

MOL #21220

Title: A common regulatory region functions bi-directionally in transcriptional activation of the human *CYP1A1* and *CYP1A2* genes

Authors: Rika Ueda, Hiromi Iketaki, Kiyoshi Nagata, Shioko Kimura, Frank J. Gonzalez, Kazutomi Kusano, Tsutomu Yoshimura and Yasushi Yamazoe

Divisions:

Division of Drug Metabolism and Molecular Toxicology, Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Japan (R.U., H.I., K.N. and Y.Y.), Comprehensive Research and Education Center for Planning of Drug Development and Clinical Evaluation, Tohoku University 21<sup>st</sup> Century “Center of Excellence” Program (Y.Y.), Laboratory of Metabolism, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA (S.K. and F.J.G.), and Drug Metabolism and Pharmacokinetics Research, Tsukuba Research Laboratories, Eisai Co., Ltd., Tsukuba, Japan (R.U., K.K. and T.Y.)

MOL #21220

Running Title: Co-induction of human CYP1A1 and CYP1A2

Corresponding author: Kiyoshi Nagata

Division of Drug Metabolism and Molecular Toxicology, Graduate School of Pharmaceutical Sciences, Tohoku University, Aramaki-Aoba 6-3, Aoba-ku, Sendai, 980-8578, Japan

Telephone number: +81-22-795-6828

FAX number: +81-22-795-6826

E-mail: [nagataki@mail.tains.tohoku.ac.jp](mailto:nagataki@mail.tains.tohoku.ac.jp)

Number of Text Pages: 29

Number of Figures: 7

Number of References: 25

Abstract: 231 words

Introduction: 641 words

Discussion: 1121 words

Non-standard Abbreviations: CYP, cytochrome P450; BNF,  $\beta$ -naphthoflavone; 3-MC, 3-methylcholanthrene; AHR, aryl hydrocarbon receptor; ARNT, AHR nuclear translocator; XRE, xenobiotic responsive element; Luc, firefly luciferase; SEAP, secreted alkaline phosphatase; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; DMSO, dimethyl sulfoxide; RL, *Renilla* luciferase;  $\beta$ -gal,  $\beta$ -galactosidase.

MOL #21220

## Abstract

The human *CYP1A1* and *CYP1A2* genes on chromosome 15 are orientated head-to-head and separated by 23 kb intergenic spacer region. Thus the possibility exists for sharing common regulatory elements contained in the spacer region responsible for transcriptional activation and regulation of the *CYP1A1* and *CYP1A2* genes. In the present study, a reporter gene construct containing -22.4 kb of the 5'-flanking region of the *CYP1A2* gene was found to support  $\beta$ -naphthoflavone (BNF) and 3-methylchoranthrene (3-MC)-mediated transcriptional activation. The responsive region was also functional in directing activation of the *CYP1A1* promoter, indicating that the region works bi-directionally to govern transcriptional activation of both the *CYP1A1* and *CYP1A2*. To simultaneously evaluate transcriptional activation of both genes, a dual reporter vector was developed in which the spacer region was inserted between two different reporter genes, firefly luciferase and secreted alkaline phosphatase. Transient transfection of the dual reporter vector in HepG2 cells revealed increases in both reporter activities after exposure of the cells to BNF and 3-MC. Deletion studies of the spacer region indicated that a region from -464 to -1829 of the *CYP1A1* gene works bi-directionally to enhance the transcriptional activation of not only *CYP1A1* but also *CYP1A2*. In addition, a negative bidirectional regulatory region was found existing from -18989 to -21992 of the *CYP1A1* gene. These data established that induction of human *CYP1A1* and *CYP1A2* are simultaneously controlled through bidirectional and common regulatory elements.

MOL #21220

The potential for induction is a typical property of many cytochromes P450 (CYP) involved in the oxidative metabolism of drugs, environmental chemicals and endogenous compounds (Denison and Whitlock, 1995). Treatment of experimental animals and humans with chemicals such as 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD),  $\beta$ -naphthoflavone (BNF) or 3-methylchorantherene (3-MC) are known to enhance expression of CYP1A1 and CYP1A2 (Nebert and Gonzalez, 1987). Most of these compounds increase transcription of both the *CYP1A1* and *CYP1A2* via activation of the aryl hydrocarbon receptor (AHR). Regulatory *cis*-elements mediating AHR activation of the *CYP1A1* gene have been extensively studied, whereas those associated with regulation of the *CYP1A2* gene are limited.

As for the transcriptional activation of the *CYP1A1*, chemicals such as TCDD and 3-MC are shown to bind to AHR, followed by translocation into nucleus (Whitlock, 1999). In the nucleus, AHR dimerizes with AHR nuclear translocator (ARNT) and interacts with xenobiotic responsible element (XRE) (5'-TNGCGTG-3') in the 5'-flanking region of the *CYP1A1* gene to activate transcription (Hankinson, 1995). Several XREs were identified in the 5'-flanking region of the human *CYP1A1* gene. Some of them interact with the AHR-complex and mediate 3-MC activation of the *CYP1A1* (Kubota et al., 1991). Based on these results, reporter gene assay systems for the assessment of human *CYP1A1* induction have been established (Bessette et al., 2005; Garrison et al., 1996; Postlind et al., 1993)

The precise molecular mechanisms responsible for the tissue-specific expression and induction of the *CYP1A2* remain unclear (Eaton et al., 1995). AHR is thought to be involved in *CYP1A2* induction, because TCDD- or 3-MC-mediated activation of the *Cyp1a2*, as well as *Cyp1a1*, was not detected in *Ahr*-null mice (Fernandez-Salguero et al., 1995; Mimura et al., 1997; Schmidt et al., 1996). However, the *cis*-element responsible for transcriptional

MOL #21220

activation of *CYP1A2* is not a typical XRE observed in the 5'-flanking region of *CYP1A1*. Analysis of the 5'-flanking region of the human *CYP1A2* revealed the existence of two regions (-2531 to -2423 and -2195 to -1987) responsible for the transcriptional activation (Quattrochi et al., 1994). One was termed X1 to which TCDD-inducible nuclear proteins bind weakly and the other X2, which does not interact with nuclear proteins. Neither of these elements was similar to the XRE nucleotide sequence found in the regulatory region of the *CYP1A1*. Although the X1 is an indispensable element for 3-MC-mediated transcriptional activation of the *CYP1A2*, gene activation was not completely abolished by the removal of X1, suggesting the involvement of additional regulatory elements in the transcriptional activation of human *CYP1A2*.

The human *CYP1A* locus is found on chromosome 15 (Jaiswal et al., 1987). The *CYP1A1* and *CYP1A2* are in head-to-head orientation and separated by more than a 20 kb of intervening DNA (Corchero et al., 2001). There is no open reading frame between the two genes, indicating that they share a 5'-flanking region. Thus, the possibility exists for distinct regulatory regions specific for each gene or common regulatory regions for both genes. That *cis*-acting elements control the tissue-specific and AHR-mediated activation of both genes was demonstrated by production of a transgenic mouse expressing both the *CYP1A1* and *CYP1A2* from a contiguous BAC genomic clone (Cheung et al., 2005; Jiang et al., 2005). To characterize the function of this spacer region, the transcriptional activation of both the *CYP1A1* and *CYP1A2* should be evaluated simultaneously. In the present study, transcriptional activation of the *CYP1A1* and *CYP1A2* were independently examined by promoter-reporter gene assays to define the 5'-flanking regions responsible for transcriptional activation of the *CYP1A1* and *CYP1A2*. A dual reporter vector containing the intergenic spacer region between human *CYP1A1* and *CYP1A2* was then produced to evaluate the

MOL #21220

regulatory regions in both directions, simultaneously. The results revealed that an XRE cluster existing near the *CYP1A1* gene works bi-directionally and is essential not only for transcriptional activation by BNF and 3-MC of the *CYP1A1* but also for the *CYP1A2*.

MOL #21220

## Materials and Methods

**Materials.** BNF and 3-MC were purchased from SIGMA-Aldrich (St. Louis, MO). DMSO was obtained from WAKO Pure Chemicals (Osaka, Japan). Oligonucleotides were prepared by SIGMA genosys Japan (SIGMA-Aldrich). Restriction endonucleases, except for *Asp* 718 (Roche Diagnostics, Basel, Switzerland), and DNA-modifying enzymes were purchased from Takara Bio (Kyoto, Japan).

**Quantitative analysis of CYP1A mRNA contents.** The human hepatocellular carcinoma cell line, HepG2, was obtained from the RIKEN cell bank (Tsukuba, Japan) and cultured in Dulbecco's modified Eagles medium containing 10% fetal bovine serum (SIGMA-Aldrich), MEM non-essential amino acid and penicillin-streptomycin-amphotericin (Invitrogen, Carlsbad, CA). HepG2 cells were seeded in 48-well plates and cultured for 40 h. BNF and 3-MC was dissolved in DMSO and added to the cells at various concentrations. The concentration of DMSO did not exceed 0.1%. Control cells were treated with 0.1% DMSO. After 40 h exposure, total RNA was extracted using ABI6100 (Applied Biosystems, Foster City, CA). Reverse transcription reactions were performed using TaqMan Reverse Transcription Reagents with oligo dT primer (Applied Biosystems). Quantitative real-time PCR was performed by ABI7900 (Applied Biosystems). Primers used for the measurement of CYP1A1 mRNA were 5'-TGGTCTCCCTTCTCTACACTCTTGT-3' and 5'-ATTTCCCTATTACATTAATCAATGGTTC-3' with SYBR Green. For the measurement of CYP1A2 mRNA, primers and TaqMan probe were used as previously described (Finnström et al., 2001). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured as an internal standard using primers 5'-GAAGGTGAAGGTCGGAGTC-3' and 5'-GAAGATGGTGATGGGATTTC-3' with SYBR Green. Values of CYP1A mRNAs were normalized by the GAPDH mRNA levels.

MOL #21220

**Isolation of a DNA segment between *CYP1A1* and *CYP1A2* genes.** As shown in Fig. 1, about 27 kb of DNA segment (from +2420 of the *CYP1A1* gene to +835 of the *CYP1A2* gene) were divided into three parts, and each fragment was amplified by PCR with *TaKaRa LA Taq* (Takara Bio). A bacterial artificial chromosome genomic clone containing the human *CYP1A1* and *CYP1A2* (Corchero et al., 2001) was used as the template. Primers used for the amplification of Fragment 1 are 5'-GCGGTCGACGGCCGGCCGGATCTCATTCTTTTTACAGCTGAATAGCACTCC-3' (forward primer) and 5'-GCGGAATTCATCTTGGAGGTGGCTGCTGAGAGAAGGTGC-3' (reverse primer). For the amplification of Fragment 2, 5'-GCGCTCGAGAGAATACCAGGCAGAAGATGGCAGAGG-3' (forward primer) and 5'-GCGACGCGTGGCCGGCCATATAGTGCATATACACAATGGAGTGCTATTCAGCTGT-3' (reverse primer) were used. Primers used for the amplification of Fragment 3 are 5'-TCCCAGCTACTCGAGAGGTTGACACACAAGAA-3' (forward primer) and 5'-CGACGCGTCCCGCTCGAGGATCCTCATAAATGGTTTAGCACCATCC-3' (reverse primer). Each fragment was subcloned into pCR-XL-TOPO (Invitrogen), and designated F1, F2 and F3, respectively. All joints in the constructs were confirmed by sequencing (Applied Biosystems).

**Construction of reporter plasmids.** A construct p1A1-204 containing bases from -204 to +1039 of the *CYP1A1* was prepared as follows (See Fig. 3): F1 was digested with *Bsp*1407 I and *Bam*H I, and the resultant fragment was inserted into the *Asp* 718 and *Bgl* II sites of pSEAP2-Basic vector (Clontech, Mountain View, CA). To obtain p1A1-887 containing bases from -887 to +2420 of the *CYP1A1*, F1 was digested with *Nhe* I and *Eco*R I, and the resultant fragment was inserted into the *Nhe* I and *Eco*R I sites of the pSEAP2-Basic vector.

MOL #21220

F1 was digested with *Spe* I and *Eco*R I, and the resultant fragment was inserted into the pSEAP2-Basic vector at *Nhe* I and *Eco*R I sites to obtain p1A1-5058. A DNA fragment from -6445 to +2420 of the *CYP1A1* gene was obtained from F1 by digesting with *Mun* I and *Eco*R I, and inserted into pSEAP2-Basic at *Eco*R I sites to construct p1A1-6445. To construct p1A1-8653, F1 was digested with *Bam*H I, and inserted into pSEAP2-Basic at *Bgl* II site. F1 was digested with *Mlu* I and *Nhe* I, and the resultant 11 kb DNA fragment was inserted into p1A1-887 at *Mlu* I (present in the vector) and *Nhe* I sites to construct p1A1-12188. Constructs p1A1-8653D and p1A1-12188D were generated by digesting p1A1-8653 and p1A1-12188 with *Bsp*1407 I and ligating themselves, respectively.

A DNA fragment from -3203 to +60 of the *CYP1A2* gene was obtained from F2 by digesting with *Kpn* I, and the resultant fragment was inserted into pSEAP2-Basic at a *Kpn* I site to construct p1A2-3203 (See Fig. 4). To construct p1A2-5221, F2 was digested with *Xho* I and *Bam*H I and the resultant fragment was inserted into pSEAP2-Basic at *Xho* I and *Bgl* II sites. F2 was digested with *Mlu* I and *Hin* dIII, and the resultant 3.4-kb fragment was inserted into pSEAP2-Basic to which an 8.8-kb fragment obtained from F2 by digesting with *Hin* dIII was inserted at the *Hin* dIII site to construct p1A2-12188. F3 was digested with *Xho* I, and about 9.5-kb fragment was inserted into p1A2-5221 at *Xho* I site to construct p1A2-14664. F1 was digested with *Spe* I, and the resultant fragment was inserted into p1A2-5221 at the *Nhe* I site to construct p1A2-5221E.

The dual reporter vector (pd-1A1/1A2) was constructed as follows: F1 was digested with *Bam*H I, and the resultant 10-kb fragment was inserted into pGL3-Basic vector (Promega, Madison, WI) at a *Bgl* II site. The resultant plasmid was digested with *Xho* I and *Sal* I, and a 12 kb fragment was obtained and inserted into p1A2-5221 at an *Xho* I site. The resultant

MOL #21220

construct was designated pd-9.5k. A 9.5 kb fragment was obtained from F3 by digesting with *Xho* I, and inserted into pd-9.5k at an *Xho* I site to construct the dual reporter vector containing the DNA segment between +1039 of *CYP1A1* and +90 of *CYP1A2*, designated pd-1A1/1A2. Using pd-9.5k, deletion mutants of the dual reporter vectors were prepared based on the standard method with the restriction sites indicated in the Figure 6. To generate p1A2-22430 (see Fig. 4), pd-1A1/1A2 was digested with *Nhe* I and ligated itself. Deletion of each XRE was conducted using a Quik-change site-directed mutagenesis kit (Stratagene, La Jolla, CA).

**Transient transfection and measurement of Luc and SEAP activities.** HepG2 cells were seeded in 48-well plates 12 h before transfection. Reporter plasmids were transfected using FuGene6 (Roche Diagnostics) according to the manufacture's instructions. pRL-SV40 or pSV- $\beta$ -gal (Promega) was co-transfected for use as internal standards. The day after transfection, BNF and 3-MC were dissolved in DMSO and added to the medium at 10  $\mu$ M and 1  $\mu$ M, respectively. The concentration of DMSO was 0.1%. Control cells were treated with 0.1% DMSO. After 40 h exposure, aliquots of the medium were collected and incubated at 65°C for 20 min to inactivate the endogenous alkaline phosphatase activity and cells were processed for luciferase assays. LumiPhos 530 (Lumigen Inc., Southfield, MI) was used as the substrate to measure SEAP activity. Luciferase activities were measured using Luciferase Assay System or Dual-Luciferase Assay Reagent (Promega).  $\beta$ -Galactosidase activity was measured using the  $\beta$ -Galactosidase Enzyme Assay System with Reporter Lysis Buffer (Promega).

**Statistics.** One-way ANOVA with Dunnet's post test was performed using Graph Pad Prism 4 (GraphPad Software Inc., San Diego, CA) for significant differences between the mean

MOL #21220

values of each group.

MOL #21220

## Results

**Effects of BNF and 3-MC on CYP1A1 and CYP1A2 mRNA levels.** As shown in Fig. 2, levels of CYP1A1 and CYP1A2 mRNAs in HepG2 cells were increased in a concentration-dependent manner after treatment with BNF or 3-MC. The levels of CYP1A1 mRNA reached about 70-fold and 25-fold higher than controls with 10  $\mu$ M BNF and 1  $\mu$ M 3-MC, respectively. CYP1A2 mRNA levels were also enhanced in response to both compounds. The levels of CYP1A2 mRNA increased about 30- and 20-fold higher than controls after treatment with 10  $\mu$ M BNF and 1  $\mu$ M 3-MC, respectively. HepG2 cells were thus used in the following experiments to study transcriptional activation of the *CYP1A1* and *CYP1A2* genes.

**5'-Flanking region necessary for transcriptional activation of the *CYP1A1* gene.** To determine the region responsible for transcriptional activation of the *CYP1A1*, various lengths of the 5'-flanking region were fused to SEAP vectors to verify elements essential for CYP1A1 induction. The largest chimeric reporter plasmid contained about the 12-kb of the 5'-flanking region of the *CYP1A1* gene (-12188 to +2420). There are eight XRE sequences within this region, as shown in the top of Fig. 3. These reporter plasmids were transiently transfected into HepG2 cells and SEAP activities were determined after 40 h treatment with 10  $\mu$ M BNF or 1  $\mu$ M 3-MC.

As shown in Fig. 3, p1A1-204 which has no XRE did not show an increase in reporter activities after treatment with BNF and 3-MC. Introduction of a single XRE (p1A1-887) showed approximately 12-fold increase of the reporter activity in the presence of BNF. 3-MC treatment also resulted in about 19-fold increase in reporter activity. Constructs p1A1-5058 and p1A1-6445 containing seven and eight XRE sequences, respectively, showed

MOL #21220

the maximum increases of 32- and 47-fold reporter activities in response to BNF and 3-MC, respectively. Reporter activity was decreased to two thirds of the maximal activation after introduction of the 5'-flanking region further than -6445 into the reporter plasmid (p1A1-8653 and p1A1-12188). With constructs having deletions from -8143 to -204 in p1A1-8653 or p1A1-12188 (p1A1-8653D and p1A1-12188D), both reporter activities were lost.

### **5'-Flanking region necessary for transcriptional activation of the *CYP1A2* gene.**

Several SEAP vectors containing various lengths of the 5'-flanking region of human *CYP1A2* gene were generated. As shown in Fig. 4, no clear enhancement of the reporter activities was observed on p1A2-3203 in the presence of BNF or 3-MC. Although p1A2-5221 had single XRE sequence, no clear increase of the reporter activities occurred after treatment with either compound. Furthermore, no response to BNF or 3-MC was obtained with p1A2-12188 or p1A2-14664 containing three additional XRE sequences. A construct p1A2-22430 containing the largest insert, however, showed about a 5-fold increase in response to BNF and 3-MC. When the 5'-flanking region from -22430 to -18237 of the *CYP1A2* gene was inserted into p1A2-5221 (p1A2-5221E), about a 4.5-fold increase in reporter activities was maintained in the presence of both compounds.

### **Co-transcriptional activation of the *CYP1A1* and *CYP1A2* genes.**

As described above, BNF- and 3-MC-mediated transcriptional activation was not detected with the constructs including approximately 14.7-kb 5'-flanking region of the *CYP1A2* gene. However, further addition of the upstream region resulted in increased activities (Fig. 4). The region enhancing *CYP1A2* transcription encompassed -887 to -5058 of *CYP1A1*, in which multiple XREs were found. This region was also effective in transcriptional activation of the *CYP1A1* gene (Fig. 3). These results suggested that the *CYP1A1* and *CYP1A2* genes are

MOL #21220

under the control of a bidirectional and common regulatory mechanism. However, simultaneous evaluation is necessary to assess the role of the common regulatory region on the transcriptional activation of both genes. Therefore, a DNA segment from +1039 of the *CYP1A1* gene to +90 of the *CYP1A2* gene was isolated (Fig. 5A) and inserted between two reporter genes (SEAP and Luc) to construct a dual reporter vector which was named as pd-1A1/1A2 (Fig. 5B). As shown in Fig. 5C, BNF and 3-MC treatment of cells transfected with pd-1A1/1A2 resulted in the appearance of Luc and SEAP activities. Luc activity derived from transcriptional activation of the *CYP1A1* gene increased about 18-fold after exposure of cells to BNF or 3-MC. SEAP activity derived from transcriptional activation of the *CYP1A2* gene increased about 2~3 times in response to BNF or 3-MC. These results suggest that the inserted DNA segment works bi-directionally for both the *CYP1A1* and *CYP1A2* transcriptional activation.

To identify a DNA region essential for activation of both *CYP1A1* and *CYP1A2* gene promoters, the regulatory region of pd-1A1/1A2 was partially deleted, and reporter activities were determined and compared with those of pd-1A1/1A2. At first, upstream region within -18096 of the *CYP1A1* gene was partially deleted as shown in Fig. 6A. Deletion from -8653 to -18096 of the *CYP1A1* gene showed a 28.3-fold increase in Luc activity in response to BNF. Deletion from -4621 to -18096 of the *CYP1A1* gene also increased Luc activity about 33-fold. However, further deletion from -887 to -18096 of the *CYP1A1* gene dramatically decreased Luc activity to 7-fold after exposure of cells to BNF. When the region from -464 to -18096 was deleted, induction of Luc activity was completely lost. 3-MC treatment of cells transfected with the dual reporter vector, which is deleted or non-deleted, resulted in similar Luc activity to BNF-treated cells. On the other hand, SEAP activity remained almost unchanged in the dual reporter vectors with DNA deleted from -887, 4621 or -8653 to -18996

MOL #21220

of the *CYP1A1* gene in response to both compounds as compared to the parental vector without deletion. However, deletion from -464 to -18096 of the *CYP1A1* gene lost inducible the SEAP and Luc activity in response to both compounds.

An upstream region within -21992 of the *CYP1A1* gene was also deleted as shown in Fig. 6B. Deletion from -18909 to -21992 of the *CYP1A1* resulted in increased Luc and SEAP activities to 40.5-fold and 9.3-fold, respectively, after treatment of cells with BNF. These values are about 2-3 times higher than pd-1A1/1A2. In addition, the vector deleted from -4621 to -21992 of the *CYP1A1* gene showed further increase in both reporter activities. Luc and SEAP activities in response to BNF reached 65-fold and 11-fold, respectively. Deletion from -1829 to -21992 of the *CYP1A1* gene resulted in no further influence on the Luc activity, whereas SEAP activity was still increased up to 17-fold. Deletion from -462 to -21992 of the *CYP1A1* gene resulted in the complete loss of both the reporter activities. 3-MC treatment of cells resulted in similar profiles of changes in both reporter activities to those obtained with BNF-treated cells.

**Influence of a single XRE deletion on dual reporter activities.** As shown in Fig. 6B, the dual reporter vector lacking from -4621 to -21992 of the *CYP1A1* gene (named as pd-4621/21992) showed high reporter activities. There were seven XRE sequences in the regulatory element of pd-4621/21992. Among them, five XREs close to the *CYP1A1* gene transcription start site were considered to be important because the dual vector deleted from -1829 to -21992, which has these five XREs, showed further increase in *CYP1A2* promoter activation. Therefore, these five XREs were designated XRE1, XRE2, XRE3, XRE4 and XRE5, and five mutants of pd-4621/21992 were generated, in which each XRE was deleted. The mutant vectors were transiently transfected into HepG2 cells and reporter activities were

MOL #21220

measured in the presence of BNF or 3-MC. The results are shown as the ratio to pd-4621/21992 (Fig. 7).

Deletion of each XRE decreased Luc activities for the *CYP1A1* promoter after the exposure to BNF. Deletion of XRE1 and XRE3 had the most significantly decreased Luc activity about 0.45-fold and 0.32-fold of pd-4621/21992, respectively (Fig 7). Deletion of XRE2, XRE4 and XRE5 showed only slight decrease of Luc activities of a maximal 0.66-fold of pd-4621/21992. On the other hand, only deletion of XRE3 produced a drastic change in *CYP1A1* promoter activity in response to 3-MC. With the *CYP1A2* promoter, as monitored by SEAP activity, the only marked decrease was obtained with disruption of XRE3 but only after treatment of cells with 3-MC. Disruption of the other XREs produced a maximal decrease of < 20% of the SEAP activity obtained with pd-4621/21992 (Fig. 7). It should be noted that these data may not reflect exactly the promoter activities that would be obtained when the complete 23.3 kb intergenic sequence is included.

MOL #21220

## Discussion

In the present study, we have analyzed the 5'-flanking region of the *CYP1A1* and *CYP1A2* genes to identify each regulatory element that mediates transcriptional response to BNF and 3-MC. As shown in Fig. 3, about -5.0 kb or -6.4 kb of 5'-flanking region of the *CYP1A1* gene containing seven or eight XREs showed the highest transcriptional activation in response to both BNF and 3-MC. These results are consistent with previous studies (Kawajiri et al., 1986; Kubota et al., 1991). About -3.2 kb of the 5'-flanking region was reported to support 3-MC-mediated transcriptional activation of the human *CYP1A2* gene by 3-MC (Quattrochi and Tukey, 1989; Quattrochi et al., 1994). However, a similar construct, p1A2-3203, did not show transcriptional activation after the exposure of cells to 3-MC (Fig. 4). The reason was unknown, but differences in the reporter vector constructs could be one of the reasons. BNF treatment also did not increase reporter activity in p1A2-3203. Up to -14.7 kb of the 5'-flanking region of the *CYP1A2* gene did not result in transcriptional activation in response to BNF and 3-MC, although several XRE sequences were included. Further upstream region encompassing -22.4 kb was needed to enhance the transcriptional activation of the *CYP1A2* gene. A similar result was observed when a DNA fragment from -22430 to -18237 of *CYP1A2* was connected to p1A2-5221 (i.e. p1A2-5221E). These results suggest that the distant regulatory region, near the *CYP1A1* gene, is necessary to support transcriptional activation of the *CYP1A2* gene. This is consistent with a recent report *in vivo*; Jiang *et al.* showed that BAC-transgenic mice carrying only human *CYP1A2* gene with -15.2 kb of the 5'-flanking region failed to increase CYP1A2 mRNA, whereas another mouse line carrying both the human *CYP1A1* and *CYP1A2* with the intact spacer region between the two genes were inducible for both genes in response to TCDD (Jiang et al., 2005).

The DNA fragment introduced in p1A2-5221E corresponds to the element from -887 to

MOL #21220

-5058 of the *CYP1A1* gene containing several XREs. This region was also effective for the transcriptional activation of the *CYP1A1* gene (Fig. 3). These data suggest the possibility that the identical regulatory elements work simultaneously for transcriptional activation of the *CYP1A1* and *CYP1A2* genes. Thus, the element seemed to have bidirectional regulatory activity, which has been recently proposed in the human genome (Trinklein et al., 2004). Therefore, to test this possibility, a dual reporter vector was produced containing the intergenic spacer region between the human *CYP1A1* and *CYP1A2* (pd-1A1/1A2). As expected, transcriptional activation of both *CYP1A1* and *CYP1A2* genes was detected by treatment of cells with BNF and 3-MC, indicating that the spacer region acts bi-directionally. However, the potency in transcriptional activation was different between the *CYP1A1* and *CYP1A2* genes. The transcriptional activation was much higher in the *CYP1A1* gene than in the *CYP1A2* gene. This result is partly consistent with the extent of induction of *CYP1A1* and *CYP1A2* mRNAs in HepG2 cell in response to BNF or 3-MC (Fig. 1).

To identify the elements essential for transcriptional activation of the *CYP1A1* and *CYP1A2* genes, several deletions of the dual reporter vector were produced. As shown in Fig 6B, both reporter activities were decreased dramatically after deletion from -464 to -1829 of the *CYP1A1* gene, which is consistent with -21492 to -22852 of the *CYP1A2* gene. The regulatory region works bi-directionally to stimulate simultaneously the transcriptional activation of the *CYP1A1* and *CYP1A2* genes. Probably all XREs found as a cluster within this region are important as revealed by their influence on the *CYP1A1* gene activation (Kubota et al., 1991). Although effect of single XRE deletion on the transcriptional activation of the *CYP1A1* and *CYP1A2* genes was different in response to BNF or 3-MC, a single XRE does not seem to govern regulation of these two gene promoters (Fig. 7). Similar results were reported on the mouse *Cyp1a1* (Fisher et al., 1990); each replacement of

MOL #21220

individual XRE in the dioxin-responsive enhancer element of the mouse *Cyp1a1* gene did not have a dramatic effect on transcriptional activation by TCDD. However, deletion of whole XRE cluster resulted in loss of transcriptional activation of the *Cyp1a1* gene. From these results, several XREs involved in the bidirectional regulatory region may work cooperatively or additively on the transcriptional activation of the *CYP1A1* and *CYP1A2*.

In the present study, we focused on the XRE, but involvement of unknown other regulatory elements could not be excluded. For example, a new regulatory element (XRE II) was identified, in which AHR-ARNT heterodimer does not directly bind, suggesting that another, yet to be identified transcriptional factor binds to the XRE II with the AHR-ARNT heterodimer acting as a coactivator (Sogawa et al., 2004). A similar mechanism might also be involved in regulation of the human *CYP1A2* gene, but the importance of the regulatory region containing the XRE cluster on the transcriptional activation of the *CYP1A1* and *CYP1A2* has been demonstrated in the present study. The XRE cluster may be the principal regulatory region governing induction of both the *CYP1A1* and *CYP1A2*.

Interestingly, a negative control region working bi-directionally on the transcriptional activation of the *CYP1A1* and *CYP1A2* genes was also found. The region of suppressive activity exists between -18909 to -21992 of the *CYP1A1* gene, which encompasses -1329 to -4412 of the *CYP1A2* gene. The nature and mechanism of this negative bidirectional regulatory element requires additional studies.

It should also be noted that in rodent models (Goldstein and Linko, 1984) and probably also in humans, *CYP1A2* is constitutively expressed in liver and not to any significant degree in extrahepatic tissues, even after treatment of animals with inducers. *CYP1A1*, on the other

MOL #21220

hand, is not constitutively expressed in liver and is inducible in liver and many extrahepatic tissues. This suggests the existence of regulatory elements that independently control the *CYP1A1* and *CYP1A2* genes. For example, binding sites for HNF1 $\alpha$  and HNF4 $\alpha$  exist in the intergenic spacer region (Corchero et al., 2001). In mice, *Cyp1a2* is regulated by HNF1 $\alpha$  (Cheung et al., 2003). The mechanisms governing constitutive regulation of the *CYP1A* gene require further study.

In conclusion, an XRE cluster in the -22.4 kb 5'-flanking region, near the *CYP1A1* gene, is necessary for the transcriptional activation of the human *CYP1A2* gene in response to BNF and 3-MC. Experiments using a dual reporter vector containing the intergenic spacer region between *CYP1A1* and *CYP1A2* genes indicate that the region encompassing -464 to -1829 of the *CYP1A1* gene works bi-directionally to affect not only *CYP1A1* induction but also *CYP1A2* induction. In addition, a negative bidirectional element is likely located within -18909 to -21992 of *CYP1A1*. These results strongly suggest that transcriptional activation of the *CYP1A1* and *CYP1A2* genes are regulated simultaneously through a common regulatory elements existing between these two genes that acts bi-directionally.

MOL #21220

## References

Bessette EE, Fasco MJ, Pentecost BT and Kaminsky LS (2005) Mechanisms of arsenite-mediated decreases in benzo[*k*]fluoranthene-induced human cytochrome P4501A1 levels in HepG2 cells. *Drug Metab Dispos* 33:312-320.

Cheung C, Akiyama TE, Kudo G and Gonzalez FJ (2003) Hepatic expression of cytochrome P450s in hepatocyte nuclear factor 1-alpha (HNF1 $\alpha$ )-deficient mice. *Biochem Pharmacol* 66:2011-2020.

Cheung C, Ma X, Krausz KW, Kimura S, Feigenbaum L, Dalton TP, Nebert DW, Idle JR and Gonzalez FJ (2005) Differential Metabolism of 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in Mice Humanized for *CYP1A1* and *CYP1A2*. *Chem Res Toxicol* 18:1471-1478.

Corchero J, Pimprale S, Kimura S and Gonzalez FJ (2001) Organization of the CYP1A cluster on human chromosome 15: implications for gene regulation. *Pharmacogenetics* 11:1-6.

Denison MS and Whitlock JP, Jr. (1995) Xenobiotic-inducible transcription of cytochrome P450 genes. *J Biol Chem* 270:18175-18178.

Eaton DL, Gallagher EP, Bammler TK and Kunze KL (1995) Role of cytochrome P4501A2 in chemical carcinogenesis: implications for human variability in expression and enzyme activity. *Pharmacogenetics* 5:259-274.

MOL #21220

Fernandez-Salguero P, Pineau T, Hilbert DM, McPhail T, Lee SS, Kimura S, Nebert DW, Rudikoff S, Ward JM and Gonzalez FJ (1995) Immune system impairment and hepatic fibrosis in mice lacking the dioxin-binding Ah receptor.[see comment]. *Science (Wash DC)* 268:722-726.

Finnström N, Thörn M, Löf L and Rane A (2001) Independent patterns of cytochrome P450 gene expression in liver and blood in patients with suspected liver disease. *Eur J Clin Pharmacol* 57:403-409.

Fisher JM, Wu L, Denison MS and Whitlock JP, Jr. (1990) Organization and function of a dioxin-responsive enhancer. *J Biol Chem* 265:9676-9681.

Garrison PM, Tullis K, Aarts JM, Brouwer A, Giesy JP and Denison MS (1996) Species-specific recombinant cell lines as bioassay systems for the detection of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-like chemicals. *Fundam Appl Toxicol* 30:194-203.

Goldstein JA and Linko P (1984) Differential induction of two 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-inducible forms of cytochrome P-450 in extrahepatic versus hepatic tissues. *Mol Pharmacol* 25:185-191.

Hankinson O (1995) The aryl hydrocarbon receptor complex. *Ann Rev Pharmacol Toxicol* 35:307-340.

Jaiswal AK, Nebert DW, McBride OW and Gonzalez FJ (1987) Human P(3)450: cDNA and complete protein sequence, repetitive Alu sequences in the 3' nontranslated region, and

MOL #21220

localization of gene to chromosome 15. *J Exp Pathol* 3:1-17.

Jiang Z, Dalton TP, Jin L, Wang B, Tsuneoka Y, Shertzer HG, Deka R and Nebert DW (2005) Toward the evaluation of function in genetic variability: characterizing human SNP frequencies and establishing BAC-transgenic mice carrying the human *CYP1A1\_CYP1A2* locus. *Hum Mutat* 25:196-206.

Kawajiri K, Watanabe J, Gotoh O, Tagashira Y, Sogawa K and Fujii-Kuriyama Y (1986) Structure and drug inducibility of the human cytochrome P-450c gene. *Eur J Biochem* 159:219-225.

Kubota M, Sogawa K, Kaizu Y, Sawaya T, Watanabe J, Kawajiri K, Gotoh O and Fujii-Kuriyama Y (1991) Xenobiotic responsive element in the 5'-upstream region of the human P-450c gene. *J Biochem (Tokyo)* 110:232-236.

Mimura J, Yamashita K, Nakamura K, Morita M, Takagi TN, Nakao K, Ema M, Sogawa K, Yasuda M, Katsuki M and Fujii-Kuriyama Y (1997) Loss of teratogenic response to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in mice lacking the Ah (dioxin) receptor. *Genes Cells* 2:645-654.

Nebert DW and Gonzalez FJ (1987) P450 genes: structure, evolution, and regulation. *Annu Rev Biochem* 56:945-993.

Postlind H, Vu TP, Tukey RH and Quattrochi LC (1993) Response of human *CYP1*-luciferase plasmids to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and polycyclic aromatic hydrocarbons.

MOL #21220

*Toxicol Appl Pharmacol* 118:255-262.

Quattrochi LC and Tukey RH (1989) The human cytochrome Cyp1A2 gene contains regulatory elements responsive to 3-methylcholanthrene. *Mol Pharmacol* 36:66-71.

Quattrochi LC, Vu T and Tukey RH (1994) The human *CYP1A2* gene and induction by 3-methylcholanthrene. A region of DNA that supports AH-receptor binding and promoter-specific induction. *J Biol Chem* 269:6949-6954.

Schmidt JV, Su GH, Reddy JK, Simon MC and Bradfield CA (1996) Characterization of a murine *Ahr* null allele: involvement of the Ah receptor in hepatic growth and development. *Proc Natl Acad Sci USA* 93:6731-6736.

Sogawa K, Numayama-Tsuruta K, Takahashi T, Matsushita N, Miura C, Nikawa J, Gotoh O, Kikuchi Y and Fujii-Kuriyama Y (2004) A novel induction mechanism of the rat *CYP1A2* gene mediated by Ah receptor-Arnt heterodimer. *Biochem Biophys Res Commun* 318:746-755.

Trinklein ND, Aldred SF, Hartman SJ, Schroeder DI, O'tillar RP and Myers RM (2004) An abundance of bidirectional promoters in the human genome. *Genome Res* 14:62-66.

Whitlock JP, Jr. (1999) Induction of cytochrome P4501A1. *Ann Rev Pharmacol Toxicol* 39:103-125.

MOL #21220

### **Footnotes**

This work was supported by Grant-in-Aid from the Ministry of Education, Sciences and Culture (Ministry of Education, Culture, Sports, Sciences and Technology), the Ministry of Health and Welfare (Ministry of Health, Labor, and Welfare) of Japan and Comprehensive Research and Education Center for Planning of Drug Development and Clinical Evaluation, Tohoku University 21<sup>st</sup> Century “Center of Excellence” Program.

Send reprint requests to: Kiyoshi Nagata, Division of Drug Metabolism and Molecular Toxicology, Graduate School of Pharmaceutical Sciences, Tohoku University, Aramaki-Aoba 6-3, Aoba-ku, Sendai, 980-8578, JAPAN

E-mail: [nagataki@mail.tains.tohoku.ac.jp](mailto:nagataki@mail.tains.tohoku.ac.jp)

MOL #21220

### Legends for figures

**Fig. 1. Schematic diagram of the *CYP1A* gene cluster on human chromosome 15.** The DNA segment between the *CYP1A1* and *CYP1A2* genes was divided into three parts, named as Fragment 1, Fragment 2 and Fragment 3. These fragments were amplified by PCR as described in Materials and Methods. Closed boxes with numbers show the exons of the *CYP1A1* and *CYP1A2* gene. Positions of XRE sequence (5'-TNGCGTG-3') are also indicated.

**Fig. 2. Effects of BNF and 3-MC on *CYP1A1* and *CYP1A2* mRNAs in HepG2 cells.** BNF and 3-MC were added to HepG2 cells at various concentrations. After 40 h exposure, total RNA was extracted and subjected to quantitative RT-PCR. GAPDH was used as the internal standard. Values are represented as ratio to DMSO-treated cells.

**Fig. 3. 5'-Flanking region necessary for transcriptional activation of the *CYP1A1* gene.** A schematic diagram of the 5'-flanking region of the *CYP1A1* gene is shown at the top of the figure. This is in the opposite direction compared with Fig. 1. Closed boxes with numbers show the exons of the *CYP1A1* gene. The reporter plasmids indicated on the left side were transiently co-transfected with p-SV- $\beta$ -gal to HepG2 cells. After 40 h exposure with 10  $\mu$ M BNF or 1  $\mu$ M 3-MC, SEAP and  $\beta$ -gal activities were measured. Values of SEAP activities were normalized by  $\beta$ -gal activities, and represented as a ratio to DMSO-treated cells. Data are the mean  $\pm$  S.E.M. from three independent experiments performed in triplicate.

**Fig. 4. 5'-Flanking region necessary for the transcriptional activation of the *CYP1A2* gene.** A schematic diagram of the 5'-flanking region of the *CYP1A2* gene is shown at the top of the figure. This is in the same direction as in Fig. 1. Closed boxes with numbers

MOL #21220

show the exons of the *CYP1A1* and *CYP1A2* gene. The reporter plasmids indicated on the left side were transiently co-transfected with p-SV- $\beta$ -gal to HepG2 cells. After 40 h exposure with 10  $\mu$ M BNF or 1  $\mu$ M 3-MC, SEAP and  $\beta$ -gal activities were measured. Values of SEAP activities were normalized by  $\beta$ -gal activities, and represented as the ratio to DMSO-treated cells. Data are the mean  $\pm$  S.E.M. from three independent experiments performed in triplicate.

**Fig. 5. Co-transcriptional activation of the *CYP1A1* and *CYP1A2* genes.** (A) The DNA segment between the *CYP1A1* and *CYP1A2* genes. Closed boxes with numbers show the exons of the *CYP1A1* and *CYP1A2* gene. (B) The region from +1039 of the *CYP1A1* gene to +90 of the *CYP1A2* gene was inserted between Luc and SEAP reporter genes to construct a dual reporter vector. (C) The dual reporter vector was transiently co-transfected with pRL-SV40 to HepG2 cell. After 40 h exposure of cells to 10  $\mu$ M BNF or 1  $\mu$ M 3-MC, SEAP, Luc and RL activities were measured. Values of SEAP and Luc activities were normalized by RL activities, and expressed as a ratio to DMSO-treated cells. Data are the mean  $\pm$  S.E.M. from three independent experiments performed in triplicate.

**Fig. 6. Influence of deletion in the regulatory region on the *CYP1A1* and *CYP1A2* gene activation.** A schematic diagram of the regulatory region between the *CYP1A1* and *CYP1A2* genes is indicated at the top of the figure. Closed boxes with numbers show the exons of the *CYP1A1* and *CYP1A2* genes. Deletion mutants of the dual reporter constructs are shown in the center. These vectors were prepared by the standard method with the restriction enzymes indicated in the diagram. The insert of pd-1A1/1A2 was deleted within -18096 (A) or -21992 (B) of the *CYP1A1* gene. Lower and upper numbers of each reporter construct represent the position from the transcriptional starting points of the *CYP1A1* and *CYP1A2*,

MOL #21220

respectively. Luc and SEAP activities are shown in the left and the right graph, respectively. Values are expressed as ratio to DMSO-treated cells. Data are the mean  $\pm$  S.E.M. from three independent experiments performed in triplicate.

**Fig. 7. Influence of a single XRE deletion on the *CYP1A1* and *CYP1A2* gene activation.**

Dual reporter vector deleted from -4621 to -21992 of the *CYP1A1* gene (designated pd-4621/21992) is shown at the top of the figure. The region from -4621 to +1039 of the *CYP1A1* gene was enlarged and shown in the center of the figure. Five XREs close to the *CYP1A1* gene were independently deleted by site-directed mutagenesis. Lower and upper numbers of each reporter construct represent the position from the transcriptional starting points of the *CYP1A1* and *CYP1A2*, respectively. Luc and SEAP activities are shown in the left and the right graph, respectively. Data are the mean  $\pm$  S.E.M. from three independent experiments performed in triplicate. \*,  $p < 0.05$  compared to pd-4621/21992.

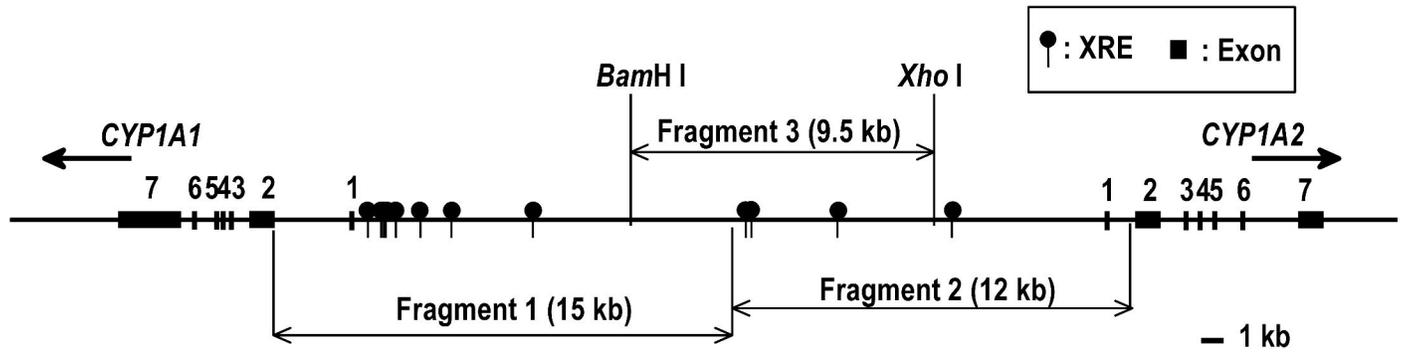


Figure 1

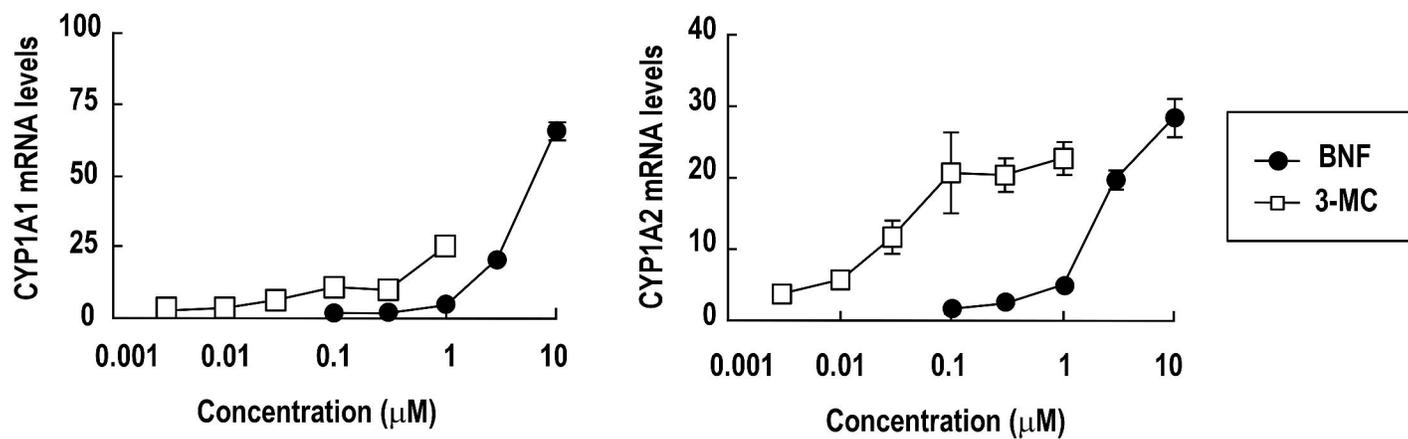


Figure 2

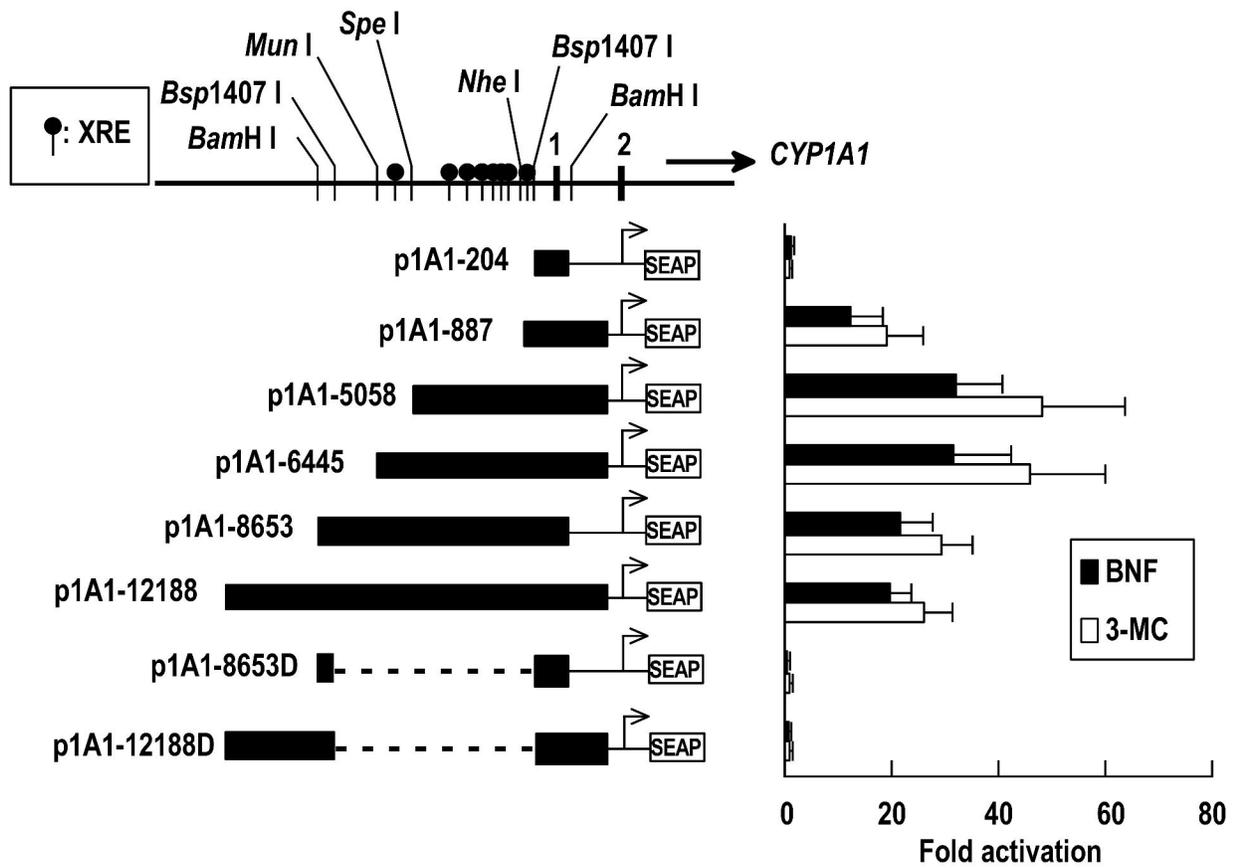


Figure 3

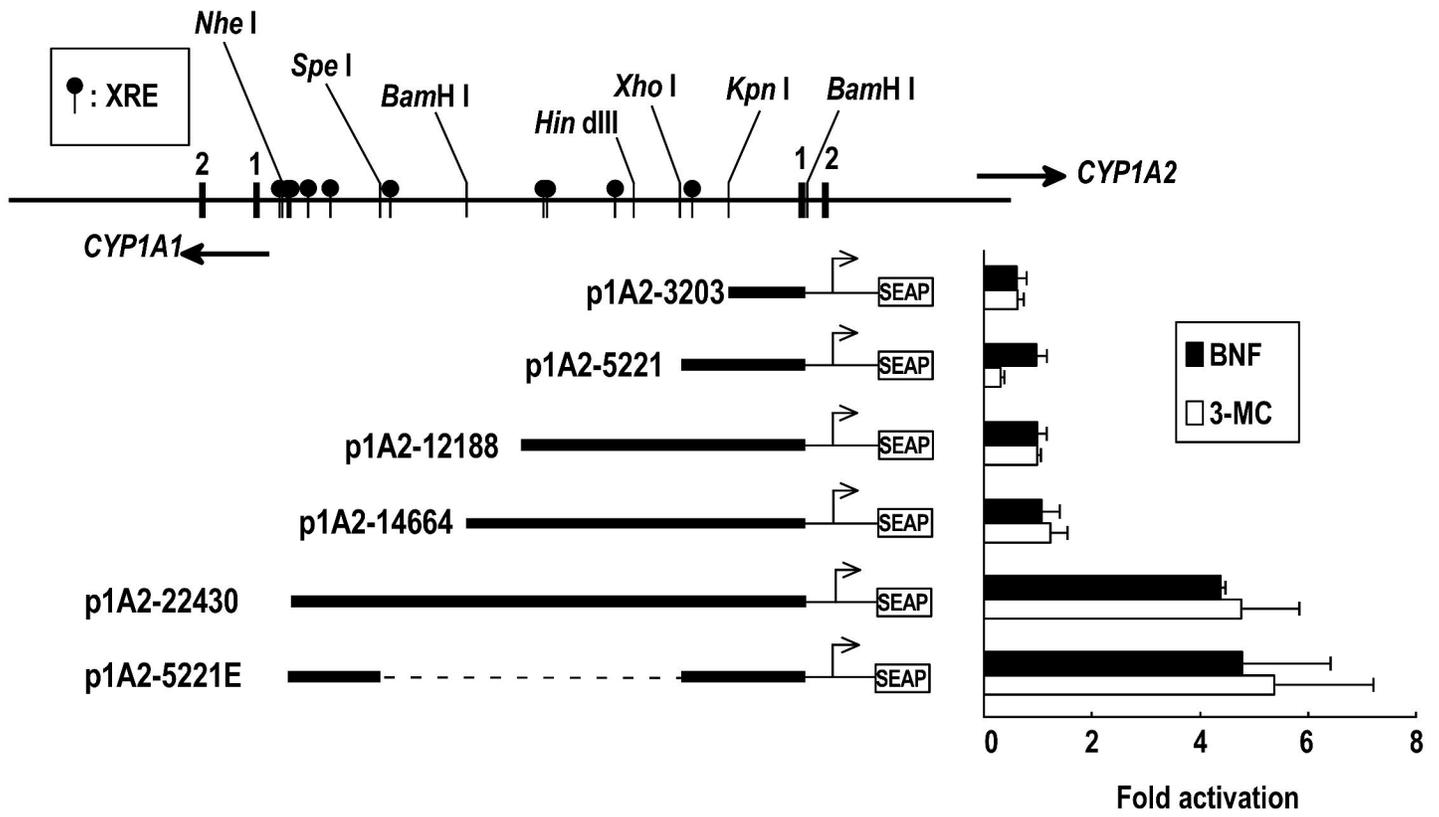


Figure 4

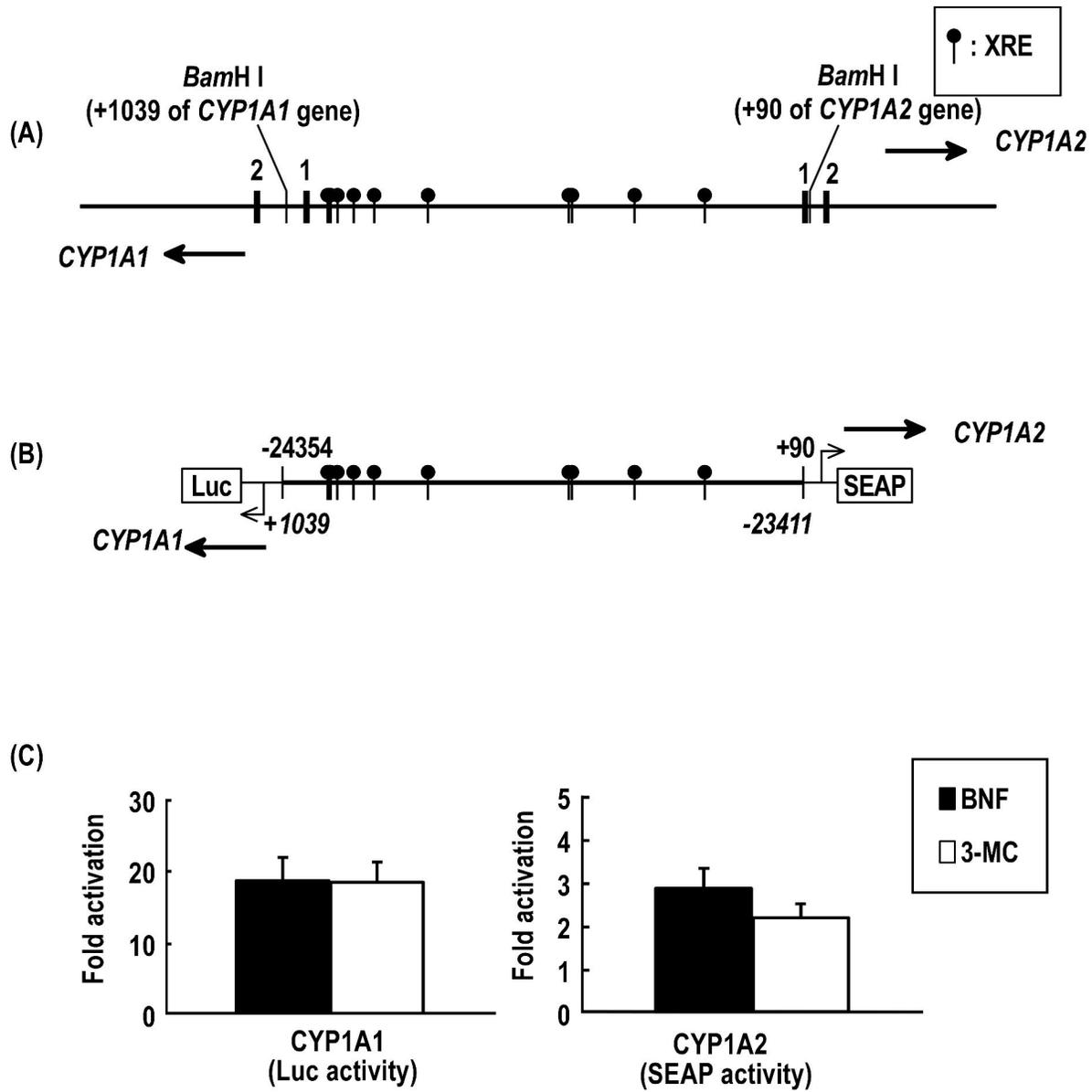


Figure 5

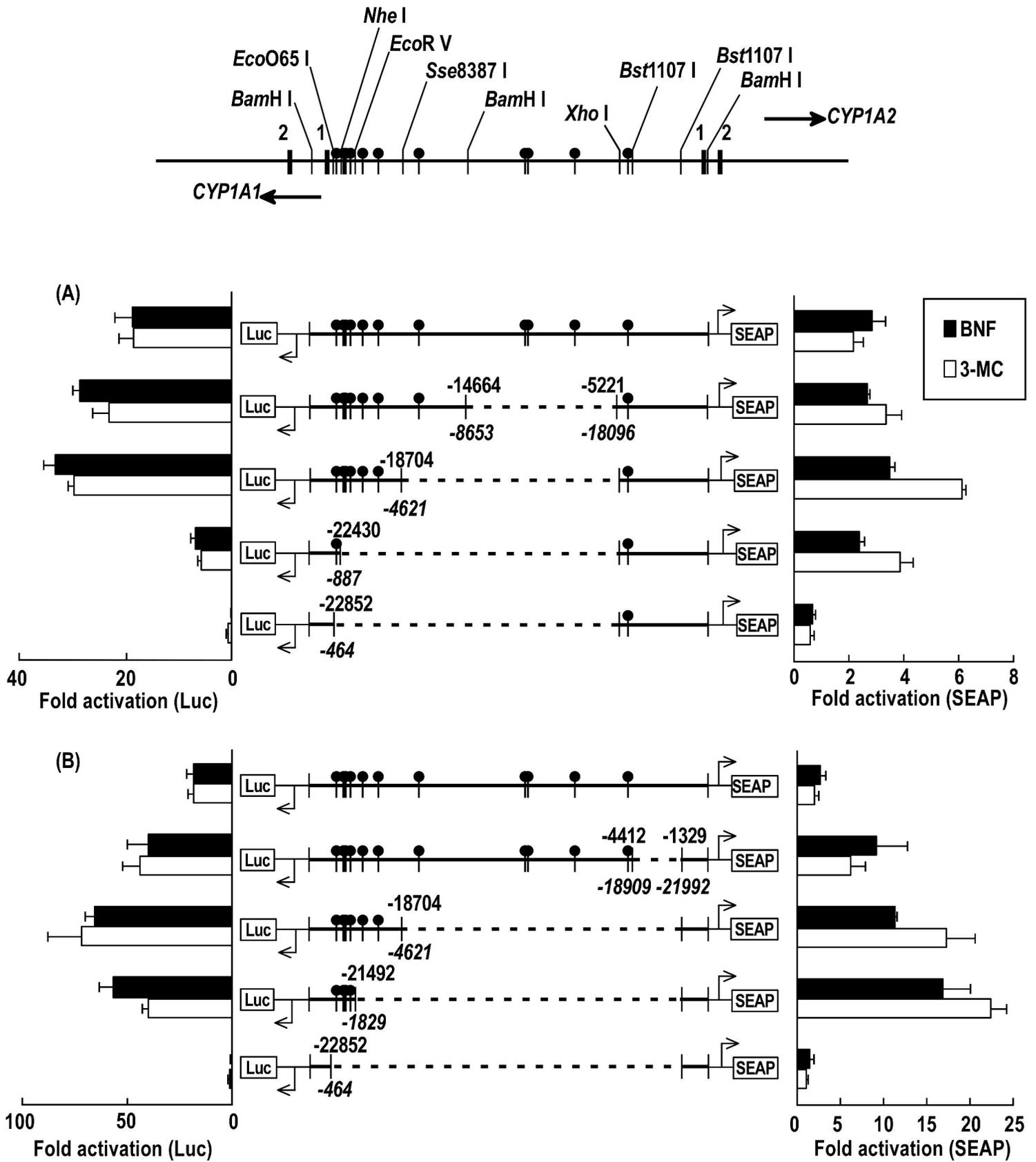


Figure 6

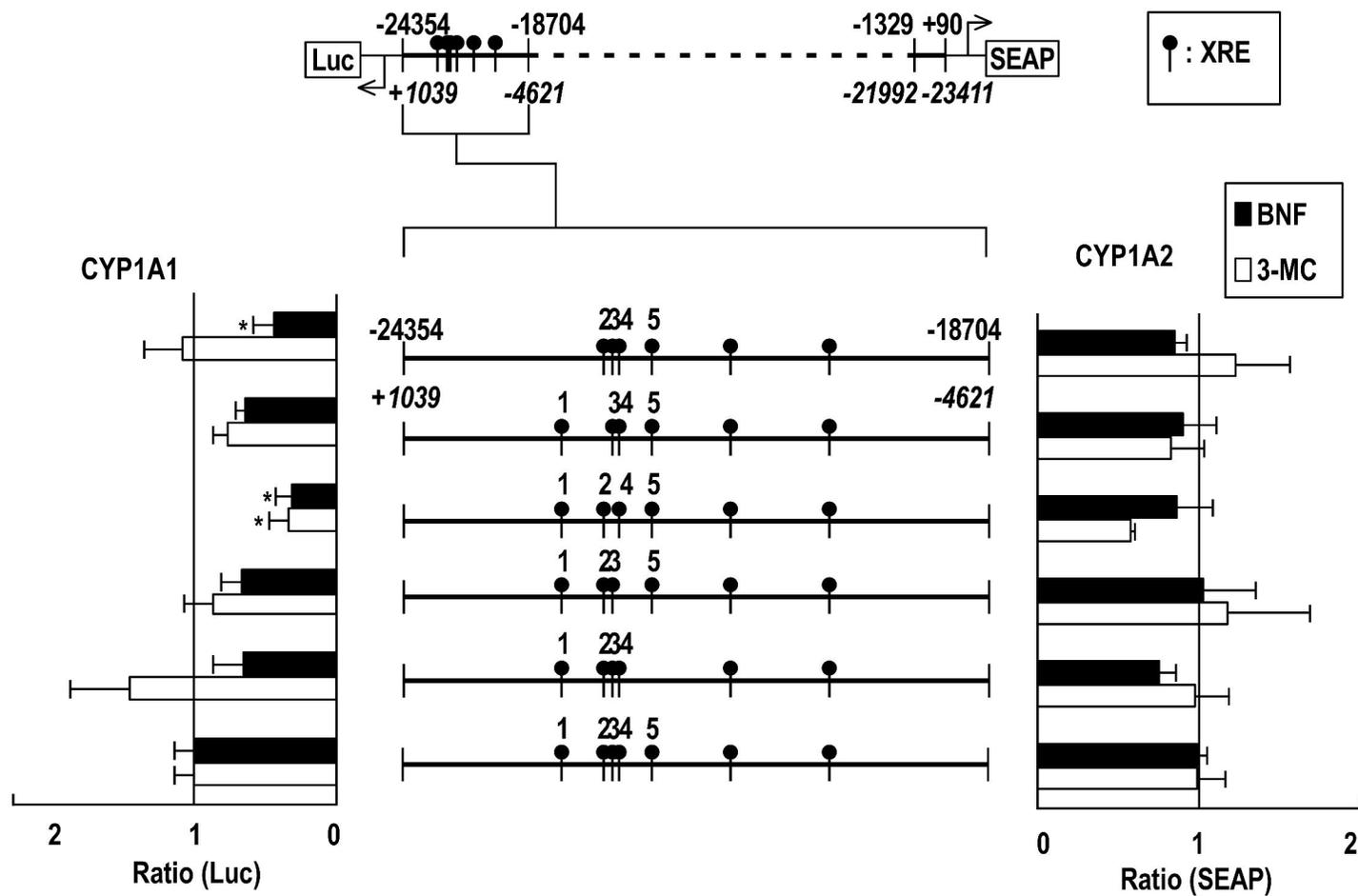


Figure 7