J. Biochem. **133**, 583–592 (2003)
DOI: 10.1093/jb/mvg075

Critical Enhancer Region to Which AhR/ARNT and Sp1 Bind in the Human *CYP1B1* Gene

Yuki Tsuchiya, Miki Nakajima and Tsuyoshi Yokoi*

Division of Drug Metabolism, Faculty of Pharmaceutical Sciences, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-0934

Received January 25, 2003; accepted February 13, 2003

Cytochrome P450 (CYP) 1B1 is known to be induced by polycyclic aromatic hydrocarbons including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). The constitutive and TCDD-inducible transcriptional expression of human CYP1B1 is known to be cell-specific. In order to identify the *cis*-elements that cell-specifically regulate the constitutive and TCDD-inducible transcription of CYP1B1, we constructed luciferase reporter plasmids containing a series of deletions of the XRE core sequence in the 5′-flanking region of the human *CYP1B1* gene. Luciferase assays were performed with MCF-7 (breast carcinoma), HepG2 (hepatocellular carcinoma), LS-180 (colon carcinoma), and OMC-3 (ovarian carcinoma) cells. Although there were large differences in the relative luciferase activity and inducibility between these four cell lines, the contribution of each reporter construct was similar. Constitutive expression increased with the regulatory elements that are present at –910 to –852 and –1652 to –1243. Potential enhancer elements for TCDD-induction were located from –1022 to –852 including three XREs, XRE3 at –853, XRE4 at –940, and XRE5 at –989. Gel shift analyses revealed binding of the AhR/ARNT heterodimer to XRE2 at –834, XRE3 at –853, XRE6 at –1024, and XRE7 at –1490. In addition, the binding of a nuclear transcriptional factor, Sp1, near XRE2 and XRE8 was observed. It was suggested that mutual interaction of XRE2 and XRE3 is important for transcriptional regulation, and that the Sp1 binding to the Sp1-like motif (–824) enhances both the constitutive and inducible transcriptional activities of the human *CYP1B1* gene.

Key words: Aryl hydrocarbon receptor, Cytochrome P450, Sp1, TCDD, Transcriptional regulation.

Abbreviations: AhR, aryl hydrocarbon receptor; ARNT, AhR nuclear translocator; CYP, cytochrome P450; CAT, chloramphenicol acetyltransferase; DRE, dioxin responsive element; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; XRE, xenobiotic responsive element.

Human cytochrome P450 (CYP) 1B1 is a comparatively new CYP isoform and its cDNA was cloned in 1994 (*1*). Human CYP1B1 metabolically activates numerous procarcinogens and promutagens including polycyclic aromatic hydrocarbons (PAHs) and aryl amines (*2*). In addition, CYP1B1 hydroxylates the endogenous substrate 17β-estradiol (*3*, *4*). The human CYP1B1 mRNA is constitutively expressed not only in extrahepatic tissues such as lung and kidney, but also in endocrine-regulated tissues including breast, uterus, and ovary (*2*, *5*). It has also been reported that CYP1B1 is expressed at a high frequency in various types of malignant tumors (*6*). CYP1B1 is induced by PAHs including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). The induction of members of the CYP1 family is controlled by the aryl hydrocarbon receptor (AhR) and AhR nuclear translocator (ARNT) (*7*–*9*). The liganded-AhR/ARNT heterodimer interacts with a 5′-CACGC-3′ DNA sequence, the core binding motif of the xenobiotic responsive element (XRE), located in the enhancer region of the AhR target genes (*10*). There are eight XREs located within 2.3 kb of the 5′-flanking region of human *CYP1B1* gene (*11*). Although it is known that the induction of CYP1B1 is also mediated by AhR/ARNT, the binding of the heterodimer to the XREs in the human *CYP1B1* gene has never been directly be proven.

In our previous study (*12*), we investigated the inducibility of CYP1B1 mRNA in various human tissue-derived cells such as HepG2 (hepatocellular carcinoma), ACHN (renal carcinoma), A549 (lung carcinoma), MCF-7 (breast carcinoma), LS-180 (colon carcinoma), HT-1197 (bladder carcinoma), HeLa (cervix of uterus adenocarcinoma), OMC-3 (ovarian carcinoma), and NEC14 (testis embryonal carcinoma). As a result, CYP1 inducible cell lines (MCF-7, HepG2, LS-180, and OMC-3) and non-inducible cell lines (ACHN, A549, HT-1197, HeLa, and NEC14) were found. However, the induction levels were not correlated with the expression levels of AhR, ARNT, and estrogen receptors α and β. There are numerous mechanisms that contribute to the regulation of cell-specific gene transcription including DNA methylation, histone acetylation, and the involvement of *trans*-acting factors. Cell-specific patterns of gene expression also depend on the combination of sequence-specific DNA-binding proteins that bind to *cis*-regulatory regions (*13*, *14*). Recent

*To whom correspondence should be addressed. Tel/Fax: +81-76-234-4407, E-mail: TYOKOI@kenroku.kanazawa-u.ac.jp

studies have suggested that a growing set of transcriptional cofactors mediate the communication between diverse upstream regulatory proteins and the core RNA polymerase II transcription complex (*15*). Shehin *et al.* (*16*) reported that the cell-specific differences in the CYP1B1 constitutive expression in MCF-7 and HepG2 are not mediated by DNA methylation or histone deacetylation. The mechanism controlling the cell-specific transcription of CYP1B1 is not known.

In the present study, to identify the 5′-flanking region involved in the cell-specific regulation of *CYP1B1* gene transcription, luciferase assays using a series of reporter constructs were performed with various human derived cell lines, *i.e.* MCF-7, HepG2, LS-180, and OMC-3 cells. We identified the XREs to which the AhR/ARNT heterodimer binds for constitutive and TCDD-inducible expression of the human *CYP1B1* gene. Furthermore, we found that Sp1 binds near XRE2, and enhances the constitutive and TCDD-inducible transcriptional activities of the human *CYP1B1* gene.

MATERIALS AND METHODS

Chemicals and Reagents—TCDD was obtained from Cambridge Isotope Laboratories (Cambridge, MA). pGL3-promoter plasmid, pRL-SV40 plasmid, Tfx-20 reagent, a dual-luciferase reporter assay system, and a double-stranded Sp1 consensus oligonucleotide were from Promega (Madison, WI). [γ-^{32}P]ATP was from Amersham (Buckinghamshire, UK). All primers and oligonucleotides were commercially synthesized at Hokkaido System Sciences (Sapporo). Taq polymerase and T4 polynucleotide kinase were from Griner Japan (Tokyo) and Toyobo (Osaka), respectively. Goat anti-human AhR polyclonal antibodies and normal goat serum were purchased from Novus (Littleton, CO) and Daiichi Pure Chemicals (Tokyo), respectively. Rabbit anti-human ARNT and rabbit anti-human Sp1 polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals and solvents were of the highest grade commercially available.

Preparation of Reporter Constructs with Serial Deletions of Human CYP1B1 5′-Flanking DNA—pGL3-basic plasmid containing the 5′-flanking region from –2299 to +25 of the human *CYP1B1* gene was kindly provided by Dr. William A. LaMarr of the University of Massachusetts Medical School (Worcester, MA). The 5′-flanking region of the human *CYP1B1* gene was transferred into the pGL3-promoter plasmid. A series of plasmids containing fragments –1652 to +25, –1243 to +25, –1022 to +25, –988 to +25, –910 to +25, –732 to +25, and –152 to +25 were constructed using restriction endonucleases (Fig. 1). One DNA fragment from –852 to +25 amplified by PCR using a 5′-upstream sense primer (5′-AAA GCC CAG CTC CGC AC-3′) and a 3′-downstream anti-sense primer (5′-TCG ATA TGT GCA TCT GTA AAA-3′) was also subcloned into the pGL3-promoter vector. The orientation of the eight constructs was verified by restriction enzyme digestion or DNA sequencing.

Mutated pGL3 plasmids (–910/XRE3 mt, –910/XRE2 mt, and –910/Sp1-like mt) were constructed by site-directed mutagenesis with a QuickChange® site-directed mutagenesis kit (Stratagene, La Jolla, CA). For –910/XRE3 mt, the forward and reverse mutagenic primers were 5′-CCA GAA GCG GCG CAC **T**CA AAG CCC AGC TC-3′ and 5′-GAG CTG GGC TTT G**A**G TGC GCC GCT TCT GG-3′, respectively (mutated sites are shown as bold letters). For –910/XRE2 mt, the forward and reverse mutagenic primers were 5′-GCC CAG CTC CGC AC**T** CAA AGG GGA GGC G-3′ and 5′-CGC CTC CCC TTT G**A**G TGC GGA GCT GGG C-3′, respectively. These primers were referred to a previous study (*16*). For –910/Sp1-like mt, the forward and reverse mutagenic primers were 5′-GCT CCG CAC GCA AAG G**TT** AGG CGA CAC GAG-3′ and 5′-CTC GTG TCG CCT **AA**C CTT TGC GTG CGG AGC-3′, respectively (mutated sites are the same as those in oligo m3). Nucleotide sequences were confirmed by DNA sequencing analyses.

Cells and Culture Conditions—The human hepatoblastoma cell line HepG2 (Riken Gene Bank, Tsukuba) was cultured in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical, Tokyo) supplemented with 10% (v/v) fetal bovine serum (FBS) (Invitrogen, Melbourne, Australia). The human breast adenocarcinoma cell line MCF-7 and the human colon adenocarcinoma cell line LS-180 (American Type Culture Collection, Rockville, MD) were cultured in DMEM supplemented with 0.1 mM non-essential amino acid (Invitrogen) and 10% (v/v) FBS. The human ovarian adenocarcinoma cell line OMC-3 (Riken Gene Bank) was cultured in Ham's F12 medium (Nissui Pharmaceutical) supplemented with 10% (v/v) FBS (Bio Whittaker, Walkersville, MD). These cells were maintained at 37°C under an atmosphere of 5% CO_2–95% air.

Transfection and Luciferase Assay—A transient expression system using the luciferase reporter gene was used to characterize the *hCYP1B1* enhancer region. The transcriptional activities of the various constructs were determined by transient transfection using Tfx-20 reagent. MCF-7, HepG2, LS-180, and OMC-3 cells were seeded into 24-well plates (4.0×10^4, 5.0×10^4, 1.0×10^5, and 3.0×10^5 cells/well, respectively) and then incubated for 24 h before transfection. MCF-7 cells were cotransfected with 0.8 μg of *CYP1B1/luc* reporter gene plasmid and 0.2 μg control reporter plasmid (pRL-SV40), the Tfx-20:plasmid DNA ratio being 1.5:1 in 200 μl of serum-free medium. HepG2 or LS-180 cells were cotransfected with 0.45 μg of *CYP1B1/luc* reporter gene plasmid and 0.05 μg pRL-SV40, the using a Tfx-20: plasmid DNA ratio being 2:1 in 200 μl of serum-free medium. OMC-3 cells were cotransfected with 0.49 μg of *CYP1B1/luc* reporter gene plasmid and 0.01 μg pRL-SV40, the Tfx-20: plasmid DNA ratio being 2:1 in 200 μl of serum-free medium. After the incubation at 37°C under an atmosphere of 5% CO_2–95% air for 1 h, the medium was added to the cells. After 24 h, the medium was replaced with medium containing 0.1% (v/v) DMSO or 10 nM TCDD/0.1% (v/v) DMSO. After 24-h treatment, the cells were resuspended in passive lysis buffer, and then the luciferase activity was measured with a luminometer (Dainippon Pharmaceutical, Osaka) using the dual-luciferase reporter assay system following the manufacturer's instructions. Transfection efficiency was monitored as *Renilla* luciferase activity with the pRL-SV40 plasmid as an internal control. Protein concentrations were determined using the Bradford protein assay reagent (Bio-Rad, Hercules, CA) with bovine serum

Probe	*Cis*-element	Sequence	Position
oligo 1	XRE1	5′-CAGGTTGTACCGAGCGTGGTTCTGGGGACA-3′	-280/-251
oligo 2	XRE2	5′-GCTCCGCACGCAAAGGGGAGGCGACACGAG-3′	-844/-815
oligo 3	XRE3	5′-ACTTTCCAGAAGCGGCGCACGCAAAGCCCAGCTCCG-3′	-874/-839
oligo 4	XRE4, E-box	5′-GCCGCCTCCGCGTGTCAGGTGCCGTGAGAA-3′	-953/-924
oligo 5	XRE5	5′-GCACCCCACGCCAAGGGTGGTGGTGG-3′	-999/-974
oligo 6	XRE6	5′-AGGCGCGACTGTGCGTGCGCAGCCGAGGGT-3′	-1,040/-1,011
oligo 7	XRE7	5′-TGGGTTAAGTCACGCAACCTCTCTGAACCC-3′	-1,504/-1,475
oligo 8	XRE8	5′-CTGGGATTACAGGCGTGAGCCACTGCGCCT-3′	-1,690/-1,661
oligo DRE	DRE (hCYP1A1)	5′-CTCCGGTCCTTCTCACGCAACGCCTGGGCA-3′	-996/-967
Sp1	Sp1 (consensus)	5′-ATTCGATCGGGGCGGGGCGAGC-3′	
oligo m1		5′-GCT**TT**GCACGCAAAGGGGAGGCGACACGAG-3′	(-841 and -840)
oligo m2		5′-GCTCCGCACGC**ACC**GGGGAGGCGACACGAG-3′	(-832 and -831)
oligo m3		5′-GCTCCGCACGCAAAGG**TT**AGGCGACACGAG-3′	(-828 and -827)

Fig. 1. **Oligonucleotides used for gel shift analysis.** The sequences and positions of XREs in the human *CYP1B1* gene are listed. Core regions (CACGC) are boxed. The E-box motif (CACGC) is double-underlined. DRE was from the human *CYP1A1* gene. Oligo m1, oligo m2, and oligo m3 are mutations of oligo 2. The mutated sites are shown as bold letters and the positions in parenthesis.

albumin as the standard. Luciferase activity was normalized as to the transfection efficiency and protein content.

Gel Shift Assay—Nuclear extracts were prepared from HepG2 or MCF-7 cells untreated (treated with 0.1% (v/v) DMSO) or treated with 10 nM TCDD for 24 h according to the method described previously (*17*). Synthetic oligonucleotides were labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The binding reactions were performed for 30 min at room temperature, the reaction mixture comprising 10 μg of the nuclear protein, 2 μg of poly [dI-dC], 1 μg of salmon sperm DNA, and 40 fmol of the radiolabelled probe (about 20,000 cpm) in a final volume of 15 μl of binding buffer [25 mM Hepes-KOH, 0.5 mM EDTA, 50 mM KCl, 10% glycerol, 0.5 mM dithiothreitol, 0.5 mM (*p*-amidinophenyl) methanesulfonyl fluoride]. To determine the specificity of the binding to the oligonucleotides, competition experiments were conducted by co-incubation with 10-, 50-, and 200-fold excesses of unlabeled competitors. In some experiments, 2 μg of anti-AhR, anti-ARNT, or anti-Sp1 polyclonal antibodies was pre-incubated with the nuclear protein on ice for 15 min. The oligonucleotide sequences are shown in Fig. 1. The sequence of the oligonucleotide containing human *CYP1A1* DRE was taken from a previous report by Masten and Shiverick (*18*). DNA-protein complexes were separated under non-denaturing conditions on 4% polyacrylamide gels with 0.5 × TBE as the running buffer. The gels were dried, and then the DNA-protein complexes were detected and quantified with a Fuji Bio-Imaging Analyzer BAS 1000 (Fuji Film, Tokyo).

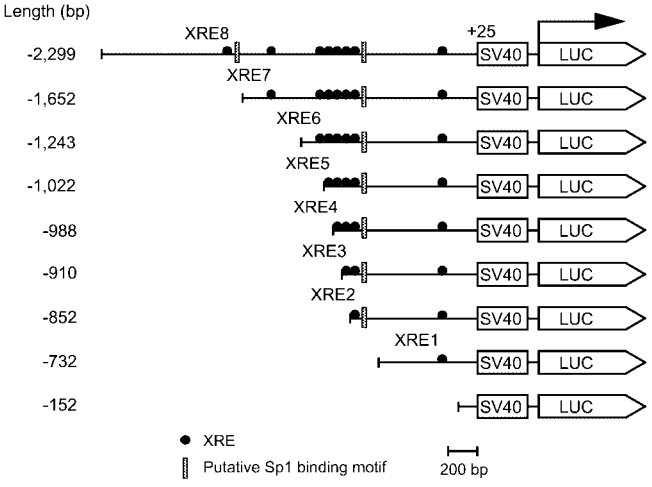

Fig. 2. **Deletion mapping of the human *CYP1B1* gene in luciferase reporter gene constructs.** The human *CYP1B1* 5′-flanking region from –2299 to +25 bp contains eight XREs. The numbers next to the schematics indicate the nucleotide positions of the 5′-ends of the constructs. Closed circles and hatched bars indicate the locations of the core XRE recognition motif and the putative Sp1 binding motif, respectively. An SV40 promoter is located upstream of the luciferase gene.

RESULTS

Comparison of Human CYP1B1 Gene Transcriptional Activities in Various Human Tissue–Derived Cell Lines—In the 5′-flanking region from –2299 to +25 of the human *CYP1B1* gene, eight XRE core sequences exist. These XREs were assigned numbers in order from the transcriptional initiation site (Fig. 2). We constructed nine plasmids each with a deleted XRE core sequence from 5′ on in order to identify the *cis*-elements that cell-specifically regulate the constitutive and TCDD-induced transcriptional activities of the *CYP1B1* gene. These constructs were transiently transfected into MCF-7, HepG2, LS-180, and OMC-3 cells, and then the luciferase activities of cell lysates were determined. In MCF-7 cells, basal reporter activity increased with increasing size of the upstream fragments, with the –910/+25 fragment show-

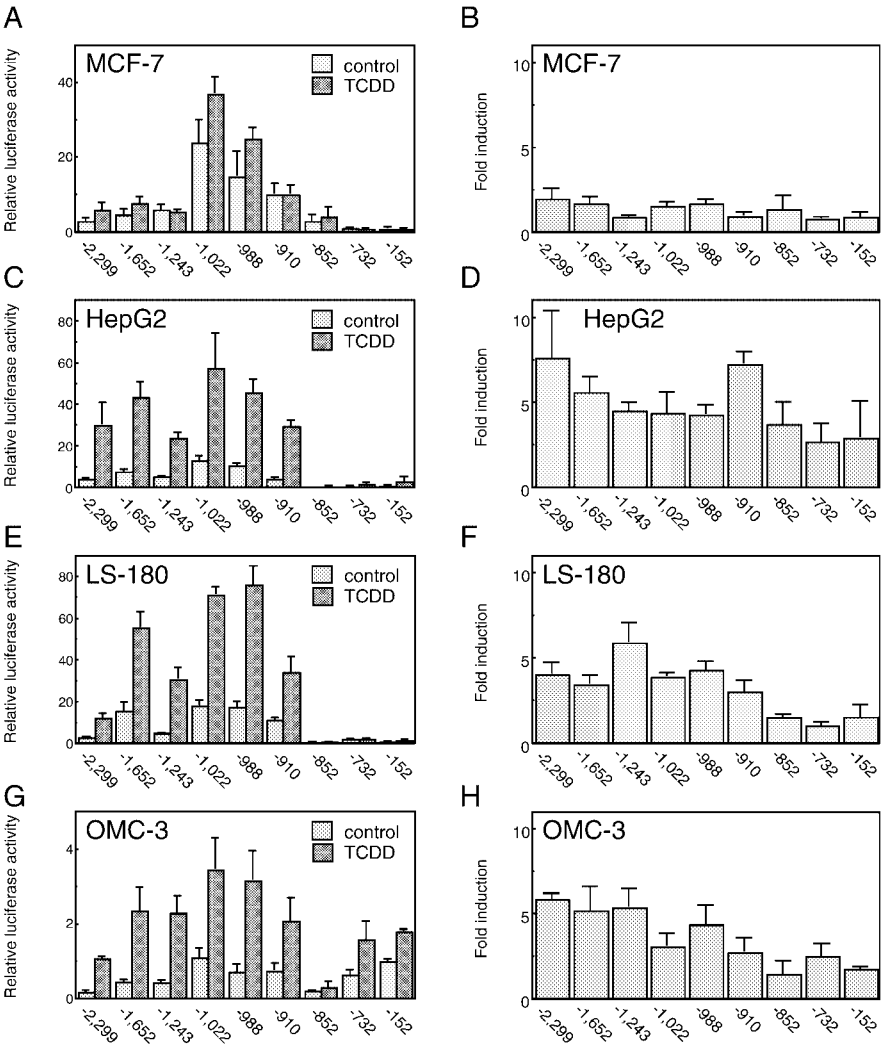

Fig. 3. **Constitutive and TCDD-induced transcriptional activities of human *CYP1B1*-luciferase constructs in various human tissue-derived cell lines.** A series of reporter constructs containing the *hCYP1B1* 5′-flanking region were transiently transfected into MCF-7 (A and B), HepG2 (C and D), LS-180 (E and F), and OMC-3 (G and H) cells. After 24 h, cells were treated with or without TCDD (10 nM) for 24 h. The relative luciferase activities were normalized as to the *Renilla* luciferase activity and the protein content of the cell lysate. Constitutive and TCDD-induced transcriptional activities were compared (A, C, E, and G). Luciferase activities relative to that with the cell lysate of the construct containing the –152/+25 fragment without TCDD-treatment are shown. Fold induction by TCDD over the control in each reporter construct is presented (B, D, F, and H). Each column represents the mean ± SD for three independent experiments.

ing a prominent increase and the –1022/+25 one showing the highest activity (Fig. 3A). The reporter activity was induced 2-fold by TCDD treatment with the reporter construct containing the –852/+25 fragment (Fig. 3B). The results suggested that XRE1 at –263 did not act as an enhancer. In HepG2 and LS-180 cells, the profile of the constitutive and TCDD-inducible reporter activities of each construct was similar to that in MCF-7 cells (Fig. 3, C and E). The maximal levels of induction with TCDD in HepG2 and LS-180 were 8-fold and 6-fold, respectively (Fig. 3, D and F). In OMC-3 cells, the relative luciferase activity was lower than those in the other cells (Fig. 3G). The constitutive reporter activities did not indicate differences between the constructs. However, the reporter construct containing the –1022/+25 fragment showed the highest TCDD-inducible reporter activity as in other cell lines. The maximal level of induction with TCDD in OMC-3 cells was 6-fold (Fig. 3H). Overall, remarkable differences in the profiles of the constitutive and TCDD-inducible reporter activities between cell lines were not observed.

Binding of Nuclear Proteins to XREs on the Human CYP1B1 Gene—The binding of nuclear proteins, particularly the AhR/ARNT complex, to various XREs of the human *CYP1B1* gene was analyzed by the gel shift assays. In Fig. 4, an oligonucleotide containing DRE on human *CYP1A1* (*18*, *19*), oligo DRE, was used as a probe, and nuclear extracts of HepG2 or MCF-7 cells untreated or treated with TCDD were used. The unlabeled oligo DRE and eight oligonucleotides each containing one of the XRE core sequences, oligo 1-oligo 8, were each used as a cold competitor in a 10-, 50-, and 200-fold molar excess (Fig. 4A). The nuclear proteins from the untreated HepG2 cells gave a weakly shifted band (lane 2). The density of the shifted band increased with the nuclear extracts of the TCDD-treated HepG2 cells (lane 3). The specificity of TCDD-induced AhR binding to DRE was confirmed by the super shift with anti-AhR antibodies (lane 4) and/or by concentration-dependent competition with a 10-, 50-, and 200-fold molar excess of unlabeled oligo DRE (lanes 5–7). In contrast to the anti-AhR antibodies, the super shifted band was not obtained with normal goat serum (Fig. 4B, lane 2). The band was also super shifted with anti-ARNT antibodies, indicating that the binding protein is the are heterodimer of AhR/ARNT (Fig. 4B, lane 3). The same shifted and super shifted bands were obtained with the nuclear extracts of MCF-7 cells (lanes 33 and 34). AhR/ARNT binding to DRE was competitively inhibited by oligo 2, oligo 3, oligo 6, and oligo 7 in a concentration-dependent manner (Fig. 4A,

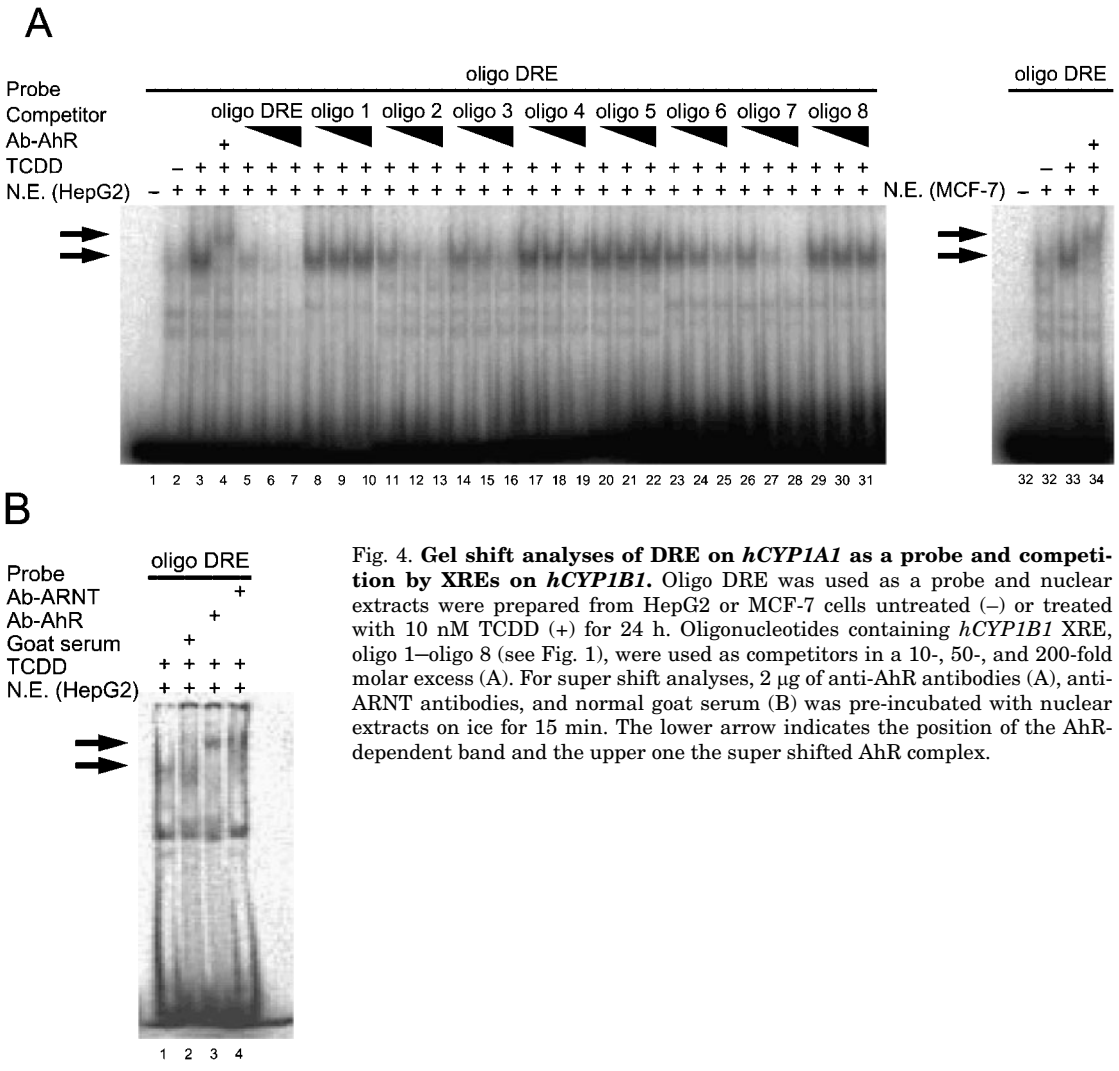

Fig. 4. **Gel shift analyses of DRE on *hCYP1A1* as a probe and competition by XREs on *hCYP1B1*.** Oligo DRE was used as a probe and nuclear extracts were prepared from HepG2 or MCF-7 cells untreated (–) or treated with 10 nM TCDD (+) for 24 h. Oligonucleotides containing *hCYP1B1* XRE, oligo 1–oligo 8 (see Fig. 1), were used as competitors in a 10-, 50-, and 200-fold molar excess (A). For super shift analyses, 2 μg of anti-AhR antibodies (A), anti-ARNT antibodies, and normal goat serum (B) was pre-incubated with nuclear extracts on ice for 15 min. The lower arrow indicates the position of the AhR-dependent band and the upper one the super shifted AhR complex.

lanes 11–16 and 23–28), but not by oligo 1, oligo 4, oligo 5, or oligo 8 (lanes 8–10, 17–22, and 29–31).

To further determine which XREs on the human *CYP1B1* gene participate in the TCDD-stimulated AhR pathway, we performed gel shift analyses with the eight oligonucleotides containing a XRE as probes (Fig. 5). In the case of oligo 3, oligo 6, and oligo 7, the nuclear protein-bound band super shifted with anti-AhR antibodies, and competitively decreased with a cold competitor, *i.e.* itself or oligo DRE (Fig. 5, C, F, and G). The AhR-dependent bound band to oligo 3 was more competitively decreased, with higher affinity, by oligo DRE than by unlabeled oligo 3 itself. In contrast, the AhR-dependent bound bands to oligo 6 and oligo 7 were more competitively decreased, with higher affinity, by unlabeled oligo 6 and oligo 7, respectively, than by oligo DRE. These bands showed the same mobility as the bands formed by the oligo DRE probe (Fig. 4). With oligo 2 as a probe (Fig. 5B), the most intense bands were obtained. However, the band did not shift with anti-AhR antibodies and was not competitively decreased by cold, excess molar oligo DRE. Using oligo 1, oligo 4, and oligo 8 as probes, non-specific bands were observed (Fig. 5, A, D, and H). With oligo 5, no prominent binding of nuclear protein was observed (Fig. 5E).

Binding of Sp1 to Oligonucleotides Containing XREs of the Human CYP1B1 Gene—The possibility of Sp1 binding to oligo 1, oligo 2, oligo 4, and oligo 8 was examined (Fig. 6A). When oligo 2 and oligo 8 were used as probes, the bands super shifted with anti-Sp1 antibodies and competed out with the unlabeled consensus Sp1 probe. However, the bands obtained with oligo 1 and oligo 4 were not affected by anti-Sp1 antibodies or the unlabeled consensus Sp1 probe.

To identify the region in oligo 2 to which Sp 1 binds, three mutants of oligo 2 (oligo m1, oligo m2, and oligo m3) were designed (Fig. 1). When oligo 2 was used as the probe, the shifted bands were competed out completely with unlabeled oligo m1 and decreased with oligo m2 , but not with oligo m3 (Fig. 6B, lanes 1–10). When oligo m1 was used as the probe, the same shifted and super shifted bands were obtained (lanes 11 and 12). The shifted bands were competed out with unlabeled oligo m1 and oligo 2 (lanes 13–18). In contrast, when oligo m2 and oligo m3 were used as probes, the band density was very weak and the super shift with anti-Sp1 antibodies was not observed (lanes 20 and 28). These results suggested

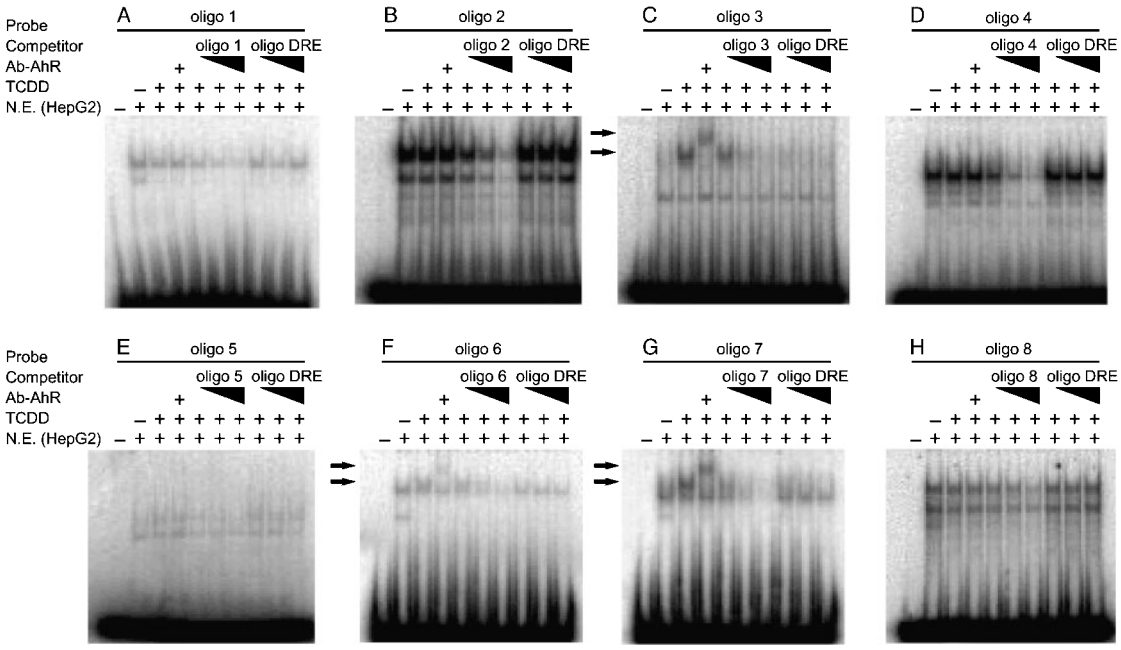

Fig. 5. **Gel shift analyses of XREs on *hCYP1B1* as probes and competition by DRE on *hCYP1A1*.** Oligonucleotides, *i.e.* oligo 1 including XRE1, oligo 2 including XRE2, oligo 3 including XRE3, oligo 4 including XRE4, oligo 5 including XRE5, oligo 6 including XRE6, oligo 7 including XRE7, and oligo 8 including XRE8, were used as probes. Nuclear extracts were prepared from HepG2 cells untreated (–) or treated with 10 nM TCDD (+) for 24 h. Each cold oligonucleotide, the same as the probe or oligo DRE probe, was used as a competitor in a 10-, 50-, and 200-fold molar excess. For super shift analyses, 2 μg of anti-AhR antibodies was pre-incubated with nuclear protein on ice for 15 min. The lower arrow indicates the position of the AhR-dependent band and the upper one the super shifted AhR complex.

that the Sp1 protein binds to oligo 2, recognizing the sequence at –832/–827. We regard the GA-rich element centered at –828/–827 as an Sp1-like motif. When oligo m1 and m2 were used as probes, the shifted bands were not affected by anti-AhR antibodies or oligo DRE (Fig. 6C, lanes 1–10). In contrast, when oligo m3 was used as the probe, the super shift with anti-AhR antibodies was observed (Fig. 6C, lane 12) and the bands were competed out with unlabeled oligo DRE (Fig. 6C, lanes 13–15). Furthermore, when oligo 2 was used as the probe, the band in the presence of an excess molar amount of unlabeled consensus Sp1 probe was super shifted with anti-AhR antibodies. These results suggested that the inhibition of binding of Sp1 to oligo 2 made AhR bind to XRE2 in oligo 2.

Effects of Mutation in XRE3, XRE2, and the Sp1-like Motif in the Human CYP1B1 Gene on the Transcriptional Activity—To investigate the roles of XRE3, XRE2, and the Sp1-like motif in the transcriptional activity, luciferase assays with mutated reporter constructs in HepG2 cells were performed (Fig. 7). The constitutive activities of the mutant-type plasmids –910/XRE3 mt, –910/XRE2 mt, and –910/Sp1-like mt were 68%, 11%, and 54% of that of the wild-type plasmid (–910/+25) (Fig. 7B). Whereas the reporter activity of –910/+25 was induced by 6.5-fold on TCDD treatment, the induction potencies of mutant-types –910/XRE3 mt and –910/XRE2 mt decreased to 3.4-fold and 3.7-fold, respectively. In contrast, the induction potency of –910/Sp1-like mt was almost the same as that of the wild-type, although the reporter activities were lower than that of –910/+25. These results suggested that mutual interaction of XRE2 and XRE3 is important for transcriptional regulation, and that Sp1 binding to the Sp1-like motif enhances both the constitutive and inducible transcriptional activities of the human *CYP1B1* gene.

DISCUSSION

Numerous mechanisms have been implicated in the cell-specific transcriptional regulation of the *CYP1B1* gene. In order to determine the cell-specific patterns of gene expression that involve *trans*-acting factors that bind to *cis*-acting elements, we constructed luciferase reporter plasmids containing a series of deletions of the XRE core sequence in the 5′-flanking region of the *CYP1B1* gene. In a previous study, we demonstrated that CYP1B1 mRNA was induced 4-fold, 16-fold, 7-fold, and 33-fold in MCF-7, HepG2, LS-180, and OMC-3 cells, respectively, by TCDD with RT-PCR (*12*). In contrast, the induction levels of the luciferase activity in MCF-7, HepG2, LS-180, and OMC-3 cells were 2-fold, 8-fold, 6-fold, and 6-fold, respectively, in the present study. *Cis*-acting elements that exist upstream from –2.3 kb might contribute to the induction of CYP1B1 transcription.

Judging from the results of the luciferase assays, potential enhancer elements are located from –1652 to –1243 and from –1022 to –852 (Fig. 3). Tang *et al.* (*11*) also reported that the fragment from –1022 to –835 exhibits enhancer activity in either orientation. Since the region from –1022 to –852 contains putative *cis*-acting elements AP-2, SF-1, and E-box in addition to three XREs (XRE3, XRE4, and XRE5), the constitutive transcriptional activity might be enhanced. The region from –1652 to –1243 contains XRE7. The binding of the AhR/

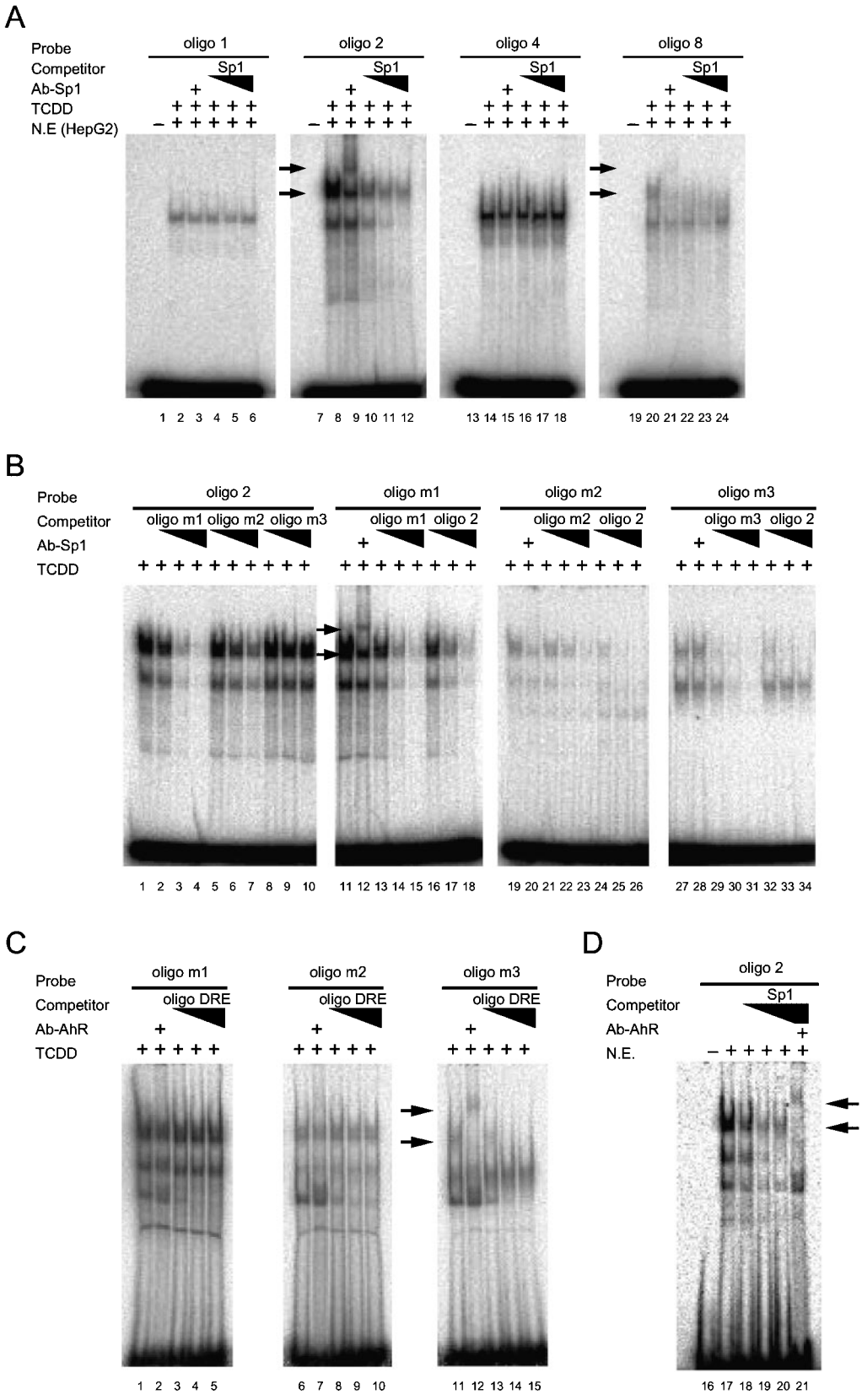

Fig. 6. **Gel shift analyses of XREs on *hCYP1B1* as probes and competition by the Sp1 consensus sequence.** Oligonucleotides, *i.e.* oligo 1, oligo 2, oligo 4, and oligo 8, were used as probes (A). Nuclear extracts were prepared from HepG2 cells untreated (–) or treated with 10 nM TCDD (+) for 24 h. The cold Sp1 consensus probe was used as a competitor in a 10-, 50-, and 200-fold molar excess. For super shift analyses, 2 μg of anti-Sp1 antibodies was pre-incubated with nuclear protein on ice for 15 min. The lower arrow indicates the position of the Sp1-dependent band and the upper one the super shifted Sp1 complex. Oligonucleotides, *i.e.* oligo 2, oligo m1, oligo m2, and oligo m3, were used as probes (B). Each cold oligonucleotide was used as a competitor in a 10-, 50-, and 200-fold molar excess. For super shift analyses, 2 μg of anti-Sp1 antibodies was pre-incubated with nuclear protein on ice for 15 min. The lower arrow indicates the position of the Sp1-dependent band and the upper one the super shifted Sp1 complex. Oligonucleotides, *i.e.* oligo m1, oligo m2, and oligo m3, were used as probes (C). The cold oligo DRE probe was used as a competitor in a 10-, 50-, and 200-fold molar excess. For super shift analyses, 2 μg of anti-AhR antibodies was pre-incubated with nuclear protein on ice for 15 min. The arrow indicates the super shifted AhR complex. Oligonucleotide oligo 2 was used as a probe (D). The cold Sp1 consensus oligonucleotide was used as a competitor in a 10-, 50-, and 200-fold molar excess. Anti-AhR antibodies were pre-incubated with nuclear protein on ice for 15 min in the presence of a 200-fold molar excess of the cold Sp1 consensus oligonucleotide. The upper arrow indicates the super shifted AhR complex.

ARNT heterodimer to XRE3 and XRE7 was also confirmed by gel shift analyses (Fig. 5). Our luciferase assay results for MCF-7 cells suggested that XRE3 at –853 is essential for the basal gene expression of CYP1B1. In contrast, Shehin *et al.* (*16*) reported that XRE2 at –834 is required for the constitutive expression of CYP1B1. As discussed below in detail, XRE2 and XRE3 might mutually regulate the constitutive and TCDD-inducible

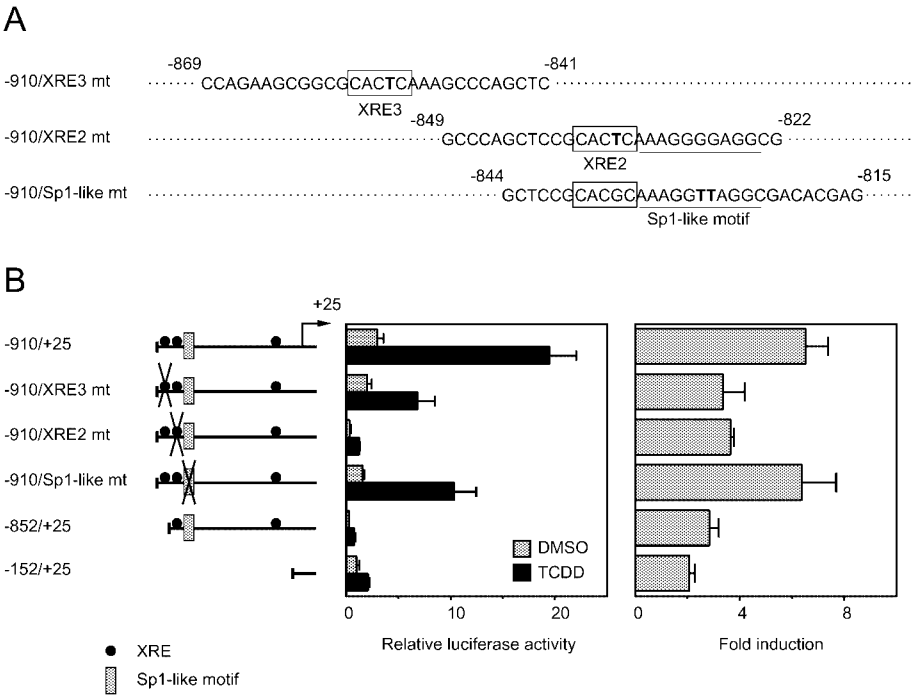

Fig. 7. **Effects of mutations of XRE3, XRE2, and the Sp1-like motif on the transcriptional activity.** Mutants –910/XRE3 mt, –910/XRE2 mt, and –910/Sp1-like mt were constructed by site-directed mutagenesis (A). XRE core regions (CACGC) are boxed, and the Sp1-like motif is underlined. Mutated sites are shown as bold letters. These plasmids were transiently transfected into HepG2 cells (B). After 24 h, cells were treated with or without TCDD (10 nM) for 24 h. The relative luciferase activities were normalized as to the *Renilla* luciferase activity and the protein content of the cell lysate. Constitutive and TCDD-induced transcriptional activities were compared (left panel). Luciferase activities relative to that with the cell lysate of the construct containing the –152/+25 fragment without TCDD-treatment are shown. Fold induction by TCDD over the control in each reporter construct is presented (right panel). Each column represents the mean ± SD for three independent experiments.

expression of human CYP1B1. Since the induction by TCDD was shown to occur upstream from –852, it is suggested that the contribution of XRE1 to the inducibility was negligible.

Since the region from –1243 to –1022 appeared to suppress the basal and TCDD-inducible activities (Fig. 3), a certain *trans*-acting factor might be involved in the negative regulation. Wo *et al*. (*20*) reported suppression of the inducibility of CYP1B1 expression with the region from –1243 to –1022, as determined by chloramphenicol acetyltransferase (CAT) assay of a human squamous cell carcinoma line, SCC12 cells. In contrast, Tang *et al*. (*11*) reported that the region from –1356 to –1022 did not show a significant decrease in the reported activity in their CAT assay using SCC12 cells. The discrepancy between these results might be due to the differences in the reported constructs. Shehin *et al*. (*16*) reported that treatment with cycloheximide enhances both the constitutive and TCDD-inducible expression of CYP1B1, suggesting the potential involvement of a labile transcriptional repressor. It has been reported that the negative regulatory element (NRE) plays a role in the decrease in human CYP1A1 gene expression (*21*, *22*). A certain *trans*-acting factor might be involved in the region from –1243 to –1022 of the *CYP1B1* gene.

Although it has been recognized that the induction of CYP1B1 is mediated by AhR/ARNT, the binding of the heterodimer to XRE in human *CYP1B1* has never been directly determined. In the present study, we showed the binding to three of the eight XREs, XRE3 (–853), XRE6 (–1024) and XRE7 (–1490), within the 2.3-kb enhancer region of the human *CYP1B1* gene, with gel shift assay (Fig. 5). In particular, the binding to XRE3 at –853 was demonstrated to have high affinity. The direct binding of the AhR/ARNT heterodimer to oligo 2 could not be detected in the gel shift assay; however, excess unlabeled oligo 2 competitively decreased the level of AhR/ARNT binding to the oligo DRE probe (Figs. 4 and 5). Furthermore, after the inhibition of binding of Sp1 to oligo 2, the binding of AhR/ARNT to oligo 2 was observed. Thus, it appears that there are TCDD-responsive and non-responsive XREs for the AhR/ARNT heterodimer in the human *CYP1B1* gene. In mice, responsive or non-responsive XREs of the *Cyp1b1* gene have been identified (*23*). Out of the four XREs of the mouse *Cyp1b1* gene at –855, –874, –973, and –1017, which correspond to XRE2, XRE3, XRE4, and XRE6, respectively, in the human *CYP1B1* gene, it has been demonstrated that the AhR/ARNT heterodimer can bind to only XRE2. In contrast, the present study on the human *CYP1B1* gene showed that the AhR/ARNT heterodimer could bind to XRE2, XRE3, XRE6, and XRE7. These results suggest possible species differences in the responsive region of XREs between the human and mouse *CYP1B1* genes.

Previously, the constitutive nuclear localization of AhR was studied, and the effects of endogenous ligands for AhR were considered (*24–26*). Shehin *et al*. (*16*) reported that XRE2 at –834 is required for the constitutive expression of CYP1B1. As shown in Fig. 7, it is suggested that XRE3 at –853, the Sp1-like motif at –824, and especially XRE2 at –834 are essential for basal gene expression. Furthermore, on comparison between the constitutive and inducible activities of –910/XRE3 mt and –852/+25, the possibility arose that there might be a region(s) from –852 to –910 responsible for the enhancement of the constitutive and TCDD-inducible transcriptional activity other than XRE3. The results of the luciferase assay (Fig. 3) showed that the basal activities were regulated in parallel with the TCDD-inducible activities in the luciferase reporter gene. In the gel shift assay (Fig. 4), AhR/ARNT binding to XREs with nuclear extracts from cells treated without TCDD was observed. Thus, it was suggested that the basal activity also requires the AhR/ARNT complex.

The present study demonstrated that transcription factor Sp1 could bind to oligo 2 and oligo 8, which contained XRE2 and XRE8, respectively. In general, Sp1 plays a role as a constitutive transcriptional factor for the expression of numerous genes to bind to GC-box sequences (GGGGCGGGG) (*27*). Recently, it was reported that Sp1 or an Sp1-like protein binds to GA-rich sequences (GGGGGAGGGG), or complementary CT-rich sequences (CCCTCCCC) as well as GC-rich sequences (*28*, *29*). For the human *CYP1B1* gene, it has been reported that two Sp1 binding sequences are located near the transcriptional initiation site (–68 and –84), which are at the optimal distance for an Sp1-dependent promoter (*20*). In the present study, it was proved that Sp1 binds to the GGGAGG motif (–824) within oligo 2. We firstly found in this study that Sp1 could bind to a distal enhancer element in the human *CYP1B1* gene. In the luciferase assays (Figs. 3 and 7), the reporter construct of –852/+25 containing XRE2 and the Sp1-like motif did not show an increase in the TCDD-inducible reporter activity, and the inclusion of XRE3 caused a prominent increase. Furthermore, the mutations in XRE2 and XRE3 decreased the inducibility of the transcription by TCDD. Therefore, it was suggested that the mutual interaction of XRE2 and XRE3 is important for transcriptional regulation of the human *CYP1B1* gene. On comparison between the constitutive and inducible activities of –910/Sp1-like mt and –910/+25 (Fig. 7), it was also suggested that the Sp1 binding to the Sp1-like motif enhances both the constitutive and inducible transcriptional activities. These results were similar to those of previous studies showing that transcription of the *CYP1A1* gene is cooperatively regulated by Sp1 and AhR (*30*, *31*).

In conclusion, we demonstrated the binding of the AhR/ARNT heterodimer to several XREs, *i.e*. XRE2 at –834, XRE3 at –853, XRE6 at –1024, and XRE7 at –1490, of the human *CYP1B1* gene. In particular, AhR/ARNT heterodimer binding to XRE2 (–834) and XRE3 (–853) is important for regulation of the constitutive and TCDD-inducible expression of the human *CYP1B1* gene. Furthermore, it is suggested that Sp1 binding to the Sp1-like motif (–824) enhances both the constitutive and inducible transcriptional activities of the human *CYP1B1* gene.

We wish to thank Dr. William A. LaMarr of University of Massachusetts Medical School (Worcester, MA) for providing the pGL3-basic plasmid containing the 5′-flanking region of the human *CYP1B1* gene, and Mr. Brent Bell for reviewing this article. This work was supported in part by a Health and Labor Sciences Research Grant from the Ministry of Health, Labor and Welfare of Japan.

REFERENCES

1. Sutter, T.R., Tang, Y.M., Hayes, C.L., Wo, Y.Y.P., Jabs, E.W., Li, X., Yin, H., Cody, C.W., and Greenlee, W.F. (1994) Complete cDNA sequence of a human dioxin-inducible mRNA identifies a new gene subfamily of cytochrome P450 that maps to chromosome 2. *J. Biol. Chem.* **269**, 13092–13099
2. Shimada, T., Hayes, C.L., Yamazaki, H., Amin, S., Hecht, S.S., Guengrich, F.P., and Sutter, T.R. (1996) Activation of chemically diverse procarcinogens by human cytochrome P450 1B1. *Cancer Res.* **56**, 2979–2984
3. Hayes, C., Spink, D., Spink, B., Cao, J., Walker, N., and Sutter, T. (1996) 17β-Estradiol hydroxylation catalyzed by human cytochrome P450 1B1. *Proc. Natl Acad. Sci. USA* **93**, 9776–9781
4. Spink, D., Spink, B., Cao, J., Gierthy, J., Hayes, C., Li, Y., and Sutter, T. (1997) Induction of cytochrome P450 1B1 and catechol estrogen metabolism in ACHN human renal adrenocarcinoma cell. *J. Steroid. Biochem. Mol. Biol.* **62**, 223–232
5. Hakkola, J., Pasanen, M., Pelkonen, O., Hukkanen, J., Evisalmi, S., Anttila, S., Rane, A., Mantyla, M., Purkunen, R., Saarikoski, S., Tooming, M., and Raunio, H. (1997) Expression of CYP1B1 in human adult and featal tissues and differential inducibility of CYP1B1 and CYP1A1 by Ah receptor ligands in human placenta and cultured cells. *Carcinogenesis* **18**, 391–397
6. Murray, G.I., Taylor, M.C., McFadyen, M.C.E., Mckay, J.A., Greenlee, W.F., Burke, M.D., and Melvin, W.T. (1997) Tumor-specific expression of cytochrome P450 CYP1B1. *Cancer Res.* **57**, 3026–3031
7. Hankinson, O. (1995) The aryl hydrocarbon receptor complex. *Annu. Rev. Pharmacol. Toxicol.* **35**, 307–340
8. Schmidt, J.V. and Bradfield, C.A. (1996) Ah receptor signaling pathways. *Annu. Rev. Cell Biol.* **12**, 55–89
9. Whitlock, J.P., Jr. (1999) Induction of cytochrome P4501A1. *Annu. Rev. Pharmacol. Toxicol.* **39**, 103–125
10. Ikuta, T., Tachibana, T., Watanabe, J., Yoshida, M., Yoneda, Y., and Kawajiri, K. (2000) Nucleocytoplasmic shuttling of the aryl hydrocarbon receptor. *J. Biochem.* **127**, 503–509
11. Tang, Y.M., Wo, Y.-Y.P., Stewart, J., Hawkins, A.L., Griffin, C.A., Sutter, T.R., and Greenlee, W.F. (1996) Isolation and characterization of the human cytochrome P450 *CYP1B1* gene. *J. Biol. Chem.* **271**, 28324–28330
12. Iwanari, M., Nakajima, M., Kizu, R., Hayakawa, K., and Yokoi, T. (2002) Induction of CYP1A1, CYP1A2, and CYP1B1 mRNAs by nitropolycyclic aromatic hydrocarbons in various human tissue-derived cells: chemical-, cytochrome P450 isoform-, and cell-specific differences. *Arch. Toxicol.* **76**, 287–298
13. Mitchell, P.J. and Tjian, R. (1989) Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science* **245**, 371–378
14. Maniatis, T., Goodbourn, S., and Fischer, J.A. (1987) Regulation of inducible and tissue-specific gene expression. *Science* **236**, 1237–1245
15. Mannervik, M., Nibu, Y., Zhang, H., and Levine, M. (1999) Transcriptional coregulators in development. *Science* **284**, 606–609
16. Shehin, S.E., Stephenson, R.O., and Greenlee, W.F. (2000) Transcriptional regulation of the human *CYP1B1* gene. Evidence for involvement of an aryl hydrocarbon receptor response element in constitutive expression. *J. Biol. Chem.* **275**, 6770–6776
17. Schreiber, E., Matthias, P., Müller, M.M., and Schaffner, W. (1989) Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res.* **17**, 6419
18. Masten, S.A. and Shiverick, K.T. (1995) The Ah receptor recognizes DNA binding sites for the B cell transcription factor, BSAP: a possible mechanism for dioxin-mediated alteration of CD19 gene expression in human B lymphocytes. *Biochem. Biophys. Res. Commun.* **212**, 27–34
19. Hines, R.N., Mathis, J.M., and Jacob, C.S. (1988) Identification of multiple regulatory elements on the human cytochrome P450IA1 gene. *Carcinogenesis* **9**, 1599–1605
20. Wo, Y-YP., Stewart, J., and Greenlee, W.F. (1997) Functional analysis of the promoter for the human *CYP1B1* gene. *J. Biol. Chem.* **272**, 26702–26707
21. Bhat, R., Weaver, J.A., Sterling, K.M., and Bresnick, E. (1996) Nuclear transcription factor Oct-1 binds to the 5′-upstream region of CYP1A1 and negatively regulates its expression. *Int. J. Biochem. Cell Biol.* **28**, 217–227
22. Piechoki, M.P. and Hines, R.N. (1998) Functional characterization of the human CYP1A1 negative regulatory element: mod-

ulation of Ah receptor mediated transcriptional activity. *Carcinogenesis* **19**, 771–780
23. Zhang, L., Savas, U., Alexander, D.L., and Jefcoate, C.R. (1998) Characterization of the mouse *Cyp1b1*: identification of an enhancer region that directs aryl hydrocarbon receptor-mediated constitutive and induced expression. *J. Biol. Chem.* **273**, 5174–5183
24. Chang, C-Y. and Puga, A. (1998) Constitutive activation of the aromatic hydrocarbon receptor. *Mol. Cell. Biol.* **18**, 525–535
25. Santiago-Josefat, B., Pozo-Guisado, E., Mulero-Navarro, S., and Fernandez-Salguero, P.M. (2001) Proteasome inhibition induces nuclear translocation and transcriptional activation of the dioxin receptor in mouse embryo primary fibroblasts in the absence of xenobiotics. *Mol. Cell. Biol.* **21**, 1700–1709
26. Ikuta, T., Watanabe, J., and Kawajiri, K. (2002) Characterization of the LxxLL motif in the aryl hydrocarbon receptor: effects on subcellular localization and transcriptional activity. *J. Biochem.* **131**, 79–85
27. Suske, G. (1999) The Sp-family of transcription factors. *Gene* **238**, 291–300
28. Ungefroren, H., Gellersen, B., Krull, N.B., and Kalthoff, H. (1998) Biglycan gene expression in the human leiomyosarcoma cell line SK-UT-1. Basal and protein kinase A-induced transcription involves binding of Sp1-like/Sp3 proteins in the proximal promoter region. *J. Biol. Chem.* **273**, 29230–29240
29. Wang, L., Liu, X., and Lenox, R.H. (2002) Transcriptional regulation of mouse MARCKS promoter in immortalized hippocampal cells. *Biochem. Biophys. Res. Commun.* **292**, 969–979
30. Robertson, R.W., Zhang, L., Pasco, D.S., and Fagan, J.B. (1994) Aryl hydrocarbon-induced interactions at multiple DNA elements of diverse sequence–a multicomponent mechanism for activation of cytochrome P4501A1 (CYP1A1) gene transcription. *Nucleic Acids Res.* **22**, 1741–1749
31. 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