Pharmacogenetics (1996) **6**, 273–277

Short communication

Characterization of *CYP1A1* gene regulatory elements in cancer-prone Atlantic tomcod*

Nirmal K. Roy, Blake Konkle and Isaac Wirgin†

Institute of Environmental Medicine, New York University Medical Center, Long Meadow Road, Tuxedo, New York 10987, USA

Received 17 July 1995 and accepted 28 September 1995

Keywords: Atlantic tomcod, CYP1A1, gene regulation, hepatocellular carcinomas

Abbreviations: AhR, aromatic hydrocarbon receptor; TCDD, tetrachlorodibenzo-*p*-dioxin; PAH, polynuclear aromatic hydrocarbon; PCB, polychlorinated biphenyl; AhRE, Ah receptor/aromatic hydrocarbon response element; TCB, tetrachlorobiphenyl; HAH, halogenated aromatic hydrocarbon; B[a]P, benzo[a]pyrene; β-NF, betanaphthoflavone; GRE, glucocorticoid responsive element

CYP1A1 gene expression in fish is induced by exposure to aromatic compounds including: PAHs, some coplanar PCBs, dioxins, and furans (Stegeman & Hahn, 1994), and is widely used to monitor levels of aromatic hydrocarbon contamination in aquatic systems. AhR protein has been identified in several fish species (Hahn & Stegeman, 1992); however, *CYP1A1* regulatory elements have yet to be functionally evaluated and their inducible protein binding profiles characterized. Atlantic tomcod (*Microgadus tomcod*) is a bottom-dwelling species which is common in Atlantic coast estuaries and has been used as a sentinel species to monitor environmental degradation (Cormier & Raccine, 1990). Tomcod from the Hudson River display an unusually high prevalence of hepatic neoplasia; over 90% of 2-year-old fish exhibit hepatocellular carcinomas whereas their frequency is less than 5% in fish from cleaner rivers (Dey *et al.*, 1993). We used hepatic *CYP1A1* gene expression as a biomarker to evaluate exposure levels of tomcod in North Atlantic coast estuaries to aromatic hydrocarbon contaminants and found significantly higher levels of CYP1A1 mRNA in tomcod from the Hudson River compared to fish from other rivers in which tomcod exhibit a much lower prevalence of hepatic tumours (Wirgin *et al.*, 1994). Furthermore, some tomcod from the Hudson River exhibited a 606 bp deletion in the 3′ untranslated region of CYP1A1 mRNA, a polymorphism which was absent in tomcod from other rivers (Roy *et al.*, 1995).

During the course of these studies we observed: 1) high levels of inter-individual variability in CYP1A1 mRNA inducibility; the magnitude of this variation was large even in calibration experiments in which tomcod matched for size, sex, and reproductive status were treated with model aromatic compounds under controlled laboratory conditions (Courtenay *et al.*, 1994); and 2) non-inducibility of CYP1A1 mRNA in tomcod which were collected from the Hudson River, extensively depurated, and subsequently treated with two halogenated aromatic compounds; 3,3′,4,4′ TCB or 2,3,7,8-TCDD (Wirgin *et al.*, 1992). In contrast, these halogenated compounds elicited high levels of CYP1A1 mRNA induction in tomcod collected from more pristine rivers (Courtenay *et al.*, 1993). Furthermore, CYP1A1 mRNA was inducible in tomcod from all rivers with two PAHs; B[a]P and β-NF,

These results suggest that CYP1A1 mRNA induction by HAHs is inhibited in tomcod from the Hudson River. Inhibition could result from: 1) persistent DNA-protein interactions in tomcod environmentally exposed to slowly metabolized halogenated compounds; or 2) genetic adaptation resulting from strong selective pressures against inducible genotypes in fish from HAH polluted environments such as the Hudson River. These results also indicate that molecular mechanisms regulating CYP1A1 induction in tomcod differ in fish treated with halogenated versus non-halogenated compounds. To initially address these issues, we sought to

*The sequence data reported in this paper has been submitted to the EMBL/GenBank Data Libraries under the accession number L41917.

†To whom correspondence should be addressed Phone: 914-351-2415. Fax: 914-351-5472. E-mail: Wirgin@charlotte.med.nyu.edu

characterize 5′ and intronic *CYP1A1* regulatory elements in tomcod from the Hudson River.

A tomcod genomic DNA library was developed and screened with a 650 bp tomcod CYP1A1 cDNA probe (Roy *et al.*, 1995) containing 115 bp of 5′ untranslated cDNA. One CYP1A1 hybridizable 4.8 kb *Pst* I fragment was identified which hybridized strongly with the tomcod CYP1A1 5′ cDNA probe and this fragment was sequenced using 76F, 85R (Table 1) and M13 primers. We report 2.7 kb of sequence from the tomcod *CYP1A1* 5′ flanking region, exon 1 and part of intron 1 in Fig. 1. With the exception of short regulatory elements, this sequence shows no similarity to *CYP1A1* 5′ flanking regions in mammalian species (Kubota *et al.*, 1991) or in rainbow trout (Berndston & Chen, 1994). This lack of similarity between tomcod and trout is surprising given the sequence similarity among some mammalian species in proximal 65–130 nucleotide (nt) tracts in *CYP1A1* 5′ flanking regions (Storm *et al.*, 1992).

The tomcod *CYP1A1* transcriptional start site was identified by primer extension analysis and is indicated in Fig. 1. The tomcod *CYP1A1* 5′ flanking region contains several domains that contribute to transcriptional activity. These include a TATA box and sequences that resemble recognition sites for CCAAT box transcription factor/nuclear factor I. The TATA box (TATAAA) was 26 nt upstream from the transcriptional start site and one CCAAT box was 18 nt upstream of the TATA box.

At least six functional AhREs have been identified in *CYP1A1* flanking regions in all mammalian species investigated which contained a core sequence of 5′-TNGCGTG-3′ (Lusska *et al.*, 1993). In comparison, the tomcod *CYP1A1* 5′ flanking region contains four core AhRE sequences which are identical to those in mammalian species. These AhRE core sequences were −762, −838, −2364, and −2459 nt upstream from the transcriptional start site. Additionally, a fifth motif at −245 nt has a 5′-GCGTG-3′ sequence, although 5′ flanking sequences differ from those in the mammalian consensus AhRE. Studies have indicated the significance of sequences immediately flanking AhREs in maintaining specificity of DNA-protein interactions and enhancement of *CYP1A1* mRNA expression (Lusska *et al.*, 1993). It was demonstrated that the AhR complex has binding specificities for flanking regions that span at least 19 nucleotides (Swanson & Bradfield, 1995). While core AhRE sequences in tomcod and mammals are identical, there is dissimilarity in sequences flanking the core motifs between these taxa. Furthermore, there is little similarity among individual tomcod AhREs in flanking sequences. Sequences similar to Sp1 sites and basic transcription elements which modulate *CYP1A1* transcription in rodents (Yanagida *et al.*, 1990) were absent in the tomcod 5′ flanking region.

Table 1. Oligonucleotide primers used in amplification and sequencing of tomcod CYP1A1 5′ flanking region

Primers	*Sequence*	*5′ Nucleotide position*
85R	5′ ATCTACAGTCCAAGTAG 3′	−342
76F	5′ ACAACAAACGAGATCCC 3′	+91
P1	5′ CAGAGGCTGCTGCTGC 3′	−2480
P2	5′ GCGCCTGTGGTGCTG 3′	−2339
P3	5′ AGAGAGAGACTATGG 3′	−875
P4	5′ GTTCCTCAACAGTTTG 3′	−720

The presence of multiple GRE consensus sequences (5′-TGT$^{T}/_{C}$CT-3′) within intron 1 of the human *CYP1A1* gene have been reported (Hines *et al.*, 1988). In tomcod, consensus GRE sequences were found at −288, −1929, and −1973 nt upstream from the tomcod *CYP1A1* transcription start site. A sequence similar to that of a GRE (5′-TGTACT-3′) was also found within the first intron (2977 nt) (Roy *et al.*, 1995). Consensus negative regulatory elements (NRE) ($G^{T}/_{G}$GCTCTG) that downregulate the *CYP1A1* promoter have been identified in the 5′ upstream region of mammalian CYP1A1 genes (Gonzalez & Nebert, 1985; Boucher *et al.*, 1993). The tomcod *CYP1A1* upstream region does not contain this sequence in either orientation. We did find 5′-GCTCT-3′ or 5′-CTCTG-3′ sequences, but their flanking sequence do not meet other criteria (e.g. absence of G-C rich regions) thought to be necessary in negative regulatory domains.

Electrophoretic mobility shift assays (EMSA) were used to initially evaluate binding between the putative tomcod AhRE and proteins in nuclear extracts prepared from the livers of tomcod treated with a dose of B[a]P previously demonstrated to significantly induce hepatic CYP1A1 mRNA expression (Courtenay *et al.*, 1994). Tomcod were collected from the Hudson River and a subset were immediately killed. The remaining fish were returned to the laboratory, depurated in clean water for a minimum of 7 d, and i.p. injected with 10 ppm B[a]P in 0.1–0.3 ml of emulphor acetone vehicle, 0.2 ml of emulphor acetone, or were untreated. Fish were killed 1 h, 2 h, 6 h, 10 h, and 24 h following treatment and their livers were excised. Additionally, reactivity of the putative fish AhRE sequences with inducible nuclear protein extracts prepared from mammalian tissue and cells was evaluated. A single Sprague Dawley rat was i.p. injected with 50 ppm B[a]P, killed 20 h later, and its liver excised. MOLT-4 is a culture derived from the blood of a human with acute lymphoblastic leukemia. These cells were grown overnight, harvested, and 10^{7} cells were added to fresh media supplemented with 0.1% DMSO or DMSO containing

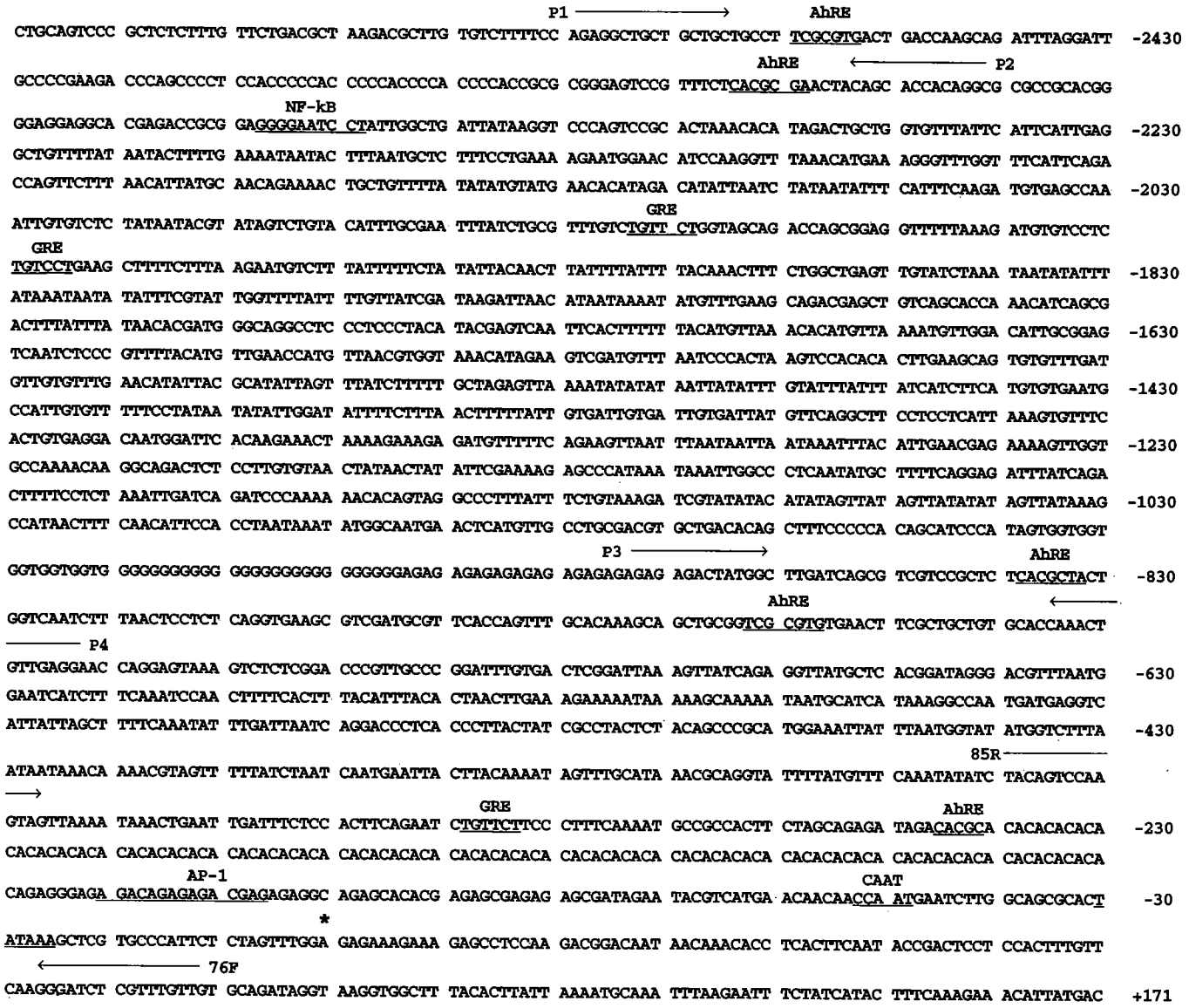

Fig. 1. The nucleotide sequence of 2.7 kb of Atlantic tomcod 5′ *CYP1A1* genomic DNA. Nucleotide positions are in relation to the 5′ end of the transcriptional start site previously determined by primer extension analysis and indicated by a (*). Nucleotides 5′ to the transcription start site are numbered consecutively with negative numbers, whereas nucleotides 3′ of the transcription start site are numbered consecutively with positive numbers. The consensus TATA box is underlined and the upstream promoter CCAAT box is labelled and underlined. Transcription factor binding sites, indicated by underlines, were determined using the transcription factor site database (TFSITES) in combination with the FINDPATTERN algorithm of GCG sequence analysis software. Five putative AhRE sequences are labelled and underlined. PCR and sequencing primer positions are indicated with horizontal arrows showing the direction of amplification.

10 μM DMBA. Treated cells were grown for 4 h and harvested.

Nuclear protein extracts were prepared from fresh livers or cells and EMSAs were conducted essentially as described (Roy *et al.*, 1991). Oligonucleotide primers were designed to amplify tomcod genomic DNA fragments containing putative ALRE elements (Table 1). Amplification products were gel purified and ^{32}P radiolabelled. Equimolar amounts of complementary oligonucleotides (5′ GATCTCTTCTCACGCAACTCCGAG-3′ and 5′ GATCCTCGGAGTTGCGTGAGAAGA 3′) containing mammalian AhREs (Shen & Whitlock, 1992) were used as competitors in gel shift assays. Reactants were separated electrophoretically in 4% non-denaturing polyacrylamide gels.

Protein binding was evaluated in a 142 bp DNA fragment (nt −2480 to nt −2339) containing putative tomcod AhREs at −2364 and −2459 nt and a 156 bp DNA fragment (nt −875 to nt −720) containing putative AhREs at −762 and −838 nt. Protein binding

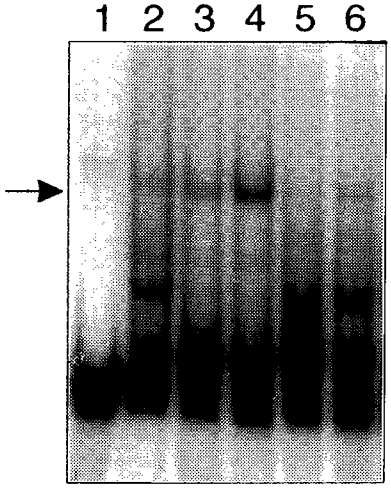

Fig. 2. An autoradiograph of an electrophoretic mobility shift assay of a 142 bp tomcod *CYP1A1* DNA fragment containing two putative AhREs at −2459 and −2364 nt (5 end) which was incubated with nuclear protein extracts prepared from tomcod livers. Tomcod were i.p. injected with a single dose of benzo[a]pyrene (B[a]P or emulphor acetone vehicle. ^{32}P labelled DNA fragment was incubated with none (lane 1), 3 μg of hepatic nuclear protein extracts of tomcod treated with vehicle (lane 2), 10 ppm of B[a]P for 2 h (lanes 3 and 5) and 6h (lanes 4 and 6). Unlabelled competitors were added at 100-fold molar excess in the binding reaction (lanes 5 and 6). DNA-protein complexes were analysed by 4% polyacrylamide gel electrophoresis. Arrow indicates shifted DNA bands.

to the 142 bp DNA fragment was observed with nuclear extracts prepared from fish treated with 10 ppm B[a]P for 2 h, 6 h, or 24 h (Fig. 2). Complexes were also observed using nuclear extracts prepared from tomcod treated with 5 ppm and 50 ppm B[a]P and killed after 24 h. Unlabelled oligonucleotides containing consensus AhRE sequences competed out nuclear protein binding and no complexes were observed with the tomcod DNA fragment (Fig. 2). This result demonstrated the specificity of inducible protein binding to AhRE-like sequences in the tomcod DNA fragment. Similarly, hepatic nuclear proteins from tomcod treated with 10 ppm B[a]P for 2 h and 6 h shifted the mobility of the 156 bp DNA fragment and one band was apparent (Fig. 3). The retardation of this complex was less than that of the 142 bp DNA fragment suggesting the likelihood that multiple protein complexes of different molecular sizes may interact with the putative tomcod AhREs. The nuclear proteins prepared from the environmentally exposed Hudson River tomcod also resulted in DNA-protein binding, however, more than one band was evident (Fig. 3). This result suggests the presence of multiple nuclear proteins which bind AhREs in environmentally exposed tomcod from the Hudson River.

The ability of nuclear proteins isolated from mammalian tissue and cells to react with the two tomcod 5′ *CYP1A1* DNA fragments was also tested. Nuclear proteins prepared from DMBA-treated rat liver and human MOLT-4 cells complexed with the tomcod *CYP1A1* fragments (data not shown). However, the electrophoretic mobility of the mammalian and tomcod DNA-protein complexes were different. This indicated the ability of mammalian nuclear proteins to recognize the putative tomcod 5′ CYP1A1 AhREs, but suggests that there are differences in structure between inducible fish and mammalian binding protein complexes.

Our results indicate that molecular mechanisms regulating *CYP1A1* gene expression in tomcod and mammalian species are similar. Earlier studies in fish models have indicated that *CYP1A1* gene expression in aromatic hydrocarbon-treated fish is regulated at the transcriptional level (Stegeman & Hahn, 1994) and the presence of a putative AhR which binds with TCDD-like ligands in the cytoplasm (Hahn & Stegeman, 1992). Our study extends these observations by demonstrating that the tomcod 5′ *CYP1A1* region contains motifs

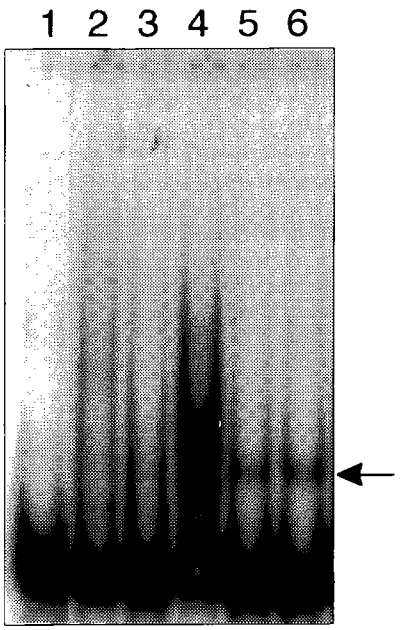

Fig. 3. An autoradiograph of an electrophoretic mobility shift assay of a ^{32}P radiolabelled 156 bp tomcod DNA fragment containing putative AhRE sequences at −838 and −762 nt (5′ end). Fragment was incubated with none (lane 1), 3 μg of nuclear protein extracts prepared from livers of; untreated tomcod (lane 2), tomcod treated with corn oil vehicle (lane 3), 10 ppm B[a]P for 2 h (lane 5), 10 ppm B[a]P for 6 h (lane 6) and tomcod collected from the Hudson River and immediately killed (lane 4). DNA-protein complexes were analysed by 4% polyacrylamide gel electrophoresis. Arrow indicates shifted DNA bands.

which are identical to AhRE core sequences in mammalian species, although their number, position, and flanking sequences are different. Furthermore, aromatic hydrocarbon-inducible nuclear proteins from both fish and mammals recognize these tomcod AhRE-like sequences. However, reporter gene assays are still needed to confirm and quantitatively evaluate the functional activities of these putative tomcod regulatory sequences. Also, the relationship between the degree of protein binding and *CYP1A1* gene induction has yet to be systematically established in this model. Nevertheless, this model should provide the opportunity to determine if non-inducibility of CYP1A1 mRNA in HAH-treated tomcod and variability in gene expression results from polymorphisms in *CYP1A1* 5′ regulatory elements or in the structure or levels of AhR pathway molecules. Furthermore, this model should provide the ability to sensitively assess the exposure history of tomcod from polluted waterways.

Acknowledgements

We thank Eli Flescher, Joseph Stabile, Lorraine Maceda, and Jannine Rosko for technical assistance and the Hudson River Foundation and NIEHS Grants ES05003, ES05541 to IW and NIEHS Center Grant ES00260 for support of this project.

References

Berndston AK, Chen TT. Two unique CYP1A genes are expressed in response to 3-methylcholanthrene treatment in rainbow trout. *Arch Biochem Biophys* 1994: **310**, 187–195.

Boucher PD, Ruch RJ, Hines RN. Specific nuclear protein binding to a negative regulatory element on the human CYP1A1 gene. *J Biol Chem* 1993: **268**, 17384–17391.

Cormier SM, Raccine, RN. Histopathology of Atlantic tomcod: a possible monitor of xenobiotics in northeast tidal rivers and estuaries. In: McCarthy JF, Shugart LR. eds. Biological markers in environmental contamination. Chelsea, MI: Lewis Publishers 1990: 59–71.

Courtenay S, Grunwald C, Kreamer G-L, Alexander R, Wirgin I. Induction and clearance of CYP1A mRNA in Atlantic tomcod caged in a bleached kraft mill effluent in the Miramichi River. *Aquat Toxicol* 1993: **27**, 225–244.

Courtenay S, Williams PJ, Grunwald C, Konkle B, Ong T-L, Wirgin II. Assessment of within-group variation in CYP1A mRNA inducibility in environmentally exposed and chemically treated Atlantic tomcod. *Environ Health Perspect* 1994: **102**(Supple. 12), 85–90.

Dey WP, Peck TH, Smith CE, Kreamer G-L. Epizoology of hepatic neoplasia in Atlantic tomcod (*Microgadus tomcod*) from the Hudson River estuary. *Can J Fish Aquat Sci* 1993: **50**, 1897–1907.

Gonzalez FJ, Nebert DW. Autoregulation plus upstream positive and negative control regions associated with transcriptional activation of the mouse P1-450 gene. *Nucl Acids Res* 1985: **13**, 7269–7288.

Hahn ME, Stegeman JJ. Phylogenetic distribution of the Ah receptor in non-mammalian species: implications for dioxin toxicity and Ah receptor evolution. *Chemosphere* 1992: **25**, 931–937.

Hines RN, Mathis JM, Jacob CS. Identification of multiple regulatory elements on the human cytochrome P450IAI gene. *Carcinogenesis* 1988: **9**, 1599–1605.

Kubota M, Sogawa K, Kaizu Y, Sawaya T, Watanabe J, Kawajiri K, Gotoh O, Fujii-Kuriyama, Y. Xenobiotic responsive element in the 5′-upstream region of the human P-450c gene. *J Biochem* 1991: **110**, 232–236.

Lusska A, Shen E, Whitlock JP Jr. Protein-DNA interactions at a dioxin-responsive enhancer. *J Biol Chem* 1993: **268**, 6575–80.

Nelson DR, Koymans L, Kamataki T, Stegeman JJ, Feyereisen R, Waxman DJ, Waterman MR, Gotoh O, Coon, MJ, Estabrook RW, Gunsalos IC, Nebert DW. P450 super family: update on new sequences, gene mapping, accession numbers, and nomenclature. *Pharmacogenetics* 1996: **6**, 1–41.

Roy RJ, Gosselin P, Guerin SL. A short protocol for micropurification of nuclear proteins from whole animal tissue. *Biotechniques* 1991: **11**, 770–777.

Roy NK, Kreamer G-L, Konkle B, Grunwald C, Wirgin I. Characterization and prevalence of a polymorphism in the 3′ untranslated region of cytochrome P4501A1 in cancer-prone Atlantic tomcod. *Arch Biochem Biophys* 1995: **322**, 204–213.

Shen ES, Whitlock JP Jr. Protein-DNA interactions at a dioxin-responsive enhancer: Mutational analysis of the DNA-binding site for the liganded Ah receptor. *J Biol Chem* 1992: **267**, 6815–6819.

Stegeman JJ, Hahn ME. Biochemistry and molecular biology of monooxygenases: current perspectives on forms, functions, and regulation of cytochrome P450 in aquatic species. In: Malins DC, Ostrander GK. eds. Aquatic toxicology: molecular, biochemical, and cellular perspectives. Florida: Lewis Publishers. 1994: 87–206.

Storm DK, Postlind H, Tukey RH. Characterization of the rabbit CYP1A1 and CYP1A2 genes: development and dioxin-inducible expression of rabbit liver p4501A1 and p4501A2. *Arch Biochem Biophys* 1992: **294**, 707–716.

Swanson H, Bradfield C. Specificity of DNA binding by the Ah-receptor, ARNT and SIM. *Fund Appl Toxicol* 1995: **15**, 92.

Wirgin II, Grunwald C, Courtenay S, Kreamer G-L, Reichert WL, Stein JE. A biomarker approach in assessing xenobiotic exposure in cancer-prone Atlantic tomcod from the North American Atlantic coast. *Environ. Health Perspect.* 1994: **102**, 764–770.

Wirgin II, Kreamer G-L, Grunwald C, Squibb K, Garte SJ, Courtenay S. Effects of prior exposure history on cytochrome P4501A mRNA induction by PCB congener 77 in Atlantic tomcod. *Mar Environ Res* 1992: **34**, 103–108.

Yanagida A, Sogawa K, Yasumoto KI, Fujii-Kuriyama Y. A novel cis-acting DNA element required for a high level of inducible expression of the rat P-450c gene. *Molec Cell Biol* 1990: **10**, 1470–1475.