[CANCER RESEARCH 64, 3119–3125, May 1, 2004]

Human *CYP1B1* Is Regulated by Estradiol via Estrogen Receptor

Yuki Tsuchiya,[1] Miki Nakajima,[1] Satoru Kyo,[2] Taro Kanaya,[2] Masaki Inoue,[2] and Tsuyoshi Yokoi[1]

[1]Division of Drug Metabolism, Faculty of Pharmaceutical Sciences, and [2]Department of Obstetrics and Gynecology, School of Medicine, Kanazawa University, Kanazawa, Japan

ABSTRACT

Human cytochrome P450 (CYP) 1B1 is a key enzyme in the metabolism of 17β-estradiol (E2). CYP1B1 is mainly expressed in endocrine-regulated tissues, such as mammary, uterus, and ovary. Because many CYP enzymes are likely to be induced by the substrates themselves, we examined whether the human CYP1B1 expression is regulated by E2 in the present study. Real-time reverse transcription-PCR analysis revealed that treatment with 10 nM E2 for 12 h induced CYP1B1 mRNA expression in estrogen receptor (ER)-positive MCF-7 cells. Luciferase reporter assays using MCF-7 cells showed a significant transactivation up to 7-fold by E2 with a reporter plasmid containing a region from −152 to +25 of the human *CYP1B1* gene. A computer-assisted homology search indicated a putative estrogen response element (ERE) between −63 and −49 in the *CYP1B1* promoter region. Specific binding of ERα to the putative ERE was demonstrated by chromatin immunoprecipitation assays and gel shift analyses. With reporter plasmids containing the wild or mutated putative ERE on the *CYP1B1* gene and the wild or mutated ERα expression vectors, luciferase assays using Ishikawa cells demonstrated that the putative ERE and ERα are essential for the transactivation by E2. Because endometrial tissue is highly regulated by estrogens, the expression pattern of CYP1B1 protein in human endometrial specimens was examined by immunohistochemistry. The staining of CYP1B1 was stronger in glandular epithelial cells during a proliferative phase than those during a secretory phase, consistent with the pattern of estrogen secretion. These findings clearly indicated that the human *CYP1B1* is regulated by estrogen via ERα. Because 4-hydroxylation of estrogen by CYP1B1 leads to decrease of the estrogenic activity but the produced metabolite is toxicologically active, our findings suggest a clinical significance in the estrogen-regulated CYP1B1 expression for the homeostasis of estrogens as well as estrogen-dependent carcinogenesis.

INTRODUCTION

Cytochrome P450s (CYP) are a multigene family of constitutive and inducible enzymes involved in the oxidative metabolic activation and detoxification of many endogenous and exogenous compounds (1, 2). CYP1B1 is a comparatively new isoform in the CYP1 family (3). Whereas CYP1B1 is capable of activating a variety of procarcinogens and promutagens including polycyclic aromatic hydrocarbons and aryl amines (4), it is induced by various polycyclic aromatic hydrocarbons as well as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (5). The induction is controlled by the aryl hydrocarbon receptor (AhR) and AhR nuclear translocator (6, 7). In our previous study (8), two xenobiotic responsive elements (XREs) that are critical for the induction were identified on the human *CYP1B1* gene.

Recent findings suggest that human CYP1B1 is a key enzyme in the metabolism of 17β-estradiol (E2; Refs. 9–11). Of particular interest is the fact that CYP1B1 is highly expressed in estrogen-related tissues such as mammary, uterus, and ovary (4, 12), suggesting that CYP1B1 is important in the localized metabolic control of estrogen homeostasis. 4-Hydroxyestradiol, a catechol metabolite formed by CYP1B1 from E2, leads to decrease of the estrogenic activity. However, it is toxicologically active and appears to play a role in tumorigenesis, because it generates free radicals from the reductive-oxidative cycling with the corresponding semiquinone and quinone forms, which cause cellular damage (13, 14). It has also been reported that the 4-hydroxyestradiol level is elevated in human endometrial and breast cancers in comparison to normal tissue (15). Thus, the metabolism of the estrogens by CYP1B1 in the uterus may be an important determinant of the toxicological as well as physiological outcomes.

In the present study, we focused on estrogens as a potential regulator of human CYP1B1, because the enzyme is highly expressed in estrogen-regulated tissues. Many CYP isoforms are likely to be induced by the substrates themselves. In addition, computer-assisted homology search identified a potential estrogen receptor (ER) binding site on the *CYP1B1* promoter. These backgrounds prompted us to investigate whether the human *CYP1B1* gene is a target of E2.

MATERIALS AND METHODS

Chemicals and Reagents. E2 and 4-hydroxytamoxifen (4OHT) were obtained from Sigma-Aldrich (St. Louis, MO). 7α-[9(4,4,5,5,5-Pentafluoropentylsulfonyl)nonyl] estra-1,3,5(10)-triene-3,17β-diol (ICI 182,780) was purchased from Tocris Cookson Inc. (Bullwin, MO). pGL3-basic plasmid, phRL-TK plasmid, Tfx-20 reagent, and a dual-luciferase reporter assay system were from Promega (Madison, WI). pSG5 vector was obtained from Stratagene (La Jolla, CA). ERα expression vector (pSG5-HE0) and mutated ERα expression vectors (HE11, HE19, and HE38) constructed previously (16, 17) were gifts from Dr. Pierre Chambon (Institut de Genetique et de Biologie Moleculaire et Cellulaire, Strasbourg, France). [γ-^{32}P]ATP was from Amersham (Buckinghamshire, United Kingdom). All of the primers and oligonucleotides were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). Recombinant human ERα was purchased from PanVera (Madison, WI). Mouse antihuman ERα monoclonal antibodies and normal mouse IgG were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit antihuman CYP1B1 polyclonal antibodies were from Alpha Diagnostic International (San Antonio, TX). All of the other chemicals and solvents were of the highest grade commercially available.

Cells and Culture Conditions. The human breast adenocarcinoma cell lines MCF-7 and MDA-MB-435 were obtained from American Type Culture Collection (Rockville, MD). The human endometrial adenocarcinoma cell line Ishikawa was a generous gift of Dr. Masato Nishida (Tsukuba University, Ibaraki, Japan). MCF-7 cells were cultured in DMEM (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 0.1 mM nonessential amino acid (Invitrogen, Melbourne, Australia) and 10% fetal bovine serum (Invitrogen). MDA-MB-435 and Ishikawa cells were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum. In the estrogen-induction assay, these cells were grown in phenol red-free DMEM medium (Invitrogen) containing 10% dextran-coated charcoal-treated fetal bovine serum for 48 h before treatment with estrogen. These cells were maintained at 37°C under an atmosphere of 5% CO_2-95% air.

Real-Time Reverse Transcription-PCR. Human CYP1B1 and glyceraldehyde-3-phosphate dehydrogenase mRNAs were quantified by real-time reverse transcription-PCR. Total RNA was isolated from the cells using ISOGEN (Nippon Gene, Tokyo, Japan), and cDNAs were synthesized as described previously (5). The forward and reverse primers for human CYP1B1 were described previously (5), and those for human glyceraldehyde-3-phosphate dehydrogenase were 5′-CCA GGG CTG CTT TTA ACT C-3′ and 5′-GCT CCC CCC TGC AAA TGA-3′. PCR was performed using the Smart Cycler (Cepheid, Sunnyvale, CA) with Smart Cycler software (Version 1.2b). The PCR condition for glyceraldehyde-3-phosphate dehydrogenase was as follows: after an initial denaturation at 95°C for 30 s, the amplification was performed by denaturation at 94°C for 4 s, annealing and extension at 64°C for 20 s for

Received 1/16/04; revised 2/21/04; accepted 2/26/04.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Tsuyoshi Yokoi, Division of Drug Metabolism, Faculty of Pharmaceutical Sciences, Kanazawa University, Takara-machi 13-1, Kanazawa 920-0934, Japan. Phone/Fax: 81-76-234-4407; E-mail: TYOKOI@kenroku.kanazawa-u.ac.jp.

45 cycles. The PCR condition for CYP1B1 was as follows: after an initial denaturation at 95°C for 30 s, the amplification was performed by denaturation at 94°C for 10 s, annealing and extension at 68°C for 20 s for 45 cycles. Amplified products were monitored directly by measuring the increase of the dye intensity of the SYBR Green I (Molecular Probes, Eugene, OR) that binds to double-strand DNA amplified by PCR. Copy number of mRNA in the cDNA samples was calculated using standard amplification curves.

Preparation of Reporter Constructs. 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). A series of plasmids containing progressive deletion fragments (−1652/+25, −910/+25, and −152/+25) were constructed previously (8). A plasmid containing the 5′-flanking region from −39 to +25 (−39/+25) was constructed using restriction endonucleases. The orientation of these constructs was verified by restriction enzyme digestion or DNA sequencing. The mutated plasmid of pGL3 (−152/+25) mt was constructed by site-directed mutagenesis with a QuikChange site-directed mutagenesis kit (Stratagene). The forward and reverse mutagenic primers were 5′-CCT GCC AG**T** TCG CGC TGC **A**CT CCT TCT ACC-3′ and 5′-GGT AGA AGG AG**T** GCA GCG CGA **A**CT GGC AGG-3′, respectively (mutated sites are shown as bold letters). Nucleotide sequences were confirmed by DNA sequencing analyses.

Transfection and Luciferase Assay. *CYP1B1/luc* reporter gene plasmid and control (phRL-TK) reporter plasmid were transiently transfected into MCF-7 or Ishikawa cells using Tfx-20 reagent. Into the Ishikawa cells, ERα expression vector or pSG5 vector (control) was also cotransfected. After the incubation with transfection mixture at 37°C under an atmosphere of 5% CO_2-95% air for 1 h, growth medium was added to the cells. After 24 h, the medium was replaced with medium containing 0.1% (v/v) DMSO or 10 nM E2/0.1% DMSO. For inhibition studies, the transfected MCF-7 cells were incubated with 1 μM of 4OHT or ICI 182,780 in the absence or presence of E2. 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, Japan) using the dual-luciferase reporter assay system.

Chromatin Immunoprecipitation (ChIP) Assay. MCF-7 cells were treated with 0.1% DMSO or 10 nM E2 for 12 h, and ChIP assay was carried out essentially using the ChIP assay kit (Upstate, Lake Placid, NY). Anti-ERα antibodies or normal mouse IgG (control) were used for immunoprecipitation of protein-DNA complexes. The forward and reverse primers for region 1 were 5′-TAC CGC ACA ATG GAA ACG T-3′ and 5′-TGA GTG GCG TCA ATT CCC A-3′, respectively. The forward and reverse primers for region 2 were 5′-AGC GGC TAC TTT TAG GGA TTC-3′ and 5′-GGT AAC CGC GCT TCA TCA CA-3′, respectively. PCR analyses were performed as follows: after an initial denaturation at 94°C for 3 min, the amplification was performed by denaturation at 94°C for 20 s, annealing at 55°C (region 1) or 58°C (region 2) for 20 s, and extension at 72°C for 30 s for 34 cycles. The PCR product was electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining.

Gel Shift Assay. Synthetic oligonucleotides were labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Toyobo, Osaka, Japan). The reaction mixture contained 3 pmol of the recombinant ERα protein, 2 μg of poly(dI·dC), 1 μg of salmon sperm DNA, and 30 fmol of the radiolabeled probe (~20,000 cpm) in a final volume of 15 μl of binding buffer [25 mM HEPES-KOH (pH 7.9), 0.5 mM EDTA, 50 mM KCl, 10% glycerol, 0.5 mM DTT, and 0.5 mM (*p*-amidinophenyl) methanesulfonyl fluoride]. Binding reactions were performed on ice for 30 min. To determine the specificity of the binding to the oligonucleotides, competition experiments were conducted by coincubation with 100- and 500-fold excesses of unlabeled competitors. In some experiments, 2 μg of anti-ERα polyclonal antibodies or normal mouse IgG were preincubated with the recombinant ERα protein on ice for 15 min. The oligonucleotide sequences are shown in Table 1. The consensus oligonucleotide for estrogen response element (ERE) was from the *Xenopus* vitellogenin A2 gene (18). DNA-protein complexes were separated under nondenaturing conditions on 4% polyacrylamide gels with 0.5 × Tris-borate EDTA 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).

Table 1 *Oligonucleotides used for gel shift analyses*

The sequence of consensus ERE[a] and putative ERE on the human *CYP1B1* gene are underlined. The sequence of cERE contains the consensus ERE in *Xenopus vitellogenin* A2 promoter. Mutated sites are shown as bold letters and the positions are in parenthesis.

Probe	Sequence	Position
cERE	5′-GTCCAAAGTCAGGTCACAGTGACCTGATCAAAGTT-3′	
1B1/ERE	5′-CCTGCCAGGTCGCGCTGCCCTCCTTCTACC-3′	−69/−39
1B1/ERE mt	5′-CCTGCCAG**T**TCGCGCTGC**A**CTCCTTCTACC-3′	(−61 and −51)

[a] ERE, estrogen response element.

Human Endometrial Tissues and Isolation of Human Endometrial Glands. Human endometrial tissues were obtained from 20 patients undergoing hysterectomy as a treatment for benign neoplasms not associated with endometrial disease at Kanazawa University Hospital. Written informed consent was obtained from all of the patients. The collected endometrial tissues were sampled for histopathological diagnosis, and the remaining normal portions of the samples were used for immunohistochemistry (19 samples) or ChIP assays (1 sample). The phase of the menstrual cycle was determined by histological examination of the tissues and also confirmed by determination of the day of the menstrual cycle. These samples were confirmed to be derived from nonsmoking patients. For ChIP assay, endometrial glands were obtained from a patient (39 years old) during the proliferative phase as follows: Minced endometrial tissue was placed on plastic dishes in DMEM containing 350 units/ml of DNase I (Takara, Ohtsu, Japan) and 180 units/ml of collagenase type 3 (Washington Biochemical Corporation, Lakewood, NJ), and gently shaken for 40 min at 37°C as described previously (19). Individual glands on the bottom of the dishes were selected under a microscope and were collected into an Eppendorf tube.

Immunohistochemistry. Immunohistochemical analyses of CYP1B1 were performed using formalin-fixed, paraffin-embedded specimens of normal human endometrial tissues. The sections were autoclaved in 1 × Antigen Retrieval Solution (Biogenex, San Ramon, CA) for 10 min and then incubated with antihuman CYP1B1 antibodies at 4°C for 16 h. For the antibodies, no significant cross-reactivity to either human CYP1A1 or CYP1A2 protein has been reported (20). Staining reactions were performed using the ABC-elite kit (Vector Laboratories, Burlingame, CA). Staining of glands in functional layers was evaluated as positive when >10% of the constituting epithelial cells in a gland were stained. The results were judged by three independent pathologists as − (<10% of the glands at the region of interest), + (>10%), or ++ (>10% with high intensity).

Statistical Analyses. Data are expressed as mean ± SD. Comparison of two groups was made with an unpaired, two-tailed Student's *t* test. In immunohistochemistry, the statistical significance of differences in the extent of staining between the different phases of the menstrual cycle was tested by Fisher's exact method. A value of $P < 0.05$ was considered statistically significant.

RESULTS

E2 Induces Human CYP1B1 mRNA Expression in ER-Positive MCF-7 Cells. The effects of E2 treatment on the CYP1B1 mRNA expression were investigated in the human breast adenocarcinoma MCF-7 and MDA-MB-435 cells, and the human endometrial adenocarcinoma Ishikawa cells. These cells were treated with 10 nM E2 for 12 h, and the mRNA expression was examined by real-time reverse transcription-PCR. As shown in Fig. 1, CYP1B1 mRNA was significantly ($P < 0.05$) induced by E2 treatment in ER-positive MCF-7 cells by ~3-fold. In contrast, no significant induction was observed in ER-negative MDA-MB-435 cells. These results suggest that E2 induced CYP1B1 mRNA expression in an ER-dependent manner. Although Ishikawa cells are ER positive, the induction of CYP1B1 by E2 was not observed.

E2 Activates Transcription of the Human *CYP1B1* Gene in an ER-Dependent Manner. To investigate the transcriptional activation of the human *CYP1B1* gene by E2, luciferase assays were performed in which a series of reported plasmids containing the 5′-flanking

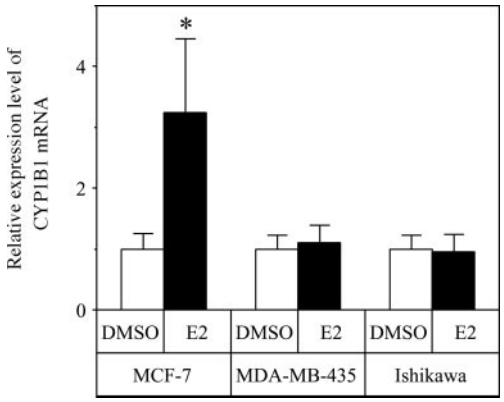

Fig. 1. Effects of 17β-estradiol treatment on the expression level of the human cytochrome P450 (CYP) 1B1 mRNA. The expression levels of the human CYP1B1 mRNA in MCF-7, MDA-MB-435, and Ishikawa cells were determined by real-time PCR as described in "Materials and Methods." These cells were treated with 0.1% DMSO or 10 nM 17β-estradiol for 12 h. The expression level of the human CYP1B1 was corrected with the expression level of glyceraldehyde-3-phosphate dehydrogenase as a control. Each column represents the mean of three independent experiments; *bars,* $\pm$SD (*, $P < 0.05$, compared with DMSO treatment).

region of the human *CYP1B1* gene were transiently transfected into MCF-7 cells (Fig. 2*A*). The reporter activity of the pGL3 (−2299/+25) plasmid was significantly ($P < 0.05$) induced by 2-fold by E2 treatment (Fig. 2*A*). Deletion from the 5′-end to −152 led to 7-fold activation of the transcription ($P < 0.01$). In contrast, the transcriptional activation was not observed with the pGL3 (−39/+25) plasmid. These results suggest the existence of an E2-responsive region between −152 and −39.

The ER dependency of the transcriptional activation by E2 was examined. MCF-7 cells transfected with the pGL3 (−152/+25) plasmid were treated with ER antagonists, 4OHT or ICI 182,780, in the absence or presence of E2 for 24 h (Fig. 2*B*). In the absence of E2, the reported activity was significantly ($P < 0.05$) increased by 4OHT or ICI 182,780 treatments. In contrast, the transcriptional activity enhanced by E2 was significantly ($P < 0.05$) inhibited by 4OHT or ICI 182,780. These results suggest that ER could be involved in the transcriptional activation of the human *CYP1B1* by E2.

ERα Is Associated with the Enhancer Region of the Human *CYP1B1* Gene. A computer-assisted homology search revealed imperfect palindromic ERE to which ERα may bind in the human *CYP1B1* gene between −63 and −49 (Fig. 3*A*). The sequence (AGGTCGCGCTGCCCT) is different from the consensus ERE (AGGTCANNNTGACCT) in two bases (Table 1). To determine whether ERα is associated with the putative ERE on the human *CYP1B1* gene, ChIP assays were performed. DNA was extracted from MCF-7 cells treated with DMSO or E2 for 12 h after cross-linking treatment and incubated with specific anti-ERα antibodies or normal mouse IgG. As shown in Fig. 3*B,* PCR was performed with a primer set for region 1 (−209 to +81) including the putative ERE or a primer set for region 2 (−534 to −206). In the presence of E2, immunoprecipitants obtained with anti-ERα antibodies generated a distinct PCR product with the primer set for region 1, whereas those obtained with mouse IgG generated no product (Fig. 3*C*). In the absence of E2, no distinct band was observed. These results indicated that the incubation with E2 facilitated the binding of ERα to the putative ERE. Using the primer set for the region 2, immunoprecipitants obtained with anti-ERα antibodies or mouse IgG did not generate the PCR product in the absence or presence of E2. These results suggest that ERα is associated with the putative ERE on the human *CYP1B1* gene.

ERα Specifically Binds to the Putative ERE on the Human *CYP1B1* Gene. To examine whether ERα binds to the putative ERE on the human *CYP1B1* gene, gel shift assays were performed (Fig. 4). Oligonucleotides used for the gel shift analyses are shown in Table 1. We first confirmed the specific binding complex formed with recombinant ERα and consensus oligonucleotide for ERE used as a probe (Fig. 4*A*). This band was competed out by the putative ERE on human *CYP1B1* gene (1B1/ERE) used as a cold competitor. The specific

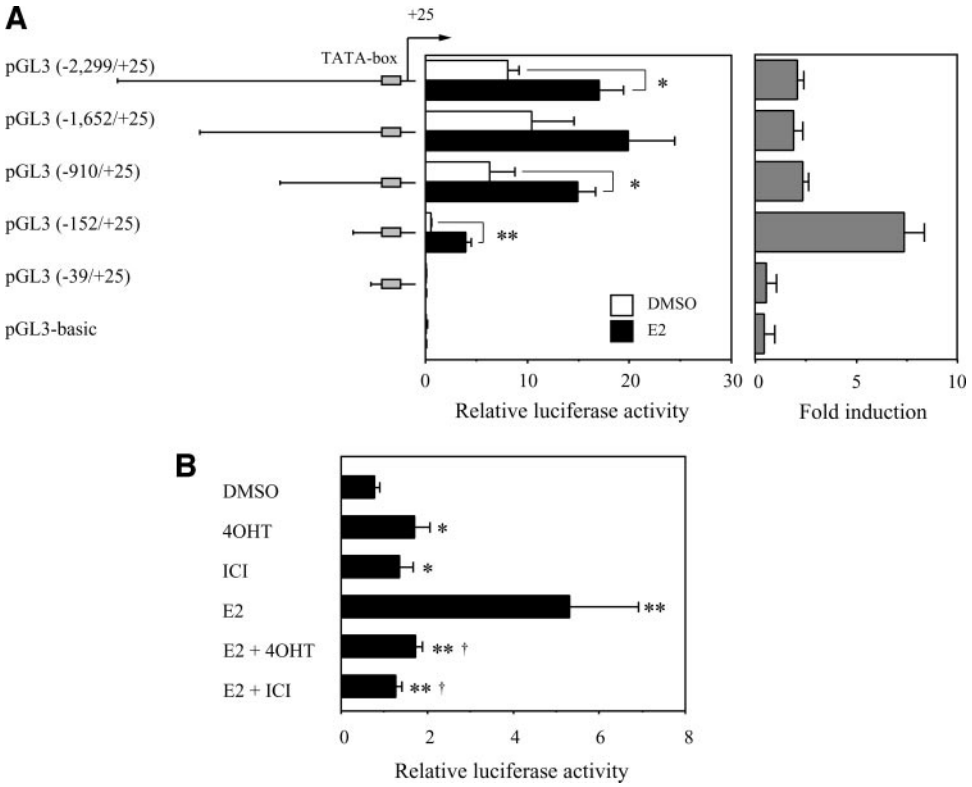

Fig. 2. 17β-estradiol (E2)-dependent transcriptional activation of the human *CYP1B1* gene in MCF-7 cells. *A*, a series of reporter constructs containing the 5′-flanking region of the human *CYP1B1* gene were transiently transfected into MCF-7. After 24 h, the cells were treated with 0.1% DMSO or 10 nM E2 for 24 h. The relative luciferase activities were normalized with the *Renilla* luciferase activities. *Right panel* shows the fold induction of the transcriptional activity by E2 treatment. Each column represents the mean of three independent experiments; *bars,* $\pm$SD (*, $P < 0.05$ and **, $P < 0.01$, compared with DMSO treatment). *B*, a reporter construct containing the region from −152 to +25 of the *CYP1B1* gene was transiently transfected into MCF-7 cells. After 24 h, the cells were treated with 1 μM of 4-hydroxytamoxifen (*4OHT*) or 1 μM of 7α-[9(4,4,5,5,5-pentafluoropentylsulfonyl)nonyl] estra-1,3,5(10)-triene-3,17β-diol (*ICI*) in the absence or presence of E2 (10 nM) for 24 h. The relative luciferase activities were normalized with the *Renilla* luciferase activities. Each column represents the mean of three independent experiments; *bars,* $\pm$SD (*, $P < 0.05$ and **, $P < 0.01$, compared with DMSO treatment and †, $P < 0.05$, compared with E2 treatment).

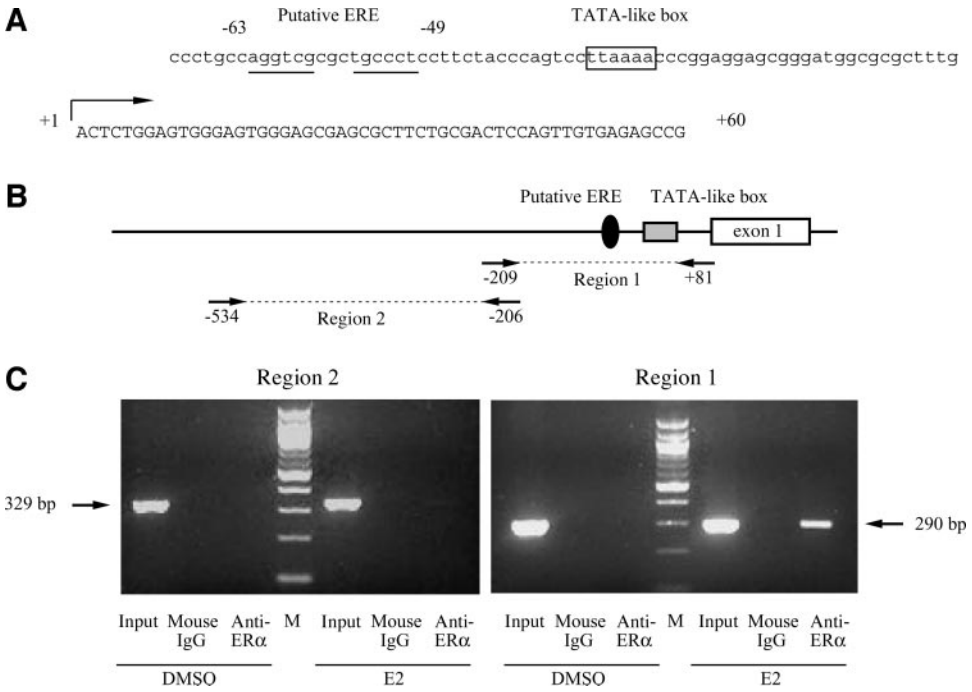

Fig. 3. Chromatin immunoprecipitation (*ChIP*) assay of the 5′-flanking region of the human *CYP1B1* gene with antiestrogen receptor (ER)α antibodies using DNA extracted from MCF-7 cells. *A,* in the sequence of the 5′-flanking region of the human *CYP1B1* gene, the TATA-like box is boxed and the position of the putative estrogen response element (ERE) binding site is *underlined*. *B,* target regions of the ChIP assay are indicated on the schematic representation of the human *CYP1B1* gene. The TATA-like box and the putative ERE are shown with a ▨ and a ●, respectively. *Numbers* indicate the 5′-end of the primers used for PCR. *Broken lines* represent the amplified regions by the PCR. *C,* MCF-7 cells were treated with 0.1% DMSO or 10 nM 17β-estradiol for 12 h. The cells were cross-linked by treatment with formaldehyde, and DNA was extracted and immunoprecipitated with anti-ERα antibodies or normal mouse IgG. After the reversal of cross-linking, DNA was purified and used as a template for PCR to amplify each target site. The PCR product was electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining. A definite PCR band was observed only when anti-ERα antibodies were used with the primer sets for region 1 targeting the putative ERE site after the 17β-estradiol treatment of MCF-7 cells. *M,* 100-bp DNA ladder marker.

band was also observed with the recombinant ERα and 1B1/ERE used as a probe that was super-shifted by anti-ERα antibodies and competed out by consensus oligonucleotide for ERE and 1B1/ERE but not by mutant 1B1/ERE (Fig. 4*B*). No significant band was observed with mutant 1B1/ERE as a probe (Fig. 4*C*). These results indicated that ERα specifically binds to the putative ERE on the human *CYP1B1* gene.

ERα and the Putative ERE Are Essential for the Transcriptional Activation of the Human *CYP1B1* Gene by E2. To investigate whether the transcriptional activation of the human *CYP1B1* gene is ERα-dependent or not, luciferase assays were performed using Ishikawa cells that expressed low levels of ERα. Notably, E2 treatment of the Ishikawa cells failed to induce the reporter activity of the pGL3 (−152/+25) plasmid containing the putative ERE. However, when ERα was overexpressed, the reporter activity was significantly ($P < 0.01$) induced up to 10-fold by the E2 treatment (Fig. 5*A*). In contrast, no activation was observed with the pGL3 (−152/+25) mt and pGL3 (−39/+25) plasmids lacking the putative ERE by E2 treatment. Therefore, the putative ERE located between −63 and −49 appeared to be indispensable for the E2-dependent transactivation.

To confirm the requirement of ERα for the transactivation of CYP1B1, various mutant ERα expression vectors were cotransfected with pGL3 (−152/+25) plasmid into Ishikawa cells (Fig. 5*B*). The transactivation by E2 was 9-fold with wild-type ERα ($P < 0.05$). However, the transactivation by E2 was not observed with the coexpressions of HE11 and HE38, which expressed mutant protein lacking the DNA-binding domain and the ligand-induced transcriptional activation domain AF-2, respectively. With the HE19 plasmid expressing mutant protein lacking the constitutive transcriptional activation domain AF-1, a significant ($P < 0.01$) transactivation by E2 was

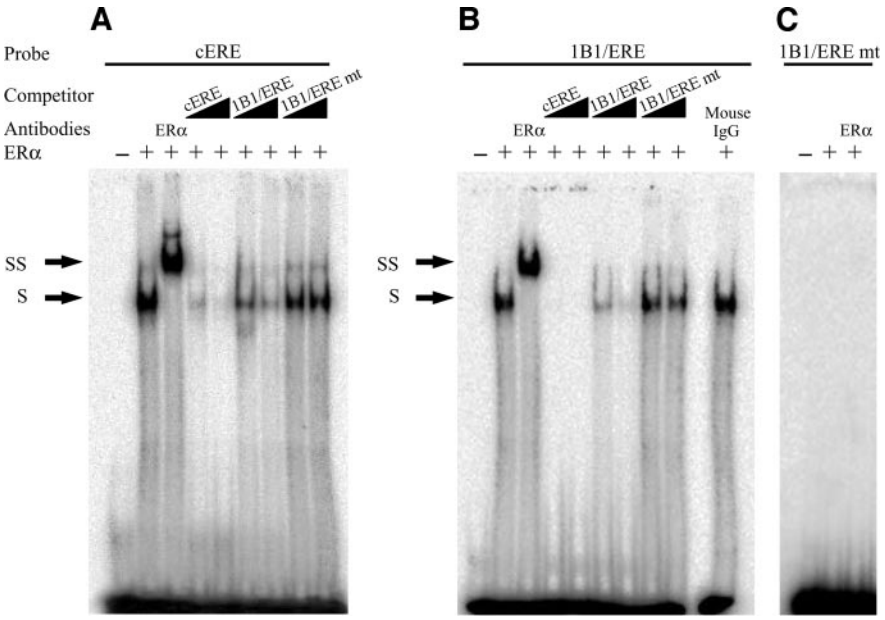

Fig. 4. Gel shift analyses of the putative estrogen response element (ERE) on the human *CYP1B1* gene with recombinant estrogen receptor (ER)α protein. Oligonucleotides of cERE (*A*), 1B1/ERE (*B*), and 1B1/ERE mt (*C*) used as probes are shown in Table 1. Cold oligonucleotides were used as a competitor in 100- and 500-fold molar excess. For super shift analyses, 2 μg of anti-ERα antibodies or normal mouse IgG were preincubated with the recombinant ERα proteins on ice for 15 min. The *lower arrow* (*S*) indicates the position of the ERα-dependent shifted band and the *upper one* (*SS*) indicates the super-shifted ERα complex.

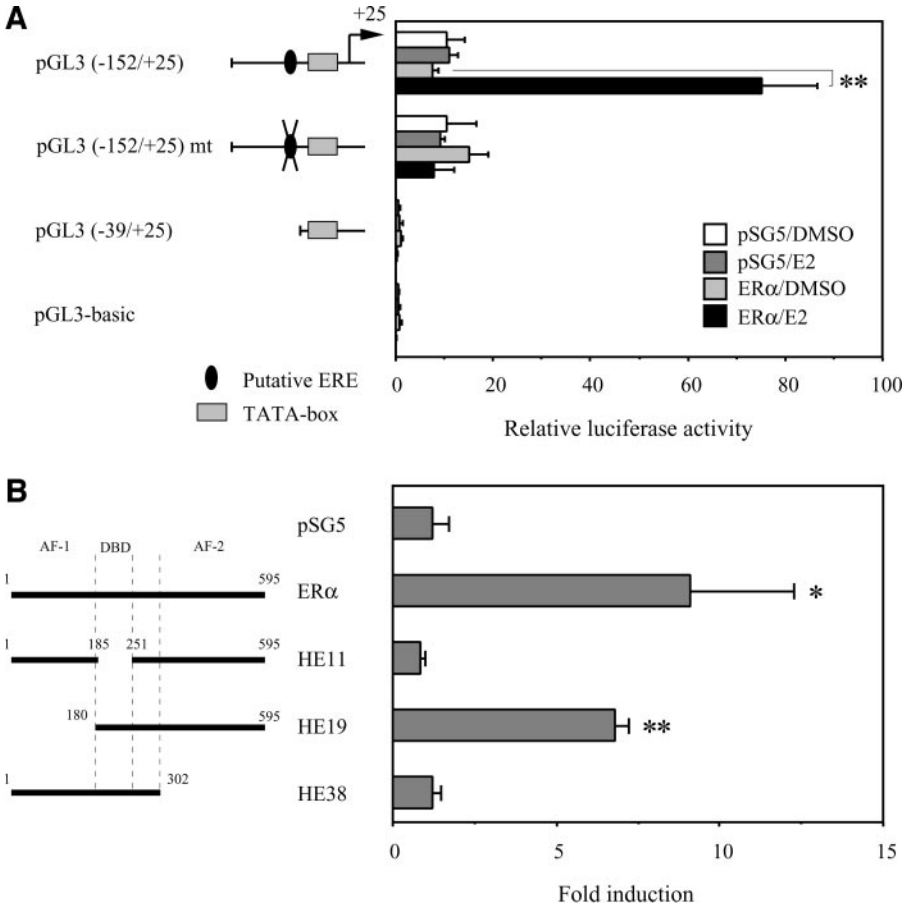

Fig. 5. Estrogen receptor (ER)α-dependent transcriptional activation of the human *CYP1B1* gene in Ishikawa cells. *A,* several deletion and mutant reporter constructs of the 5′-flanking region of the human *CYP1B1* gene were transiently transfected into Ishikawa cells with the ERα expression vector or pSG5 vector (*control*). After 24 h, the cells were treated with 0.1% DMSO or 10 nM 17β-estradiol for 24 h. The relative luciferase activity was normalized to the *Renilla* luciferase activity. Each column represents the mean of three independent experiments; *bars,* $\pm$SD (**, $P < 0.01$, compared with DMSO treatment). *B,* wild or three mutated ERα expression vectors (*EH11, EH19,* and *EH38*) were transiently transfected into Ishikawa cells with the reporter construct of the 5′-flanking region from -152 to $+25$ of the human *CYP1B1* gene. After 24 h, the cells were treated with 0.1% DMSO or 10 nM 17β-estradiol for 24 h. The relative luciferase activity was normalized with the *Renilla* luciferase activity. Each column represents the mean of three independent experiments; *bars,* $\pm$SD (*, $P < 0.05$ and **, $P < 0.01$, compared with pSG5 transfection).

observed (6.7-fold). Therefore, the DNA-binding domain and the ligand-dependent transcriptional activation domain AF-2 appeared to be required for the E2-dependent transcriptional activation of CYP1B1.

ERα Is Associated with the Putative ERE on the Human *CYP1B1* Gene in Intact Human Endometrial Epithelial Cells. Because CYP1B1 is expressed in uterus and the endometrium is highly regulated by estrogens, human endometrial glandular epithelial cells were used for ChIP assays to investigate the association of ERα with the *CYP1B1* promoter in intact cells. As shown in Fig. 6, the immunoprecipitant obtained with anti-ERα antibodies generated a distinct PCR product when the primer set for region 1 was used, but not with mouse IgG. Using the primer set for region 2, the immunoprecipitants obtained with anti-ERα antibodies or mouse IgG generated no PCR product. The association of ERα with the putative ERE on the human *CYP1B1* gene was demonstrated in human endometrial cells.

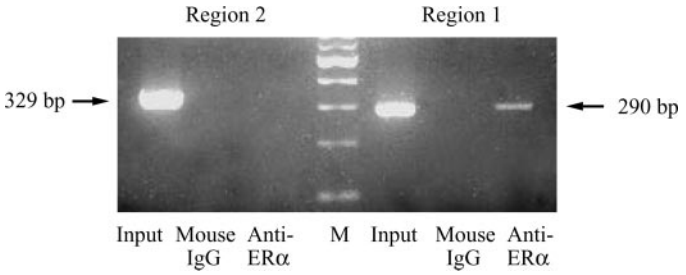

Fig. 6. Association of estrogen receptor (ER)α with the putative estrogen response element on the human *CYP1B1* gene in normal human endometrium. Chromatin immunoprecipitation assay of the 5′-flanking region of the human *CYP1B1* gene with anti-ERα antibodies was performed using the endometrial glandular epithelial cells. Isolated glandular epithelial cells were cross-linked by treatment with formaldehyde, and DNA was extracted and immunoprecipitated with anti-ERα antibodies or normal mouse IgG. After the reversal of cross-linking, the DNA was purified and used as a template for PCR to amplify target sites as shown in Fig. 3*B*. The PCR product was electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining. A definite PCR band was observed only when anti-ERα antibodies were used with the primer sets for region 1 targeting the putative estrogen response element site. *M,* 100-bp DNA ladder marker.

Expression of CYP1B1 Protein in Normal Human Endometrial Tissues Is Dependent on the Menstrual Cycle. The endometrium would be a suitable tissue to investigate the regulation of CYP1B1 by E2. Immunohistochemistry of CYP1B1 was performed in normal endometrial tissues obtained from 19 patients at different phases of the menstrual cycle. Immunostaining of CYP1B1 was detected in both the glandular epithelial or stromal cells (Fig. 7). We evaluated the staining of CYP1B1 in the glandular epithelial cells in functional layers, because they are mostly susceptible to E2. CYP1B1 protein was strongly stained in glandular epithelial cells in the proliferative phase (Fig. 6*A*). It was still apparent in the early secretory phase, but with subsequent diminishment with the progression to the secretory phase. Of 11 endometria in the proliferative phase, 6 and 5 samples were judged as ++ and +, respectively (Table 2). Of 5 endometria in the early secretory phase, 2 and 3 samples were judged as ++ and +, respectively. Three endometria in the late secretory phase were judged as $-$. The expression level of CYP1B1 in the glandular epithelial cells was significantly ($P < 0.001$) different according to the phase of the menstrual cycle (Table 2).

DISCUSSION

In the present study, we demonstrated that E2 induced the expression of human CYP1B1 mRNA in ER-positive cells, which is medi-

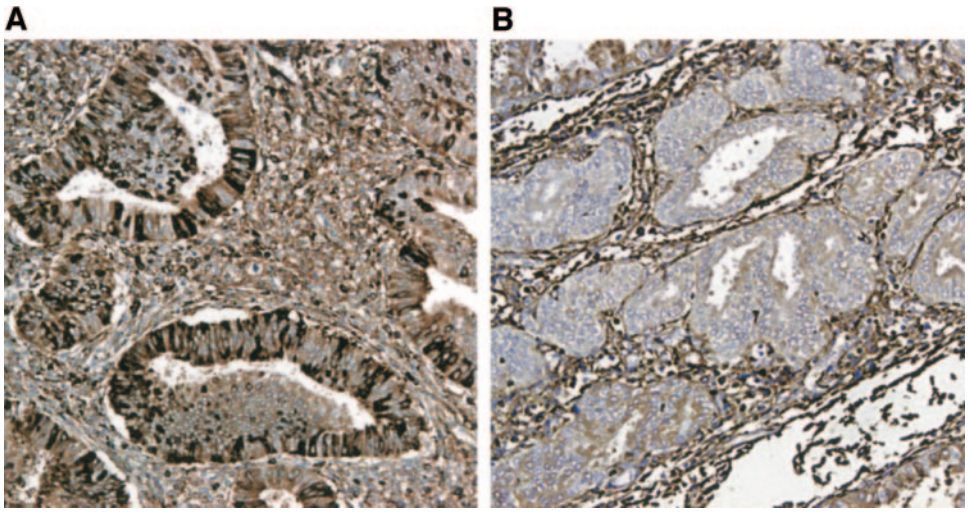

Fig. 7. Immunohistochemical staining of cytochrome P450 (CYP) 1B1 protein in normal human endometrial tissues. Representative results of immunohistochemistry of CYP1B1 in endometrial tissues during the proliferative phase (*A*) and the late secretory phase (*B*) of the menstrual cycle are shown. Each specimen was incubated with anti-CYP1B1 antibodies (*brown*). The specimens were counterstained with hematoxylin (*purple-blue*). Higher immunoreactivity of CYP1B1 is observed in glandular epithelial cells during proliferative phase (*A*) rather than those during the secretory phase (*B*). Original magnification is ×100.

ated by the direct interaction of ERα with an ERE on the *CYP1B1* gene. This is the first study in which the specific activation of estrogen-metabolizing enzyme by estrogen itself was demonstrated. The phenomenon was highly dependent of the function of the ER. Whereas ER-positive MCF-7 cells sensitively responded to E2, ER-negative MDA-MB-435 cells did not. Of particular interest is the poor responsiveness of ER-positive Ishikawa cells to E2. This cell line is well known to express ER but at low levels (21, 22). The overexpression of ERα in this cell line was able to induce CYP1B1 transcriptional activity in the presence of E2. Even MCF-7 cells that constitutively express high levels of ER exhibited dramatic transcriptional activation of CYP1B1 up to 80-fold when ER was overexpressed (data not shown). ER-antagonist 4OHT or ICI 182,780 clearly inhibited the E2-mediated CYP1B1 transcriptional activation. Taken together, these findings support that ER expression plays crucial roles in the E2-mediated activation of human CYP1B1.

It is well known that the *CYP1B1* gene is under the regulatory control of the AhR. Many polycyclic aromatic hydrocarbons are known to induce CYP1B1 and their own metabolism through binding to and activation of the AhR. A recent report (23) demonstrated that AhR might be responsible for E2-induced CYP1B1 expression in ER-positive cells. In that study, AhR expression was significantly increased in response to E2 stimulation. Because AhR is known to interact with XRE on the *CYP1B1* gene and activate the transcription without exogenous AhR ligand, it was speculated that E2-induced CYP1B1 expression was mediated by the AhR function activated by E2. Our results do not support this hypothesis for the following reasons. First, the pGL3 (−910/+25) plasmid containing two critical XRE sites (8) for AhR-dependent transactivation showed low E2-responsiveness in our reporter assays. Second, the introduction of a mutation in XRE in the pGL3 (−910/+25) plasmid did not affect the E2 responsiveness (data not shown). Third, there is no XRE sequence up to −152 bp in the *CYP1B1* gene in which prominent ER responsiveness was observed, and our ChIP assays and gel shift analyses failed to confirm binding of the AhR to the E2-response element on the *CYP1B1* promoter. We thus concluded that the ER-mediated pathway, rather than the AhR-pathway, plays essential roles in E2-induced CYP1B1 expression.

Table 2 *CYP1B1-specific staining of normal human endometrium during proliferative and secretory phase*

Staining of glands in the functionalis was evaluated as positive when >10% of the constituting epithelial cells in a gland were stained. The results were judged by three independent pathologists as − (<10% of the glands at the region of interest), + (>10%), or ++ (>10% with high intensity). The statistical significance of differences in the extent of staining in the different phases of the menstrual cycles was tested by Fisher's exact method ($P < 0.001$).

	Staining of glandular epithelial cells		
Menstrual phase	++	+	−
Proliferative (n = 11)	6	5	0
Early secretory (n = 5)	2	3	0
Late secretory (n = 3)	0	0	3

It has been reported that some estrogen-regulated genes are indirectly regulated by the cooperation of Sp1 and ER within a GC-box and ERE half site (24, 25). For the human *CYP1B1* gene, two Sp1 binding sequences have been reported to be located at −84 and −68 (26) that are near the putative ERE identified in the present study. It will be important to clarify whether Sp1 and ER cooperatively regulate the transcription of the human *CYP1B1* gene.

Our immunohistochemical analyses revealed predominant staining of CYP1B1 in endometrial glandular cells in a proliferative phase. The staining was attenuated with the progression to the secretory phase. This pattern of expression is consistent with change in the local concentration of E2 in endometrium (27), increasing E2 levels peaking at the ovulatory phase, with a subsequent decrease in the secretory phase, which supports the presence of an E2-dependent activation pathway of CYP1B1. Furthermore, it was clearly demonstrated with the ChIP assays using endometrial glandular cells that ERα binds to the *CYP1B1* promoter *in vivo*.

An interesting aspect of our result is the potential pathway of E2-induced carcinogenesis in estrogen-target tissues, especially in endometrium. CYP1B1 catalyzes the 4-hydroxylation of estrogens (9). The induced expression of CYP1B1 in the endometrium would result in the increased production of 4-hydroxyestradiol. 4-Hydroxyestradiol is a catechol estrogen that can lead to carcinogenesis by the generation of free radicals (13, 14). This notion is supported by several previous studies, showing elevated levels of 4-hydroxyestradiol in breast tumors and endometrial adenocarcinoma (15, 28). Therefore, the enhanced metabolism of the estrogens by CYP1B1 in the uterus may facilitate carcinogenesis. Of particular interest is the action of tamoxifen, a specific antagonist of ER. In our reporter assays, 4OHT effectively inhibited E2-induced CYP1B1 expression. However, in the absence of E2, this reagent exhibited an agonistic function, up-regulating the *CYP1B1* promoter. Whereas tamoxifen effectively blocks the action of E2, it increases the risk of endometrial cancer (29), although the precise molecular mechanisms remain unclear. The up-regulation of CYP1B1 by 4OHT may therefore be one component of the carcinogenesis pathway in the endometrium.

In summary, we proposed that the human *CYP1B1* gene is a novel target of estrogen. ERα plays critical roles in this regulation. Significant levels of CYP1B1 expression were observed in uterus and were tightly correlated with the local levels of E2. These findings may provide insights into the molecular mechanisms of the homeostasis of estrogens as well as estrogen-dependent carcinogenesis.

ACKNOWLEDGMENTS

We thank Dr. William A. LaMarr of University of Massachusetts Medical School (Worcester, MA) for providing pGL3-basic plasmid containing 5′-flanking region of human *CYP1B1* gene. We are grateful to Dr. Pierre Chambon of Institut de Genetique et de Biologie Moleculaire et Cellulaire (Strasbourg, France) for the gift of ERα expression vectors (pSG5-HE0, HE11, HE19, and HE38). We also thank Dr. Masato Nishida of Tsukuba University (Ibaraki, Japan) for providing Ishikawa cells. We acknowledge Brent Bell for reviewing the manuscript.

REFERENCES

1. Gonzalez FJ. The molecular biology of cytochrome P450s. Pharmacol Rev 1988;40:243–88.
2. Guengerich FP, Shimada T. Oxidation of toxic and carcinogenic chemicals by human cytochrome P-450 enzymes. Chem Res Toxicol 1991;4:391–407.
3. Sutter TR, Tang YM, Hayes CL, et al. 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 1994;269:13092–9.
4. Shimada T, Hayes CL, Yamazaki H, et al. Activation of chemically diverse procarcinogens by human cytochrome P450 1B1. Cancer Res 1996;56:2979–84.
5. Iwanari M, Nakajima M, Kizu R, Hayakawa K, Yokoi T. 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 2002;76:287–98.
6. Hankinson O. The aryl hydrocarbon receptor complex. Annu Rev Pharmacol Toxicol 1995;35:307–40.
7. Schmidt JV, Bradfield CA. Ah receptor signaling pathways. Annu Rev Cell Biol 1996;12:55–89.
8. Tsuchiya Y, Nakajima M, Yokoi T. Critical enhancer region to which AhR/ARNT and Sp1 bind in the human *CYP1B1* gene. J Biochem (Tokyo) 2003;133:583–92.
9. Hayes C, Spink D, Spink B, Cao J, Walker N, Sutter T. 17β-Estradiol hydroxylation catalyzed by human cytochrome P450 1B1. Proc Natl Acad Sci USA 1996;93:9776–81.
10. Spink DC, Spink BC, Cao JQ, et al. Induction of cytochrome P450 1B1 and catechol estrogen metabolism in ACHN human renal adenocarcinoma cells. J Steroid Biochem Mol Biol 1997;62:223–32.
11. Lee AJ, Cai MX, Thomas PE, Conney AH, Zhu BT. Characterization of the oxidative metabolites of 17β-estradiol and estrone formed by 15 selectively expressed human cytochrome p450 isoforms. Endocrinology 2003;144:3382–98.
12. Hakkola J, Pasanen M, Pelkonen O, et al. Expression of CYP1B1 in human adult and fetal tissues and differential inducibility of CYP1B1 and CYP1A1 by Ah receptor ligands in human placenta and cultured cells. Carcinogenesis 1997;18:391–7.
13. Han X, Liehr JG. DNA single-strand breaks in kidneys of Syrian hamsters treated with steroidal estrogens: hormone-induced free radical damage preceding renal malignancy. Carcinogenesis 1994;15:997–1000.
14. Newbold RR, Liehr JG. Induction of uterine adenocarcinoma in CD-1 mice by catechol estrogens. Cancer Res 2000;60:235–7.
15. Liehr JG, Ricci MJ, Jefcoate CR, Hannigan EV, Hokanson JA, Zhu BT. 4-Hydroxylation of estradiol by human uterine myometrium and myoma microsomes: implications for the mechanism of uterine tumorigenesis. Proc Natl Acad Sci USA 1995;92:9220–4.
16. Kumar V, Green S, Staub A, Chambon P. Localisation of the oestradiol-binding and putative DNA-binding domains of the human oestrogen receptor. EMBO J 1986;5:2231–6.
17. Kumar V, Green S, Stack G, Berry M, Jin JR, Chambon P. Functional domains of the human estrogen receptor. Cell 1987;51:941–51.
18. Kyo S, Takakura M, Kanaya T, et al. Estrogen activates telomerase. Cancer Res 1999;59:5917–21.
19. Tanaka M, Kyo S, Takakura M, et al. Expression of telomerase activity in human endometrium is localized to epithelial glandular cells and regulated in a menstrual phase-dependent manner correlated with cell proliferation. Am J Pathol 1998;153:1985–91.
20. Muskhelishvili L, Thompson PA, Kusewitt DF, Wang C, Kadlubar FF. In situ hybridization and immunohistochemical analysis of cytochrome P450 1B1 expression in human normal tissues. J Histochem Cytochem 2001;49:229–36.
21. Ignar-Trowbridge DM, Teng CT, Ross KA, Parker MG, Korach KS, McLachlan JA. Peptide growth factors elicit estrogen receptor-dependent transcriptional activation of an estrogen-responsive element. Mol Endocrinol 1993;7:992–8.
22. Ignar-Trowbridge DM, Pimentel M, Teng CT, Korach KS, McLachlan JA. Cross talk between peptide growth factor and estrogen receptor signaling systems. Environ Health Perspect 1995;103:35–8.
23. Spink DC, Katz BH, Hussain MM, Pentecost BT, Cao Z, Spink BC. Estrogen regulates Ah responsiveness in MCF-7 breast cancer cells. Carcinogenesis 2003;24:1941–50.
24. Petz LN, Nardulli AM. Sp1 binding sites and an estrogen response element half-site are involved in regulation of the human progesterone receptor A promoter. Mol Endocrinol 2000;14:972–85.
25. Saville B, Wormke M, Wang F, et al. Ligand-, cell-, and estrogen receptor subtype (α/β)-dependent activation at GC-rich (Sp1) promoter elements. J Biol Chem 2000;275:5379–87.
26. Wo, Y-YP, Stewart J, Greenlee WF. Functional analysis of the promoter for the human *CYP1B1* gene. J Biol Chem 1997;272:26702–7.
27. Alsbach GP, Franck ER, Poortman J, Thijssen JH. Subcellular distribution of estradiol and estrone in human endometrium and myometrium during the menstrual cycle. Contraception 1983;27:409–21.
28. Lemon HM, Heidel JW, Rodriguez-Sierra JF. Increased catechol estrogen metabolism as a risk factor for nonfamilial breast cancer. Cancer 1992;69:457–65.
29. Levenson AS, Jordan VC. Selective oestrogen receptor modulation: molecular pharmacology for the millennium. Eur J Cancer 1999;35:1974–85.

Human *CYP1B1* Is Regulated by Estradiol via Estrogen Receptor

Yuki Tsuchiya, Miki Nakajima, Satoru Kyo, et al.

Cancer Res 2004;64:3119-3125.