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Stimulation of Trout *CYP1A* Gene Expression in Mouse HEPA-1 Cells by 3-Methylcholanthrene

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Trout *CYP1A*-CAT expression construct was generated by cloning ~3.5 Kb 5' flanking DNA of trout liver *CYP1A* gene in front of CAT gene at pCAT-basic vector. Hepa 1 cells, which are known to contain a functional arylhydrocarbon receptor' were transfected with trout *CYP1A*-CAT using lipofectin. 3-Methylcholanthrene (1 nM) was added into hepa 1 cells in culture in order to examine if 5' flanking DNA of trout *CYP1A* gene could interact with mouse transactivating factors to bring about transcription of the chloramphenicol acetyltransferase(CAT) reporter gene. The level of CAT protein was measured by CAT ELISA and the level of CAT mRNA was determined by RTPCR. The treatment of 1 nM 3-methylcholanthrene resulted in two fold increases in CAT protein as well as CAT mRNA compared to untreated control hepa 1 cells. These data indicate that arylhydrocarbon receptors of mouse hepa 1 cells are functional to activate exogenously transfected trout *CYP1A*-CAT construct in terms of both transcription and translation of CAT. We also examined the effect of 3-methylcholanthrene on endogenous *cyp1a1* activity in hepa 1 cell. 3-Methylcholanthrene (1 nM) treatment to hepa 1 cells transfected with trout *CYP1A*-CAT construct stimulated the level of cyp1a1 mRNA by two folds and the activity of ethoxyresorufin-*O*-deethylase by two fold compared to that of control cells. In this study we reported that trout *CYP1A*-CAT reporter gene expression construct could be expressed by 3-methylcholanthrene treatment in mouse hepa 1 cells. Thus trout *CYP 1A*-CAT could serve as a good model to study the mechanism of regulation of *CYP1A1* gene expression.

Key words : Trout *CYP1A*, 3-Methylcholanthrene, Hepa 1 cells, Ethoxyresorufin-*O*-deethylase, Chloramphenicol acetyltransferase (CAT)

INTRODUCTION

Cytochrome P450s collectively constitute a gene super family, with the evolution of a large number of P 450s that mediate various monooxygenase reactions (Gonzalez, 1989; Nebert and Gonzalez, 1987; Nelson *et al.*, 1993). The functional characteristics of many different P450s are often accentuated in animals by the administration of agents that increased the physical abundance of the proteins, a phenomenon, called induction. Depending upon the type of chemicals used to enhance P450 inducibility, the mechanisms contributing to the rate limiting events have been shown to be controlled at different cellular and molecular levels. Events that are controlled at the level of gene transcription (Denison and Whitlock, 1995; Whitlock, 1986), and posttranscriptional regulation such as mRNA and protein stabilization (Tukey *et al.*, 1981; Kimura *et al.*, 1984; Hankinson *et al.*, 1985), have all been implicated in P450 inducibility. In adult animals, treatment with polycyclic aromatic hydrocarbons (PAH) such as 3-methylcholanthrene (3-MC) or halogenated aromatic hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) resulted in the induction of the expression of *CYP1A1* and *P4501A2* (Hines *et al.*, 1988; Jaiswal *et al.*, 1987; Gonzalez *et al.*, 1988; Sogawa *et al.*, 1986; Strom *et al.*, 1992). While previous reports suggested that PAHs induced *P4501A2* at the level of posttranscription, recent reports have demonstrated that TCDD results in the induction of *P4501A1* and *P4501A2* mRNAs from transcriptional activation of the *cyp1a1* and *cyp1a2* genes (Nebert and Nelson, 1991). Analysis of *P4501A1*-dependent benzo(a)pyrene hydroxylase activity and quantification of RNA levels indicate that the induction of *P4501A1* can occur in many different tissues (Amdur *et al.*, 1996) based on analysis of *P4501A1* benzo(a) pyrene hydroxylase activity and the level of mRNA, suggesting that tight tissue specific control of the *CYP 1A1* gene did not exist. The molecular events associated with transcriptional activation of the *CYP1A1*

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gene involved binding of TCDD or 3MC with high affinity to the cytosolic dioxin Ah-receptor, followed by nuclear translocation and binding to specific enhancer sequences flanking the *CYP1A1* gene promoter (Whitlock, 1986). Interestingly, *CYP1A2* mRNA have been observed only in liver and nasal mucosa (Gillner *et al.*, 1987; Lund *et al.*, 1985; Dahl, 1995), indicating that the regulation of *CYP1A2* gene is controlled by factors that influence the tissue-specific expression of this gene. Transfection and expression of the human *CYP1A2* gene in HepG2 and not in the breast cancer MCF-7 cells confirmed that the expression of *CYP1A2* was regulated by tissue-specific factors (Quattrochi and Tukey, 1989). Differences have also been observed in the developmental expression of *CYP1A1* and *P4501A2* in response to inducers such as 3-MC. The expression of the *CYP1A1* had been detected constitutively in fetal rodent tissues (Omiecinski *et al.*, 1990; Dey *et al.*, 1989). Exposure of pregnant animal to 3-MC resulted in a differential pattern of expression of the *CYP1A* genes in the newborns, with *P4501A2* mRNA expression detectable 1-2 days following birth (Ikeda *et al.*, 1983).

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 has been hypothesized that the only a single gene must exist in fish (Nebert and Gonzalez, 1987). We have cloned *CYP1A1* gene from rainbow trout liver (Roh and Sheen, 1996). In this study, we constructed expression system of fish *P4501A* by cloning trout 5'flanking DNA in front of CAT gene. Hepa 1 cells were transfected with trout *P4501A*-CAT plasmid, followed by 3-MC treatment resulted in stimulation of both CAT protein and CAT mRNA.

MATERIALS AND METHODS

Materials

Restriction enzymes, MEM media, fetal bovine serum, lipofectin, trypsin-EDTA, dNTPs, and superscript reverse transferase were obtained from BRL (Gaithersburg, MD, USA). The CAT ELISA kit was obtained from BMS (Boehringer Mannheim, Germany). Ethoxyresorufin, and resorufin were purchased from Sigma Chemical Co. (St.Louis, MO, USA). Tissue culture dishes were supplied from Corning (New york, NY USA). Primers were synthesized from the Seoul National University (Seoul, Korea) and Taq polymerase was prepared in the laboratory.

Plasmid DNA preparation

Single colony from ampicillin containing LB agar plate was picked and inoculated into 100 ml of LB medium. Cells were grown for overnight at 37°C in the presence of ampicillin (50 μg/ml). Cells were alkali lysed and plasmid DNA was prepared (Ritter *et al.*, 1991).

Construction of trout *CYP1A*-CAT expression vector

A 3.5 Kb DNA *Pst1* fragment from Puc19 plasmid was isolated and subcloned into pCAT basic at *Pst1* site. Plasmid DNA was transformed into *E. coli* DH 5α cells and white colony as a positive clone, was selected from the agar plate containing X-gal and IPTG.

Cell culture

Mouse liver cell line, and hepa 1 cells were cultured in MEM medium containing 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (1 mg/ml) at 37°C under 5% CO_2 atmosphere. Trypsin-EDTA solution was used for harvesting hepa 1 cells.

Transfection

Supercoiled plasmid DNA (2 μg) was isolated by CsCl gradient (Ritter *et al.*, 1991) and 10 μl of lipofectin (Gibco BRL) and 190 μl of serum free medium were added. After the 15 minutes, 800 μl of serum free medium and the DNA-lipofectin mixture were added to hepa 1 cells. 5~24 hours after the addition of DNA, culture medium was changed with 10% serum containing medium.

Total RNA preparation

Confluent (80%) hepa 1 cells were harvested and washed with PBS (phosphate buffered saline). Cells were lysed with 1.5 ml of 0.5% sarcosyl, 2% β-mercaptoethanol and homogenized in the presence of CsCl (0.1 g/ml). After the CsCl gradient centrifugation at 60,000 rpm for 12 hours (Ti 70.1 rotor), pellets were collected and dissolved in 400 μl of 0.5% sarcosyl, 5 mM EDTA, and 2% β-mercaptoethanol and purified using phenol and chloroform.

Reverse transcription polymerase chain reaction (RTPCR)

Cells were lysed by boiling at 90°C for 15 minutes and centrifuged at 12000×g for 30 seconds. Ten microliter of supernatant was subjected to reverse transcription by adding random primers and 0.5 mM dATP, dCTP, dGTP, and dTTP in the presence of 200 units of superscript TM II at 42°C for 40 minutes. PCR reactions were carried out in a final volume of 200 μl containing PCR buffer [25 mM Tris HCl (pH 8.3 at 25°C)], with heating at 94°C, annealing at 54°C, and

extention at 72°C. One set of primers with 17 nucleotides was used for PCR reaction which amplified DNA between 151 nucleotide and 739 nucleotide of CAT gene. The PCR product of 588 bps was analyzed on 1% agarose gel electrophoresis. For the quantification of PCR product, DNA was eluted from the agarose gel and O.D at 260 nm was measured.

CAT ELISA

A CAT ELISA kit from Boehringer Mannheim was used. After cells were lysed and centrifuged at 18000 ×g for 5 minutes, supernatant was incubated with polyclonal CAT antibody at 37°C for 1 hour, and futher incubated with digoxigenin-labeled anti-CAT antibody at 37°C for one hour. After the washing, peroxidase-labeled antidigoxigenin-antibody (150 mU/ml) and the peroxidase substrate were added. After one hour incubation, optical density was measured at 405 nm.

Measurement of ethoxyresorufin O-deethylase (EROD) activity

Hepa I cells were prepared as previously reported (Han and Sheen, 1996). Microsomes were incubated with 5 μM ethoxyresorufin and 250 μM NADPH for 1 minute at 37°C, and enzymatic activity was monitored based on time-related changes in fluorescence intensity. After the 15 minutes, enzymatic activity was calculated from the area under the peak. (excitation 530 nm, emission 579 nm).

RESULTS AND DISCUSSION

Construction of trout *CYP1A*-CAT expression plasmid

The 5' flanking DNA, 3.5 kb *Pst*I fragment of trout liver *CYP1A* gene was isolated as described previously (Roh and Sheen, 1996), and cloned into pCAT-basic at *Pst*I site. As shown in Fig. 1, transformation of ligation mixture with *Pst*I fragment trout *CYP1A* DNA and Pst1 digested pCAT-basic vector resulted in white colonies on LB-agar plate containing X-gal and IPTG.

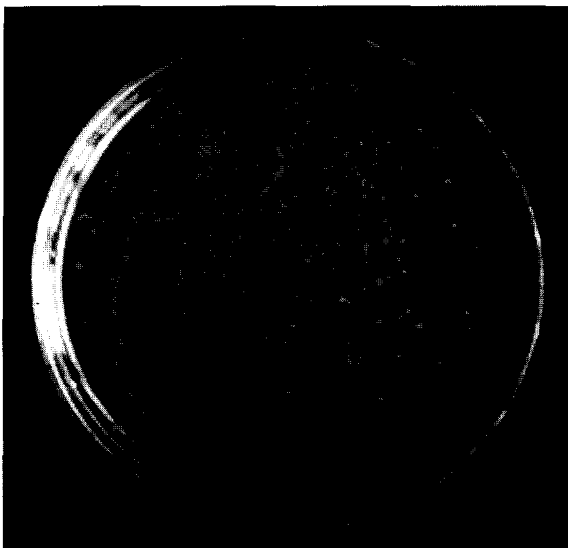
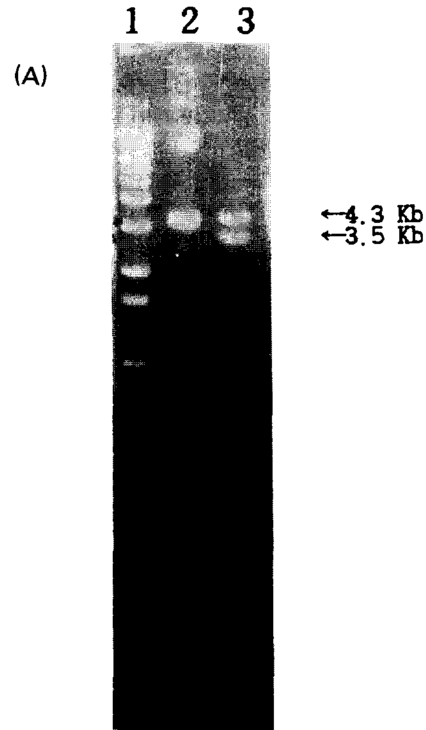


Fig. 1. Plate of *E.coli* HB101 cells transformed with pCAT-basic plasmid containing *Pst*I fragment of trout *CYP1A* 5'-flanking region as an insert DNA.



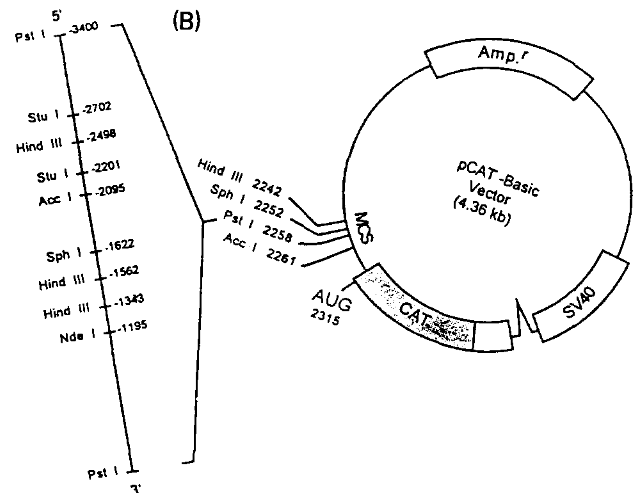


Fig. 2. (A) Analysis of subcloned pCAT-basic plasmid containing *Pst*I fragment of trout *CYP1A* 5'-flanking region as an insert DNA on 1% agarose gel. lane 1: DNA size marker, lane 2: pCAT-basic plasmid containing *PstI* fragment of trout *CYP1A* 5'-flanking region as an insert DNA, lane 3: Pst1 digested pCAT-basic plasmid containing *PstI* fragment of trout *CYP1A* 5'-flanking region as an insert DNA. 4.3 Kb band represents pCAT basic and 3.5 Kb band represents insert DNA. (B) pCAT-basic plasmid containing *Pst*I fragment of trout *CYP1A* 5'-flanking region as an insert DNA.

A white colony was inoculated to obtain large scale culture from which plasmid DNA was prepared and the orientation of the inserted DNA was identified via restriction enzyme digestion of plasmid DNA (Fig 2). Plasmid containing right orientation of 5' flanking DNA of *CYP1A* in respect to CAT gene could be a useful tool to study the role of 5' untranslational region of *CYP1A* gene in the regulation of *CYP1A* gene expression as well as the transactivating factors for *CYP1A* gene expression.

Expression of trout *CYP1A*-CAT in hepa 1 cells

Mouse hepatocyte cell line, hepa 1 cells were known to have functional arylhydrocarbon receptors (Denison and Whitlock, 1995) and to respond to inducers such as 3-MC, TCDD. In order to study 5' flanking DNA of trout *CYP1A* gene, trout *CYP1A*-CAT was transfected into hepa 1 cells and examined if 3-MC induced the expression of CAT gene. We measured the CAT protein using CAT ELISA in hepa 1 cells treated with 3-MC for 24 hours. As shown in Fig. 3, 3-MC increased the CAT protein by 2.3 fold over untreated hepa 1 cells. This result strongly suggested that mouse transacting system was working with trout 5' flanking DNA to regulate CAT gene expression.

We further examined if trout 5' flanking DNA of *CYP1A* gene could drive CAT gene by measuring the CAT mRNA level in hepa 1 cells treated with 3-MC for 24 hours. Fig. 4 shows the CAT mRNA level. As shown in Fig. 4, 3-MC increased the level of CAT mRNA by 2.5 fold (Table I), which indicates that the transcriptional activating system in mouse was working with trout 5' flanking DNA to activate the transcription of CAT gene. From these data, it is likely that hepa 1 cells could be a good model to study the role of trout 5' flanking DNA in the regulation of the trout *CYP1A* gene expression. In addition, it would be a new finding that *CYP1A* gene expression took place in the heterologous system, i.e. trout *CYP1A* gene versus mouse hepa 1 cells.

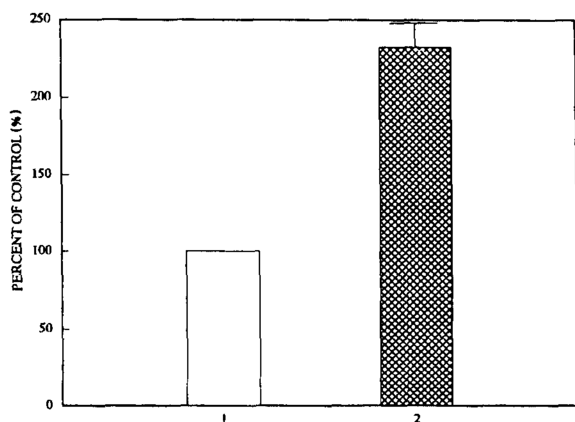


Fig. 3. The CAT protein of hepa-1 cell transfected with trout *CYPIA*-CAT expression construct. Hepa 1 cells were transfected with trout *CYP1A*-CAT construct and treated with or without 1 nM 3-MC for 24 hours. Cells were lysed and CAT protein was measured as described in Materials and Methods. lane 1:control, lane 2:1nM 3-MC, Bars represent mean S.E. (n=3).

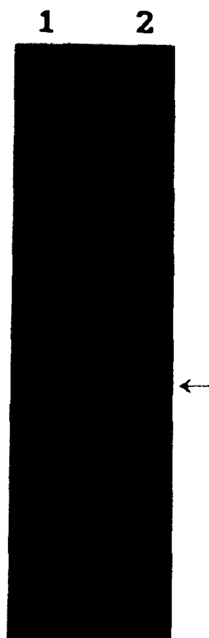


Fig. 4. Effect of 3-MC on the level of CAT mRNA in hepa-1 cells transfected with trout *CYP1A*-CAT construct. CAT expression constructs were transfected into hepa-1 cells and treated with or without 1 nM 3-MC for 24 hours. Cells were lysed and RTPCR was used for measurement of CAT mRNA expression as described in Materials and Method. The arrow indicates the PCR product of CAT mRNA and lower band represents the PCR product of β-actin mRNA. lane 1: control, lane 2:lnM 3-MC.

EROD in hepa 1 cells containing trout CYP1A-CAT

EROD activities were determined with the hepa 1 cells containing trout *CYP1A*-CAT from which both CAT protein and CAT mRNA were measured. As shown in Table I, 3-MC treatment resulted in the stimulation of EROD activity by 2-3 folds. 3-MC treatment also stimulated endogenous *CYP1A* gene expression as well as trout *CYP1A* gene derived CAT gene expression that was transfected exogenously (Table I). Data from this study suggest that transactivating proteins in mouse have been evolutionary conserved so that they can interact with fish 5' flanking DNA of trout *CYP1A* gene, leading to the transcriptional activation of the down stream gene. Since hepa 1 cells contained functional arylhydrocarbon receptors for fish *CYP1A* gene, it would be a very useful system to study the mechanism of the regulation of trout *CYP 1A* gene expression in comparison with those of mu-

Table I. The quantifications of fluorimetry of ethoxyresorufin-O-deethylase activity in microsome, CAT protein and CAT mRNA in hepa-1 cell treated with 1nM 3-MC

Treatment	EROD activity* (pmol/mg protein/min)	CAT protein* (% of control)	CAT mRNA* (% of control)
control	3.2±0.8	100	100
1 nM 3-MC	6.2±1.0	235±14	250±54

*Mean±S.D., n=6

rine and mammalian *CYP1A* gene expression. It have been known that arylhydrocarbons induce two types of *CYP1A* mRNAs, *CYP1A1* mRNA and *CYP1A2* mRNA when given to mouse *in vivo*, whereas in the case of fish, only one *CYP1A* mRNA was induced (Fujusawa-Sehara *et al.*, 1988; Marie *et al.*, 1988; Heilman *et al.*, 1988). Considering this, it would be very important to examine what caused the difference between aquatic animals and terrestrial animals in terms of the regulation of *CYP1A* gene expression.

One way of answering this question would be looking at transactivating factors in both fish and mouse hepatocytes, and another way would be looking at the cis-element, 5' flanking DNA of *CYP1A*. Since transacting factors in mouse cells were working for fish *CYP1A* DNA in terms of activating transcription, it would be worth while studying 5' flanking DNA of *CYP1A* gene in order to understand the mechanism of the regulation of *CYP1A* gene expression. Also, this reporter gene expression system could be used for toxicological application for screening for contamination with arylhydrocarbons such as, TCDD and benzo(a)pyrene in the environment. For detection of arylhydrocarbon contamination, this reporter gene system might be more sensitive than conventional chemical analytical methods. In this study, we presented trout *CYP1A*-CAT reporter gene system which can be studied in mouse hepa-1 cells for toxicological and molecular biological mechanism of action of arylhydrocarbons and other chemicals on *CYP1A* gene expression.

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