0026-895X/99/020279-09$3.00/0
Copyright © by The American Society for Pharmacology and Experimental Therapeutics
All rights of reproduction in any form reserved.
MOLECULAR PHARMACOLOGY, **55:**279–287 (1999).

MOL PHARM

A Novel Positive Regulatory Element That Enhances Hamster *CYP2A8* Gene Expression Mediated by Xenobiotic Responsive Element

KOUICHI KUROSE, MASAHIRO TOHKIN, and MORIO FUKUHARA

Department of Pharmaceutical Sciences, National Institute of Public Health, Minato-ku, Tokyo, Japan

Received August 24, 1998; accepted November 10, 1998

This paper is available online at http://www.molpharm.org

ABSTRACT

CYP2A8 is a major form of cytochrome P-450 inducible by 3-methylcholanthrene in Syrian hamster liver. To identify DNA elements necessary for the transcriptional activation of the *CYP2A8* gene, we analyzed the regulatory region of the *CYP2A8* gene and conducted transient transfection experiments of *CYP2A8*-luciferase fusion plasmids in primary cultures of hamster hepatocytes. We analyzed up to −5 kb of the 5′-flanking region and found the region sufficient for the 3-methylcholanthrene-inducible gene expression. This region contained a consensus sequence for xenobiotic responsive element (XRE) between −2366 and −2349, which was shown to be essential for induction of the gene expression. Furthermore, we found a novel positive regulatory element for XRE-mediated gene expression (PREX) located upstream of the XRE. This element is not identified in any genes inducible by 3-methylcholanthrene so far reported. Without PREX, the XRE-mediated promoter activity was enhanced nearly 10-fold, whereas with PREX, the activity was enhanced 20-fold over the basal level. Gel mobility shift assays revealed specific binding of nuclear proteins to PREX. Mutations and deletions of PREX caused a loss of the binding and promoter-enhancing activities, respectively. Moreover, transient expression experiments showed that the enhancing activity of PREX was not observed in *Drosophila* Schneider's line 2 cells, which were shown to lack the PREX binding proteins.

MOLECULAR PHARMACOLOGY

Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

The cytochrome P-450 (CYP) superfamily consists of various isozymes that are classified into gene families and subfamilies based on the similarity in their primary amino acid sequences (Nelson et al., 1996). The isozymes catalyze xenobiotics including drugs and environmental chemicals as well as endogenous steroids (Gonzalez, 1989; Porter and Coon, 1991; Guengerich, 1997). Several members of CYP families are known to be induced markedly by these substances, including aromatic hydrocarbons, barbiturates, peroxisome proliferators, and steroids (Denison and Whitlock, 1995). Aromatic hydrocarbons such as 3-methylcholanthrene (3-MC) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin induce several CYPs including 1A1, 1A2, 1B1, and 2A8 (Fukuhara et al., 1989b; Denison and Whitlock, 1995). Although the induction mechanisms of these CYPs have not yet been fully elucidated, transcriptional activation mechanism of one of these genes, *CYP1A1*, has been studied extensively (Whitlock et al., 1996). The inducer binds to aryl hydrocarbon receptor (AhR) and it heterodimerizes with AhR nuclear translocator (Arnt). AhR-Arnt complex then binds to the enhancer element termed xenobiotic responsive element (XRE) located in the 5′-flanking region of the *CYP1A1* gene.

In our laboratory, we have studied CYPs of the Syrian hamster and cloned and characterized CYP2A8, 3A31, and 2A9 (Fukuhara et al., 1989a; Alabouch et al., 1998; Kurose et al., 1998). These studies showed that induction mode of CYPs in the Syrian hamster is markedly different from that of the rat and mouse. Notably, the induction of CYP2A subfamily of the hamster is unique. In the rat and mouse liver, CYP1A1 and 1A2 are the major forms induced by 3-MC-type inducers (Conney, 1982; Nebert and Gonzalez, 1987; Whitlock, 1987), whereas in the hamster liver, it is not CYP1A1 but CYP2A8 that is a major form induced by 3-MC-type inducers (Sunouchi et al., 1988; Fukuhara et al., 1989b; M. Fukuhara, unpublished data). Expression of CYP2A isozymes is known to differ depending on species and isozymes (Honkakoski and Negishi, 1997). However, the mechanisms by which CYP2A isozymes are induced have been scarcely studied and any studies have not identified transcriptional elements on the

This work was supported by a Grant 1508 from the Japan Health Sciences Foundation and by Grants-in-Aid for Cancer Research from the Ministry of Health and Welfare of Japan and for Scientific Research from the Ministry of Education, Culture, Sport, and Science of Japan. K.K. and M.T. contributed equally to this work.

ABBREVIATIONS: AP-1, activator protein-1; AhR, aryl hydrocarbon receptor; Arnt, AhR nuclear translocator; BTE, basic transcription element; CYP, cytochrome P-450; 3-MC, 3-methylcholanthrene; SL2, *Drosophila* Schneider's line 2; PREX, positive regulatory element for XRE-mediated gene expression; 5′-RACE, rapid amplification of 5′-cDNA ends; XRE, xenobiotic responsive element.

MOL PHARM

MOLECULAR PHARMACOLOGY

CYP2A genes that may regulate the expression, except for nasal transcriptional activating element on *CYP2A3* gene (Zhang and Ding, 1998).

To understand the mechanism of induction of the hamster CYP2A8, we studied the induction mode of CYP2A8 in primary hepatocyte cultures, which suggested the involvement of XRE-mediated *CYP2A8* gene expression (Tohkin et al., 1996). Hence, in the present study, to further elucidate the regulation mechanism of the *CYP2A8* gene expression, we analyzed the 5′-flanking region of the gene. We identified the functional XRE and furthermore, a novel positive regulatory element (PREX) that enhanced the XRE-mediated promoter activity of the *CYP2A8* gene. We also demonstrated the presence of specific nuclear factors involved in the activation by PREX.

Materials and Methods

Animals, Cells, and Materials. Female Slc:Syrian hamsters (Nippon SLC Inc., Hamamatsu, Japan), aged between 6 to 8 weeks, were used for the preparation of primary cultures of hepatocytes. *Drosophila* Schneider's line 2 (SL2) cells were obtained from American Type Culture Collection (Rockville, MD). Schneider's *Drosophila* medium, fetal bovine serum, OPTI-MEM, Lipotectin and 5′ rapid amplification of cDNA ends (RACE) System kit were purchased from GIBCO BRL (Gaithersburg, MD). Synthetic oligonucleotides were purchased from Greiner Japan Inc. (Tokyo, Japan). Luciferase reporter vector pGL2-Basic was obtained from Promega (Madison, WI) and λEMBL3 vector and pBluescript SK- vector were from Stratagene (La Jolla, CA). Human AhR, Arnt, and Sp1 expression plasmids (pGEMAct-hAhR, pGEMAct-Arnt, pGEMAct-Sp1) (Kobayashi et al., 1996) and anti-rat AhR IgG (Matsushita et al., 1993) were kindly donated by Dr. Fujii-Kuriyama (Tohoku University, Sendai, Japan). All other reagents used were as described previously (Tohkin et al., 1996).

Cloning of CYP2A8 Genomic DNA. Syrian hamster genomic DNA prepared from spleens was partially digested with *Sau*3A I and ligated into the *Bam*HI site of λEMBL3 vector. Approximately 10^6 phage plaques were screened initially with a 5′-end *Eco*RI fragment (0.6 kb) of CYP2A8 cDNA (Fukuhara et al., 1989a) as a probe. Four independent clones were isolated, and the genomic clone DNAs were further analyzed by restriction mapping and Southern hybridization with a *CYP2A8* specific 5′-end oligonucleotide (5′-TGCCACCATGCTGGTGTCC-3′). One of the clones, designated L4, contained a 1.85-kb *Sal*I fragment that hybridized to the 5′-end oligonucleotide probe. This fragment was then subcloned into the pBluescript SK- vector and sequenced. This sequence showed that the fragment had contained the first coding exon. Because the L4 phage also contained a further 15.6-kb upstream region, some fragments of the phage DNA were subcloned into the pBluescript SK- vector, which were used in subsequent promoter studies. To determine the DNA sequence and to construct *CYP2A8*-luciferase reporter gene fusion plasmids, a series of deletions of the *CYP2A8* gene 5′-flanking region were generated by the exonuclease III/mung bean nuclease method. The 5.6-kb *Xba*I-*Sal*I fragment (−6.8 k to −1.2 k) was subcloned into the pBluescript SK-. After digestion with *Bst*XI (in the multiple cloning site located at the upstream of the *Xba*I site) and *Xba*I, the DNA was incubated with exonuclease III for appropriate time intervals at 30°C. The remaining single-stranded DNAs were removed by mung bean nuclease treatment followed by self-ligation. The resulting deletion clones were then sequenced and used for construction of luciferase fusion plasmids.

DNA Sequencing and Analysis. Nucleotide sequences were determined by the dideoxy chain termination method using a DSQ1000 DNA sequencer (Shimadzu, Kyoto, Japan). The nucleotide sequences were analyzed and compared using GeneWorks version 2.5 sequence analysis software (Oxford Molecular Group Inc., CA). Transcriptional regulatory elements were searched through TRANSFAC database (Heinemeyer et al., 1998).

Nucleotide Sequence Accession Number. The nucleotide sequence data reported in this article has been submitted to the DDBJ/Gene Bank/EMBL Data Bank with the accession number AB001516.

Determination of Transcription Start Site. To determine the transcription start site of the *CYP2A8* gene, the 5′-RACE experiment was performed using the 5′ RACE System kit essentially according to the manufacturer's instructions. Forty nanograms of poly(A)$^+$ RNA from female Syrian hamster liver was reverse-transcribed by a CYP2A8 cDNA-specific antisense primer (5′-GTATGAGAAAGCTGGTCTC-3′). The first-strand cDNA was amplified by PCR using the anchor primer provided with the system and a nested CYP2A8 cDNA-specific antisense primer (5′-ACTTCTCTGTGTCCAGCTCC-3′). A single band of about 230 bp was gel purified and sequenced directly with a upstream CYP2A8 cDNA-specific antisense primer (5′-TCTCCTCTGCCTCCACACAGAC-3′).

Luciferase Reporter Gene Construction. A series of constructs were prepared, in which various lengths of the 5′-flanking region of the *CYP2A8* gene were cloned upstream of the luciferase reporter gene in plasmid pGL2′-Basic. This plasmid was constructed by digestion of the luciferase reporter vector pGL2-Basic with *Sal*I followed by self-ligation of the blunt-ended site. First, we subcloned the 1.2 kb *Sal*I -*Bst*XI fragment of the 1.85 kb *Sal*I fragment of the L4 clone into pGL2′-Basic. Because the *Bst*XI site includes translation initiation codon ATG, we eliminated the ATG codon as follows. The plasmid that contained the 1.85-kb *Sal*I fragment was digested with *Bst*XI and blunt ended with T4 DNA polymerase to remove the ATG, then digested with *Sal*I. The resulting 1.2-kb fragment (−1199 to +26, relative to the *CYP2A8* gene transcriptional start site) was inserted into the *Sal*I-blunt ended *Xho*I site of pBluescript SK-. Then, plasmid pGL2A8-1.2 was constructed by insertion of the 1.2-kb fragment, which was cut out with *Sac*I and *Xho*I into the *Sac*I-*Xho*I site of pGL2′-Basic. Plasmid pGL2A8-2.1 was constructed by insertion of the *Sac*I-*Sal*I fragment (−2073 to −1198) of the 5′-flanking region into the *Sac*I-*Sal*I site of the pGL2A8-1.2. Plasmid pGL2A8-2.2 was constructed by insertion of the *Pst*I-*Sal*I fragment (−2137 to −1198) of the 5′-flanking region into the *Pst*I-*Sal*I site of the pGL2A8-1.2. To prepare a series of deletions of the 5′-flanking region-luciferase fusion constructs, the deletion constructs described in the section *Cloning of CYP2A8 Genomic DNA* above were excised by digestion with *Sac*I (in the vector and at −2073) or with *Sac*I (in the vector) and *Sal*I (at −1198). The series of deletion fragments were then subcloned into the *Sac*I site or *Sac*I-*Sal*I site of pGL2A8-2.1. Plasmid pGL2A8-Δ1 was constructed by deletion of the *Pvu*II fragments of one of the deletion constructs pGL2A8-2.6 (−2668) followed by self-ligation. Plasmid pGL2A8-Δ2 was constructed by insertion of double-stranded synthetic oligonucleotide OL3 (−2452 to −2421) into the *Pvu*II site of pGL2A8-Δ1. Plasmid pGL2A8-Δ3 was constructed by insertion of a *Pvu*II fragment (−2437 to −2208) into the *Pvu*II site of pGL2A8-Δ1. Plasmid pGL2A8-Δ4 was constructed by insertion of a *Pvu*II fragment (−2437 to −2208) into the *Pvu*II site of pGL2A8-1.2. Plasmid pGL2A8-Δ5 was constructed by insertion of a *Pst*I fragment (−2626 to −2138) into the *Pst*I site of pGL2A8-1.2.

Transient Transfection of Plasmids into Hepatocytes. Primary cultures of Syrian hamster hepatocytes were prepared as described previously (Tohkin et al., 1996) and 1×10^6 cells were plated in 35-mm collagen-coated plastic culture dishes in 2 ml of Waymouth's MB752/1 medium containing bovine serum albumin (2%), insulin (0.5 mg/liter), transferrin (0.5 mg/liter), selenium (0.5 μg/liter), and dexamethasone (1 nM). After cultivation for 24 h, the dishes were washed with 1 ml of serum free OPTI-MEM and then exposed to a mixture consisting of 5 μl of Lipofectin with 2.5 μg of pGL2A8 and 5 μg of pSV-β-galactosidase plasmids in 1 ml of serum free OPTI-MEM. After 17 h of incubation, the Lipofectin-plasmid mixture was removed and the cells were cultured with 2 ml of Waymouth's MB752/1 medium for 5 h, and then the medium was

Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

MOL PHARM

MOLECULAR PHARMACOLOGY

replaced with Waymouth's MB752/1 medium containing 1 μM 3-MC in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide in the culture medium was 0.1%. After 24 h of treatment with 3-MC, luciferase activities in cell extracts were measured by a luciferase assay kit. Differences in transfection efficiencies between dishes within given experiments were normalized by the β-galactosidase activity.

Transient Transfection of Plasmids into *Drosophila* SL2 Cells. SL2 cells were maintained in the *Drosophila* Schneider's medium supplemented with 10% fetal bovine serum. Plasmids (3 μg of DNA) were introduced into SL2 cells (5×10^6 cells/2 ml of culture medium/35-mm plastic culture dish) by the calcium-phosphate method as described by Di Nocera and Dawid (1983). After incubation for 72 h, 3-MC or dimethyl sulfoxide alone was added to the cell culture. Cells were harvested 24 h later and collected by centrifugation. Cells were washed two times with phosphate-buffered saline and luciferase activities in cell extracts were measured.

Nuclear Extract Preparation and Gel Mobility Shift Assay. Nuclear extracts from Syrian hamster livers and SL2 cells were prepared according to the procedure of Sierra et al. (1993). The extracts were dialyzed against 25 mM HEPES-KOH (pH 7.6) containing 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10% glycerol (v/v), 40 mM KCl, and 0.1 mM EDTA. Double-stranded synthetic oligonucleotides were end-labeled by filling the recessed 3′-termini with [α-^{32}P]dCTP using a large fragment of DNA polymerase I and purified using a Sephadex G-25 column. The labeled oligonucleotide probes (20,000–30,000 cpm) were mixed with 2 μg of poly(dI-dC)poly(dI-dC), 1 μg of nuclear extract, and competitor oligonucleotides, if necessary, in a final volume of 20 μl of solution containing 25 mM HEPES-KOH (pH 7.8), 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10% glycerol (v/v), 50 mM KCl, and 0.5 mM EDTA. The solutions were incubated at 25°C for 30 min and electrophoresed on 4% nondenaturing polyacrylamide gels using 50 mM Tris-HCl (pH 8.5), 0.38 M glycine, and 2 mM EDTA as a running buffer. Gels were dried and exposed to X-ray film for autoradiography.

Results

Analysis of the 5′-Flanking Region of *CYP2A8* Gene. We constructed a Syrian hamster genomic library, and isolated a genomic clone containing about 16.8 kb of 5′-flanking region, the first and second exons, the first intron, and part of the second intron of the *CYP2A8* gene. The start site of transcription on the *CYP2A8* gene was determined by the 5′-RACE method with poly $(A)^+$ RNA prepared from hamster livers. Sequence analysis identified the transcription start site at 27 bp upstream of the ATG start codon. The nucleotide sequences of the 5′-flanking region were analyzed up to −3322 (relative to the *CYP2A8* gene transcription start site) and the sequences from −2672 to −2123 and −172 to +128 are shown in Fig. 1. A putative TATA box, TATAAA, was located at −30 bp upstream of the transcription start site. Upstream of the TATA box, a putative basic transcription element (BTE; GC box-like sequence) was found at position −50 to −36. We found a consensus sequence for XRE locating between −2366 and −2349 that contained the full AhR-Arnt responsive element including two core sequences, CACGC, repeated in tandem.

XRE-Mediated Gene Regulation. We previously showed that the expression of the *CYP2A8* gene by 3-MC was observed both in the primary hepatocyte cultures (Tohkin et al., 1996) and in in vivo experiments (Fukuhara et al., 1989b). Using this hepatocyte culture, we studied the regulatory elements that participate in the transcriptional activation by 3-MC. Various luciferase reporter constructs containing successive 5′-deletions of the *CYP2A8* regulatory region were introduced into cultured hepatocytes, and the transient expression of the luciferase enzyme driven by the constructs was determined in the presence or absence of 3-MC. The plasmids carrying 5′-flanking sequences longer than −2.6 kb had high transcriptional inducibilities by 3-MC showing approximately 20-fold induction, whereas those with length shorter than −2.3 kb failed to respond to 3-MC (Fig. 2). These results suggest that the sequence between −2.6 kb and −2.3 kb is necessary to the transcriptional activation of the gene by 3-MC treatment.

To identify the elements in this region essential for the gene activation, we performed further successive deletions of the region (from −2668 to −2309) in luciferase fusion plasmids and examined their transcriptional activation by 3-MC. As shown in Fig. 3A, the constructs containing −2468 or more retained approximately 20-fold induction level, whereas further deleted construct starting from −2363, which lacked the 5′-part of the consensus XRE, completely lost 3-MC-inducible expression. Interestingly, the construct starting from −2409, although it contains the consensus XRE, lost more than 50% of the induction compared with that from −2468. We further analyzed the responsible regions for the 3-MC-inducible expression. As shown in Fig. 3B, the internal deletion mutants of pGL2A8-Δ1 and -Δ2, which lack the region including the consensus XRE, resulted in elimination of 3-MC-induced luciferase activity, whereas almost no decrease of induction was observed in the deletion mutants pGL2A8-Δ3 and -Δ5, both containing the consensus XRE. These results confirm that the region including the consensus XRE plays the role as functional XRE. To confirm that AhR-Arnt certainly binds to the XRE of *CYP2A8*, we performed gel mobility shift assay with the XRE oligonucleotide as a probe. Using nuclear extracts from AhR-Arnt-expressed or nonexpressed *Drosophila* SL2 cells that are devoid of AhR and Arnt proteins (Kobayashi et al., 1996), the specific DNA-protein complex was detected only in the AhR-Arnt-expressed nuclear extracts (data not shown). Furthermore, we confirmed that the XRE-protein complex formation in hamster liver nuclear extracts was abolished by the anti-AhR antibody, which was reported to inhibit the binding of the AhR-Arnt to the XRE (Matsushita et al., 1993; data not shown).

Positive Regulatory Element for PREX. As described above (Fig. 3A), the construct starting from −2409 lost more than 50% of the induction compared with the full activity. This indicated the existence of positive regulatory elements other than XRE that are necessary for the full activation of the gene expression. As shown in Fig. 3B, pGL2A8-Δ4, which has the XRE but lacks the sequence upstream of −2437, had also less than half the ability of pGL2A8-Δ3 and -Δ5 to activate transcription. This result restricts the location of the positive regulatory element between −2437 to −2626. On the other hand, pGL2A8-Δ2, which contains the positive regulatory region but not the consensus XRE, did not show induction of the gene expression (Fig. 3B). Furthermore, the positive regulatory region exerted no influence to basal transcription level (Fig. 3). These results mean that the positive regulatory effect of the element depends on the XRE. Therefore, we designated the novel positive regulatory element for the XRE-mediated *CYP2A8* gene expression as

Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

aspet

MOL PHARM

MOLECULAR PHARMACOLOGY

PREX. The fact that pGL2A8-2468 had the full ability of the gene activation (Fig. 3A), whereas pGL2A8-Δ4, which lacks the regions upstream of −2437, had less than half of ability of the gene activation (Fig. 3B), suggests that the region between −2468 and −2437 includes the essential part of PREX that potentiates the XRE-mediated promoter activity.

Cooperative Transcriptional Enhancement of AhR-Arnt with Sp1. We found XRE and BTE in the 5′-flanking region of the *CYP2A8* gene. Using a model *CYP1A1* promoter, Kobayashi et al. (1996) reported that AhR-Arnt complex, the *trans*-acting factor on XRE, enhanced the transcription activity in cooperation with Sp1, the *trans*-acting factor on BTE. Therefore, we studied whether these factors are certainly involved in the activation of the *CYP2A8* gene transcription or not. We examined the transcriptional activity of *CYP2A8*-luciferase fusion genes (pGL2A8-2668, pGL2A8-Δ3, pGL2A8-Δ4, and pGL2A8-Δ5) in the transient expression system using *Drosophila* SL2 cells, which are devoid of AhR, Arnt, and Sp1 proteins (Kobayashi et al., 1996). As shown in Fig. 4, 3-MC did not induce luciferase expression from each construct in SL2 cells transfected with Sp1 expression plasmid alone. The expression of AhR and Arnt activated the transcription only slightly in all cases. In contrast, cotransfection of the AhR and Arnt expression plasmid with the Sp1 expression plasmid enhanced luciferase expression by 6-fold in the presence of 3-MC in all cases. This means that AhR-Arnt activates the gene expression by 3-MC in cooperation with Sp1. The induction of luciferase expression was observed not only in the SL2 cells transfected with pGL2A8-2668 but also those transfected with pGL2A8-Δ3, which lacked most of the internal region (−2207/−131) of pGL2A8-2668. This indicates that the internal region deleted is not essential for the enhancement by AhR-Arnt and Sp1. Although the induction of luciferase expression of pGL2A8-Δ4 by 3-MC was less than half that of pGL2A8-Δ5 in hepatocytes (Fig. 3B), induction of the two constructs by 3-MC was almost

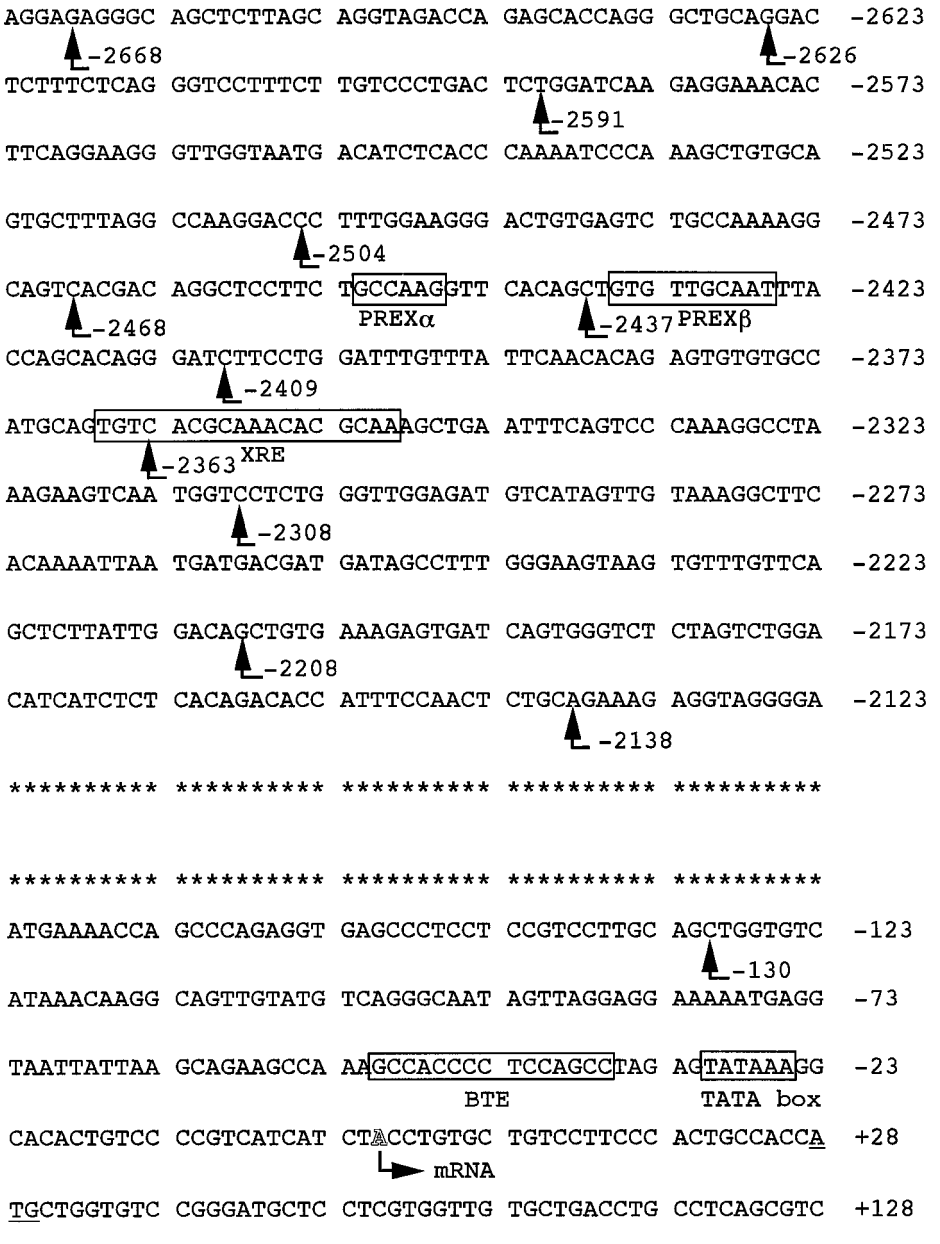

Fig. 1. Nucleotide sequence of 5′-flanking region of *CYP2A8*. Sequence of the 5′-flanking region up to −172 and the sequence from −2672 to −2123 are shown. Nucleotide sequences of TATA box, BTE (GC box-like sequence), XRE, and positive regulatory elements (PREXα and -β) are enclosed in boxes. Arrows show 5′-end point of deletion mutants.

Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet

equivalent in SL2 cells transfected with AhR, Arnt, and Sp1 expression plasmid (Fig. 4, C and D). These results indicate that PREX could not enhance the XRE-mediated *CYP2A8* gene in SL2 cells and that the nuclear factors for PREX would be deficient in SL2 cells.

Gel Mobility Shift Assays of PREX. To examine whether any nuclear factors could bind to PREX, we performed gel mobility shift assays using hamster liver nuclear extracts with the labeled PREX as a probe. Because the region between −2468 and −2437 included the essential part of PREX, we used three double-stranded synthetic oligonucleotides OL1 (−2481/−2453), OL2 (−2470/−2435) and OL3 (−2452/−2421) as probes (Fig. 5A). As shown in Fig. 5B, two DNA-protein complexes were observed only when OL3 was used as a probe. The addition of a 50-fold excess of unlabeled OL3 as a competitor prevented the formation of the DNA-protein complexes. To further determine the core element necessary for the protein binding, competition experiments were carried out using a series of competitors. As shown in Fig. 6, the m1 competitor failed to compete absolutely, indicating that the sequence region mutated in the m1 is essential for the formation of the specific complexes. The regions

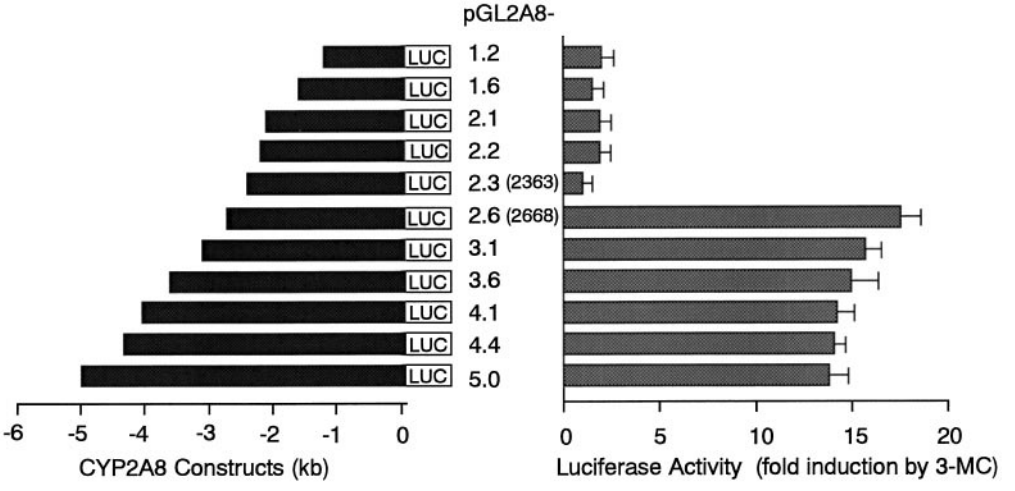

Fig. 2. Structure of successive 5′-deletion mutants of *CYP2A8* regulatory region and their luciferase activities in transfected primary hepatocyte cultures. Illustration shows luciferase reporter constructs containing various 5′-deletions. Numbers given to deletion plasmids indicate 5′-ends of the 5′-flanking sequences of the *CYP2A8* gene from the transcription start site. These plasmids were introduced into primary cultures of hepatocytes and luciferase assays were performed after 17 h of 3-MC treatment. Fold induction refers to ratio of luciferase activity of 3-MC-treated cells to untreated cells. Values are means ± S.E. of three independent experiments.

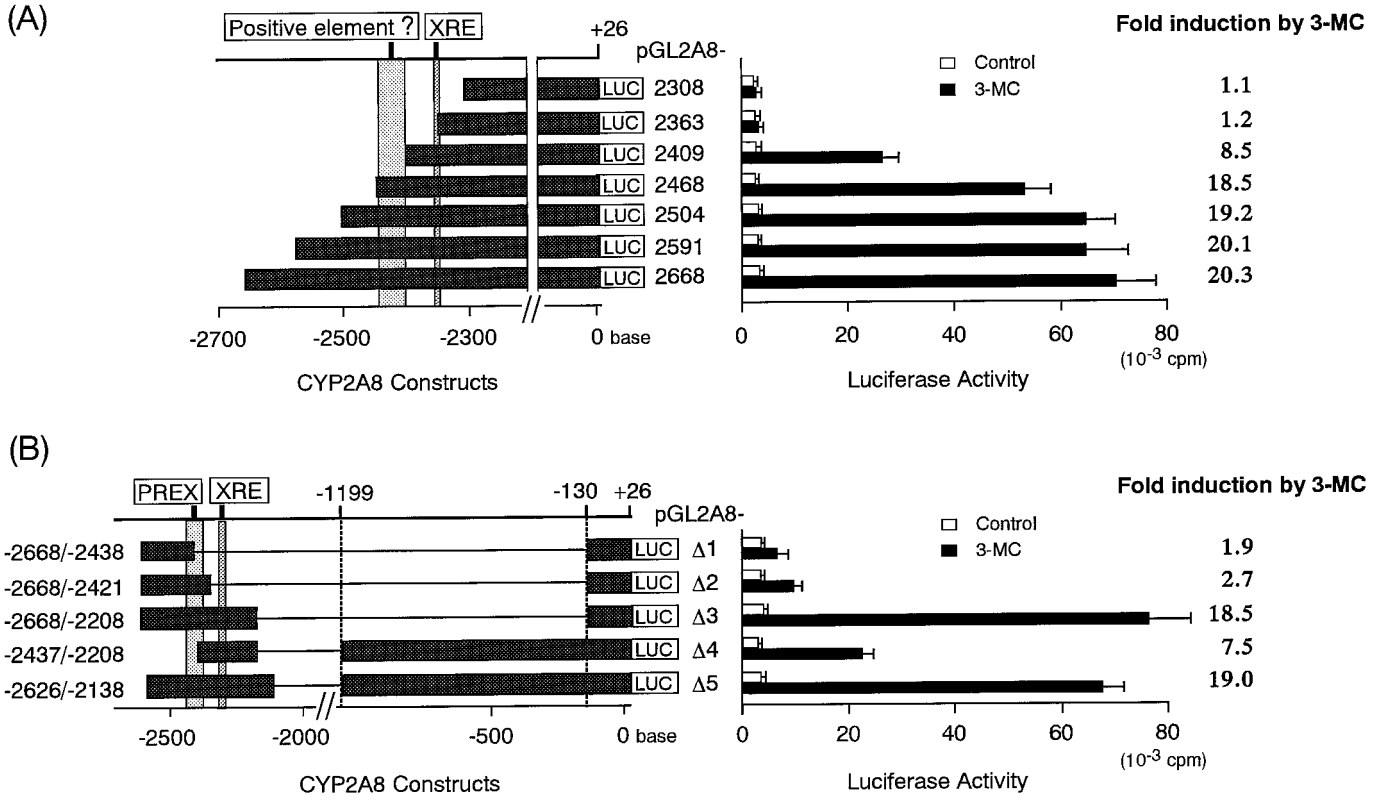

Fig. 3. Structure of deletion mutants of *CYP2A8* regulatory region and their luciferase activities in transfected primary hepatocyte cultures. Illustrations show the pGL2 fusion construct of several successive 5′-deletions (A) and internal deletion mutants (B). Numbers given to deletion plasmids indicate 5′-ends of the 5′-flanking sequences of the *CYP2A8* gene from the transcription start site. These plasmids were introduced into primary hepatocyte cultures and luciferase assays were performed after 17 h of 3-MC treatment. Fold induction refers to ratio of luciferase activity of 3-MC-treated cells to untreated cells. Values are means ± S.E. of three independent experiments.

Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet

mutated in the m4 and m5 are also involved in the complex formation to some extent. From these results, we conclude that the sequences from −2450 to −2446 and from −2435 to −2426 are both essential for the complex formation. Thus, we named these sequences as PREXα and PREXβ, respectively. The involvement of both the sequences for the binding was also supported by the fact that no shifted bands were observed using OL2, which contains PREXα alone and not PREXβ, as a probe (Fig. 5).

As described earlier (Fig. 4), PREX did not enhance the XRE-mediated *CYP2A8* gene expression in SL2 cells, suggesting that some of the nuclear factors for PREX should be deficient in SL2 cells. Therefore, we performed gel mobility shift assays using SL2 cell nuclear extracts with labeled OL3 as a probe, but no binding proteins to OL3 in SL2 cell nuclear extracts were observed (Fig. 7). The results coincided with the finding that PREX had no effect on the transcriptional activation in SL2 cells observed in the transient transfection experiments (Fig. 4).

Discussion

The present study has partly elucidated the mechanism by which the *CYP2A8* gene is transcriptionally activated by 3-MC in the Syrian hamster hepatocytes. The mechanism was shown to involve the XRE-mediated induction as observed in other 3-MC-inducible gene expressions. In addition, we found that the XRE alone was not sufficient for the full activation of the gene expression but that another element, PREX, which we found, was necessary for it. This was shown first by luciferase reporter gene assays that demonstrated that the transcription enhancing activity decreased to less than a half when PREX or 5′-part of PREX (PREXα) was deleted (Fig. 3). Secondly, gel mobility shift assays demonstrated that nuclear factors bound to PREX (Fig. 5) and two sites in this region (PREXα and PREXβ) were essential for the binding because mutation of either one of the two sites resulted in loss of the binding activities of the nuclear factors to PREX (Fig. 6). Moreover, PREX did not enhance the XRE-mediated promoter activation in SL2 cells (Fig. 4) that are lacking in binding proteins for PREX (Fig. 7). These results indicate that the binding proteins would function as *trans*-acting factors for PREX in hamster livers. Because PREX did not activate the transcription of the *CYP2A8* gene without XRE (Fig. 3B), it is likely that the nuclear factors for PREX interacted with AhR-Arnt complex and/or Sp1 directly or indirectly, resulting in the enhancement of the XRE-mediated *CYP2A8* gene expression.

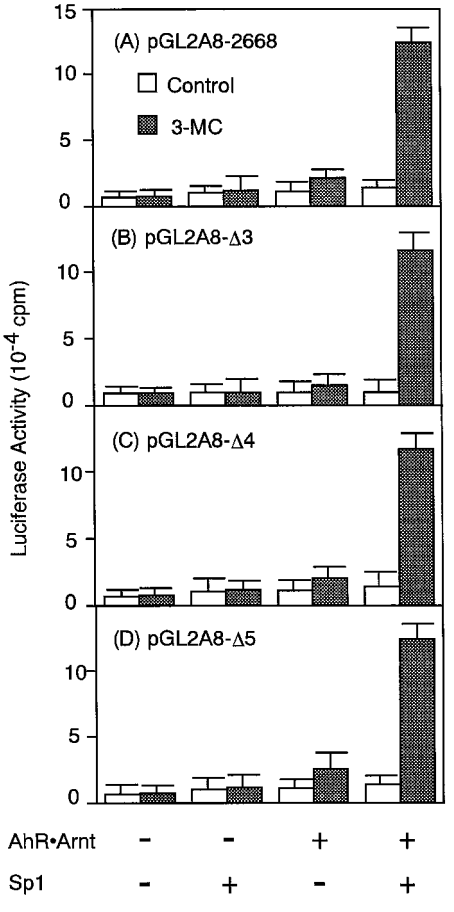

Fig. 4. Functional property of AhR-Arnt on enhancement of transcriptional activity of *CYP2A8* in cooperation with Sp1. AhR, Arnt, and/or Sp1 expression plasmids (each 3 μg) were introduced into SL2 cells with different deletion mutants of *CYP2A8*-luciferase fusion plasmids. Fusion plasmids are schematically shown in Fig. 3. Cells were cultured with or without 3-MC for 24 h and luciferase activities of cell extracts were measured. Open columns represent untreated cells and closed columns represent 3-MC-treated cells. Values are means ± S.E. of three independent experiments.

To know whether some of the known regulatory elements are involved in PREX region, we analyzed the sequence of PREX region. A search of TRANSFAC database revealed that three regulatory elements for AP-4, HSF, and C/EBPβ were found in PREX (OL3) region (11–20, 12–8, and 16–29 of OL3 sequence, respectively). However, it is obvious that the PREXα site is essential for the protein-binding (Fig. 6), whereas none of the three sites in PREX have PREXα site. Therefore, the three sites would not participate in the binding of the PREX-binding proteins.

Two specific protein-PREX complex formations were observed in gel mobility shift assays (Fig. 5 and 6). We can postulate the mode of binding of nuclear factors to PREX as follows. First, there might be two nuclear factors that can bind to PREX. Second, these factors could form a dimer complex and one factor could bind to PREX and the other could associate with the DNA-binding factor, which might dissociate during electrophoresis. Third, one nuclear factor that is easily degraded might bind to PREX. In this case, the nuclear factor might be degraded at the position that was not correlated to the DNA binding region. Another possibility is that each nuclear factor might recognize the PREXα and PREXβ, respectively. But this possibility is negligible because the mutation of one of the two binding sites resulted in the complete loss of the bindings of the nuclear factors.

In the analysis of the 5′-flanking region of the *CYP2A8* gene, we found BTE in addition to XRE, both of which were also reported in the genes of other 3-MC-inducible drug-metabolizing enzymes including CYP1A1, CYP1B1, NAD(P)H:quinone oxidoreductase, glutathione *S*-transferase Ya subunit, class 3 aldehyde dehydrogenase, and UDP-glucuronosyltransferase family 1 (Fujisawa-Sehara et al., 1987; Rushmore et al., 1990; Favreau and Pickett, 1991; Asman et al., 1993; Tang et al., 1996; Emi et al., 1996). As observed in *CYP1A1* promoter, we also demonstrated that the AhR-Arnt was involved in the induction of *CYP2A8* gene expression in cooperation with Sp1 using *Drosophila* SL2 cells (Fig. 4). The distance of XRE from the transcription start site of *CYP2A8*

Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

MOL PHARM

MOLECULAR PHARMACOLOGY

is quite far compared with that of the other 3-MC-inducible genes. However, it was shown to be not important for XRE-mediated transcriptional activation because the internal deletion mutant of *CYP2A8*-luciferase fusion plasmids (pGL2A8-Δ3 and pGL2A8-Δ5), in which the XRE was located near the transcription start site, had transcriptional activities equivalent to that of the pGL2A8-2668, in which the XRE located quite far from the start site (Fig. 3).

Various factors have been reported to be involved in the transcriptional regulation of *CYP* genes. We previously reported that activator protein-1 (AP-1) was involved in the 3-MC-induced *CYP2A8* expression in hamster hepatocytes (Tohkin et al., 1996). In the present study, sequence analysis up to −3322 revealed seven possible AP-1 binding sites in the 5′-flanking region (data not shown). Therefore, to know how AP-1 would act on the transcriptional regulation of *CYP2A8*, we examined the effects of okadaic acid, which is an AP-1 inducer (Thevenib et al., 1991; Haby et al., 1994), and c-Jun, which acts as AP-1 with a homodimer, on 3-MC-inducible luciferase expressions using several reporter gene constructs. Although both okadaic acid and c-Jun potentiated 3-MC-inducible CYP2A8 expression in the hepatocytes (Tohkin et al., 1996), we were unfortunately unable to obtain the activation effects of AP-1 on luciferase induction (data not shown). Thus, it is likely that AP-1 plays a role at the upstream region of more than −5 kb, although the mechanism by which AP-1 participates in the transcription of *CYP2A8* remains to be elucidated. It has been reported that a negative regulatory element located in the 5′-flanking region of hamster, rat, and human *CYP1A1* genes contains an octamer binding motif (Boucher et al., 1993; Sterling et al., 1993; Sagami et al., 1994), and that Oct-1 is a negative regulator of rat *CYP1A1* expression via the octamer sequence (Sterling and Bresnick, 1996). Although we found analogous sequences to the Oct-1 motif on −2561 to −2552 of *CYP2A8* upstream region, transient transfection experiments indicated that this element did not function as a negative regulatory element for 3-MC-inducible *CYP2A8* expression in hamster hepatocytes (Fig. 3A).

All these results indicate that the induction mechanism mediated by XRE and BTE seems to be common in the 3-MC-inducible genes, however, the mechanism of CYP2A8 differs from that in other 3-MC-inducible genes such as CYP1A1. Notably PREX, a positive regulatory element found in the 5′-regulatory region in the *CYP2A8* gene, is unique among those so far identified in the activation of 3-MC-inducible genes.

In summary, we found a new positive regulatory element, PREX that potentiated XRE-mediated *CYP2A8* gene expression by 3-MC in Syrian hamster hepatocytes. As for the mechanism of the gene expression, at least four regulatory factors are involved, which include AhR, Arnt, Sp1, and nuclear factor(s) for PREX. The mechanism by which PREX enhances XRE-mediated gene activation would be further clarified by characterization of the nuclear proteins that interact with PREX.

Acknowledgments

We thank Dr. Fujii-Kuriyama (Tohoku University, Sendai, Japan) for the kind donation of anti-AhR IgG, pGEMAct-hAhR, pGEMAct-

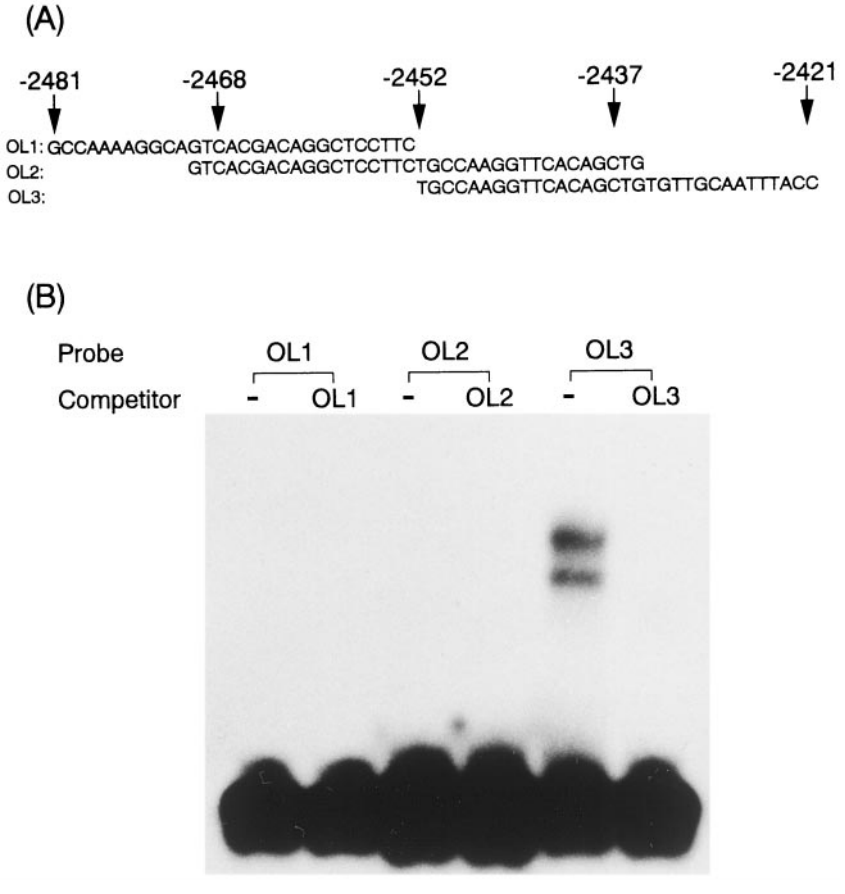

Fig. 5. Binding of hamster liver nuclear factors to PREX. A, nucleotide sequences used for probes and competitors. Numbers given to oligonucleotides indicate position of 5′-flanking sequences of the *CYP2A8* gene from the transcription start site. B, labeled oligonucleotides were incubated with Syrian hamster liver nuclear extracts in the presence or absence of a 50-fold excess of unlabeled oligonucleotides as shown on the top of each lane.

aspet

Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

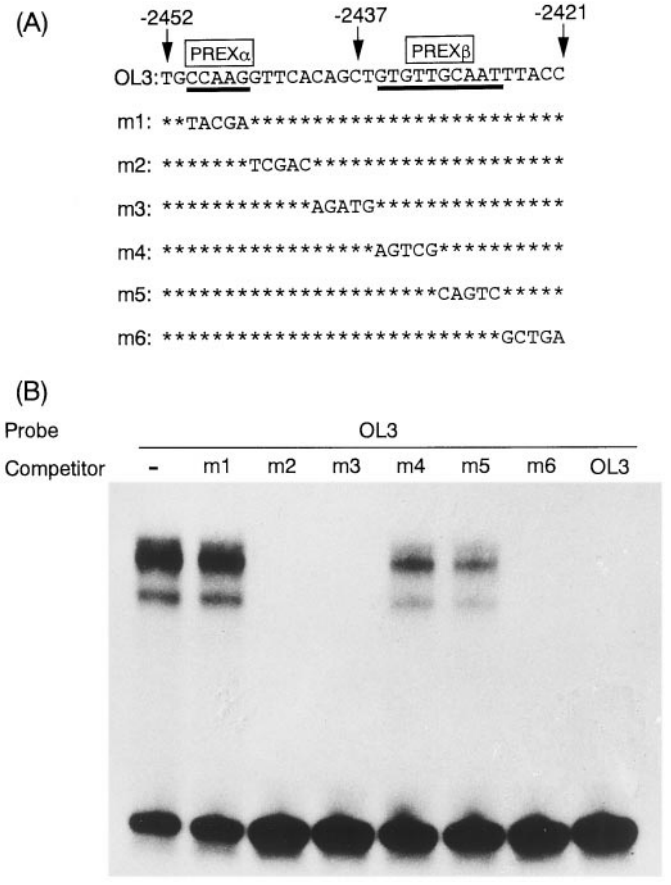

Fig. 6. Competition by mutant PREXs for binding of hamster liver nuclear factors to PREX. A, nucleotide sequences of wild-type and mutant PREXs. Numbers given to oligonucleotide indicate position of 5′-flanking sequences of the hamster *CYP2A8* gene from the transcription start site. Underlines indicate protein-binding elements. Asterisks indicate the same nucleotides as OL3. B, labeled OL3 was incubated with Syrian hamster liver nuclear extracts in the presence or absence of a 50-fold excess of unlabeled mutated PREXs as shown in A.

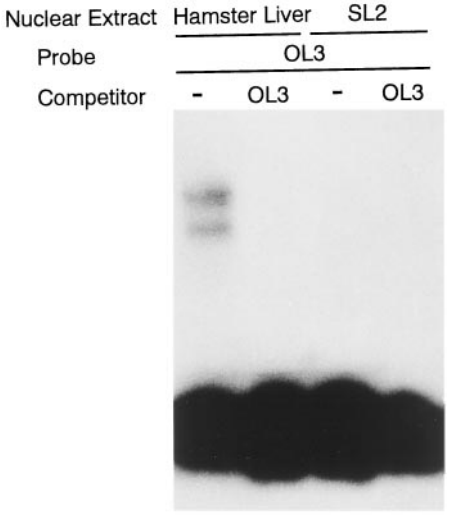

Fig. 7. Gel mobility shift assay using hamster liver nuclear extracts and SL2 cell nuclear extracts with PREX. Labeled OL3 was incubated with Syrian hamster liver nuclear extracts or SL2 cell nuclear extracts in the presence or absence of a 50-fold excess of unlabeled OL3 as shown on the top of each lane.

Arnt, and pGEMAct-Sp1. We also thank the Human Genome Center, Institute of Medical Science, the University of Tokyo, Japan, for use of the database search system.

References

Alabouch S, Kurose K, Tohkin M, Bani M-H, Fukuhara M, Nagata K and Yamazoe Y (1998) cDNA cloning and expression of a novel CYP3A from the Syrian hamster, CYP3A31. *Biochim Biophys Acta* **1397:**9–13.

Asman DC, Takimoto K, Pitot HC, Dunn TJ and Lindahl R (1993) Organization and characterization of the rat class 3 aldehyde dehydrogenase gene. *J Biol Chem* **268:**12530–12536.

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

Conney AH (1982) Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons. *Cancer Res* **42:**4875–4917.

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

Di Nocera PP and Dawid IB (1983) Transient expression of genes introduced into cultured cells of Drosophila. *Proc Natl Acad Sci USA* **80:**7095–7098.

Emi Y, Ikushiro S and Iyanagi T (1996) Xenobiotic responsive element-mediated transcriptional activation in the UDP-glucuronosyltransferase family 1 gene complex. *J Biol Chem* **271:**3952–3958.

Favreau LV and Pickett CB (1991) Transcriptional regulation of the rat NAD(P)H: quinone reductase gene. Identification of regulatory elements controlling basal level expression and inducible expression by planar aromatic compounds and phenolic antioxidants. *J Biol Chem* **266:**4556–4561.

Fujisawa-Sehara A, Sogawa K, Yamane M and Fujii-Kuriyama Y (1987) Characterization of xenobiotic responsive elements upstream from the drug-metabolizing cytochrome P-450c gene: A similarity to glucocorticoid regulatory elements. *Nucleic Acids Res* **15:**4179–4191.

Fukuhara M, Nagata K, Mizokami K, Yamazoe Y, Takanaka A and Kato R (1989a) Complete cDNA sequence of a major 3-methylcholanthrene-inducible cytochrome P-450 isozyme (P-450AFB) of Syrian hamsters with high activity toward aflatoxin B_1. *Biochem Biophys Res Commun* **162:**265–272.

Fukuhara M, Nohmi T, Mizokami M, Sunouchi M, Ishidate M Jr. and Takanaka A (1989b) Characterization of three forms of cytochrome P-450 inducible by 3-methylcholanthrene in golden hamster livers with special reference to aflatoxin B_1 activation. *J Biochem* **106:**253–258.

Gonzalez FJ (1989) The molecular biology of cytochrome P450s. *Pharmacol Rev* **40:**243–288.

Guengerich FP (1997) Comparisons of catalytic selectivity of cytochrome P450 subfamily enzymes from different species. *Chem-Biol Interact* **106:**161–182.

Haby C, Aunis D and Zwiller J (1994) Okadaic acid induces activator protein 1 activity and immediate early gene transcription in rat pheochromocytoma cells. Mechanism of action. *Biochem Pharmacol* **48:**819–825.

Heinemeyer T, Wingender E, Reuter I, Hermjakob H, Kel A, Kel O, Ignatieva E, Ananko E, Podkolodnaya O, Kolpakov F, Podkolodny N and Kolchanov N (1998) Databases on transcriptional regulation: TRANSFAC, TRRD, and COMPEL. *Nucleic Acids Res* **26:**364–370.

Honkakoski P and Negishi M (1997) The structure, function, and regulation of cytochrome P450 2A enzymes. *Drug Metab Rev* **29:**977–996.

Kobayashi A, Sogawa K and Fujii-Kuriyama Y (1996) Cooperative interaction between AhR. Arnt and Sp1 for the drug-inducible expression of *CYP1A1* gene. *J Biol Chem* **271:**12310–12316.

Kurose K, Tohkin M, Ushio F and Fukuhara M (1998) Cloning and characterization of Syrian hamster testosterone 7α-hydroxylase, CYP2A9. *Arch Biochem Biophys* **351:**60–65.

Matsushita N, Sogawa K, Ema M, Yoshida A and Fujii-Kuriyama Y (1993) A factor binding to the xenobiotic responsive element (XRE) of P-4501A1 gene consists of at least two helix-loop-helix proteins, Ah receptor and Arnt. *J Biol Chem* **268:**21002–21006.

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

Nelson DR, Koymans L, Kamataki T, Stegeman JJ, Feyereisen R, Waxman DJ, Waterman MR, Gotoh O, Coon MJ, Estabrook RW, Gunsalus IC and Nebert DW (1996) P450 superfamily: Update on new sequences, gene mapping, accession numbers, and nomenclature. *Pharmacogenetics* **6:**1–42.

Porter TD and Coon MJ (1991) Cytochrome P450: Multiplicity of isoforms, substrates, and catalytic and regulatory mechanisms. *J Biol Chem* **266:**13469–13472.

Rushmore TH, King RG, Paulson KE and Pickett CB (1990) Regulation of glutathione S-transferase Ya subunit gene expression: Identification of a unique xenobiotic-responsive element controlling inducible expression by planar aromatic compounds. *Proc Natl Acad. Sci USA* **87:**3826–3830.

Sagami I, Kikuchi H, Ikawa S and Watanabe M (1994) Characterization of hamster CYP1A1 gene: Inducible expression and negative regulation. *J Biochem* **116:**801–810.

Sierra F, Tian J-M and Schibler U (1993) In vitro transcription with nuclear extracts from differentiated tissues, in *Gene Transcription: A Practical Approach* (Hames BD and Higgins SJ eds) pp 117–144, Oxford University Press, London.

Sterling K and Bresnick E (1996) Oct-1 transcription factor is a negative regulator of rat CYP1A1 expression via an octamer sequence in its negative regulatory element. *Mol Pharmacol* **49:**329–337.

Sterling K, Weaver J, Ho K-L, Xu L-C and Bresnick E (1993) Rat *CYP1A1* negative regulatory element: Biological activity and interaction with a protein from liver and hepatoma cells. *Mol Pharmacol* **44:**560–568.

Sunouchi M, Fukuhara M, Ohno Y and Takanaka A (1988) Effects of various inducers on the activities of cytochrome P-450-mixed function oxidases and aflatoxin B_1 activation in microsomes of hamster livers. *J Toxicol Sci* **13:**193–204.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

Tang YM, Wo Y-YP, Stewart J, Hawkins AL, Griffin CA, Sutter TR and Greenlee WF (1996) Isolation and characterization of the human cytochrome P450 *CYP1B1* gene. *J Biol Chem* **271:**28324–28330.
Thevenib C, Kim S-J and Kehr JH (1991) Inhibition of protein phosphatases by okadaic acid induces AP1 in human T cells. *J Biol Chem* **266:**9363–9366.
Tohkin M, Kurose K and Fukuhara M (1996) Okadaic acid potentiates 3-methylcholanthrene-induced *CYP2A8* gene expression in primary cultures of Syrian hamster hepatocytes: Possible involvement of activator protein-1. *Mol Pharmacol* **50:**556–564.
Whitlock JP Jr (1987) The regulation of gene expression by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Pharmacol Rev* **39:**147–161.
Whitlock JP Jr., Okino ST, Dong L, Ko HP, Clarke-Katzenberg R, Ma Q and Li H (1996) Induction of cytochrome P450 1A1: A model for analyzing mammalian gene transcription. *FASEB J* **10:**809–818.
Zhang J and Ding X (1998) Identification and characterization of a novel tissue-specific transcriptional activating element in the 5′-franking region of the *CYP2A3* gene predominantly expressed in rat olfactory mucosa. *J Biol Chem* **273:**23454–23462.

Send reprint requests to: Dr. Kouichi Kurose, Department of Pharmaceutical Sciences, National Institute of Public Health, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8638, Japan. E-mail: kurose@iph.go.jp

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012