *Kpn*I, *Pst*I, *Pvu*II, and *Sal*I, electrophoretically separated in 1.2% agarose gels, transferred to Maximun Strength Nytran Plus membranes, and hybridized (35) to ^{32}P radiolabeled (33), full-length 4.6-kb tomcod AhR cDNA probes, and to four individual cDNA probes that span different regions in tomcod AhR, including 1.24 kb (exons 1–7; nt 1–1240), 0.86 kb (exon 10; nt 1568–2428), 0.7 kb (exon 10; nt 2429–3131), and 1.4 kb (exons 10–11; nt 3242–4626). The 0.86-kb probe is exclusively in exon 10 and the 1.4-kb probe includes the 3′ end of exon 10 and all of exon 11. Two final wash stringencies were used for each hybridization, 1.0× SSC/0.1% SDS and 0.1× SSC/0.1% SDS at 65°C for 1 h and membranes were exposed to X-ray film following each wash.

RESULTS

Inducible Hepatic Protein Binding to Tomcod DREs

We initially compared the levels of inducible hepatic nuclear protein binding to four pair of oligonucleotides containing DREs 5′ to tomcod CYP1A1 with hepatic protein extracts prepared from depurated Hudson River and Miramichi River tomcod that were untreated or treated with an HAH (TCB), a PAH (B[a]P), or vehicle controls (Fig. 1). This enabled us to evaluate the effects of population differences and chemical treatments on levels of inducible hepatic protein binding to tomcod DREs. Identical results were obtained with all

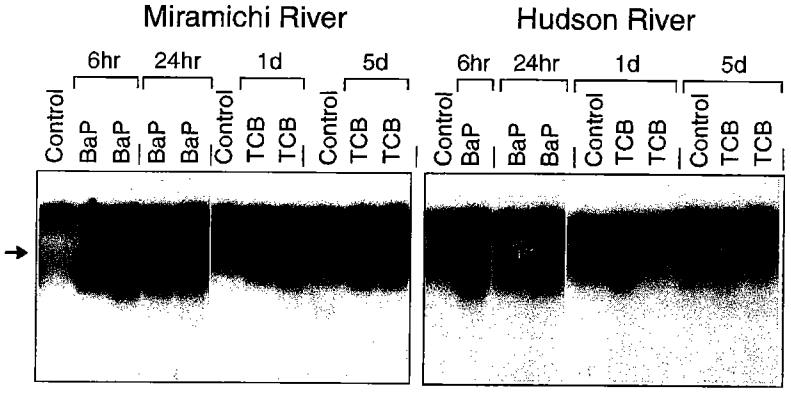

FIG. 1. Gel mobility shift assays of inducible hepatic protein extracts prepared from Atlantic tomcod from the Miramichi River and the Hudson River that were treated with B[a]P (for 6 and 24 h), or TCB (for 1 and 5 days) or that were treated with vehicle control (emulphor acetone or DMSO, respectively) and incubated with ^{32}P-radiolabeled oligonucleotides containing tomcod DREs. Arrow indicates shifted DNA fragments.

four pairs of oligonucleotides. In tomcod that were untreated or were treated with vehicle controls, low, but detectable levels of protein binding to the DREs were observed. However, no difference in levels of protein binding to DREs was observed between tomcod from the two populations. High levels of protein binding to DREs were observed in tomcod from both the Hudson and Miramichi rivers that were treated with B[a]P for 6 or 24 h. Levels of protein binding were higher in fish treated for 24 h than for 6 h; but no difference was observed between treated fish from the two populations at either time. In contrast, levels of inducible protein binding to DREs differed between tomcod from the Hudson and the Miramichi rivers that were treated with TCB for 1 or 5 days. At both time points, levels of protein binding were higher with protein extracts prepared from Miramichi River than Hudson River tomcod. However, even extracts from TCB-treated Hudson River tomcod showed higher levels of protein binding than seen in untreated or vehicle control-treated tomcod from either river. Addition of excess unlabeled competitor oligonucleotides abolished all inducible protein complexes confirming the specificity of protein binding to the tomcod DRE sequences.

Characterizations of Tomcod AhR Genomic and cDNA

To investigate the reasons for the reduced binding of inducible protein extracts prepared from livers of Hudson River tomcod treated with TCB, we characterized the structure and expression of tomcod AhR. A tomcod-specific, 136-bp AhR 7th exon probe was developed by PCR amplification of tomcod genomic DNA using primers derived based on conserved sequences and this probe was used to screen the tomcod genomic and cDNA libraries. Tomcod genomic DNA (5.6 kb) was characterized which extended from the 3′ end of intron 4 through the end of exon 11. Absolute sizes of tomcod AhR introns 5–10 were similar to those in mammals; 504, 326, 1980, 334, 349, and 228 bp, respectively; however, their nucleotide sequences were highly divergent. GT-AG exon-intron boundary sequences that are conserved among eukaryotic genes were also observed in tomcod AhR genomic DNA.

A 1.6-kb tomcod genomic DNA fragment that contained AhR exons 5–7 was then used to screen the tomcod cDNA library. Six clones proved positive through tertiary screenings, insert sizes were determined for four of these and they were identical at slightly more than 4.6 kb. Two were sequenced and proved identical. It should be emphasized that the complete tomcod AhR cDNA sequence was obtained from these two clones and 3′ sequence was confirmed by AhR genomic DNA sequence which extended from exon 5 through exon 11.

AhR gene organization is highly conserved between tomcod and mammals. Each contains 11 exons and the relative sizes of AhR exons, with the exception of exons 3, 10, and 11 are similar among tomcod, mouse, rat, and humans (Table III). In fact, the absolute sizes of exons 4, 7, 8, and 9 in tomcod and mammals are identical. The smaller overall size of the tomcod AhR compared to the mammalian forms is due to the truncated size of the untranslated region in exon 11. The tomcod cDNA sequence includes a putative poly(A) signal sequence (AATTAA) which is 127 nucleotides upstream of the 9-nt-long poly(A) sequence.

The full-length tomcod AhR cDNA (4626 bp) (Fig. 2) is somewhat smaller than that in mouse Hepa 1c1c7 cells (5524 bp) (17) and humans (5228 bp) (36). Overall nucleotide sequence similiarity between tomcod and mouse, and between tomcod and human AhR coding sequences was 67.9 and 64.4%, respectively. Nucleotide

TABLE III

A Comparison of Sizes (bp) of AhR Exons in Tomcod, Mouse, Rat, and Humans

Exon	Tomcod	Mouse[a]	Rat[b]	Human[c]
1	444	437	<62	440
2	191	188	188	188
3	89	104	104	107
4	90	90	90	90
5	121	112	124	124
6	134	131	131	131
7	203	203	203	203
8	110	110	110	110
9	142	142	142	142
10	2042	1263	1270	1243
11	1060	2747	<150	2450
Total	4626	5527	2574	5228

[a] From (17).
[b] Genbank Accession No. U09000.
[c] From (65).

sequence similarity between tomcod and mammals was much higher in exons 1–9, than in exons 10–11. For example, overall sequence similarity in exons 1–9 between tomcod and human was 65%, but significant sequence similarity in exons 10 and 11 between tomcod and human was only found in a 57-bp stretch at the 3′ end of the untranslated region (in exon 11) which includes the poly(A) stretch.

Arrangement of domains which encode for putative DNA, protein, and ligand binding sites are identical between tomcod and mammals and their absolute sizes are also highly conserved. For example, in tomcod, the basic region (90 nt) is in exons 1 and 2, the helix–loop–helix domain (123 nt) is in exon 2, the PAAS A domain (168 nt) is in exons 4 and 5, and the PAAS B domain (162 nt) is in exons 7–9. Nucleotide sequence similarity between tomcod and mouse was moderately conserved at the basic (65%), helix–loop–helix (70%), PAAS A (77%), and PAAS B (71%) domains.

Primer extension analysis was performed with tomcod primer AhRT19 to determine whether the cDNA contained all of the 5′ untranslated region. To characterize the size of the extension products, a dideoxy sequencing reaction of a recombinant plasmid containing tomcod AhR cDNA and using tomcod primer AHRT19 was electrophoresed along with the extension products. A 59-mer extension product was observed which aligned with the first nucleotide (A) of the isolated tomcod AhR cDNA (Fig. 3). This confirmed that the isolates contained all of the 5′ untranslated region.

An open reading frame of 2472 bp was found in tomcod AhR cDNA and the putative methionine translation initiation codon is preceded by nucleotides that fall within the Kozak consensus sequence (37). The putative translational start codon was 59 bp upstream of the first exon-intron boundary at nucleotide 386 and the stop codon was 710 bp upstream from the tenth exon-intron boundary at nucleotide 2857. The overall size of the deduced tomcod AhR peptide was 824 amino acids (Fig. 2), a size which is within the range of those observed in humans (849 amino acids) (36) and murine cell lines (805, 848, and 883 amino acids) (18). The molecular weight of the deduced tomcod AhR protein is 90.4 kDa. Overall amino acid similarity between tomcod and mouse AhR was 60.3% and between tomcod and human AhR was 58%. However, amino acid sequence similarity between tomcod and mammals was much higher at amino terminus domains critical to DNA, protein, or ligand binding. For example, AhR amino acid sequence similarity between tomcod and mouse Hepa 1c1c7 cells (17) at the basic region was 75%, at the helix–loop–helix region 81%, at PAAS A 87%, and at PAAS B 87% (Fig. 4). Amino acid sequences in the PAAS A and PAAS B domains in tomcod were imperfect repeats of 43 and 54 amino acids, repectively, that were separated by 107 residues. Amino acid sequence similarity between PAAS A and PAAS B in tomcod was 58%, slightly higher than between the two PAAS regions in mouse (48%).

Similarity of amino acid sequences between tomcod and mammals was quite high for the first 28 residues of exon 10, but thereafter sequences were highly divergent. The Q-rich region in mammalian exon 10 is characterized by stretches of 2 to 4 glutamine repeats. For example, there are 20 and 14 glutamine residues, respectively, within exon 10 of mouse and human AhR (within residues 600 to 638 in the human sequence). However, we found no evidence of a Q-rich region in exon 10 of tomcod; there are no stretches of glutamine repeats and the total number of glutamine residues between residues 600 and 638 in tomcod exon 10 is only three.

The deduced amino acid sequence was used to identify possible sites for modification of the AhR protein (36). Five putative phosphorylation sites for protein kinase C (S/T - R/K), nine for protein kinase A (R/K R/K - S/T), and ten for casein kinase II (S/T - - D/E) were identified. However, their genic locations were dissimilar from that in mouse and human AhR (36) with the exception that the vast majority were in the amino half of the molecule. There was no evidence of a nuclear localization signal sequence (KKKGK) (38) in the tomcod PAAS domains; the most closely related sequence (MKKRKK) was at residues 10–15 within the basic region.

RFLP Analysis

Partial characterization of AhR cDNA in killifish suggested the possiblity of duplication of the AhR locus (39) in other fish taxa, although sequence similarity

```
ATCACTTTATATCCAACCAA AAAGTGACTCTGAATAACGA GGCAGTGTTGTTTATACCGG GGCTGGACACAGGCTGATTA CAAAAGGGAGAAAACTTTCC GCAAGATATCCATCGGTAAT  120
                                          <- AHRT19

CACGCTATGCAGCTCTATTG AAGGAAAAACTCTCCAGAAC TTCCTTCCATCCCACGAGTT TCACCCGCACATCGATCGAA GGTCGTTGTGAATGCTGGGG AACAGTCAATGCCTTACGAT  240

TTGTGGTTGATTCATAATTG ACTTTGCCTTTTGAGGGATA TTTGAAATTATGGCAGAGTA GGCTAGTGTGATCATATGGA TAAAGCGCCTCTATTACAGA CTGGATTTTACCTTCTTGTC  360

ATTGAACAATAAGCCGAGTG ACACCATGTTGGGTAACGCT GGGACTTACGCTATGAAGAA GCGGAAGAAGCCCGTTCAAA AACCCAAGAAGCTCCCTGGA GTGGATGGGGTCATCAAGTC  480
                      M  L  G  N  A   G  T  Y  A  M  K  K   R  K  K  P  V  Q  K   P  K  K  L  P  G   V  D  G  V  I  K  S  (32)

AAACCCTTCCAAACGCCACA GGGATCGGCTGAACGGGGAG CTGGATCGCCTGACCGACCT GCTACCCTTCTCCGAGGACA TCCGTACGCGCCTGGACAAA CTGTCCGTGCTCCGACTCAG  600
 N  P  S  K  R  H  R   D  R  L  N  G  E   L  D  R  L  T  D  L   L  P  F  S  E  D  I   R  T  R  L  D  K   L  S  V  L  R  L  S  (72)

CGTGGGCTACCTGAGAGTCA AGGGCTTCTTTAAAGCCACC ATGAAGAAGAACAATGCCCC GATTGGACAGGGGCGGAATG CGCTGGACCTTGCCACCATA TCTGAGGGAGACCTCCTGCT  720
 V  G  Y  L  R  V  K   G  F  F  K  A  T   M  K  K  N  N  A  P   I  G  Q  G  R  N  A   L  D  L  A  T  I   S  E  G  D  L  L  L  (112)

CCAGGCGCTGAACGGCTTTG TGATCGTAGTCACTGCCGAG GGATTGGTCTTCTACTCCTC TTCCACAATCCAAGACTACC TGGGCTTCCATCAGTCGGAC GTGGTCCACCAGAGCGTGTA  840
 Q  A  L  N  G  F  V   I  V  V  T  A  E   G  L  V  F  Y  S  S   S  T  I  Q  D  Y  L   G  F  H  Q  S  D   V  V  H  Q  S  V  Y  (152)
                                                                                                          <- AHRT10

CGAGCTCATCCACACTGATG ACCGGGCCATGTTCAGAGAA CAGCTTCACTTCGCTCTCAA CCCCAAACTCTACGCAGCCG AACAAGGTGGAGACGCGTTG GCCTTGCAGTGCAACAGCGA  960
 E  L  I  H  T  D  D   R  A  M  F  R  E   Q  L  H  F  A  L  N   P  K  L  Y  A  A  E   Q  G  G  D  A  L   A  L  Q  C  N  S  D  (192)

CCAGGTGAAGTATGACCCCG AGAGACTCCCCCCGGAGAAC TCCTCCTTCCTGGAGCGGAG CTTCGTCTGCCGTTTCCGCT GTCTCCTGGACAACTCCTCT GGCTTCCTGGCTTTGAAATT 1080
 Q  V  K  Y  D  P  E   R  L  P  P  E  N   S  S  F  L  E  R  S   F  V  C  R  F  R  C   L  L  D  N  S  S   G  F  L  A  L  K  F  (232)
                                                                                                  <- AHRT9 AHRT11 ->

CCAAGGGCGACTGAAGTACC TCCACGGCCAGAGCATGATG AGCGACGACGGCACGCGGGT CCAGTCTCAGCTAGCCCTGT TCAGCATCGCCGTCCCCGTC CAGACTCCATCCATCCTGGA 1200
 Q  G  R  L  K  Y  L   H  G  Q  S  M  M   S  D  D  G  T  R  V   Q  S  Q  L  A  L  F   S  I  A  V  P  V   Q  T  P  S  I  L  E  (272)
                         AHRT1 ->

GATCAGGGCCAAAACACTTA TCTTCCAGACCAAGCATCAG CTGGACTTCACCCCGATGGG CATCGACAACAGGGGGAAAG TGGTTCTCGGGTACTCAGAG CTTGAGTTGTGTATGCGAGG 1320
 I  R  A  K  T  L  I   F  Q  T  K  H  Q   L  D  F  T  P  M  G   I  D  N  R  G  K  V   V  L  G  Y  S  E   L  E  L  C  M  R  G  (312)
                      <- AHRT2 AHRT3 ->

CTCGGGGTACCAGTTCATCC ATGCAGCTGACATGATGTAC TGCGCAGACAACCACTTACG CATGATAAAGACAGGCGAGA GTGGGCTGACTGTTTTCCGA CTGCTGTCCAAATCAAGTGG 1440
 S  G  Y  Q  F  I  H   A  A  D  M  M  Y   C  A  D  N  H  L  R   M  I  K  T  G  E  S   G  L  T  V  F  R   L  L  S  K  S  S  G  (352)
                                                                                                  AHRT18 ->

CTGGGTGTGGGTGCAGGCCA ACGCCAAGCTGGTGTACAAA GGAGGAAGACCGGACTTCAT CATTGCACGGCAGAGAGCTT TAGTCAATGCCGAGGGAGAG GAGCACCTCAGGCAGCGCCG 1560
 W  V  W  V  Q  A  N   A  K  L  V  Y  K   G  G  R  P  D  F  I   I  A  R  Q  R  A  L   V  N  A  E  G  E   E  H  L  R  Q  R  R  (392)

GCTCCAGCTGCCCTTCAGCT TCACCACGGGGGAGGCCATG CTCTATGAGGTGGGCCCCAG CCTTGACGTCACGCAGATCC AGACCAGCCAAACCTTCAAC AGCAGCCAGCCGGAGGAGGT 1680
 L  Q  L  P  F  S  F   T  T  G  E  A  M   L  Y  E  V  G  P  S   L  D  V  T  Q  I  Q   T  S  Q  T  F  N   S  S  Q  P  E  E  V  (432)
                                                                                        <- AHRT17

GGGCGGCCTACTAGGATGCA ACCAGGACAAGAACGTCTAC ATCCAGGACTCGGAGGCCCA GCTCCCGGTGGACCAGGTGT TCATGGAGAGCCGGGCCTGG TCAATGTGCCCAGCGACACC 1800
 G  G  L  L  G  C  N   Q  D  K  N  V  Y   I  Q  D  S  E  A  Q   L  P  V  D  Q  V  F   M  E  S  R  A  W   S  M  C  P  A  T  P  (472)

TGGCAGGCCGTCGGTTGCGG GCGGACGACGGCGGGCACGT GATGATCAAGGAGGAGGGCA GCCTTCGGTGACGGCCATGA TCGACGCCCTGGAGGACTTT GTGGAGGGCGGGGAGCTGGT 1920
 G  R  P  S  V  A  G   G  R  R  R  A  R   D  D  Q  G  G  G  Q   P  S  V  T  A  M  I   D  A  L  E  D  F   V  E  G  G  E  L  V  (512)

GTCGCTAGAGGGGCTGGACG TGGACCCCAGCGAGCTGATG GAGTGGGAGAACACGCTCAA GAAGCTCAGTCAGGAGGAGA ACGGTGACAACGGTGACCAC ACCAAGTATGAGCTGGAGAG 2040
 S  L  E  G  L  D  V   D  P  S  E  L  M   E  W  E  N  T  L  K   K  L  S  Q  E  E  N   G  D  N  G  D  H   T  K  Y  E  L  E  S  (552)

CCTGCTCAGTAACGACATTT TTGCCTACGTAGACAATGTT TTGTTTAAGGAAATCGCAGA GGCTAATTTAAATACGAGCC AGTCTAGCTGCTTTTCGTCC GTCAACAATAATCAAGCCGA 2160
 L  L  S  N  D  I  F   A  Y  V  D  N  V   L  F  K  E  I  A  E   A  N  L  N  T  S  Q   S  S  C  F  S  S   V  N  N  N  Q  A  D  (592)

CCTCTTCGATCAGACGGCGC ACTACGCTGGCTCCGGGGAC ACTTGTGAAATGATGCTGTT CCAGTCGCCTAGCGATGGCG CAAAAGCTCACTTCCACGCA AGGCCTCAGCCCTGCGGTGG 2280
 L  F  D  Q  T  A  H   Y  A  G  S  G  D   T  C  E  M  M  L  F   Q  S  P  S  D  G  A   K  A  H  F  H  A   R  P  Q  P  C  G  G  (632)
                         <- AHRT13

TGCCGCAGCCCACGCACAAC CGGCCGGCAAACTTAGCGGC CAAGGCTTGGCCACACAAAC CCCGGCGATGTTTAACAGCA CTCAGAAACTCTCCCATTAT GGACCCGCCATCCCTGAGGC 2400
 A  A  A  H  A  Q  P   A  G  K  L  S  G   Q  G  L  A  T  Q  T   P  A  M  F  N  S  T   Q  K  L  S  H  Y   G  P  A  I  P  E  A  (672)

CGTCCCCCAGCTTCCCACCA CCCCTCAGCTGCTCACAAAC TTTTTCAACCCTTCCGTCAA CCTCCCGGGCCTCAACCTTC CCAAGCTGCCGTTGGCCAGC AACGACCTACGGTCCTTTGA 2520
 V  P  Q  L  P  T  T   P  Q  L  L  T  N   F  F  N  P  S  V  N   L  P  G  L  N  L  P   K  L  P  L  A  S   N  D  L  R  S  F  E  (712)

ACCTTGTGGACAGGCGTTGA TCAGCCACTATCAAGGGCTG CCCAGCAACGCGATGTCCAA CCAGACGCTGTCCAACCAGA CACTGTCCAACCAGACGCTG CCCAACCAGACGCTGTTCCA 2640
 P  C  G  Q  A  L  I   S  H  Y  Q  G  L   P  S  N  A  M  S  N   Q  T  L  S  N  Q  T   L  S  N  Q  T  L   P  N  Q  T  L  F  Q  (752)

ACCCGACGCTGTCCAACCAG ACTCTGGCAAACCAGATGCT CTCCAACCAGACGCTGTCCA ACCAGATGGTCTCCAACCAG ATGCTATCCAACCAGATGCA CTCTACACACAACGACTGCT 2760
 P  D  A  V  Q  P  D   S  G  K  P  D  A   L  Q  P  D  A  V  Q   P  D  G  L  Q  P  D   A  I  Q  P  D  A   L  Y  T  Q  R  L  L  (792)

GATCAACCGAGAAGTGGTGT CCAGGGACACGAGCTGGTCG GCCCCAGTCCCTCCATGCCC CCTCACCGGAAGGCCAGCAG CCTCCGATGATGGGTAACGG ACACTTTCTCCAGGGTTCCA 2880
 I  N  R  E  V  V  S   R  D  T  S  W  S   A  P  V  P  P  C  P   L  T  G  R  P  A  A   S  D  D  G  *                              (824)

TTCACGCACCGGGATCCACG TTGGACCCAATGTGGTGGCC CCGGCGGCCCACCCTCCCTC AAAATGACTTTTGCTTGCCG ACCAATTCCAACGAAAACAG GGCTCTCTTTACTGGCAACT 3000
                                          <- AHRT14

ACATGGTGCAGGGATGCGCC CCATTTCAAACACACAGCAA CCACAGAGCACCGCAATGGC AGCAGGACCTCCAGCACCAT CATCAAGACTCTACCCCATG CCAGCGTGGCTCAGAATCCA 3120
                                                                                                          AHRT12 ->

CACACTGCCAGCTGCCCACA GCCAAGCCTTTGAGAGCCAG CGACTTCTCGTCTGGGCACA GAACTATAACCAACCTCCTC CGCACCGAGGCCTGGCGCCC TCTGCGGACCAATCCCAGCA 3240

GCTGCATGCTGGACAAGCCC TTACACCCCCTCGGCCATCC TCACCCCCTCACCAACGGCA ACCTGGCCAGCGCCAAGGGA ACGGGGCTCGGCCCATGCCG CTGTGCCAGAGGGCCAACGA 3360

GGCCGGCCCTGCACCAGAGC CCTCCGAAGGCTACGTCCAA TGGGGCCAGGGTCCAGGTGT GCCTCGTTGGGCACGCCGCC ATGGGCCAGGAGAACGCCGC CTTCGGAGCGAGGCCGGCGC 3480

AGCTACTGCCGGCTAACATT TCCTCGGGCGCGCCGGACGA CATGGCTGCCATGCCGCATT ACCTGGACGCCAACAAACAC ACACAGATGCTGAGCCTCCC TGCTGAAGACAACGATCTGC 3600

TCGCCATCCCTCCCCTCATC GACGGAAATATTAACTTTTC CGATCAGTCACAACTCAACT GTTGCAACTTCTGAGTTTGA AACGCATCGTCTCCTTAAAC CACAGCGAGGGCGAGCGCGG 3720

GCGAGCGAGGGCGAGCGAGG GCGAGCGATGGCGAGCGATG GCGAGCGAGGGGCGAGCGAG GGGCGAGCGATGGCGAGCGC GGGCGTGGACAACGTTTACT TAACAAAGCCCCGTTTCAGT 3840

GCTGGGCCACGCTTCTTCAG ATGTGATCATCAAAGAAAGA TTATCCTTATAATTAATATT ATGTACTGTATAAAAAAAGA GCAGTAGCCTGAGAGCCTTG AGTAATAATGAAGTATGGAG 3960

CCACAAGTGCATTTATTAAG GAAGGAACTTGAGCCAGCTA GGGACATTGGCTAAGTTCCT CCTCTTTACCAAAGGACTTC AGTTTTAAAGAGGGTGAGAA ATTTATCAATTAAAAAAACA 4080

AGAAAACAAGAATTTCATTC CAAAATGGCCTCTCTAAAAT GTACAAAGTGCCACATCTGC TAATACCAAATGGTGGCTTC CATTGAATTGAACACTCGTT ACAAGTATAACAAAACATGT 4200

TGATCTAAAGATTCCACCCA GCTAACAAATGTAAGCCCTT ACACAAAAAAGACAGTGCCT TTGTTTTTCTCCTTGCCCCA CTGTATATATAGCATTTTGG AATGAAACACTTACACAGGA 4320

TTTATATCTTTGTTAAAAGT CAAATTTGTGGAATCTTTAT TTGTGTATTTTTATTGTCAG TGTCCTGAACATCCTAATTG TCTGTCGTGTTTGTGTCAAT GAGTAACCTTGCACTTGATC 4440
                                                                  AHRT16 ->

TATATACAATAAGAAGCAGC ATAAGAAACAAAGTGTGCGT CTGGAAAAAATACAATTAAC AACTATACTTTGTTTCATAT TTAGTCCGGTTTTGGCTTGA TTAATTGTATCCACCGACAT 4560

GCACAAAATGCTTTTTTTGT GTGATACATGTTTTTATAAA AAAAAAAAGAAATGACTAAA AAAAAA
```

FIG. 2. The nucleotide sequence of Atlantic tomcod AhR cDNA. Deduced amino acid sequence is shown in bold beneath the nucleotide sequence. Amino acid positions are indicated in parenthesis. The location of tomcod-specific primers used in PCR amplification and DNA sequencing are indicated. The * at the end of amino acid sequence indicates termination codon.

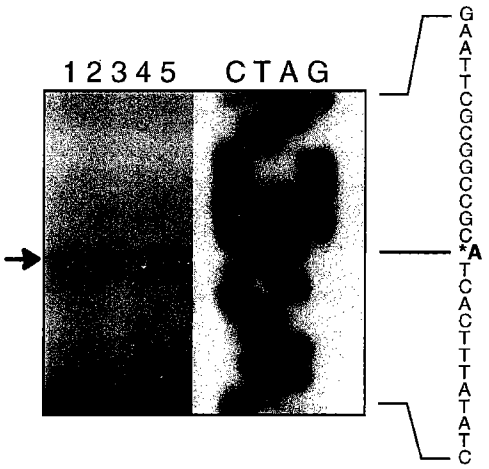

FIG. 3. Primer extension analysis to identify the 5′ end of Atlantic tomcod AhR mRNA using total hepatic RNA and primer AHRT19. Lanes 1–5 show reverse transcriptase reaction products obtained by using RNA from five fish. * Indicates the 5′ end of AhR mRNA. Sequencing reactions of tomcod cDNA using radiolabeled primer AHRT19 are shown in the four right lanes along with the actual nucleotide sequence.

between the two AhR isoforms in killifish was low (65%). We used RFLP analysis to determine if there is evidence of duplication of AhR in tomcod and if there is variation in AhR structure among tomcod from four populations; the Hudson River and three rivers in Canada (Margaree, Miramichi, St. Lawrence). When the full-length AhR cDNA (4.6 kb) was used as probe, only a single AhR hybridizable DNA fragment was evident in the *Kpn*I and *Sal*I digests, even when relatively low stringency final wash conditions were used (1.0× SSC/0.1% SDS at 65°C). This suggests the absence in tomcod of a duplicated AhR locus with moderate levels of sequence similarity to the full-length tomcod AhR cDNA probe or if there are duplicated loci they share identical sizes and nucleotide sequences at the recognition sites for these enzymes.

In contrast, *Hae*III, *Hin*dIII, *Pst*I, and *Pvu*II revealed polymorphisms in genomic DNA among tomcod from the four rivers. Tomcod from each river shared multiple polymorphic AhR hybridizable DNA fragments that were absent in tomcod from the other three rivers. The consistency of the differences in the electrophoretic mobilities of individual fragments among samples digested with different restriction endonucleases suggested that many of the polymorphisms resulted from large (>200 bp) additions/deletions. The majority of the variation was among river samples; however, there were also polymorphisms among specimens from within single rivers, including the Hudson and Margaree rivers.

To further characterize these polymorphisms, DNA from tomcod from the four populations was digested again with *Pvu*II and hybridized to four different tomcod AhR cDNA probes; (a) 1.2 kb containing exons 1–7, (b) 0.86 kb containing the 5′ end of exon 10, (c) 0.7 kb containing the middle of exon 10, and (d) 1.4 kb containing the 3′ end of exon 10 and all of exon 11. Banding patterns among populations for the 1.2- and 0.7-kb probes were invariant, but polymorphisms were observed for the 0.86- and 1.4-kb probes. The 0.86-kb probe hydridized to fragments exclusively in exon 10 and recognized two alleles, 0.86 and 0.67 kb. The size of the 0.86-kb allele is consistent with predictions based on the mapping of *Pvu*II sites in the tomcod AhR cDNA and genomic DNA clones. Three different genotypes were observed; homozygotes for both alleles and a heterozygote (Fig. 5A). The 1.4-kb probe recognized sequence that extended from the end of exon 10 through at least the end of exon 11 and included a 228 intron. Based on sequence from a 4.6-kb AhR genomic DNA clone, there are no *Pvu*II sites within intron 10 or exon 11, therefore it is unknown whether this variation maps to exon 11, intron 10, or 3′ flanking sequence. The 1.4-kb probe revealed at least seven different polymorphic DNA fragments among tomcod samples from four rivers (Fig. 5B). All of the Hudson River tomcod displayed a 3.5-kb fragment which was absent in tomcod from other rivers and is consistent with the size of the *Pvu*II fragment containing AhR exon 11 in the 11.0-kb tomcod genomic DNA clone. DNA fragments in tomcod from other populations ranged up to 7.0 kb in size.

RNA Analyses

A single AhR hybridizable band of approximately 5.0 kb was observed in Northern blot analysis of tomcod poly(A)+ RNA. In slot blot analysis, little or no hybridization signal was observed under low stringency wash conditions between the tomcod AhR probe and total RNA isolated from rat brain or heart. These results supported the specificity of the hybridization signal in slot blot analysis as AhR transcript. Levels of hepatic AhR mRNA were quantified in individual fish to determine if variation in AhR mRNA expression between tomcod from the Hudson River and Miramichi River populations could explain population differences in CYP1A1 mRNA inducibility. Initially, we compared levels of hepatic AhR mRNA expression between untreated or vehicle-treated tomcod from the two rivers (Hudson $\bar{x} = 0.43 \pm 0.049$; Miramichi ($\bar{x} = 0.45 \pm 0.081$) and no difference was observed ($P = 0.35$) (Fig. 6). Next, we determined if a single treatment with B[a]P (fish sacrificed after 6 or 24 h) or TCB (24 or 120 h) would alter levels of hepatic AhR mRNA, but no difference was observed between chemically treated ($\bar{x} = 0.32 \pm 0.047$) and untreated tomcod ($\bar{x} = 0.45 \pm 0.081$) from the Miramichi River ($P = 0.26$). Levels of hepatic AhR

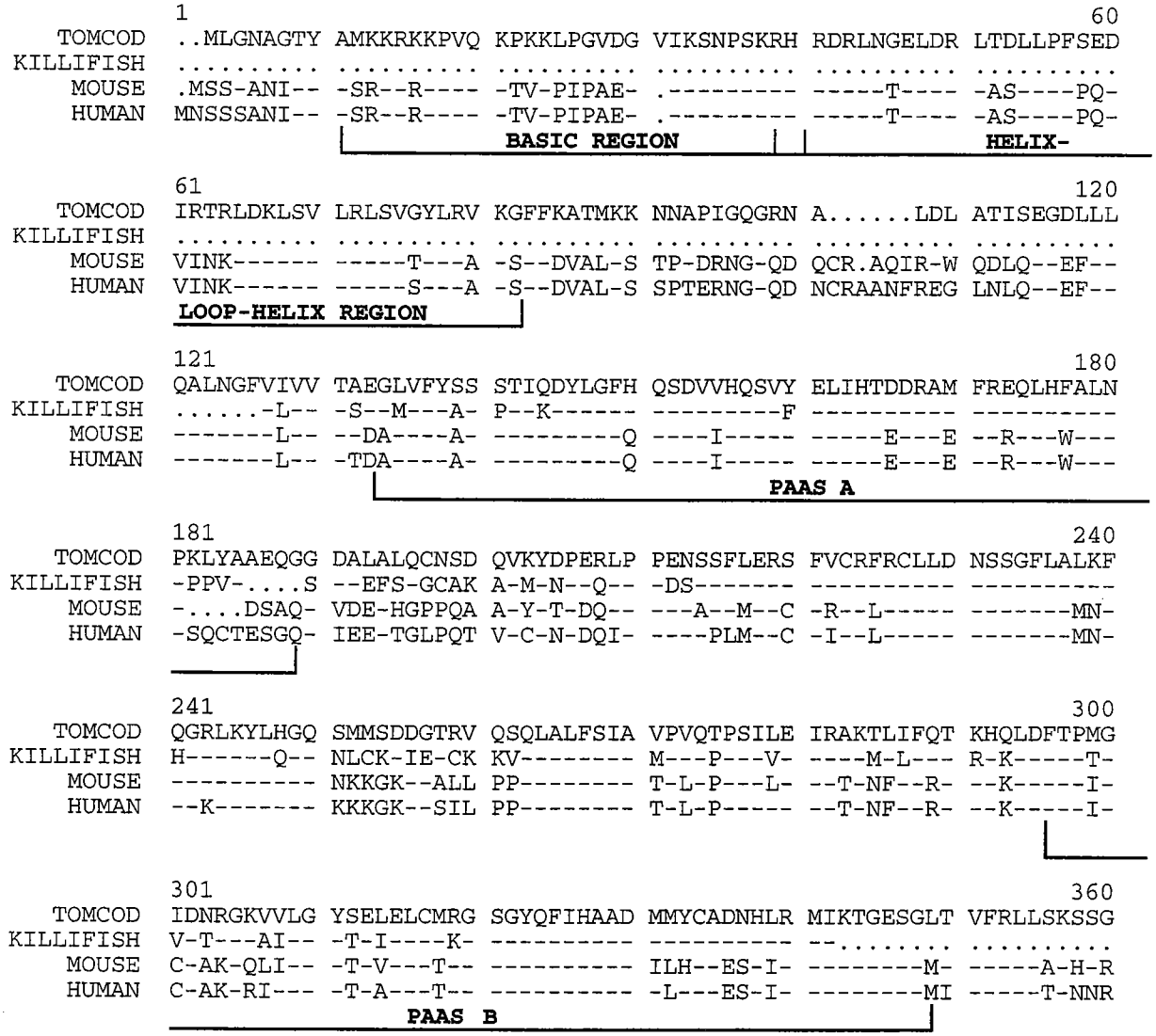

FIG. 4. A comparison of the deduced peptide sequence at the amino terminus for Atlantic tomcod, *F. heteroclitus* (killifish), mouse, and human AhR genes. Basic, helix–loop–helix, PAAS A and PAAS B regions are indicated. Identical amino acids are indicated (–). Gaps (.) were introduced to facilitate sequence alignment.

mRNA were also compared between untreated and TCB or β-NF-treated tomcod from the Hudson and the Miramichi rivers with the expectation that lower levels of gene expression would be observed in tomcod from the Hudson; however, no difference in levels of AhR mRNA expression was observed between fish from the two populations.

In additional experiments, levels of AhR mRNA expression were compared among tissues taken from Hudson and Miramichi tomcod that were untreated or treated with B[a]P or TCB and sacrificed after 6 and 24 h (B[a]P) and 1 and 5 days (TCB). Large differences in AhR mRNA levels were observed among tissues (Fig. 7). Highest levels of AhR mRNA expression were observed in gill, somewhat lower levels in heart, kidney, and spleen, and the lowest levels were seen in liver and brain. For example, mean AhR mRNA levels were 4-fold higher in gills than in liver. These same blots were then rehybridized to a tomcod CYP1A1 cDNA probe to investigate the relationship between tissue levels of AhR mRNA and CYP1A1 mRNA expression. We found no correspondance between tissue levels of AhR mRNA and CYP1A1 mRNA. Highest levels of CYP1A1 mRNA expression were observed in liver, next highest levels were in heart, and lower levels were seen in the other tissues. For example, in AH-treated fish, CYP1A1 mRNA levels were 13-fold higher in liver than in kidney and 11-fold higher than in gills. Similarly,

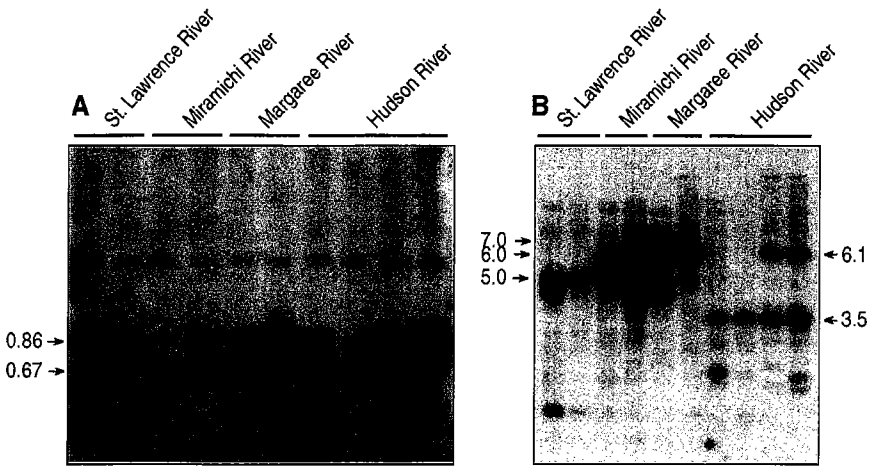

FIG. 5. Autoradiographs of Southern blot hybridization of genomic DNA from Atlantic tomcod from the Hudson, St. Lawrence, Miramichi, and Margaree rivers digested with *Pvu*II and hybridized to tomcod AhR cDNA probes. Final wash conditions were 0.1× SSC/0.1% SDS at 65°C for 1 h. Polymorphic fragments are indicated by arrows and molecular sizes (bp) are indicated. A depicts fragments in AhR exon 10 that were visualized by hybridization to an 0.86-kb cDNA probe and B depicts fragments that were visualized by hydridization to a 1.4-kb cDNA probe that extends from the 3′ end of exon 10 through the end of exon 11.

CYP1A1 mRNA levels were 3-fold higher in heart than in kidney. Interindividual variation in levels of AhR mRNA within tissue types were small. In contrast, interindividual variation within tissue types in levels of CYP1A1 mRNA were large.

DISCUSSION

This study was initiated to determine the mechanistic bases of variation in CYP1A1 mRNA inducibility between tomcod from a population that is chronically challenged with xenobiotics (Hudson River) and from a population that is exposed to much lower levels of AH compounds (Miramichi River) (3, 13). The importance of determining the mechanistic basis of noninducibility of CYP1A1 extend beyond that of CYP1A1-mediated DNA damage and cancer-related endpoints because of the central role of the AhR pathway in mediating most, if not all, toxic consequences resulting from exposure to AH compounds (15). For example, the importance of the AhR pathway in hepatic cell proliferation (40), teratogenicity (41), immunological impairment (42), normal liver development (43), and early life stage mortality (44) have been demonstrated. We hypothesize that noninducibility of CYP1A1 mRNA in tomcod from the Hudson River results from down-regulation of the AhR pathway through either genetic or epigenetic mechanisms and could therefore impact on a host of higher level biological endpoints in tomcod from the Hudson River population.

A comparison of inducible hepatic protein binding, presumably AhR complex, to tomcod CYP1A1 DREs suggested that variation in CYP1A1 mRNA inducibility between tomcod from the Hudson and the Miramichi rivers may in part result from differences in levels of AhR expression or the ability of AhR complex to bind DREs. Temporal profiles of induction and clearance of CYP1A1 and levels of maximum-fold induction are very similar between tomcod from the Hudson and

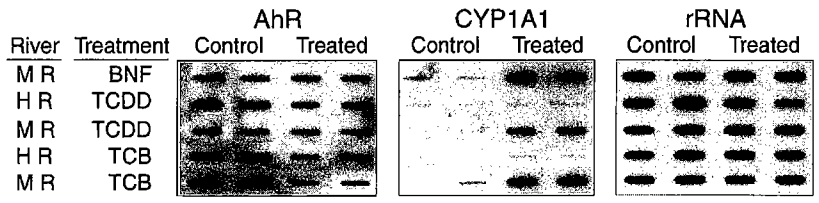

FIG. 6. Autoradiographs of a slot blot of hepatic AhR mRNA, CYP1A1 mRNA, and 18S rRNA from Atlantic tomcod from the Hudson River (HR) and the Miramichi River (MR) that were treated (i.p.) with vehicle controls (corn oil and DMSO) or aromatic hydrocarbon compounds (β-naphthoflavone (β-NF), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), or 3,3′,4,4′-tetrachlorobiphenyl (TCB). The slot blot was originally hybridized to a tomcod AhR cDNA probe, then to a tomcod CYP1A1 cDNA probe, and finally to a rat 18S rRNA probe. Final wash conditions were 0.4× SSPE/0.1% SDS at 65°C for 1 h. Vehicle controls for chemical treatments were: β-NF-corn oil; TCDD-DMSO; and TCB-DMSO. Each slot contains RNA from a different fish.