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Binding of Steroidogenic Factor-1 to the Regulatory Region Might Not Be Critical for Transcriptional Regulation of the Human *CYP1B1* Gene

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Cytochrome P450 (CYP) 1B1, which catalyzes 17β-estradiol 4-hydroxylation, is expressed in steroid-related tissues including ovary, testis, and adrenal gland. Generally, the expressions of steroidogenic CYPs are transcriptionally regulated by steroidogenic factor-1 (SF-1) and cAMP response element (CRE) binding protein (CREB). In the present study, we examined the possibility that the human *CYP1B1* gene might be regulated by SF-1 and CREB. Gel shift analyses revealed that *in vitro* translated SF-1 can bind to the putative SF-1 binding sites, SF-1a (at –1722) and SF-1b (at –2474), on the *CYP1B1* gene. *In vitro* translated CREB barely binds to the putative SF-1 binding sites. Luciferase analysis revealed that a reporter plasmid, pGL3 (–2623/+25), containing the SF-1a and SF-1b elements is transactivated by the concomitant co-expression of SF-1 and protein kinase A (PKA). However, the transcriptional activity is induced by PKA alone. Mutations in the SF-1a and SF-1b elements did not affect the luciferase activity. Thus, the binding of SF-1 to the putative SF-1 binding sites of the human *CYP1B1* gene might not be essential for transcriptional regulation. Interestingly, deletion and mutation analyses indicated that the PKA signaling pathway is involved in the xenobiotic responsive element (XRE)–mediated transactivation of the human *CYP1B1* gene.

Key words: CREB, CYP1B1, gene regulation, PKA; SF-1.

Abbreviations: AhR, aryl hydrocarbon receptor; ARNT, AhR nuclear translocator; CRE, cAMP response element; CREB, CRE binding protein; CYP, cytochrome P450; PKA, protein kinase A; SF-1, steroidogenic factor-1; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; XRE, xenobiotic responsive element.

Cytochrome P450 (CYP)s comprise a multigene family of constitutive and inducible enzymes involved in the oxidative metabolic activation and detoxification of many endogenous and exogenous compounds (*1*, *2*). Human CYP1B1 is mainly expressed in lung, kidney, ovary, uterus, breast, prostate, and adrenal gland (*3*, *4*). Its function involves not only the metabolic activation of a variety of procarcinogens and promutagens, including polycyclic aromatic hydrocarbons (PAHs) and aryl amines (*4*), but also the hydroxylation of the endogenous substrate 17β-estradiol in human (*5*, *6*).

In rat, CYP1B1 expression has been reported to be increased by adrenocorticotropic hormone (ACTH) and cAMP (*7*, *8*). In addition, Zheng *et al.* (*9*, *10*) recently reported that the transcriptional regulation of the rat *CYP1B1* gene is mediated by steroidogenic factor-1 (SF-1) and cAMP response element binding protein (CREB). SF-1 is a member of the orphan nuclear receptor family. SF-1 is expressed in adrenal cortex, testis, ovary, pituitary gonadotrope cells, and hypothalamus (*11*, *12*), and is essential for adrenal development and sexual differentiation (*13*, *14*). SF-1 is activated by the cAMP-dependent protein kinase A (PKA) and plays an important role in regulating the expression of various steroidogenic genes, such as steroidogenic acute regulatory protein (*15*), CYP11A1 (*16*), CYP11B1 (*17*), CYP17 (*18*), and CYP19 (*19*). In the 5′-flanking region of these target genes, SF-1 binds to a consensus sequence (PuPuAGGTCA or PyCAAGGPyPy) as a monomer and enhances the transcriptional activation (*20*).

CREB is a member of the leucine zipper family, which is also activated by the PKA signaling pathway, and recognizes the cAMP response element (CRE) core sequence (TGACGTCA) of the target gene (*21*). SF-1 is involved in cAMP-regulated gene expression, since SF-1 interacts with CREB mediated by the PKA pathway (*22*, *23*).

In the rat *CYP1B1* gene, four SF-1 binding sites and two potential CREs have been identified at –5298 to –5110 (Far Upstream Enhancer Region, FUER) (*9*, *10*). Although the corresponding FUER rat homolog does not exist in the human *CYP1B1* gene, we found two putative SF-1 binding sites at –1722 and –2474 in the human *CYP1B1* gene. This prompted us to investigate the possibility that human CYP1B1 expressed in steroidogenic tissues might also be controlled by SF-1 and CREB. In the present study, we examined the binding of SF-1 or CREB to the putative SF-1 binding sites on the 5′-flanking region of the human *CYP1B1* gene by gel shift analyses. In addition,

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luciferase analyses were performed using various reporter constructs containing the 5′-flanking region of the human *CYP1B1* gene to investigate the involvement of these factors in the transcriptional regulation.

MATERIALS AND METHODS

Chemicals and Reagents—The pGL3-basic plasmid and luciferase reporter assay system were from Promega (Madison, WI). The pCMV-CREB and pCMV-PKA plasmids, which contain the full-length cDNA of human CREB and the catalytic subunit of human PKA, respectively, were purchased from BD Biosciences Clontech (Palo Alto, CA). The pRc/RSV-SF-1 plasmid containing bovine SF-1 cDNA and rabbit anti–bovine SF-1 antiserum were kindly provided by Dr. Ken-Ichirou Morohashi (National Institute of Basic Biology, Okazaki, Japan). [γ-^{32}P]ATP was from Amersham (Buckinghamshire, UK). All primers and oligonucleotides were commercially synthesized by Hokkaido System Sciences (Sapporo, Japan). The mouse anti–human CREB monoclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals and solvents were of the highest grade commercially available.

In Vitro Transcription/translation and Gel Shift Analyses—One microgram of the pTNT-SF-1 plasmid and pTNT-CREB plasmid were incubated at 30°C for 90 min with the T7 TNT quick-coupled transcription/translation system (Promega) in the presence of methionine. The *in vitro* synthesized products were then subjected to gel shift analyses. Synthetic oligonucleotides were labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Toyobo, Osaka, Japan). The oligonucleotide sequences are shown in Table 1. The sequence of the consensus SF-1 binding site (cSF) was from the bovine *CYP11B1* gene (*20*) and the sequence of the consensus cAMP response element (CRE) was provided by Santa Cruz Biotechnology. The reaction mixture contained 1 μl of the *in vitro* translated product, 2 μg of poly [dI-dC], 1 μg of salmon sperm DNA, and 30 fmol of radiolabeled probe (~20,000 cpm) with binding buffer [25 mM Hepes-KOH (pH 7.9), 0.5 mM EDTA, 50 mM KCl, 10% glycerol, 0.5 mM dithiothreitol, 0.5 mM (*p*-amidinophenyl) methanesulfonyl fluoride] in a final volume of 15 μl. The binding reaction was performed at 25°C for 30 min. To determine the specificity of the binding to the oligonucleotides, competition experiments were conducted by co-incubation with excess amounts of unlabeled competitors. For super-shift analyses, rabbit anti–bovine SF-1 antiserum or anti–human CREB monoclonal antibody were pre-incubated with the *in vitro* translated product on ice for 15 min, and then each radiolabeled probe was added. DNA-protein complexes were separated under nondenaturing conditions in 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, Japan).

Plasmid Constructs—The pTNT-SF-1 plasmid was constructed by cloning the full-length bovine SF-1 cDNA digested with *Sac*II and *Xba*I from the pRc/RSV-SF-1 plasmid into the *Xho*I and *Xba*I-digested pTNT vector (Promega). The pTNT-CREB plasmid was constructed by cloning the full-length human CREB cDNA digested with *Eco*RI and *Mlu*I from the pCMV-CREB into the *Eco*RI and *Mlu*I-digested pTNT vector. A series of pGL3-basic plasmids containing the 5′-flanking region of the human *CYP1B1* gene (–2299/+25, –1652/+25, –1022/+25, –910/+25, and –732/+25) and mutated plasmids (–910/XRE3 mt, –910/XRE2 mt, and –910/Sp1-like mt) were constructed in our previous study (*24*). A plasmid containing the 5′-flanking region of the human *CYP1B1* from –2623 to +25 was constructed as follows: a DNA fragment containing the sequence from –2623 to –869 was amplified by PCR using the forward primer adapted to include a *Mlu*I site at the 5′ end, 5′-CGC GTA TCT AAG TTC CCC ATC ATG-3′, and the reverse primer, 5′-GAA AGT CGG CTC CAG TCA TAT CC-3′. After digestion with *Eco*RI, the PCR product was cloned into the *Mlu*I and *Eco*RI-digested pGL3-basic (–2299/+25) plasmid. The orientation of the construct was verified by restriction enzyme digestion or the nucleotide sequence was confirmed by DNA sequencing analysis. Mutated plasmids (–2623/SF-1a mt, –2623/SF-1b mt, and –2623/SF-1a/b mt) were constructed by site-directed mutagenesis with a QuikChange® site-directed mutagenesis kit (Stratagene, La Jolla, CA). For the –2623/SF-1a mt construct, the forward and reverse mutagenic primers were 5′-GGT CTC GAA CTC CTG AAA TCA AGT GAT CCG CCC GC-3′ and 5′-GCG GGC GGA TCA CTT GAT TTC AGG AGT TCG AGA CC-3′, respectively (mutated sites are underlined). For the –2623/SF-1b mt construct, the forward and reverse mutagenic primers were 5′-GGT GGA TCA CCT GAA ATC AGG AGT TTG AGA CCA GCC-3′ and 5′-GGC TGG TCT CAA ACT CCT GAT TTC AGG TGA TCC ACC-3′, respectively. Nucleotide sequences were confirmed by DNA sequencing analysis. The p0.2 plasmid containing the 0.2 kb proximal promoter region of the rat *CYP1B1* gene and the pFUER/0.2 plasmid containing the far upstream enhancer region (FUER) from –5298 to –5188 of the rat *CYP1B1* gene in addition to the 0.2 kb proximal promoter region were previously constructed (*9*).

Cells Culture and Luciferase Assay—The human ovarian granulosa–like tumor cell line KGN (*25*) and the mouse adrenal tumor cell line Y-1 were obtained from Riken Gene Bank (Tsukuba, Japan) and American Type Culture Collection (Rockville, MD), respectively. KGN cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% (v/v) fetal bovine serum (FBS) (Invitrogen). Y-1 cells were cultured in DMEM supplemented with 10% FBS. These cells were maintained at 37°C under an atmosphere of 5% CO_2–95% air. For the luciferase assays, cells were seeded into 24-well plates (8×10^4 cells/well) and incubated for 24 h before transfection. KGN cells were transfected with 400 ng of *CYP1B1/luc* reporter gene plasmid and 50 ng of pRc/RSV-SF-1 plasmid or pRc/RSV vector in the presence of 50 ng of pCMV-PKA plasmid or pCMV vector using Superfect Transfect Regents (QIAGEN, Hilden, Germany) at a reagent:DNA ratio of 3:1 in 200 μl of serum-free culture medium. After incubation at 37°C for 3 h under an atmosphere of 5% CO_2–95% air, growth medium was added to the cells. Y-1 cells were transfected with the same plasmids as above using Tfx-20 reagent (Promega) at a reagent:DNA ratio of 2.5:1 in 200 μl of

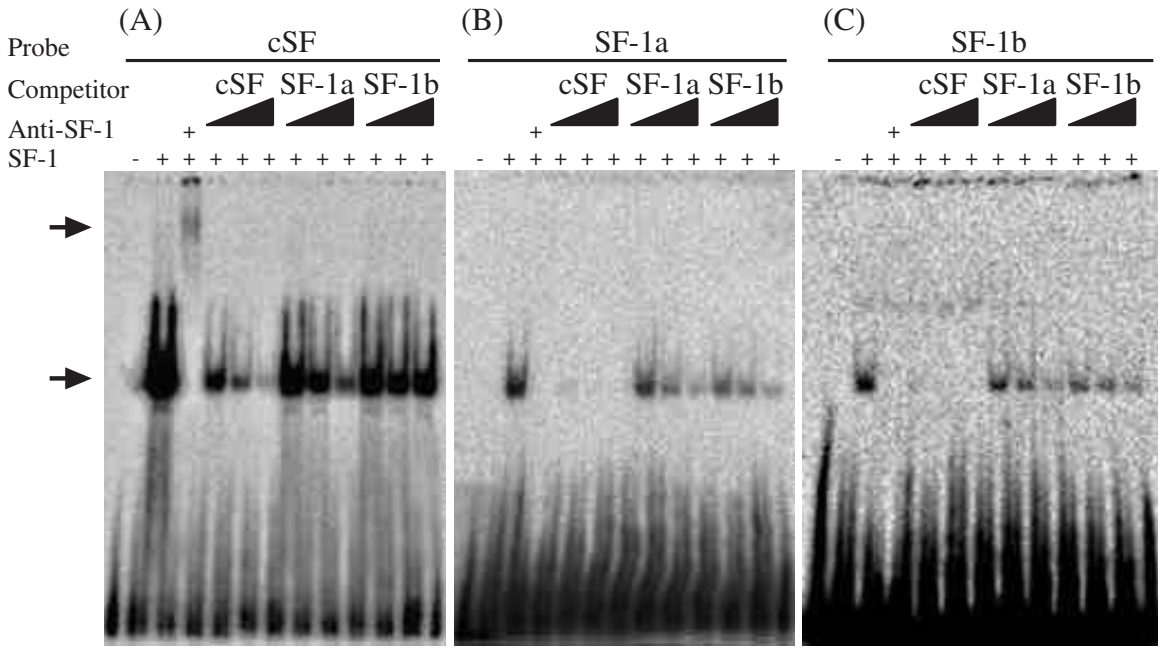

Fig. 1. **Gel shift analyses of the putative SF-1 binding sites on the human *CYP1B1* gene with SF-1**. Radiolabeled cSF (A), SF-1a (B), and SF-1b (C) were used as probes and each cold oligonucleotide was used as a competitor at 200-, 500-, and 1,000-fold molar excesses. For super-shift analyses, rabbit anti–bovine SF-1 serum was pre-incubated with *in vitro* translated SF-1 protein on ice for 15 min, and then the radiolabeled probe was added. DNA-protein complexes were separated under nondenaturing conditions in 4% polyacrylamide gels. The lower arrow indicates the position of the SF-1–dependent shifted band and the upper arrow indicates the super-shifted SF-1 complex.

serum-free culture medium. After incubation at 37°C for 1 h under an atmosphere of 5% CO_2–95% air, growth medium was added to the cells. After 48 h, the cells were resuspended in passive lysis buffer, and then the luciferase activity was measured with a luminometer (WALLAC, Turku, Finland) using a luciferase reporter assay system (Promega). Protein concentrations were determined using the Bradford protein assay regent (Bio-Rad, Hercules, CA) with bovine γ-globulin as the standard. The luciferase activity was normalized to the protein content.

Statistical Analyses—Data are expressed as mean ± SD of triplicate determinations. Statistical significance was determined by analysis of variance and Scheffe's test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Binding of SF-1 to the Putative SF-1 Binding Sites on the Human CYP1B1 Gene—A computer-assisted homology search revealed two putative SF-1 binding sites, SF-1a at –1722 and SF-1b at –2474, in the 5′-flanking region of the human *CYP1B1* gene. The SF-1a and SF-1b sequences are different from the consensus SF-1 binding sequence (PuPuAGGTCA) in one base. To determine whether SF-1 can bind to the SF-1a or SF-1b sequence on the human *CYP1B1* gene, gel shift analyses were performed (Fig. 1). Oligonucleotides used for the gel shift analyses are shown in Table 1. Using an oligonucleotide containing the consensus SF-1 binding sequence (cSF) as a probe, we confirmed that the *in vitro* translated SF-1 binds to cSF (Fig. 1A). A super-shifted band was observed with anti–SF-1 antiserum. However, the super-shifted band was not observed with normal rabbit IgG (data not shown). The band density was decreased by a cold cSF competitor. In contrast, the competition with the cold competitors of SF-1a and SF-1b was weak. Using SF-1a or SF-1b as a probe, the shifted band was also observed with SF-1, although the intensity was weak (Fig. 1, B and C). The shifted bands were abolished with anti–SF-1 antiserum, and excessive cold cSF competitors robustly diminished the DNA-protein complexes for SF-1a and SF-1b. In contrast, the competition with cold competitors of SF-1a and SF-1b was weak. These results indicate that SF-1 binds to the putative SF-1 binding sites on the human *CYP1B1* gene.

Table 1. **Oligoprobes used for gel shift analyses.**

Probe	Sequence	Position
SF-1a	5′-CGAACTCCTGACCTCAAGTGA-TCCGCCCGC-3′	–1737/–1708
SF-1b	5′-GTGGATCACCTGAGGTCAGGA-GTTTGAGAC-3′	–2491/–2462
cSF	5′-ACATACCCAAGGTCCCCTTT-3’	–337/–318
cCRE	5′-AGAGATTGCCTGACGTCAGA-GAGCTAG-3′	Artificial

Sequence and position of SF-1 binding sites in the human *CYP1B1* gene are listed. Each core region is underlined. SF-1a is actually the reverse complement of the SF-1 binding site. The consensus SF-1 binding site (cSF) sequence is that of the bovine *CYP11B1* gene. The consensus CRE (cCRE) sequence was created by Santa Cruz Biotechnology.

Binding of CREB to the Putative SF-1 Binding Sites on the Human CYP1B1 Gene—The sequences of SF-1a and SF-1b resemble that of the cAMP response element

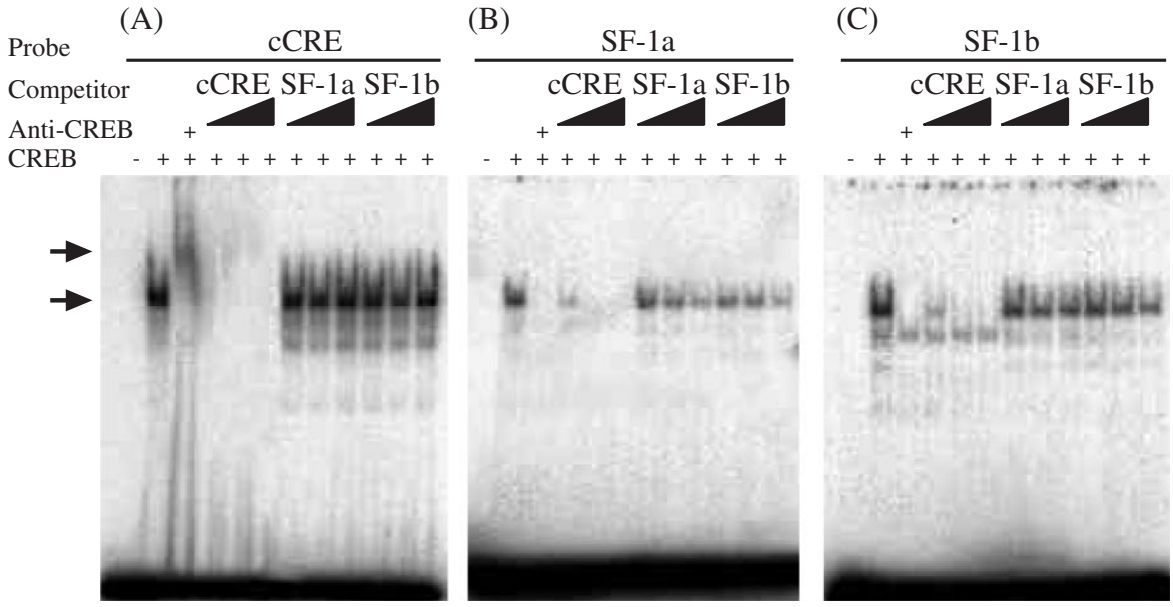

Fig. 2. **Gel shift analyses of the putative SF-1 binding sites on the human *CYP1B1* gene with CREB.** Radiolabeled cCRE (A), SF-1a (B), and SF-1b (C) were used as probes and each cold oligonucleotide was used as a competitor at 200-, 500-, and 1,000-fold molar excesses. For super-shift analyses, mouse anti–human CREB antibodies were pre-incubated with *in vitro* translated CREB protein on ice for 15 min, and then the radiolabeled probe was added. DNA-protein complexes were separated under nondenaturing conditions in 4% polyacrylamide gels. The lower arrow indicates the position of the CREB-dependent shifted band and the upper arrow indicates the super-shifted CREB complex.

(CRE: TGACGTCA), suggesting possible binding of CREB. To investigate whether CREB can bind to the SF-1a or SF-1b sites on the human *CYP1B1* gene, gel shift analyses were performed (Fig. 2). Using an oligonucleotide containing the consensus CRE sequence (cCRE), it was confirmed that the *in vitro* translated CREB binds to the sequence (Fig. 2A). A super-shifted band was observed with anti-CREB antibody. In contrast, no super-shifted band was observed with normal mouse IgG (data not shown). The band was competed out by cCRE when used as a cold competitor, but not by cold competitors of SF-1a and SF-1b. Using SF-1a or SF-1b as a probe, a shifted band was also observed with CREB (Figs. 2B and 2C). The shifted band was abolished with anti-CREB antibody, although no super-shifted band was observed. Thus, CREB barely binds to the putative SF-1–binding sites on the human *CYP1B1* gene.

Transcriptional Activities of the Human CYP1B1 Gene with the Co-Expression of SF-1 and PKA in KGN Cells—SF-1–dependent transcriptional regulation is known to be activated by PKA. To investigate whether SF-1 is involved in the transcriptional activation of the human *CYP1B1* gene via the putative SF-1 binding sites, luciferase assays were performed (Fig. 3). A luciferase reporter plasmid containing the 5′-flanking region from –2623 to +25 of the human *CYP1B1* gene was transiently transfected into KGN cells co-transfected with pRc/RSV-SF-1 plasmid, pCMV-PKA plasmid or in combination. Although the transcriptional activity was not affected by the co-expression of SF-1, it was significantly (5.9-fold) increased by the concomitant co-expression of SF-1 and PKA. However, the co-expression of PKA alone also produced similar transcriptional activation (6.7-fold). Using the mutated plasmids pGL3 (–2623/SF-1a mt), pGL3 (–2623/SF-1b mt), and pGL3 (–2623/SF-1a/b mt), the role of the putative SF-1 binding sites in the transcriptional activities was determined. The mutations did not affect the transcriptional activities in either the basal or PKA-dependent activities. These results suggest that the SF-1a and SF-1b sequences do not function in the transcriptional regulation of the human *CYP1B1* gene.

A series of deleted pGL3 plasmids were transfected into KGN cells co-transfected with the SF-1–expression vector in the absence or presence of the PKA-expression vector (Fig. 3). Using the pGL3 (–1652/+25), pGL3 (–1022/+25), and pGL3 (–910/+25) plasmids, stimulation of the transcriptional activities by PKA was observed in each plasmid (6.0-, 4.0-, and 4.9-fold, respectively), although no effects of the co-expression of SF-1 were observed (Fig. 3). Further deletion to –732 abolished the PKA-dependent transactivation. When CREB was co-expressed instead of SF-1 for each reporter construct, similar results were obtained (data not shown). These results suggest that the transcription of the human *CYP1B1* gene is regulated by PKA in the region –910 to–732.

Comparison of KGN Cells and Y-1 Cells in the Transcriptional Activities of the CYP1B1 Gene with the Co-Expression of SF-1 and PKA—As described above, SF-1 failed to transactivate the human *CYP1B1* gene. To investigate the responsiveness of SF-1 in KGN cells, a reporter plasmid containing the enhancer region of the rat *CYP1B1* gene (pFUER/0.2), which is known to be transactivated by SF-1 (*10*), was transfected (Fig. 4A). In contrast to the plasmids containing the human *CYP1B1* gene, the reporter activity of pFUER/0.2 was slightly increased by the co-expression of SF-1 (7.1-fold). Furthermore, the concomitant co-expression of SF-1 and PKA produced a maximum

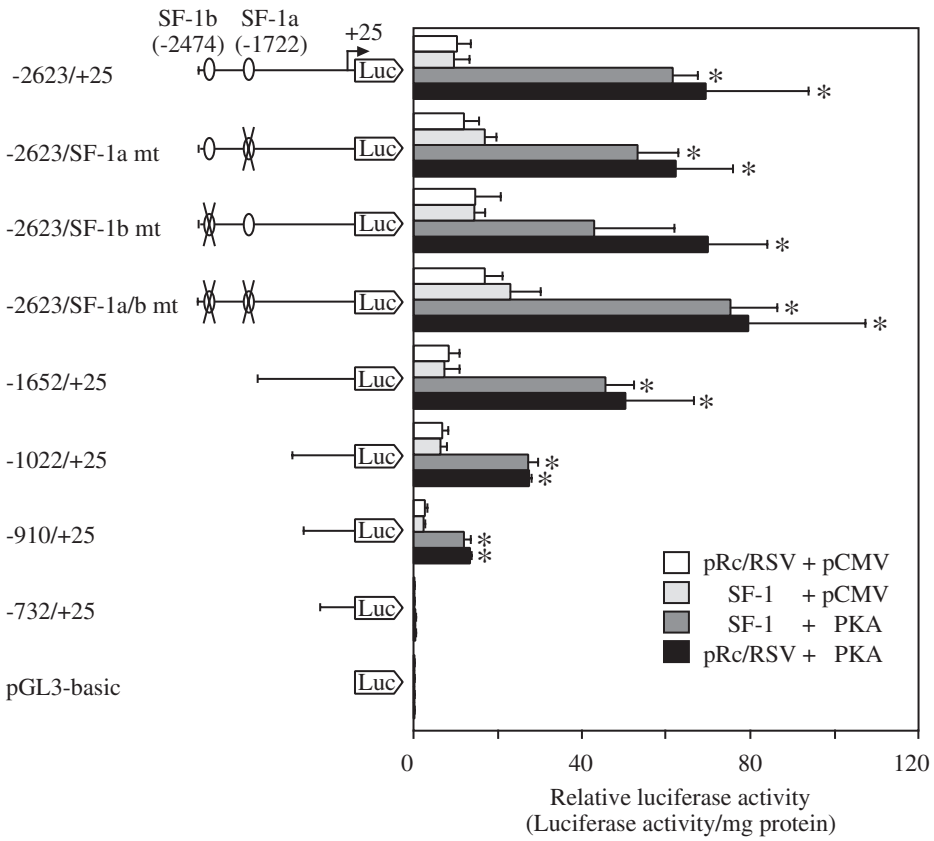

Fig. 3. **Transcriptional activity of the human *CYP1B1* gene in KGN cells.** A series of reporter constructs containing the 5′-flanking region of the human *CYP1B1* gene were transiently transfected into KGN cells with pRc/RSV-SF-1 plasmid (SF-1), pCMV-PKA plasmid (PKA), or in combination. To adjust the total amount of transfected plasmid, an empty vector (pRc/RSV or pCMV) was transfected as a control. After 48 h, luciferase activity and protein content were determined for the harvested cellular extracts. Relative luciferase activities are expressed as luciferase activity per mg protein. Each column represents the mean ± SD of three independent experiments $^*P < 0.05$, compared with control (pRc/RSV and pCMV vector co-transfection).

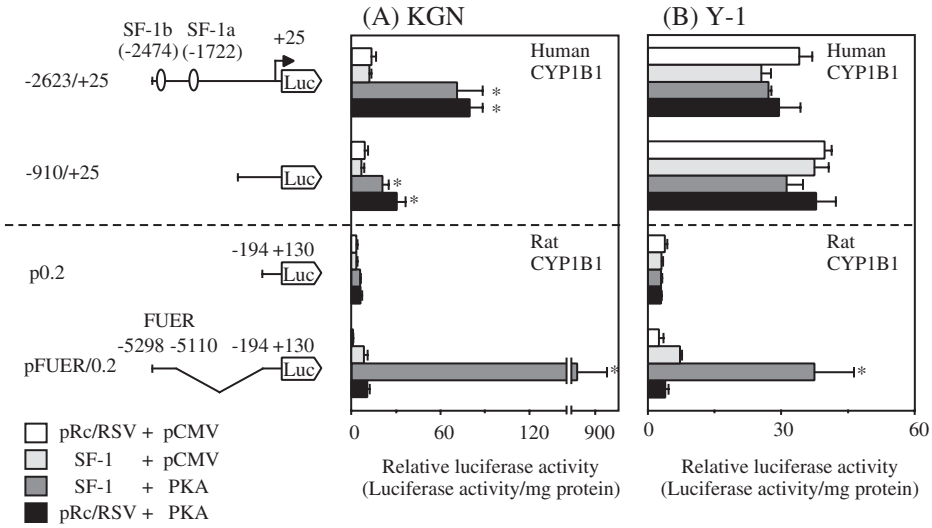

Fig. 4. **Transcriptional activity of the human or rat *CYP1B1* gene in KGN or Y-1 cells.** The reporter plasmids were pGL3 (–2623/+25) and pGL3 (–910/+25) containing the 5′-flanking region of the human *CYP1B1* gene, as well as pFUER/0.2 and p0.2 containing the 5′-flanking region of the rat *CYP1B1* gene. These reporter plasmids were transiently transfected into KGN cells (A) and Y-1 cells (B) with pRc/RSV-SF-1 plasmid (SF-1), pCMV-PKA plasmid (PKA), or in combination. To adjust the total amount of transfected plasmid, an empty vector (pRc/RSV or pCMV) was transfected as a control. After 48 h, the luciferase activity and protein content were determined for the harvested cellular extracts. Relative luciferase activities are expressed as luciferase activity per mg protein. Each column represents the mean ± SD of three independent experiments $^*P < 0.05$, compared with control (pRc/RSV and pCMV vector co-transfection).

transcriptional activation of 697-fold. The reporter activity of the p0.2 construct was not affected by the co-expression of SF-1. These results suggest that SF-1 can function in KGN cells.

Mouse adrenal tumor Y-1 cells are frequently used for the examination of SF-1–regulated transactivation of target genes. Therefore, the SF-1-dependent transactivation of the human *CYP1B1* gene was investigated using Y-1

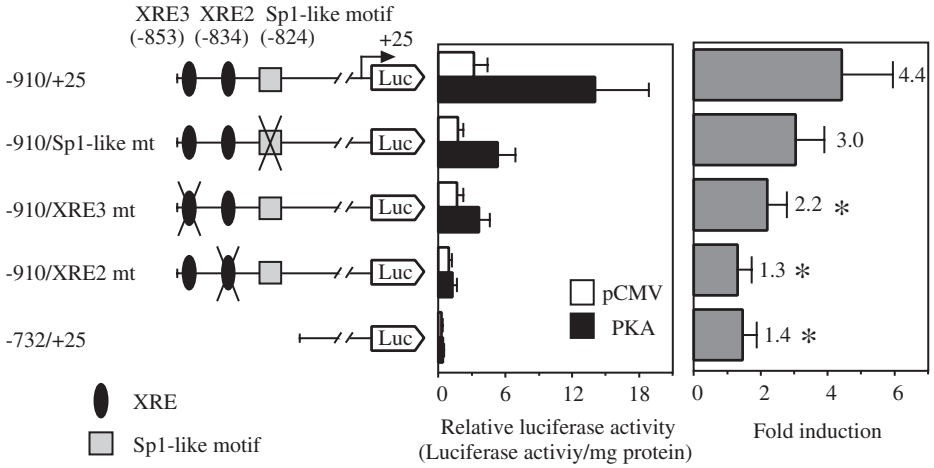

Fig. 5. **Effects of mutations of XRE3, XRE2, and the Sp1-like motif in the 5′-flanking region of the human *CYP1B1* gene on the transcriptional activity by PKA.** A series of reporter plasmids were transiently transfected into KGN cells with the pCMV-PKA plasmids. To adjust the total amount of transfected plasmid, the pCMV vector was transfected as a control. After 48 h, the luciferase activity and protein content were determined for the harvested cellular extracts. Relative luciferase activities are expressed as luciferase activity per mg protein. Constitutive and PKA-inducible transcriptional activities were demonstrated (left panel), and fold induction by PKA over control in each reporter construct was demonstrated (right panel). Each column represents the mean ± SD of three independent experiments $^*P < 0.05$, compared with pGL3 (–910/+25).

cells (Fig. 4B). In accordance with the previous report (*10*), the reporter activity of pFUER was increased by the co-expression of SF-1 (2.9-fold) and concomitant co-expression of SF-1 and PKA (15-fold). In contrast, reporter plasmids containing the human *CYP1B1* gene were not transactivated by the co-expression SF-1 or PKA in Y-1 cells. These results suggest that the regulation of the rat and human *CYP1B1* genes by SF-1 is not similar.

PKA-Dependent Transactivation Is Mediated by XRE— The *CYP1B1* gene is well known to be regulated by a ligand-activated aryl hydrocarbon receptor (AhR) *via* the xenobiotic responsive element (XRE). In our previous study (*24*), it was demonstrated that two XRE sequences at –853 and –834 on the 5′-flanking region of *CYP1B1* gene cooperatively regulate the constitutive and ligand-inducible transcriptional regulation of *hCYP1B1* with the Sp1-like motif at –824. To examine the possibility that the PKA-dependent transactivation is associated with these elements, luciferase analyses with mutated reporter constructs were performed (Fig. 5). The constitutive activities of the mutated reporter plasmids pGL3 (–910/Sp1-like mt), pGL3 (–910/XRE3 mt), and pGL3 (–910/XRE2 mt) were 56%, 52%, and 30% that of wild-type pGL3 (–910/+25), respectively. The reporter activities of pGL3 (–910/+25) and pGL3 (–910/Sp1-like mt) were induced 4.4-fold and 3.0-fold by the co-transfection of PKA, respectively. In contrast, mutations in XRE3 and XRE2 significantly reduced the PKA-dependent transactivation to 2.2-fold and 1.3-fold, respectively, and the PKA-dependent transactivation was not observed with the pGL3 (–732/+25) plasmid (1.4-fold). These results suggest that the PKA-dependent transcription of the human *CYP1B1* gene is mediated by XRE3 and XRE2.

DISCUSSION

Steroidogenic CYPs such as CYP11A1 (*16*), CYP11B1 (*17*), CYP17 (*18*), and CYP19 (*19*) possess a common cAMP responsive enhancer region in the 5′-flanking region of the genes. These are regulated by SF-1 and/or CREB (*26*). It has been reported that PKA directly phosphorylates SF-1 (*27*). It was recently reported that PKA phosphorylates mitogen-activated protein kinase phosphatase-1 (MKP-1), which dephosphorylates SF-1 (*28*). Although the functions of the phosphorylation and/or dephosphorylation of SF-1 are controversial, the involvement of PKA in the activation of SF-1 would be plausible. CREB can bind to the target gene after the phosphorylation by PKA, which is activated by cAMP (*29*). CYP1B1 is also highly expressed in steroidogenic tissues such as ovary and adrenal gland, and acts in the metabolism of 17β-estradiol. It has been reported that rat CYP1B1 is regulated by SF-1 and CREB (*9*, *10*). In the present study, we investigated the possibility that SF-1 or CREB might be involved in the transcriptional regulation of the human *CYP1B1* gene.

The binding of SF-1 and CREB to two SF-1 binding sites (SF-1a and SF-1b) on the human *CYP1B1* gene was demonstrated by gel shift analyses. However, luciferase analyses revealed no transactivation of the human *CYP1B1* gene with SF-1. A similar phenomenon has been reported for human CYP11B2, aldosterone synthase, expressed in adrenal zona glomerulosa (*30*). Although SF-1 binds to the Ad4 sequence on the promoter region of the *CYP11B2* gene, SF-1 does not stimulate the transcriptional activity. Thus, if the binding affinity of SF-1 and CREB to DNA is low, these factors might be unable to transactivate.

In the rat *CYP1B1* gene, two potential CREs to which CREB can bind were identified at –5122 and –5255 (*9*). Furthermore, four SF-1 binding elements to which SF-1 can bind were identified at –5298 to –5110 (Far Upstream Enhancer Region, FUER) of the rat *CYP1B1* gene (*10*). Zheng and Jefcoate (*10*) also reported that SF-1 and CREB cooperatively transactivate the rat *CYP1B1* gene. In the present study, the SF-1– and PKA-dependent transactivation of the rat *CYP1B1* gene, but not the human *CYP1B1* gene, was observed in both KGN cells and Y-1

cells. When the sequences of the human and rat *CYP1B1* genes are compared, the homology is greater than 90% in the 5′-flanking region –1058 to –802 containing XRE. Since both the human and rat *CYP1B1* genes are up-regulated by AhR *via* XRE, this region is critical for human and rat *CYP1B1* gene regulation. However, far from –1 kbp in the 5′-flanking region, the homology between the human and rat *CYP1B1* genes decreases prominently, and the corresponding FUER rat homolog (–5298/–5110) is not found in the human *CYP1B1* gene. In the present study, we focused on two putative SF-1 binding sites at –1722 and –2474 in the *hCYP1B1* gene. Although rat FUER has multiple binding regions for SF-1 and CREB in a short sequence (189 bp), the SF-1a and SF-1b sites in the human *CYP1B1* gene exist separately. Thus, the dissimilarity in the sequences in the regulatory regions of the human and rat *CYP1B1* genes might be the cause of the low contribution of SF-1 to the regulation of the human *CYP1B1* gene. Taking our present data into consideration, SF-1 would not be a major regulator of human CYP1B1 expression. However, we cannot exclude the possibility that SF-1 might act indirectly if SF-1 modifies another transcriptional factor(s) that regulates human CYP1B1.

We found that PKA activates the transcription between –910 to –732 on the 5′-flanking region of the human *CYP1B1* gene (Fig. 3). This region includes two XRE sites and an Sp1-like motif, which play roles in the regulation of *CYP1B1* in constitutive and AhR-ligand dependent expression (*24*). It is well known that AhR binds to XRE after heterodimerization with AhR nuclear translocator (ARNT) (*31*, *32*). Interestingly, mutations in XRE3 at –853 and XRE2 at –834 inhibit the PKA-dependent activation (Fig. 5). As shown in Fig. 4, this PKA-dependent transactivation of the human *CYP1B1* gene was observed in KGN cells, but not in Y-1 cells. In KGN cells, AhR mRNA is expressed and can induce the CYP1B1 mRNA in the presence of ligands (data not shown). In contrast, AhR mRNA is not expressed in Y-1 cells (*9*). Thus, AhR is likely to be involved in PKA-dependent transactivation. A number of early studies reported the phosphorylation of AhR; especially, the phosphorylation of AhR via protein kinase C (PKC) is well known to be essential for the binding of AhR to XRE (*33*, *34*). Until now, the association of PKA with the phosphorylation of AhR activation was unknown. However, it has been reported that PKA augments the transactivation potential of ARNT, a partner of AhR (*35*). The findings of our study suggest that the PKA signaling pathway is partially involved in the transcriptional regulation of the human *CYP1B1* gene *via* XRE, although further studies are necessary.

In conclusion, we demonstrated the binding of SF-1 and CREB to the putative SF-1 binding sites of the human *CYP1B1* gene, but these factors do not activate transcription. Thus, SF-1 and CREB might not be essential for the regulation of the human *CYP1B1* gene. Interestingly, it was found that PKA signaling is involved in the XRE-mediated transactivation in the human *CYP1B1* gene.

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