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Human *CYP1A2* Promoter Fused-Luciferase Gene Constructs Hardly Respond to Polycyclic Hydrocarbons in Transient Transfection Study in HepG2 Cells

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ABSTRACT *: In previous study, both constitutive expression and 3-methylcholanthrene (3MC)-mediated elevation of CYP1A2 mRNA were demonstrated in human hepatoma HepG2 cells by reverse transcription-polymerase chain reaction (RT-PCR), suggesting that HepG2 cells would be appropriate for the study of human CYP1A2 regulation(Chung and Bresnick, 1994). Further studies were conducted to determine the basis of this induction phenomenon that is observed in HepG2 cells. Since CYP1A1 gene, another polycyclic hydrocarbon(PH)-inducible gene, is regulated by PHs through their interactions via receptors with cis-elements, the 5'-flanking region of human CYP1A2 gene was analyzed to search such responsive elements. The promoter activity of various lengths of CYP1A2 gene sequence (-3203/+58 bp) was measured in transiently-transfected HepG2 cells by fusion constructs containing the CAT, hGH or luciferase genes as a reporter. This region of the CYP1A2 gene, although containing a XRE, was only weakly responsive (less than 2 fold induction) to 10 nM of TCDD or 1 μM 3 MC treatment. This small enhancement of promoter activity is inconsistent with the previous observation, i.e., 12 to 14 fold-enhanced CYP1A2 mRNA from 1 μM 3 MC treated HepG2 cells, suggesting that additional mechanisms would exist for PH-mediated induction of CYP1A2 in these cells.*

Key Words *: Cytochrome P4501A2, CYP1A2, Polycyclic hydrocarbons, Gene regulation, 3MC, TCDD*

I. INTRODUCTION

Cytochrome P450s are superfamily of heme-containing enzymes that are responsible for biotransformation of numerous endogenous and exogenous compounds including drugs and environmental pollutants. The cytochrome P450 family consists of constitutively expressed enzymes and those that require prior exposure of the animal to an inducer. Administration of polycyclic hydrocarbons, *i.e.*, benzo(a)pyrene, 3-methylcholanthrene, and dioxins such as [1]TCDD to animals leads to an induction mostly of the P4501 family which consists of CYP1A1 and CYP1A2 (Conney, 1967; Gelboin, 1967; Gillette *et al.*, 1972; Ioannides and Parke, 1990).

Previous studies demonstrated the constitutive expression of CYP1A2 in HepG2 cells. Furthermore, the level of induction of CYP1A2 by 3MC was determined as 1.7-fold at 12 hr, 12-fold at 24 hr, and 14-fold at 48 hr after 3MC treatment (Chung and Bresnick, 1994). However, in contrast to *CYP1A1*, the molecular mechanism for induction of CYP1A2 is less well understood. Dioxins and PAH's are known to induce CYP1A1 by increasing transcription initiation, which is mediated by cytosolic receptors. One of such receptors is Ah receptor. Ah receptor complexes with hsp90 in the cytosol. Once the ligand binds the Ah receptor, hsp90 dissociates from the complex, allowing the Ah receptor nuclear translocator (ARNT) protein to interact with the ligand-bound Ah receptor. The ligand-bound Ah receptor/ARNT complex undergoes an additional transformation which is followed by translocation into the nucleus. This heterodimer, in turn, acts as a transcriptional factor by binding to XREs or DREs present in the 5'-flanking region of *CYP1A1*, resulting in activation of *CYP1A1* expression (Nebert and Jones, 1989; Hoffman *et al.*, 1991; Reyes *et al.*, 1992; Burbach *et al.*, 1992). In addition

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to the Ah receptor, a cytosolic 4S PAH-binding protein which interacts with B(a)P, B(e)P and 3MC in a saturable and high-affinity fashion has been implicated as another receptor (Bresnick *et al.*, 1988; Raha *et al.*, 1990; Sterling *et al.*, 1994). The 4S PAH-binding protein has been identified as glycine N-methyltransferase (GNMT), which appears to have multiple functions including mediating the *CYP1A1* gene expression (Raha *et al.*, 1994, 1995).

These observations provided the basis for the examination of the 5'-flanking region of human *CYP1A2*, a polycyclic hydrocarbon (PH)-inducible gene. Thus, the present studies were designed to uncover *cis*-acting elements that could be responsible for the PH-mediated induction of human CYP1A2. Transient transfection experiments with fusion gene constructs that contained reporter gene with the 3.2 kb of 5'-flanking region of human *CYP1A2* as a promoter were carried out to determine if any PH-response sequences were present.

II. MATERIALS AND METHODS

1. Plasmid DNA constructs

All the constructs used in this study are depicted in Fig. 1. The plasmids, p1A2GH and p1A2CAT, were constructed as follows: a 2.9 kb of a *Bam*H I fragment which included the region -2800 to +85 bp relative to the transcription start site was isolated from a human CYP1A2 genomic clone (generous gift from Dr. Frank Gonzalez, National Cancer Institute, U.S.A.) and was subcloned into the *Bam*H I site of pGEM7Z (Promega), to generate p1A27Z. The plasmid, p1A2GH, was made by ligation of the 2.9 kb *Bam*H I fragment from p1A27Z and *Bam*H I-digested pGH vector (Seldon *et al.*, 1986) which contained the hGH gene as a reporter gene (Seldon *et al.*, 1986). A 2.9 kb *Kpn I/Hind* III fragment from p1A27Z was fused to the *Kpn* I/*Hin*d III sites of pL9CAT (Magnuson *et al.*, 1987) after removing PEPCK promoter to yield p1A2CAT that included the *cat* reporter.

Human *CYP1A2*-luciferase fusion gene plasmids were constructed as follows: a 3261 bp *Kpn* I fragment of *CYP1A2*, from -3203 to +58 bp relative to the transcription start site, was isolated from H1A2-4-10 and subcloned into the *Kpn* I site of the luciferase expression vector, pXP1 (Nordeen, 1988), to generate pICluc (Chung and Bresnick, 1995). 5'-end deleted *CYP1A2*-luciferase fusion constructs were prepared as previously reported (Chung and Bresnick, 1995). In order to construct pICluc△1161.1461, in which 1161 bp from -1461 to -300 bp had been deleted, pICluc was digested with *Bgl* II and the larger *Bgl* II/*Bgl* II segment was redigested with *Stu* I. A 6 kb *Bgl* II/*Stu* I fragment containing *luc* and the smaller *Bgl* II/*Bgl* II fragment were blunt end-ligated after treatment with Klenow polymerase and T4 DNA ligase. The correct orientation of all constructs was verified by restriction endonuclease mapping and sequence analysis.

2. Cell culture

Human hepatoma HepG2 cells were maintained in a humidified atmosphere of 5% CO2/95% O_2 at 37°C in Dulbecco's-modified Eagle's medium (GIBCO BRL) and supplemented with 10 % fetal bovine serum (GIBCO BRL) and gentamycine at 1 μg/ml.

3. Transfection and reporter gene assays

Transient DNA transfections were carried out by calcium phosphate precipitation method (Sambrook *et al.*, 1989). Cells were plated on 6 cm dishes at an initial concentration of 0.3×10^6 cells/dish and the medium was changed 20 h later. Four hours later, the DNA which had been coprecipitated with calcium phosphate was introduced to the cells. Each dish received 10 μg of test plasmid, pSluc (Nordeen, 1988) for positive control, or pXP2 for negative control and 2 μg of the β-galactosidase expression vector, pCH110 (Pharmacia LKB Biotechnology, Inc.) per dish. The latter served as a means for normalizing transfection efficiency. The cells that were exposed to the calcium phosphate-DNA precipitate for 24 hr, were subjected to a 15% glycerol shock for 2 min; the xenobiotics were then introduced in a complete medium. 72 h after initial transfection, the cells were harvested and assayed for the reporter enzyme activities. The hGH (Nichols Institute), CAT (Neumann *et al.*, 1987), luciferase (Brasier *et al.*, 1989) and β-galactosidase (Norton and Coffin, 1985) assays were performed as described. All plasmids were prepared by equilib-

rium sedimentation in CsCl gradients. All transfection experiments were performed a minimum of three times, and at least two different preparations of plasmid DNA were tested. Within an experiment, each construct was studied in triplicate. To correct for differences in transfection efficiencies between dishes within a given experiment, the luciferase activity in a cell extract was normalized to the β-galactosidase activity and protein concentration. Statistical analysis was performed using Student's *t* test.

III. RESULTS AND DISCUSSION

Initially, p1A2GH and p1A2CAT (Fig. 1), which contained a 2.9 kb (from -2800 to +85 bp) of 5'-flanking region of human *CYP1A2*, were tested to see whether this region of DNA had *cis* elements that could respond to TCDD and/or B(a)P upon their transfection into HepG2 cells. Throughout these experiments, the transfection efficiency of every plate was normalized by the expression of β-galactosidase activity which resulted from cotransfection of the cells with pCH110. A 1.2- and 2-fold induction, respectively in

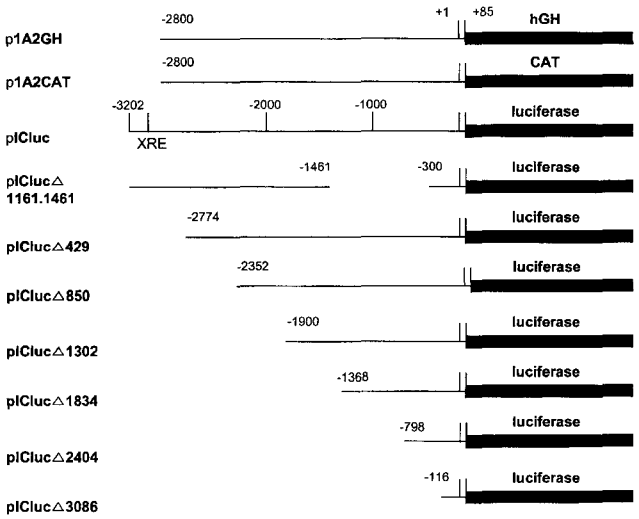

Fig. 1. Schematic presentation of the human *CYP1A2* plasmid constructs. Constructs are presented with the sense orientation relative to the reporter genes. The *CYP1A2* promoter is shown as a solid line while the reporter genes, e.g., h*GH*, *cat* and luciferase, are indicated as solid bars. The numbers represent the position of the nucleotide relative to the transcription start site. The 5'-direction of the plasmid is on the left. The deletion mutants are indicated with a △, and the number following this symbol represents the extent of the deletion in bp, e.g., pICluc△850 indicates a deletion of 850 bp.

cat and *hGH* expression, was detected upon addition of 1 μM B(a)P and 10 nM TCDD to the hepatoma cells. Those levels of induction were too low to allow the definition of the regulatory elements in this region of *CYP1A2*. On the other hand, the concentration of xenobiotics used in the study was sufficient to induce CYP1A1 in hepatoma cells without cytotoxicity (our observation).

Since HepG2 cells retained inducibility of endogenous CYP1A2 mRNA by 1 μM 3MC and a region of homology to the XRE sequence, CACGC, was reported at -2903 bp (Quattrochi and Tukey, 1989), pICluc containing 5'-sequences of *CYP1A2* from -3203 to +58 bp with respecte to the transcription start site was examined. HepG2 cells into which pICluc had been transfected were treated with 1 μM 3MC in order to determine if a XRE present in the promoter sequences was functional. As shown in Fig. 2, no significant inducible luciferase activity was detected when pICluc-transfected cells were treated with 1 μM 3MC. In addition, the inclusion of 10 nM TCDD, the most potent inducer among PHs, in pICluc transfected cells showed no further induction of luciferase activity than 2 fold.

A mutant plasmid, pICluc △1161.1461, that lacked an internal sequence of 1161 bp from -1461 to -300

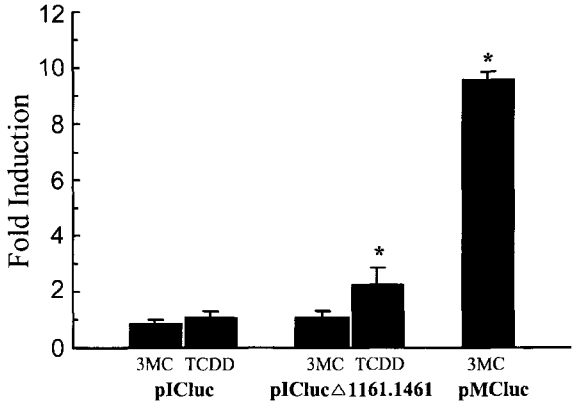

Fig. 2. Induction of luciferase activity after 3MC or TCDD treatment in pICluc- and pICluc△1161.1461- transfected HepG2 cells. Cells were transiently transfected with test plasmids by calcium phosphate coprecipitation method and incubated with 1 μM 3MC, 10 nM TCDD or vehicle for 24 h. The luciferase activity in a cell extract was normalized to the β-galactosidase activity to correct for differences in transfection efficiencies. The data are presented as the ratio of luciferase activity of the vehicle *versus* xenobiotic-treated cells. pMCluc contains 5' regulatory sequences (-1175 to +2545 bp) of the rat *CYP1A1*. *$P<0.05$, compared with vehicle-treated cells.

bp, was also tested for expression of reporter gene activity after administration of the inducers. A small increase in luciferase activity was noted with HepG2 cells that had been transfected with this mutant plasmid after administration of the inducers. However, the level of induction was only within a 2-fold range (Fig. 2). On the other hand, cells transfected with the positive control plasmid, pMCluc, showed an elevation in luciferase activity of 10-fold under the same conditions. pMCluc contains the 5'-upstream region of the rat *CYP1A1*, from -1175 to +2545 bp.

To assess the presence of any DNA sequence that would repress inducibility, a number of 5'-end deletion mutations (Chung and Bresnick, 1995) were tested for inducibility potential in the HepG2 cells that had been treated with 3MC. However, no more than a 2-fold induction was observed in these transfected cells (Fig. 3). These results indicated that the 5'-flanking region from -3203 to +58 bp is barely able to responds to 3MC or TCDD in these cells.

We have shown that the detection of both constitutive and 3MC-mediated elevation of CYP1A2 mRNA by RT-PCR method in HepG2 cells. *CYP1A2* expression was shown to be elevated by 8-12-fold upon administration of 3MC, which implicates that the machinery for PH-mediated induction of the CYP1A2 does exist in this hepatoma cell (Chung and Bresnick, 1994). In regard to the regulation of constitutive

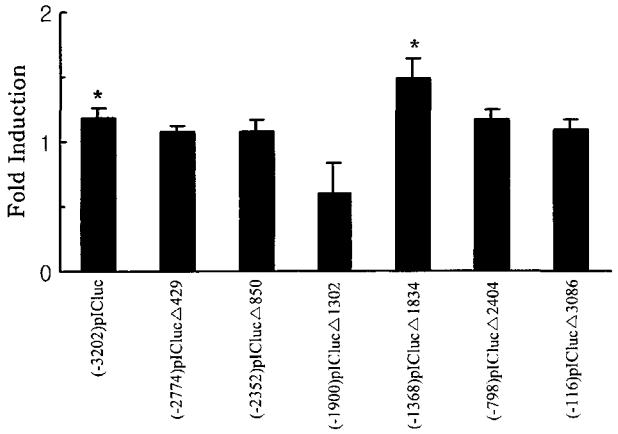

Fig. 3. Induction of luciferase activity after 3MC treatment in pICluc and 5'-end deletion mutants transfected HepG2 cells. Cells were transfected with 10 μg of test plasmid by calcium phosphate precipitation technique. Cells were exposed to DNA for 24 h followed by treatment with 1 μM 3MC for 48 h. Data are represented as the ratio of luciferase activity of the vehicle *versus* xenobiotic-induced cells. *$P<0.05$, compared with vehicle-treated cells.

expression of *CYP1A2*, at least a negative and a positive *cis*-elements are involved. It also appears that a hepatocyte nuclear factor-1 (HNF-1) does play a role in positive regulation of human *CYP1A2* gene expression (Chung and Bresnick, 1995, 1997). In the present study, we further investigated the basis of this induction phenomenon. In contrast to *CYP1A1*, the results of transient expression experiments revealed that the 5'-flanking region from -3203 to +58 bp only weakly responded to PHs regardless of the presence of XRE at -2903 bp. Present results are consistent with the results of Postlind *et al.* (1993) who observed no inductions by PHs in HepG2 cells that had been stably transfected with the same human *CYP1A2* regulatory sequence as incorporated into pICluc construct. Moreover, the rabbit 5'-sequence which bears a consensus XRE at -493/-499 bp, a position that is much closer to the promoter than in the human *CYP1A2*, failed to respond to TCDD or 3MC in transiently-transfected HepG2 cells, suggesting its non-functional nature in this rabbit regulatory region (Strome *et al.*, 1992). It would appear that parameters other than the core sequence are required for functionality of the XRE, as occurs in *CYP1A1*.

The XRE-like *cis*-elements that was responsible for 3MC-mediated enhancement in *CYP1A2* promoter activity have been reported (Quattrochi *et al.*, 1994). They were able to find these element by using of 10 μM of 3MC, a concentration that is 10 times higher than that used in the present study. However, they also observed 2-3 fold increased reporter gene activity when they used 1 μM 3MC.

Since the present constructs did not include the XRE that occurs in intron 1, it still remains uncertain whether the latter sequence could participate in 3MC induction. It is also possible that other functional XREs might exist that are further upstream from the transcription start site, *i.e.*, >3.2 kb. These possibilities are all predicted on the model that was developed for *CYP1A1* regulation. However, other mechanisms as suggested for the rat and mouse *CYP1A2*, such as an increase in precursor RNA processing (Silver and Krauter, 1990), and mRNA stabilization (Kimura *et al.*, 1986), respectively, might be responsible for the 3MC-mediated induction of the human gene. In HepG2 cells, it also appeared that 3MC somewhat prevented the rapid diminution of CYP1A2 tran-

scripts in HepG2 cells which would have been transiently increased in respond to serum-containing medium (Chung and Bresnick, 1994). Recently, Raffalli-Mathieu *et al.*, (1997) have characterized two nuclear proteins, *i. e.*, 37 kDa and 46 kDa proteins, which interact with mouse Cyp1a2 mRNA. According to their speculations, the 37 kDa protein binds to the 3' untranslated region of CYP1A2 for the maintenance of uninduced levels of CYP1A2 mRNA, while the 46 kDa protein could be important in the maturation of elevated levels of CYP1A2 pre-mRNA during induction by 3MC (Raffalli-Mathieu *et al.*, 1997).

Why PHs-bound receptors preferentially interact with promoter of *CYP1A1* to that of *CYP1A2*? The refractory responsiveness of the *CYP1A2* promoter may have been explained by Fisher *et al.*, (1990) who suggested that differences in the number of Ah receptor binding sites and the distance between these sites would determine the level of induction of a gene by polycyclic hydrocarbons. *CYP1A1* 5'-flanking regions contain the clustered Ah receptor recognition sites in many species (Nebert and Jones, 1989), whereas few have been found in the 5'-flanking regions of *CYP1A2*. Indeed no such motifs have been found in the mouse and rat (Ikeya *et al.*, 1989), and only one XRE is located in the 5'-regulatory region of the human (Quattrochi and Tukey, 1989) and the rabbit *CYP1A2* genes (Strome *et al.*, 1992). Furthermore the mouse *CYP1A1* promoter contains an additional GC box between two XREs that can augment the enhancer function once the Ah receptor binds to XREs (Fisher *et al.*, 1990).

In summary, an XRE found in the 5'-flanking regulatory region of human *CYP1A2* appears to be nonfunctional in PH-mediated human *CYP1A2* regulation at least in HepG2 cells. The weak effect of 3MC on *CYP1A2* promoter activity was not related to the absence of receptors or other accessory proteins in HepG2 cells since *CYP1A1* promoter was highly inducible. The slight enhancement of promoter activity could not account for the induction of endogenous CYP1A2 in HepG2 cells. Additional studies are needed to fully establish the mechanisms by which 3MC upregulates *CYP1A2* expression.

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FOOTNOTES

[1]The following abbreviations are used: ARNT, Ah receptor nuclear translocator; B(a)P, benzo(a)pyrene; CAT, chloramphenicol acetyltransferase; CYP1A1, cytochrome P4501A1; *CYP1A1*, CYP1A1 gene; CYP1A2, cytochrome P4501A2; *CYP1A2*, CYP1A2 gene: DRE, dioxin-response element; hGH, human growth hormone; luc, luciferase; 3MC, 3-methylcholanthrene; PAH, polycyclic aromatic hydrocarbon; PH, polycyclic hydrocarbon; RT-PCR, reverse transcription-polymerase chain reaction; TCDD. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; XRE, xenobiotic responsive element.