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Isolation of 5'-Untranslational Region of Trout *Cyp1A1* Gene

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The genomic DNA was prepared from trout liver which was treated with 3-methycholanthrene, and cloned into lambda EMBL3 at *BamH*I site. The genomic library was constructed via infections of these recombinant phages into *E. coli* K802, and screened by the most 5'-portion of trout CYP1A1 cDNA. After the screening of 10^9 clones of the amplified library, 12 positive clones were isolated, and subjected to further screenings. The results of southern blot hybridization of genomic DNA prepared from the positive clone showed the presence of a single gene of *CYP1A1*, and 3.5 Kb *Pst*I fragment that hybridizes with the most 5'-region DNA of CYP1A1 cDNA. The restriction map of *Pst*I fragment was determined by the restriction digestion with various enzymes. The nucleotide sequence of the upstream genomic DNA of *CYP1A1* was determined by DNA sequencing of exonuclease III unidirectionally deleted *Pst*I fragment DNA using [^{35}S]dATP. This paper presented the upstream genomic DNA of CYP1A1 contained a part of coding region which was about 351 base pairs (from ATG to *Pst*I site at 3563).

Key words : Trout, CYP1A1, 5'-flanking, Cloning, 3-methylcholanthrene

INTRODUCTION

Monooxygenase reactions which mediated via cytochrome P450 enzymes occurred in microorganisms, plants, and animals (Andrew *et al.*, 1996). The substrates for cytochrome P450 enzymes were widespread ranging from physiologically occurring lipids such as steroids and prostaglandins to biologically and chemically synthesized xenobiotics such as plants metabolites, therapeutic medicines, and pesticides (Nelson *et al.*, 1993).

Many lipophilic organic xenobiotics such as polyaromatic hydrocarbon were metabolized by enzymes encoded in the *CYP1* gene family (Nebert, and Gonzalez 1987; Nebert and Nelson, 1991). Although the enzymes encoded in the *CYP1* gene family were involved in detoxification, some of intermediate metabolized by the enzyme were more toxic than parent compounds (Nebert, and Gonzalez, 1987). Two different *CYP1* genes, *CYP1A1* and *CYP1A2*, were found in human, mouse, rat and rabbit species (Hines, *et al.*, 1988; Jaiswal, 1987; Gonzalez, 1989; Sogawa *et al.*, 1986; Strom *et al.*, 1992). The expressions of *CYP1A1* and *CYP1A2* were induced by polyaromatic hydrocarbons such as 3-methylcholanthrene (3MC) and 2,3,7,8,-tetrachlorodibenzo-p-dioxin (TCDD) (Nebert and Gonzalez, 1987). In rodents, the induction of *CYP1A1* gene expression had been studied extensively and was known to involve in: (i) binding of the ligand to the aromatic hydrocarbon receptor, (ii) translocation of the occupied receptor into the nucleus, and (iii) binding of the receptor complex to specific DNA sequences upstream of the *CYP1A1* promoter (Witlock, 1986).

Full expression of *CYP1A1* gene was required for the interaction of proteins binding to the proximal element with proteins binding to the distal element (Denison and Whitlock, 1995). This interaction resulted in a rapid increase in: (i) transcription of CYP1A1 mRNA, (ii) translocation of CYP1A1 protein, and (iii) induced CYP1A1 enzyme activity (Tukey *et al.*, 1981; Kimura *et al.*, 1984; Hankinson *et al.*, 1985). In contrast, the induction of *CYP1A2* gene expression in rodents was not well understood. Although the induction was thought to involve in binding of arylhydrocarbon receptor (Nebert and Gonzalez, 1987).

Very little was known about the *CYP1* gene family in fish. The *CYP1A1* and *CYP1A2* genes in mammals were thought to have originated by a gene duplication event and diverged no more than 250 million years ago. Lower vertebrates such as fish were thought to have diverged from land animals before this time, therefore it was hypothesized that the only a single gene must exist in fish (Nebert and Gonzalez, 1987). We had been conducted the molecular clon-

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ing and characterization of CYP1A1 cDNA in rainbow trout (Heilman *et al.*, 1988). A single 2.59 Kb cDNA was isolated from a cDNA library generated from 3MC treated rainbow trout liver. The isolation of a single cDNA species supported the hypothesis that only one *CYP1A1* gene was present in fish. Although it had not been determined conclusively if only a single gene was present and expressed in fish. Therefore, the purpose of this study was to clone 5'-flanking region of *CYP1A1* gene in rainbow trout in order to understand the regulation of the *CYP1A1* gene expression in fish. If the mechanism of expression of *CYP1A1* gene was identical throughout the phylogeny.

MATERIALS AND METHODS

Materials

Restriction enzymes, T_4 DNA ligase, Klenow fragment, T_4 DNA polymerase and ExoIII nuclease, S1 nuclease were obtained from BRL (Gaithersburg, USA). [α-^{32}p]dCTP (~3,000 ci/mmol), [γ-^{32}p]ATP (~6,000 ci/mmol), and [^{35}S]dATP (500~600 ci/mmol) were purchased from Amersham International (Chicago, USA).

Construction of genomic library

Trout genomic DNA was isolated from the liver rainbow trout that was treated with 3MC (100 mg/kg) for 48hrs (Ritter *et al.*, 1991). *BamH*I digested EMBL3 lambda DNA and the isolated trout genomic DNA that was partially digested with *BamH*I were ligated using T_4 DNA ligase. *E. coli* K802 was transfected with the packaged DNA and the library was amplified.

Generation of trout cDNA probe

The most 5'-region of trout CYP1A1 cDNA was prepared by *Pvu*II digestion of pUC19 plasmid containing complete coding sequence of *CYP1A1* gene. A 224bp DNA fragment was isolated and labeled with [α-^{32}P]dCTP by nicktranslation

Screening of genomic library

Amplified genomic library was plated and plaques were transferred onto nitrocellulose paper, followed by denaturation and renaturation. Nitrocellulose filters were hybridized with ^{32}P-labeled *Pvu*II fragment DNA as a probe at 65°C for overnight, washed at 42°C with 2×SSC and autoradiographed.

Southern blot hybridization

Lambda DNA was isolated from the positive clone after the tertiary screening gemonic library with cDNA probe. Lambda DNA was digested with various restriction enzymes and analyzed on 1% agarose gel electrophoresis, which then blotted onto nitrocellulose paper. Filters were hybridized with ^{32}P labeled cDNA probe for overnight at 65°C. Washing the filter at 42°C with 2×SSC, autoradiograph was conducted.

Cloning of 5'-flanking DNA of trout *CYP1A1* gene

A 3.5 Kb *Pst*I fragment DNA was the largest DNA band that hybridized to cDNA probe, and which was isolated and subcloned into pUC19 at *Pst*I site. Plasmid DNA was transformed into *E. coli* DH5α cells and white colonies as a positive clones were selected from agar plate containing X-gal and IPTG.

DNA sequencing of 5'-flanking DNA of trout *CYP1A1* gene

pUC19 plasmid containing *Pst*I fragment DNA was digested with *Nco*I and *Sst*I. Unidirectional deletion constructs were generated by ExoIII digestion followed by ligation. Using unidirectional deleted double straned DNA, dideoxynucleotide sequencing was conducted with [^{35}S]-dATP (Ritter *et al.*, 1991).

RESULTS AND DISCUSSION

Construction of genomic library

Genomic DNAs from 3MC treated trout liver were isolated and DNAs corresponding to 10 Kb~20 Kb in size were fractionated with 5~40% sucrose density

Fig. 1. Result of primary screening of genomic library. Few hundreds plaques of amplified genomic library were plated on agar plate and were transferred onto nitrocellulose paper, followed by denaturation and renaturation. Nitrocellulose filters were hybridized with ^{32}P-labeled *Pvu*II fragment DNA as a probe at 65°C for overnight. And Filters were washed at 42°C with 2×SSC and autoradiographed. Circled plaques were isolated for the further screening.

gradient centrifugation. Purified genomic DNAs were cloned into lambda vector, EMBL3 and transfected into *E. coli* K802. The Library was amplified so that the titer of library was about 10^9.

Genomic library screening

10^9 genomic plaques were screened with nick-translated 224bp cDNA fragment that was the most 5'-region of CYP1A1 cDNA, and 12 plaques were identified as positive clones from filters (Fig. 1). These positive clones indicated as circles in figure 1 were subjected to secondary and tertiary screening with the same cDNA probe. Results of tertiary screening of duplicated filters of genomic library of trout liver with the most 5'-region of cDNA were shown in figure 2 and all of the plaques undergone to the tertiary screening turned out to be positive clones (Fig. 2).

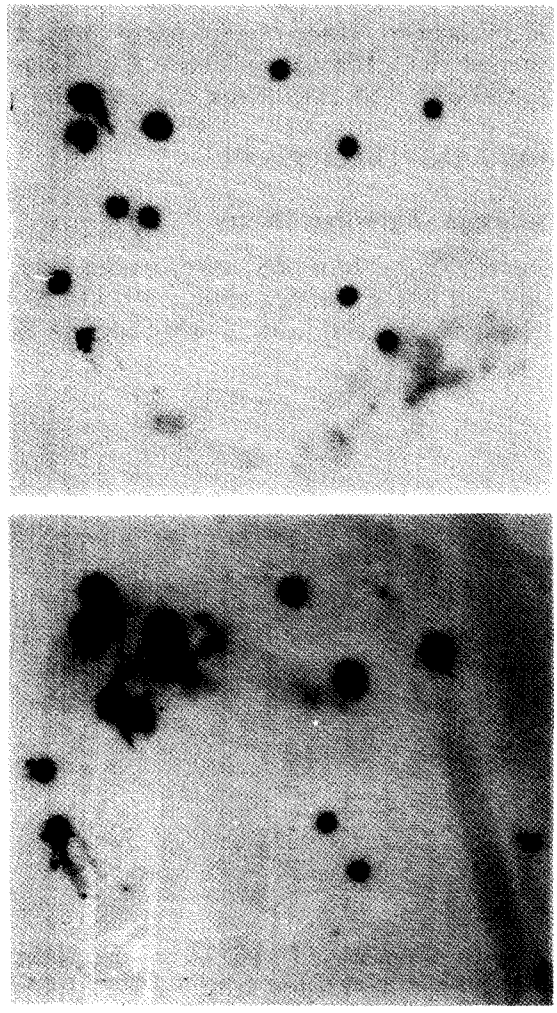

Fig. 2. Result of tertiary screening of genomic library. Few hundreds plaques of amplified positive clones from secondary screening were plated on agar plate and screening was carried out the same way as the primary screening. Autoradiography of duplicated filters were shown.

Southern blot hybridization

One positive clone from the tertiary screening of trout liver genomic library with cDNA probe (Fig. 2) was amplified and lambda DNA from the positive clone was isolated. Digestion of the lambda DNA with *Pst*I and *Pvu*II resulted in restriction enzyme digestion pattern shown in Fig. 3A. Southern blotting of the restriction enzyme digested lambda DNA onto nitrocellulose paper followed by hybridization with the most 5'region of trout CYP1A1 cDNA (224 bps) showed *Pst*I digested 3.5 Kb DNA and *Pvu*II digested 0.6 Kb DNA fragments of trout liver genomic DNA appeared to hybridize with cDNA probe (Fig. 3B). And double restriction enzyme digestion of genomic DNA with both *Pst*I and *Pvu*II enzymes resulted in only 0.6 Kb DNA fragment that hybridized to the cDNA probe (Fig. 3B). From these data it was clear that *Pvu*II fragment DNA resided within *Pst*I fragment of trout liver genomic DNA. And also, the intensity of DNA band on southern blot hybridization analysis in-

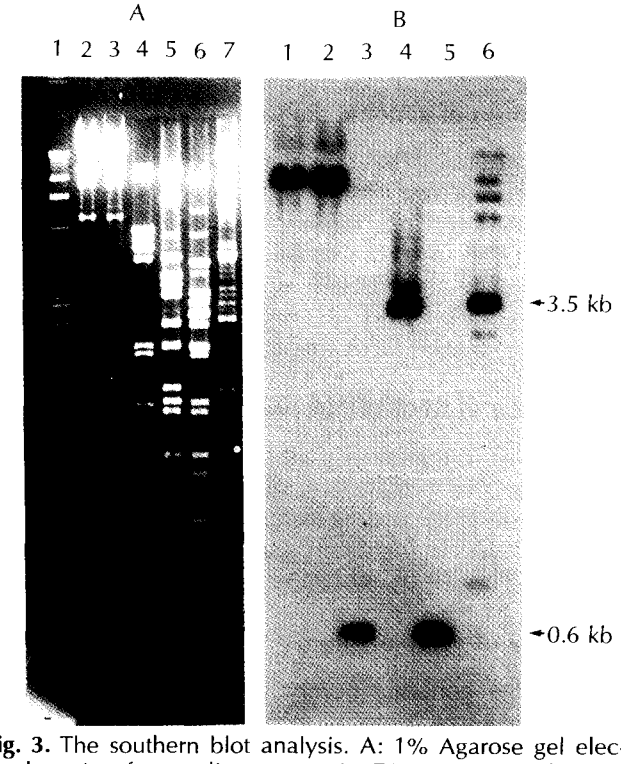

Fig. 3. The southern blot analysis. A: 1% Agarose gel electrophoresis of trout liver genomic DNA (1.5 ug) digested with various restriction enzymes. (1: *Hind*III cut lambda DNA, 2: *Sal*I, 3: *Sal*I+*Puv*II, 4: *Pst*I, 5: *Pst*I 6: *Puv*II+*Pst*I, 7: *Sal*I+*BamH*I) B: Nitrocellulose filters to which trout liver genomic DNA (1.5 ug) digested with various restriction enzymes were blotted on after the electophoresis on 1% agarose gel (1: *Sal*I, 2: *Sal*I+*Pvu*II, 3: *Pvu*II, 4: *Pst*I, 5: *Pvu* II+*Pst*I, 6: *Sal*I+*BamH*I). Nitrocellulose filters were hybridized with ^{32}P-labeled *Pvu*II fragment DNA as a probe at 65°C for overnight. And Filters were washed at 42°C with 2× SSC and autoradiographed.

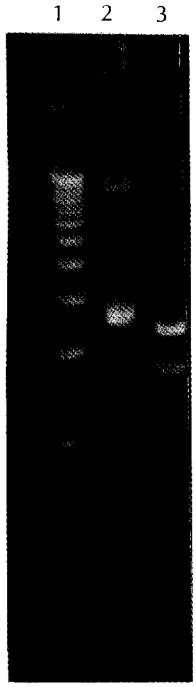

Fig. 4. Cloning of PstI fragment into pUC19 vector. Trout liver genomic DNA (1.5 ug) was digested with *Pst*I at 37°C for overnight and analyzed on 1% agarose gel. 3.5 Kb DNA fragment was gel isolated and ligated to calf intestinal phoshatase treated *Pst*I cut pUC19 vector. Ligation mix was transformed *E. coli* DH5α. From the white colony from IPTG and X-gal plate, plasmid DNA was isolated. 1: *Hind*III digested lambda DNA, 2: *Pst*I fragment cloned pUC19 plasmid, 3: pUC19 vector

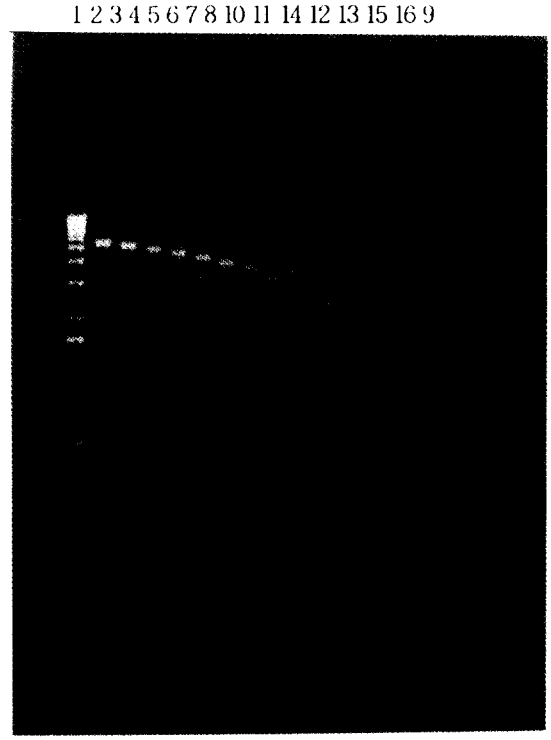

Fig. 5. The exonucleaseIII deletion of plasmid. pUC19 plasmid containing *Pst*I fragment DNA was digested with *Nco*I and *Sst*I. Double restriction enzyme digested *Pst*I fragment DNA containing plasmids were digested with exonuclease III for various time. 1% of agarose gel electrophoresis of unidirectionally deleted DNAs were shown (1: 1Kb ladder, 2:0 sec, 3:30 sec, 4:1 min, 5:1.5 min, 6:2 min, 7: 2.5 min, 8:3 min, 9:3.5 min, 10:4.0 min, 11:4.5 min, 12:5 min, 13:5.5 min, 14:6.0 min, 15:6.5 min 16:7.0 min).

dicated that there might be a single gene of cytochrome *P4501A1* gene in trout liver. Dr. Nebert hypothesized that the only a single gene would exist in fish (Debert and Gonzalez, 1987), and our finding seemed to support his hypothesis.

5'-flanking DNA of trout liver *CYP1A1* gene

*Pst*I digested genomic DNA fragment (3.5 Kb) was isolated from southern blot and subcloned into pUC19 vector, and plasmid was transformed *E. coli* DH5α. 1% agarose gel analysis of plasmid DNA confirmed the subcloning of *Pst*I fragment DNA into pUC19 vector (Fig. 4). In order to determine the nucleotide sequence of 5'-flanking DNA of trout liver *CYP1A1* gene, pUC19 plasmid containing *Pst*I fragment DNA was digested with *Sst*I and *Nco*I. And these double restriction enzyme digested DNAs were 3'-unidirectionally deleted with exonucleaseIII (Fig. 5). Each deleted DNA was ligated and transformed into *E. coli* DH5α cells and each deleted plasmid DNA was undergone to DNA sequencing using ^{35}S-dATP. From the DNA sequence data (not shown), the restriction enzyme map of 5'-flanking DNA of trout liver *CYP1A1* was genereated (Fig. 6). The start site of mRNA synthesis, ATG was located at 3189 and second *Pvu*II site located at 3413 which was used to generate probe of the most 5'-portion of trout liver CYP1A1 cDNA via *Pvu*II digestion. These data showed the cloned *CYP1A1* genomic DNA from 3MC treated trout liver contained a part of coding region of cytochrome P4501A1 cDNA, which was about 351bps (from ATG to Pst1 site at 3563). In other words, we cloned the trout liver *CYP1A1* gene encoded the trout liver CYP1A1 cDNA that was used for screening trout liver genomic library. Our previous study showed trout liver CYP1A1 mRNA was increased by arylhydrocarbons, such as 3MC (Heilman, *et al.*, 1988). It would be interesting to know how this cloned 5'-flanking region of *CYP1A1* gene play a role for the regulation of gene expression by inducers, such as 3MC. Based on [^{3}H]TCDD binding analysis on sucrose gradient, it was clear that arylhydrocarbon receptors were present in trout liver (Heilman *et al.*, 1988), although it needed to be examined if the arylhydrocarbon receptors would be functional as the same way as those were in mouse liver. One could

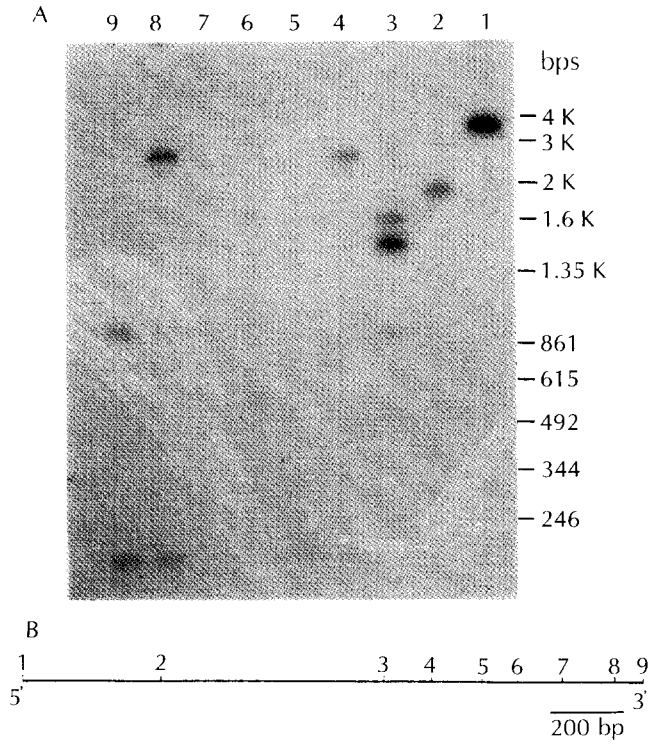

Fig. 6. The restriction map of the PstI fragment DNA. A: Aoutoradiograph of 1% agarase gel of 5'-labeled with [^{32}P] dATP PstI fragment digested with various restriction enzyme (1: *EcoR*I, s: *Hind*III, 3: *Nco*I, 4: *Nde*I, 5: *Pvu*II, 6: *Nco*I+*Pvu*II, 8: *Nde*I+*Pvu*II, 9: *Nco*I+*Nde*I+*Pvu*II), B: Map of restiction enzyme. (1: *Pst*I, 2: *Hind*III, 3: *Hind*III, 4: *Hind*III, 5: *Nde*I, 6: *Pvu*II, 7: *Nco*I, 8: *Pvu*II, 9: *Pst*I)

clone this 5'-flanking DNA of trout liver *CYP1A1* gene in front of reporter gene such as chloramphenicol acetyltransferase and then transfect this construct into arylhydrocarbon receptor positive cells in order to study the mechanism of the regulation of *CYP1A1* gene expression. Cloning of 5'-flanking DNA of trout liver *CYP1A1* would be significantly useful for toxicological and environmental studies as well as molecular biological studies of eucaryotic gene expression regulation.

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