Estrogen Receptor α, Fos-Related Antigen-2 and c-Jun co-ordinately regulate human UDP glucuronosyltransferase 2B15 and 2B17 expression in response to 17β-estradiol in MCF-7 cells.

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**ABBREVIATIONS:** AP-1; activator protein 1; AR, androgen receptor; BSA, bovine serum albumin; CCD, charcoal dextran; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assays; ER, estrogen receptor; ERE, estrogen response element; ERU, estrogen response unit; FBS, fetal bovine serum; Fra-2, Fos related antigen 2; GREB, gene regulated in breast cancer; PBS, phosphate buffered saline; PMA, phorbol 13-myristate 12-acetate; RT-PCR, reverse transcription-polymerase chain reaction; TPA, 12-O-tetradecanoylphorbol 13-acetate; TRE, TPA response element; UGT, UDP-glucuronosyltransferase.
ABSTRACT

UDP-glucuronosyltransferase 2B15 and 2B17 expression is up-regulated by 17β-estradiol in MCF-7 breast cancer cells, as assessed by quantitative real-time PCR. Using 5′-deletion mapping and site-directed mutagenesis, we demonstrate that 17β-estradiol activation of UGT2B15 gene transcription is mediated by a 282-bp fragment positioned -454 to -172 nucleotides from the translation start site. This region contains two putative activator protein-1 (AP-1) elements, one imperfect estrogen response element (ERE), and two consensus ERE half-sites. We propose that these five sites act as an estrogen response unit (ERU), as mutation in any site reduces activation of the UGT2B15 promoter by 17β-estradiol. Despite the presence of two AP-1 elements, the UGT2B15 promoter is not responsive to the AP-1 activator, phorbol 13-myristate 12-acetate. Although electrophoretic mobility shift assays (EMSA) indicate that the AP-1 proteins, c-Jun and Fos-related antigen 2 (Fra-2), bound to the distal AP-1 site, binding of Jun or Fos family members to the proximal AP-1 site was not detected by EMSA. Chromatin immunoprecipitation (ChIP) assays showed a 17β-estradiol-induced recruitment of estrogen receptor (ER) α, c-Jun, and Fra-2 to the 282-bp ERU. The involvement of these three transcription factors in the stimulation of UGT2B15 gene expression by 17β-estradiol was confirmed by siRNA silencing experiments. Mutagenesis and siRNA experiments indicate that UGT2B17 expression is also regulated by 17β-estradiol via the ERU, which is fully conserved in both promoters. As UGT2B15 and UGT2B17 inactivate steroid hormones by glucuronidation, the regulation of their genes by 17β-estradiol may maintain steroid hormone homeostasis and prevent excessive estrogen signalling activity.
Estrogen signalling is mediated by estrogen receptors (ERα and ERβ) via genomic and non-genomic pathways (Marino et al., 2006). In the classic genomic pathway, ER binds as a dimer to an estrogen response element (ERE) in the regulatory regions of target genes to control gene transcription. The ideal or consensus ERE is a 13-bp palindrome (5′-GGTCAnnnTGACC-3′; n, any nucleotide), which was first discovered in the 5′-flanking region of the Xenopus vitellogenin A2 gene (Klein-Hitpass et al., 1986). However, genomic regions termed Estrogen Response Units (ERU) are also capable of enhancing transcription in the presence of estrogen. These ERUs may extend over several hundred nucleotides and contain many imperfect EREs (these deviate by 1-3bp from the consensus) and ERE half-sites, which act synergistically to achieve high estrogen inducibility (Klein-Hitpass et al., 1988; Klinge, 2001). Indeed, mapping of human ER binding sites in the MCF-7 genome showed that 25% of the identified in vivo ERα binding sites bore only half-EREs or imperfect EREs (Klinge, 2001; Lin et al., 2007). ER can also regulate gene transcription by modulating the activities of other transcriptional factors, such as activator protein-1 (AP-1), nuclear factor-kappa B and stimulating protein-1 (Kushner et al., 2000; Marino et al., 2006; Safe and Kim, 2008). In this non-classical pathway, ER does not bind DNA, but interacts with bound transcription factors via protein-protein contacts to stabilize DNA-protein complexes and/or recruit co-regulators.

Signalling initiated by 17β-estradiol binding to ER, is essential for epithelial cell proliferation and ductal development in the breast. Unfortunately, this capacity of 17β-estradiol to stimulate cell proliferation is also a contributor to breast cancer development and progression (Bocchinfuso and Korach, 1997). The factors that control the balance between these beneficial and potentially adverse effects of 17β-estradiol on the breast are poorly
understood, but appear to depend on complex interactions between both male and female hormones and growth factors.

Androgen signalling through the androgen receptor is an important modifier of estrogen action (Murphy and Watson, 2002). Breast cells contain androgen receptor (AR) and accumulating evidence supports a significant role for androgens as negative modulators of estrogen action in the mammary gland. This evidence includes their capacity to antagonize the effects of estrogens in normal breast development (i.e., to act as anti-estrogens), and their ability to inhibit the basal and estradiol-stimulated growth of AR-positive breast cancer cell lines, including MCF-7 (Ando et al., 2002). Given this antagonistic interaction between androgen and estrogen signalling pathways in breast cells, the differential inactivation and elimination of androgens and estrogens may be an important determinant of the cell or organ’s response to these hormones.

The capacity of steroid hormones to bind to their receptors is inhibited by glucuronidation. In addition, steroid glucuronides are more hydrophilic and more readily excreted than steroids. These two outcomes of glucuronidation ensure termination of steroid signalling effects, as aptly illustrated in the prostate (Chouinard et al., 2007). There are 19 functional human UGTs grouped into one family containing 9 members (UGT1A1, 1A3-10) and a second composed of 10 members (UGT2A1-3, UGT2B4, 2B7, 2B10, 2B11, 2B15, 2B17 and 2B28) (Mackenzie et al., 2005). However, only a few of these 19 UGTs glucuronidate biologically active steroids to any significant extent. The active androgens, testosterone and dihydrotestosterone are exclusively glucuronidated by UGT2B15 and UGT2B17, whereas the biologically active estrogen, 17β-estradiol, is primarily glucuronidated by UGT1A1, 1A3 and 1A10 (Chouinard et al., 2007; Chouinard et al., 2006; Itaaho et al., 2008). The 17β-OH group of the active androgens is the site of glucuronidation,
whereas the major metabolite of 17β-estradiol is the 3-OH glucuronide, although recent evidence suggests that UGT2B7 and UGT2B17 are also quite active in forming the 17β-OH glucuronide of this estrogen (Itaaho et al., 2008). Given that UGT2B15 and 2B17 are the only UGTs capable of terminating the effects of active androgens, the mechanisms that control their expression in breast are likely to be of considerable importance in modulating this organ’s response to hormones. However, little is known about these mechanisms.

A recent study reported that UGT2B15 expression in MCF-7 cells is upregulated by estradiol (Harrington et al., 2006). However, the mechanisms mediating this regulation have not been identified. In the present study we show that UGT2B17, in addition to UGT2B15 is induced by 17β-estradiol in MCF-7 cells, and demonstrate that this process is mediated by an ERU containing multiple AP-1 and ERE elements, which are completely conserved in both promoters.
MATERIALS AND METHODS

Cell culture, RNA extraction and reverse transcription

The breast cancer cell line, MCF-7, obtained from the American Type Culture Collection (ATCC) was maintained in RPMI medium 1640 (Invitrogen) supplemented with 5% (v/v) fetal bovine serum (FBS) at 37°C in a 5% CO₂ atmosphere. For 17β-estradiol treatments, MCF-7 cells were cultured for 3 days in phenol red-free RPMI medium containing 5% charcoal-dextran (CCD)-stripped FBS and then plated into 6-well plates at a density of 5x10⁵ cells/well in 3 ml of fresh medium. After 72 h, cells were treated with either 10 nM 17β-estradiol or 0.1% ethanol (vehicle) for a further 24 h. Total RNA was extracted using the RNeasy Midi Kit (Qiagen) according to the manufacturer’s instructions. Reverse transcription (RT) was carried out using reagents from Invitrogen. Briefly, total RNA (~1 µg) was treated with DNase I at room temperature for 15 min, and then reverse-transcribed at 50°C for 50 min in a final volume of 20 µl of 1x first strand buffer (50 mM Tris-HCl, pH 8.0, 75 mM KCl, and 3 mM MgCl₂) containing random hexamer primers, 50 units of RNaseOut recombinant ribonuclease inhibitor, and 50 units of Superscript™ III. The resulting cDNAs were treated by 50 units of RNase H at 37°C for 20 min and diluted 5 times in RNase-free H₂O prior to quantitative real-time PCR as described below.

Quantitative real-time PCR

Most of real-time PCR primers used in this study for quantifying mRNAs of UGT enzymes were previously published (Congiu et al., 2002), including both forward and reverse primers for 18S rRNA and UGT1A1, 1A4, 1A6, 1A9, 2B4, 2B7, 2B10, 2B11, 2B15, and 2B17, and the reverse primers for UGT1A10 and 2B28. The forward primer for UGT1A10 was described elsewhere by (Strassburg et al., 1997). Primers for UGT1A3, 1A7, and 1A8,
and the forward primer for UGT2B28 were designed in this study as shown in Table 1. A set of gene-specific reference templates were prepared to generate standard curves for quantification. Templates containing full length target cDNAs included pEF-IRES-derived templates (UGT1A3, 1A7, 1A8, and 1A10), a pBS-derived template for UGT1A4, and a pCR-blunt-template for UGT2B28. The templates for the remaining target genes (18S rRNA, UGT1A1, 1A6, 1A9, 2B4, 2B7, 2B10, 2B11, 2B15, and 2B17) were all pCR-blunt-derived plasmids containing a single copy of the gene-specific RT-PCR product amplified from cDNA samples as described above. All primers were synthesized by Sigma-genosys (Castle Hill, New South Wales, Australia).

Real-time PCR was performed using a RotorGene 3000 instrument (Corbett Research, NSW, Australia) in 20 µl of 1x QuantiTect Sybr Green PCR master mix (Sigma-Aldrich) containing 2 µl (~40 ng) of each aforementioned cDNA sample and a pair of gene-specific primers (~500 nM for each primer). The amplification conditions consisted of an initial activation step of 95°C for 15 min, and 40 cycles of 95°C for 10 seconds, 56-60°C for 15 seconds, and 72°C for 20 seconds. Data were obtained during the 72°C extension phase of each cycle and analysed by the program RotorGene 6.1. Each sample was amplified in duplicate and the resultant mean values were used for analysis. Four serial 10x diluted samples containing known copy numbers (e.g., 6000, 600, 60, and 6 for all UGTs) of gene-specific reference templates were included in each real-time PCR to generate a standard calibration curve, in order to calculate copy numbers of target transcripts in the samples amplified in the same run. Copy numbers of 18S rRNA transcripts were used as a reference to normalize the amount of total RNA amplified in each reaction. Quantification of mRNA expression was expressed as the relative copy number of each target transcript per $10^3$ or $10^9$ copies of 18S rRNA transcript.
Construction of Luciferase reporter constructs

For the sake of simplicity, the “A” of the initiation codon (ATG) was numbered +1 to describe the positions of nucleotides in the promoter throughout this article. The fragment from nucleotides -3 to -2716 of the UGT2B15 promoter was amplified from human genomic DNA (Roche) by PCR with primers 2B15-2716For and 2B15-3Rev (Table 1) and ligated into the KpnI and MluI sites of pGL3-basic vector to generate the luciferase reporter construct (2B15-2716/-3Luc). The promoter region from nucleotides -3 to -747 was amplified from this construct with primers 2B15-747For and 2B15-3Rev (the common reverse primer) (Table 1) and cloned into the same position of pGL3-basic vector to create the construct 2B15-747/-3Luc. Constructs of 2B15-705/-3Luc, 2B15-595/-3Luc, 2B15-556/-3Luc, 2B15-458/-3Luc, 2B15-412/-3Luc, 2B15-253/-3Luc, 2B15-202/-3Luc, 2B15-154/-3Luc, and 2B15-54/-3Luc were prepared in a similar manner using the construct 2B15-747/-3Luc as a template and specific primers as detailed in Table 1. The promoter sequences of all reporter constructs were confirmed by sequencing.

Mutagenesis of putative AP-1 and ERE binding sites in reporter constructs

Mutagenesis was carried out using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and complementary pairs of oligonucleotide primers with the desired mutation(s) in the middle of the primer. The sense sequences of the primers are detailed in Table 1. The mutation from “TGAGTCA” to “CAGAGCA” at the 5’-AP-1 site with the primer 5’AP-1m and the mutation from “TGTGTCA” to “CAGAGCA” at the 3’-AP-1 site using the primer 3’AP-1m were separately or simultaneously introduced into three different UGT2B15 promoter constructs, namely 2B15-458/-3Luc (Fig. 3B, MT-6, MT-7, and MT-8), 2B15-747/-3Luc (Fig. 3B, MT-9, MT-10, and MT-11), and 2B15-2716/-3Luc (Fig. 3B, MT-12, MT-13, and MT-14). The 3’-ERE half-site was mutated from “GGTCA” to
“AAAAA” in both 2B15-412/-3Luc (Fig. 3B, MT-1) and 2B15-458/-3Luc (Fig. 3B, MT-3) using the primer 3’EREhm. The imperfect ERE full-site mutation from “TGTCAaggGCACC” to “AAAAAGaggGCACC” was introduced into both 2B15-412/-3Luc (Fig. 3B, TM-2) and 2B15-458/-3Luc (Fig. 3B, TM-5) with the primer EREfm. The 5’-ERE half-site of “GGTCA” was changed to “TTTTT” in 2B15-458/-3Luc (Fig. 3B, MT-4) using the primer 5’EREhm. The three sites, 3’-AP-1, 5’-AP-1, and the 3’-ERE half-site were all mutated in TM-15 (Fig. 3B) by introducing a mutation at the 3’-ERE half-site in the mutant TM-8 (Fig. 3B). A mutation was created in the imperfect ERE full site in TM-8 to generate the mutant TM-16 (Fig. 3B), which contained mutations at both AP-1 sites and the imperfect ERE full-site. Additionally, the proximal AP-1 site (starting at nucleotide -372) of the UGT2B17 promoter in the construct 2B17-650/+42Luc was mutated using primer 3’AP-1mα to create the mutant 2B17-650/+42LucMut (Fig. 9B). The identities of all mutants were verified by sequencing.

**Transient transfection**

MCF-7 cells were plated into 96-well plates at a density of 2.5 x 10⁴ cells/well in 200 µl of phenol red-free RPMI with 6% FBS. After 24 h, cells were washed twice using 1x PBS prior to transfections. Transfections were carried out in 50 µl of phenol red-free RPMI without serum using 100 ng of each reporter construct with (co-transfection) or without 2.5 ng of ERα expression plasmid HEGO/pSG5 and 0.4 µl of LipofectAMINE2000 per transfection according to the manufacturer’s instructions (Invitrogen). After 5 h, the transfection mix was removed and cells were treated with either 10 nM 17β-estradiol or 0.1% ethanol (vehicle). 40 h post-treatment, cells were harvested in 100 µl of 1x passive lysis buffer and assayed for firefly luciferase activities using the Luciferase Reporter Assay system (Promega) and a Packard TopCount luminescence and scintillation counter (Parkard, Mt Waverly, Victoria,
Australia). The plasmid HEGO/pSG5 contained the full cDNA sequence of human estrogen receptor alpha cloned into the pSG5 vector and was a kind gift from Dr Amelia Peters of the University of Adelaide, Australia.

**Electrophoretic mobility shift and supershift assays**

Nuclear extracts were prepared from MCF-7 cells as previously reported (Gardner-Stephen et al., 2005) except that cells were preincubated for 6 days in phenol red-free RPMI supplemented with 5% CCD-stripped FBS and treated for a further 24 h with 10 nM 17β-estradiol. Recombinant human ERα was prepared with the ERα expression plasmid HEGO/pSG5 using the TNT Quick Coupled Transcription/Translation kit according to the manufacturer’s instructions (Promega). The probes for electrophoretic mobility shift assays (EMSAs) were generated with the same primers as used in mutagenesis, except that a different pair of primers was designed for the probe analysing the proximal AP-1 site (Table 1). Briefly, probes were prepared by annealing complementary sense and antisense oligonucleotides, followed by end-labelling with (γ-32P) ATP (Perkin Elmer) using T4 polynucleotide kinase (New England Biolabs) and purification through G25 columns (GE Healthcare, Australia). The EMSA reactions consisted of 1 µg of poly (dI-dC) (Sigma) and either 5 µg of MCF-7 nuclear extract or 1 µl of in vitro transcribed/translated ERα protein in a 15 µl TM-buffer containing 25 mM Tris-HCl, pH 7.6, 100 mM KCl, 5 mM MgCl₂, 0.5 mM EDTA, and 10% Glycerol. After incubation on ice for 10 min, 50,000 cpm of probe (~1 ng) was added and the reaction was incubated at room temperature for a further 30 min. For competitive assays, unlabelled wild type or mutated probes were added into the reaction in a 10-100 molar excess of labelled probe before the initial 10-min incubation on ice. For supershift assays, 2 µg of antibody (~1 µl) was added immediately after the addition of labelled probes. DNA-protein complexes were resolved in 0.5x Tris borate-EDTA on 4%
nondenaturing polyacrylamide gels. The gels were dried and exposed to X-ray films followed by autoradiography. All antibodies were purchased from Santa Cruz Biotechnology (USA), including anti-ERα (HC-20, sc-543), anti-c-fos (H125, sc-7202), anti-c-fos (K-25, sc-253), anti-c-fos (4) (sc-52), anti-Fra-1 (N-17, sc-48), anti-Fra-2 (L-15, sc-171), FosB (102, sc-48), anti-c-Jun (N, sc-45), and anti-c-Jun (D, sc-44). As shown in Fig. 4, the two specific anti-c-Fos antibodies, namely H125 and (4) sc-52, were designated as Ab_c-Fos1 and Ab_c-Fos2, respectively. The anti-c-fos (K-25, sc-253) was designated as Ab_Fos family as it recognizes all Fos family members, whereas the anti-c-Jun (D, sc-44) recognizes all Jun family members, hence it was designated Ab_Jun-family.

**siRNA knockdown experiments**

SiGenome SMARTpool siRNAs were purchased from Dharmacon (USA), including siRNAs directed against ERα (M-003401-04), c-Jun (M-003268-03), and Fra-2 (M-004110-00), and the scramble siRNA (non-targeting siRNA Pool #1 D-001206-13) as a negative control. MCF-7 cells pre-cultured in phenol red-free RPMI medium supplemented with 5% CCD-stripped FBS for 4 days were plated into 6-well plates at a density of 5x10⁵ cells per well in 1.5 ml of fresh medium containing 8 ul of LipofectAMINE2000 (Invitrogen) and 100 nM target siRNA. 5 h post-transfection, the medium was changed and cells were cultured in fresh phenol red-free RPMI supplemented with 5% CCD-stripped FBS. 48 h post-transfection, cells were treated with 10 nM 17β-estradiol for a further 24 h and then harvested for assessing the mRNA levels of each target gene by quantitative real time RT-PCR with gene-specific primers (Table 1).
Chromatin immunoprecipitation assay

MCF-7 cells were cultured for 6 days in phenol red-free RPMI containing 6% CCD-stripped FBS and treated with either 10 nM 17β-estradiol or 0.1% ethanol (vehicle) for 2 h or 24 h. Cross-linking was achieved by treating the cells with 1% formaldehyde at 37°C for 30 min and subsequently quenched by incubating the cells at 37°C for 10 min in 1x PBS solution containing 125 mM glycine. Cells from three T175 flasks (~90% confluence) were washed twice with 1x PBS and lysed in 10 ml of buffer (15 mM Tris-HCl, pH 8.0, 1% Nonidet P40, 0.5 mM EGTA, 15 mM NaCl, 60 mM KCl, 300 mM sucrose, and 0.5 mM β-mercaptoethanol) containing 1x protease inhibitor mixture (Roche). The resultant lysates were incubated on ice for 10 min and nuclei pellets were collected by centrifugation at 4000 rpm at 4°C for 10 min. The pellets were resuspended in 1.5 ml of nuclear lysis buffer (1% SDS, 10 mM EDTA, 0.5 mM PMSF, and 50 mM Tris-HCl, pH 8.0) containing 1x protease inhibitor mixture (Roche), and incubated on ice for at least 15 min, followed by sonication to yield fragments ranging in size from 500 bp to 1200 bp. Supernatants of the sonicated products were collected by centrifugation at 14000 rpm for 10 min at 4°C, diluted 6 times in dilution buffer (16.7 mM Tris-HCl, pH 8.0, 0.01% SDS, 1% Triton X-100, 1.2 mM EDTA, 150 mM NaCl, and 1x protease inhibitor mixture), and then immuno-precleared at 4°C for 2 h with 300 µl of 50% Protein A Sepharose CL-4B (GE Healthcare, Australia) containing 50 µg of salmon sperm DNA and 20 µg of BSA. The pre-cleared chromatin samples were recovered after centrifugation at 4°C for 1 min, and ~ 0.8 ml aliquots saved as input DNA or subjected to immuno-precipitation at 4°C overnight with 10 µg of each antibody, or equivalent amounts of pre-immune IgG serum (designated as IgG) as a negative control. After antibody treatment, 100 µl of 50% protein A Sepharose CL-4B containing 17 µg of salmon sperm DNA and 7 µg of BSA was added to each sample, and the incubation was continued for another 2 h. The precipitates were collected by centrifugation as described above and then washed sequentially
for 1 min, twice in dilution buffer, high salt buffer (16.7 mM Tris-HCl, pH 8.0, 0.01% SDS, 1% Triton X-100, 1.2 mM EDTA, and 500 mM NaCl), LiCl buffer (16.7 mM Tris-HCl, pH 8.0, 0.01% SDS, 1% Triton X-100, 1.2 mM EDTA, and 250 mM LiCl), and TE buffer (16.7 mM Tris-HCl, pH 8.0 and 1.2 mM EDTA). The protein-DNA complexes were eluted from the beads in 200 µl of elution buffer (1% SDS and 0.1 M NaHCO₃). Cross-linking was reversed by heating the eluates at 65°C overnight. The supernatants containing the precipitated DNA were collected after centrifugation and digested with proteinase K at 37°C for 6 h, followed by phenol-chloroform extraction and ethanol precipitation. The DNA pellets were resuspended in 50 µl of TE buffer. Quantitative real-time PCR was performed using 2 µl of each of the resultant DNA samples and primers (Table 1), which were respectively specific to the *UGT2B15* promoter, the coding region of the *HCG3* gene (NM_001001394) on chromosome 2 (2q37) as a reference gene for data normalization, or the promoter of the *trefoil factor 1* gene (*pS2/TFF1*) as a positive control. Data obtained from the *HCG3* gene were used to normalize the starting amounts of the immuno-precipitated DNA samples seeded in each PCR reaction.

**Phorbol 13-myristate 12-acetate treatment and analysis of gene expression**

Phorbol 13-myristate 12-acetate (PMA, Sigma) (100 µM) was prepared with ethanol as a stock solution. MCF-7 cells were pre-incubated for 3 days in phenol red-free RPMI with 6% CCD-stripped FBS, and then plated into 6-well plates in the same medium. Three days post-plating, cells were treated respectively with 0.1% ethanol (vehicle), 100 nM PMA for 6 h, 10 nM 17β-estradiol for 24 h, and a combination of 10 nM 17β-estradiol for 24 h and 100 nM PMA for 6 h. After treatment, cells were harvested, total RNA was extracted and target mRNAs were quantified by quantitative real-time RT-PCR as above.
RESULTS

17β-Estradiol induces expression of UGT2B15 and UGT2B17 in MCF-7 cells.

A recent study identified the human UGT2B15 gene as an estrogen-responsive gene in MCF-7 breast cancer cells (Harrington et al., 2006). To see whether estrogens also regulate other UGT genes, we treated MCF-7 cells for 24 h with 0.1% ethanol (vehicle) or 17β-estradiol at three different concentrations (0.1 nM, 1 nM, and 10 nM), and then quantified the mRNA levels of eight UGT1A and seven UGT2B enzymes by quantitative real-time RT-PCR.

Vehicle-treated MCF-7 cells contained high levels of UGT1A6 mRNA, low levels of UGT1A1, 1A4, 1A7, 1A8, 1A9, 1A10, and 2B15 mRNA, and barely detectable levels of UGT2B17 mRNA. The mRNA levels for UGT1A3, 2B4, 2B7, 2B10, 2B11, and 2B28 were below the detectable level (Fig. 1). As expected, treatment of MCF-7 cells with 17β-estradiol at all three concentrations (0.1 nM, 1 nM, and 10 nM) elevated UGT2B15 mRNA levels by approximately 39, 63, and 72 fold, respectively, as compared to that in the absence of 17β-estradiol (Fig. 1). Surprisingly, treating MCF-7 cells with 0.1 nM 17β-estradiol also increased UGT2B17 mRNA levels by 21 fold as compared to that in vehicle-treated cells. This 17β-estradiol-mediated induction was further enhanced by 2 and 3.7 fold in cells treated with 17β-estradiol at 1 nM and 10 nM, respectively (Fig. 1). However, the levels of UGT2B17 mRNA were much lower than those of UGT2B15, both in the presence and absence of 17β-estradiol (Fig. 1). Treatment with 17β-estradiol (0.1–10 nM) had little effect on the mRNA levels of the remaining thirteen UGT enzymes and β-actin, a house-keeping gene used as an internal control (Inset of Fig. 1).
Overall, it appears that not only UGT2B15, but also UGT2B17 are estrogen-targeted genes, as both are up-regulated by 17β-estradiol in a dose-dependent manner in MCF-7 cells. As UGT2B15 expression was much higher than UGT2B17 expression, this study focused on UGT2B15 in an attempt to define the mechanism underlying this selective 17β-estradiol-induced UGT expression.

The proximal promoter of the UGT2B15 gene responds to 17β-estradiol through an Estrogen Response Unit containing multiple AP-1 and estrogen response elements

To determine whether induction of UGT2B15 expression by 17β-estradiol is mediated through the UGT2B15 promoter, a series of proximal promoter fragments of various lengths were incorporated into the luciferase gene reporter pGL3-basic vector and tested for their capacity to drive transcription in MCF-7 cells in transient transfection experiments.

In the absence of 17β-estradiol, the UGT2B15 promoter constructs tested gave very low basal activities of only 2- to 5-fold over that of the negative control, the promoterless pGL3-basic vector (Fig. 2). Stimulation by 17β-estradiol (10 nM) had small effects on the activities of promoters ≤ 412 bp in length, whereas the activities of promoters ≥ 458 bp in length were markedly increased, with the construct 2B15-458/-3Luc giving the highest activity of about 18-fold compared to that of pGL3-basic. Treatment by 17β-estradiol in combination with transfected ERα further increased the activities of promoters ≥ 458 bp in length. The activity of construct 2B15-458/-3Luc was 200-fold greater than that of the control pGL3-basic vector. This was equivalent to a 36-fold induction in promoter activity over that of the controls treated with vehicle alone. These results suggested the presence of estrogen-
responsive cis-activating element(s) in the promoter region from nucleotides -412 to -458. Sequence analysis of this 47-bp region showed two putative AP-1 sites (separated by 16 bp); one consensus site from nucleotides -448 to -454 (5'-TGAGTCA-3') (designated as 5'-AP-1) and the other from nucleotides -425 to -431 (5'-TGTGTCA-3') (designated as 3'-AP-1). The latter deviates from the consensus by only one nucleotide (Fig. 3A).

We mutated the two AP-1 sites separately or both together in three promoter constructs of varying lengths, namely 2B15-458/-3Luc, 2B15-747/-3Luc, and 2B15-2716/-3Luc (Fig. 3B), and examined the effects of these mutations on 17β-estradiol/ERα-activated promoter activities. Compared to that (set at a value of 100%) of the wild-type (WT) constructs, activities were decreased to 21-32% for mutants carrying mutations at either the 5'-AP-1 site (MT-6, MT-9, and MT-12) or the 3'-AP-1 site (MT-7, MT-10, and MT-13), and further, to about 4-10% for mutants with mutations at both AP-1 sites (MT-8, MT-11, and MT-14). These results demonstrate the importance of both AP-1 sites in mediating 17β-estradiol-stimulated UGT2B15 expression. To the best of our knowledge, this is the first evidence that a native promoter responds to 17β-estradiol via two adjacent AP-1 binding sites.

The activities of constructs carrying a promoter region between nucleotides -154 and -412 were also moderately enhanced by 5- to 10-fold over the control pGL3-basic vector following 17β-estradiol treatment and transfection of ERα (Fig. 2). Construct 2B15-412/-3Luc gave a moderate induction of 7-fold compared to that of the vehicle-treated controls. These results imply that additional estrogen-inducible elements might also be present in the promoter region from nucleotides -154 to -412. As shown in Fig. 3A, sequence analysis of this region revealed one imperfect ERE site between nucleotides -334 and -346 (5'-TGTCAGaggGCACC-3') (designated as Imperfect ERE site) and two putative ERE half-sites...
from nucleotides -246 to -250 (5’-GGTCA-3’) (5’-ERE half-site) and from -172 to -176 (5’-GGTCA-3’) (3’-ERE half-site), respectively. The importance of these sites for 17β-estradiol/ERα-dependent induction was also investigated by mutagenesis. Compared to the wild-type constructs, mutations of the 3’-ERE half-site in both constructs 2B15-412/-3Luc (Fig. 3B, MT-1) and 2B15-458/-3Luc (Fig. 3B, MT-3) decreased the promoter activity to a similar level of ~17%. When the 5’-ERE half-site was mutated in construct 2B15-458/-3Luc (Fig. 3B, MT-4), promoter activity was significantly reduced to about 8%. Mutation of the Imperfect ERE site resulted in a 60% reduction in promoter activity of construct 2B15-412/-3Luc (Fig. 3B, MT-2), and almost abolished the activity of construct 2B15-458/-3Luc (Fig. 3B2, MT-5). Collectively, our data clearly demonstrate that these three ERE/half-EREs respond cooperatively to 17β-estradiol. Given their close proximity to each other (only 164 bp apart), they probably act as a cooperative unit, as was observed with the *Xenopus* vitellogenin B1 and B2 genes and the chicken ovalbumin gene (Kato et al., 1992; Klein-Hitpass et al., 1988).

Additionally, although mutations in both AP-1 sites reduced 17β-estradiol/ERα-activated promoter activities to about 10-13% that of the unmutated construct (Fig. 3B, MT8), further mutations in the 3’-ERE half-site and imperfect ERE reduced activity to only 2-3% (Fig. 3B, MT-15 and MT-16). Thus it appears that the 2 AP-1 and 2 ERE-half sites, as well as the imperfect ERE of the *UGT2B15* promoter, act synergistically in response to 17β-estradiol and hence constitute an estrogen response unit.
c-Jun and Fra-2 bind to the distal AP-1 site in the *UGT2B15* proximal promoter

We next sought to use EMSAs to determine if AP-1 factors bound to the two AP-1 sites within the *UGT2B15* promoter. AP-1 factors are leuzine-zipper (bZIP)-containing transcription factors of the Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fra-1 and Fra-2) families. Members of the Jun family can form homodimers (e.g., c-Jun/c-Jun), whereas members of the Fos family only form heterodimers with Jun family members (e.g., c-Jun/c-Fos, c-Jun/Fra-2, and JunD/Fra-2) (Hess et al., 2004). To determine whether AP-1 proteins were able to bind to the distal AP-1 site (5'-AP-1), an EMSA was performed by incubating a probe encompassing the distal AP-1 site, corresponding to nucleotides from -469 to -437 of the *UGT2B15* promoter, with nuclear extracts from 17β-estradiol-stimulated MCF-7 cells. As shown in Fig. 4A, lane 1, three major complexes (labelled a, b, and c) were observed with the wild-type probe (Table 1, 5'AP-1wt). Mutation of this site from “TGAGTCA” to “CAGAGCA” (Table 1, 5’AP-1m) completely abolished the formation of complexes a and b and only slightly decreased the formation of complex c (lane 6). The formation of complexes a, b, and c were completely inhibited in the presence of a 10- to 100-fold molar excess of the unlabelled wild-type probe (lanes 2 and 3). However, only the formation of complex c was reduced by the addition of a 10- to 100-fold molar excess of the unlabelled mutated probe (lanes 4 and 5). These results indicate that proteins in MCF-7 nuclear extracts specifically bind to the distal AP-1 site, forming complexes a and b.

To identify the proteins in complexes a and b, supershift assays were performed using antibodies to ERα and members of the Jun and Fos families. The formation of complexes a and b was decreased by the addition of either the anti-Jun family antibody (lane 12) or the anti-c-Jun antibody (lane 9), suggesting the presence of c-Jun in both complexes a and b. The
anti-Fos family antibody inhibited the formation of complex b and resulted in the formation of a larger supershifted complex (labelled SS) (lane 11). The formation of complex b was also reduced by the addition of anti-Fra-2 antibody (lane 13), implying the presence of Fra-2 in complex b. Changes in the amounts of complexes a and b were not observed in a series of supershift assays with the addition of antibodies to ERα (lane 10), and other members of the Fos family, including Fra-1 (lane 14), FosB (lane 15), and c-Fos, with two different antibodies c-Fos1 (lane 8) and c-Fos2 (lane 16). Collectively, these results suggest that c-Jun and Fra-2 bind to the distal AP1 site in the UGT2B15 promoter, mainly as AP-1 heterodimers c-Jun/Fra-2 in complex b or possibly homodimers c-Jun/c-Jun in complex a.

The above experiments were repeated with a probe encompassing the proximal AP-1 site (3’-AP-1) within the UGT2B15 promoter. As shown in Fig. 4B, EMSAs with the labelled wild-type probe (Table 1, 3’AP-1wtβ) showed a DNA/protein complex (labelled d) (lane 1), which migrated slightly faster than complex c, formed with the probe containing the distal AP-1 site (lane 17). We demonstrated that the formation of complex d was 3’-AP-1 site-specific by showing that it did not form on a labelled mutated probe (Table 1, 3’AP-1mβ) (lane 6) and that its formation was completely abolished in the presence of either a 10- to 100-fold molar excess of the unlabelled wild type probe (lanes 2 and 3) but unaffected by a 10- to 100-fold molar excess of unlabelled mutated probe (lanes 4 and 5). However, despite extensive supershift assays with antibodies to ERα and members of the Jun and Fos families, the identities of the proteins in complex d could not be resolved as shown in Fig. 4B, lanes 8-16.
**ERα does not appear to interact with probes containing each of the three putative imperfect and half EREs of the *UGT2B15* promoter in EMSAs**

We further performed EMSAs with in-vitro transcribed/translated recombinant human ERα proteins to see whether ERα was able to bind to the imperfect ERE and two ERE-half sites within the *UGT2B15* ERU. As shown in Fig. 5A, the incubation of recombinant ERα with a probe containing the consensus ERE sequence (VitERE) of the *Xenopus* Vitellogenin A2 gene resulted in major ERα/DNA complexes (labelled A, lane 2), which were supershifted (labelled SS) by the addition of the anti-ERα antibody (lane 3). However, these complexes were not observed with any of the probes containing the 3’-ERE half-site (lane 5), the 5’-ERE half-site (Lane 8), and the Imperfect ERE full-site (lane 11) (Table 1, 3’EREhwt, 5’EREhwt, EREfwt). We repeated these experiments with 17β-estradiol-stimulated MCF-7 nuclear extracts and similar results were observed (data not shown). These results suggest that the binding of ERα might be too weak to be detected in standard EMSAs, which is in agreement with previous observations that the binding of ER to single half-EREs *in vitro* is 50- to 100-fold weaker than that to a perfect ERE (Kato et al., 1992; Tora et al., 1988).

We next performed competitive EMSAs to see whether the binding of ERα to VitERE was competed with a 100-fold molar excess of unlabelled probes containing the individual imperfect ERE and ERE-half sites. As shown in Fig. 5B, the 3’-ERE half-site (lane 5) and the 5’-ERE half-site (Lane 7) demonstrated significant abilities to compete for ERα binding to VitERE. The competition for ERα binding was abolished when the sequence (5’-GGTCA-3’) of each ERE half-site was mutated to be either 5’-TTTTT-3’ (Table 1, 5’EREhm) at the 5’-ERE half-site (lane 8) or 5’-AAAAA-3’ (Table 1, 3’EREhm) at the 3’-ERE half-site (lane 6), suggesting that such competition was specific to the intact ERE half-site. These results
provide further evidence for a role of these two ERE half-sites in 17β-estradiol-stimulated UGT2B15 transcription.

The imperfect ERE full-site (EREfwt) did not compete for ERα binding to VitERE (Fig. 5B, lane 9) despite evidence that mutation of this site almost completely abolished 17β-estradiol-induced activity of the UGT2B15 promoter (Fig. 3B, TM-5). The reason for this is unknown, but is consistant with reports that an increasing number of natural and synthetic imperfect palindromic EREs are found to be estrogen-inducible in transient transfections but show very weak or no ERα binding in vitro as assayed by EMSAs (Kato et al., 1992; Klinge, 2001; Tora et al., 1988).

**siRNA-mediated knockdown of ERα, Fra-2, and c-Jun reduces the capacity of 17β-estradiol to activate the UGT2B15 gene**

As mutagenesis implicated the involvement of an imperfect ERE, two ERE-half sites and two AP-1 sites in the process of induction of UGT2B15 promoter activity by 17β-estradiol, we sought to determine whether the relevant transcription factors that may bind to these sites are involved, by altering their levels in MCF-7 cells using siRNA knockdown strategies. As our EMSAs already demonstrated the binding of c-Jun and Fra-2 to the distal AP-1 site (Fig. 4A), we selected three siRNAs directed against c-Jun, Fra-2, and ERα, and introduced them into MCF-7 cells by transient transfection, followed by measuring target mRNAs using quantitative real-time RT-PCR. As shown in Fig. 6A, treatment of MCF-7 cells with ERα siRNA successfully reduced the levels of endogenous ERα transcripts to 43% of that in cells treated with scrambled siRNAs. This reduction of ERα mRNA resulted in an approximately 10-fold decrease in UGT2B15 mRNA levels, whereas there was no effect on
the mRNA levels of three other genes, including GAPDH, UGT1A6, and Fra-2. The levels of c-Jun mRNA were decreased by half, which is consistent with the reported activation of c-Jun transcription by estrogen (Weisz et al., 1990). As shown in Fig. 6B, down-regulation of c-Jun mRNA with siRNA to a level that was 51% that of the controls, reduced UGT2B15 mRNA levels down to 43%, without significantly altering the mRNA levels of GAPDH, ERα, and Fra-2. c-Jun siRNA treatment also reduced the mRNA levels of UGT1A6 by about 3-fold, which is consistent with the proposed activation of the UGT1A6 gene by AP-1 transcription factors (Heurtaux et al., 2006). As shown in Fig. 6C, down-regulation of Fra-2 with siRNA to a level that was 6% that of the controls, decreased UGT2B15 mRNA levels by 5-fold, whereas the mRNA levels of three other genes including GAPDH, UGT1A6, and c-Jun were not significantly changed. Of note, this treatment also reduced ERα mRNA levels down to 52%, a level similar to that of cells treated with ERα siRNA (Fig. 6A), suggesting that Fra-2 may play a role in ERα gene expression. Collectively, these results indicate that ERα, c-Jun, and possibly Fra-2 are involved in the up-regulation of UGT2B15 expression by 17β-estradiol in MCF-7 breast cancer cells.

**ERα, c-Jun, and Fra-2 are recruited to the UGT2B15 promoter in MCF-7 cells after 17β-estradiol stimulation**

To determine whether ERα, c-Jun, and Fra-2 bind to the endogenous UGT2B15 promoter, we performed ChIP assays and analysed specific regions of the UGT2B15 promoter by quantitative real-time PCR with primers as detailed in Table 1. We first demonstrated the efficacy of the methodology by showing the reported 17β-estradiol-stimulated recruitment of ERα and AP-1 factors, namely c-Jun and Fra-2, to the pS2/TFF1 promoter, which has an ERE and an AP-1 response element (Fig. 7A and B) (Baron et al.,
Initially, we tried to quantify enrichment of a 435-bp region (Table 1, primers 2B15-ChipF3/R1) of the UGT2B15 promoter, which covered all of the five cis-acting elements involved in up-regulation of the UGT2B15 promoter by 17β-estradiol, as demonstrated by mutagenesis. However, this strategy proved ineffective, due to the extremely low efficiency of amplification of the 435-bp fragment by real-time PCR (data not shown).

Since our supershift assays already demonstrated in vitro binding of c-Jun and Fra-2 to the distal AP-1 site (Fig. 4A), we then focused on analysis of a 180-bp region (Table 1, primers 2B15-ChipF2/R3) of the UGT2B15 promoter covering only the two AP-1 sites. After MCF-7 cells were treated with 10 nM 17β-estradiol for 2 h, the binding of ERα, c-Jun, and Fra-2 to this region was increased by 8 (p < 0.001), 2 (p < 0.05), and 1.7 (p < 0.05) fold respectively, compared with controls precipitated by equivalent amounts of the irrelevant preimmune-IgG serum (designated as IgG) (Fig. 7CD1). This recruitment of ERα, c-Jun, and Fra-2 to the UGT2B15 promoter was also observed in the analyses of another two promoter regions, namely a 129-bp region (Table 1, primers 2B15-ChipF1/R2) covering only the Imperfect ERE site (Fig. 7CD2), and a 247-bp region (Table 1, primers 2B15-ChipF1/R1) containing all three ERE/half-ERE sites (Fig. 7CD3). In contrast, there was no statistically significant occupancy (p > 0.05 in all cases) of ERα, c-Jun, and Fra-2 on the UGT2B15 promoter in the vehicle-treated cells for any of the three regions being analysed (Fig. 7D). We repeated the experiments after 24 h of 17β-estradiol stimulation and found that the binding of ERα, c-Jun, and Fra-2 to the UGT2B15 promoter remained at a statistically significant level (data not shown). Taken together, these results indicate that ERα, c-Jun, and Fra-2 are recruited to the UGT2B15 promoter in MCF-7 cells upon 17β-estradiol stimulation.
The AP-1 sites of the UGT2B15 promoter do not function as classic TPA response elements.

It is well known that a number of genes are induced by 12-O-tetradecanoylphorbol 13-acetate (TPA, also known as phorbol 12-myristate 13-acetate, PMA) through AP-1 binding sites in their promoters. Because of their TPA-inducibility, these AP-1 sites are termed TPA response elements (TREs) (Lacroix et al., 2004). As the UGT2B15 promoter contains two potential AP-1 sites and AP-1 factors bound to the distal site, we investigated whether these AP-1 sites acted as classic TREs.

Treating MCF-7 cells with 100 nM PMA slightly increased UGT2B15 mRNA levels by 1.5 fold, suggesting a marginal effect of PMA on UGT2B15 expression (Fig. 8). In contrast, PMA treatment resulted in a dramatic induction of the mRNA levels of our positive control, the matrix metalloproteinase 1 (MMP-1) gene, a well-known PMA-inducible gene (Lacroix et al., 2004) (Fig. 8, inset). Surprisingly, PMA decreased the activation UGT2B15 expression by 17β-estradiol nearly 8 fold. It has been reported that a large number of genes including ERα and estrogen-induced genes are down-regulated by PMA (Lacroix et al., 2004). Thus, our observed down-regulation of UGT2B15, might be simply a result of the PMA-mediated decrease in ERα expression. This assumption was confirmed as we observed an approximately 3-fold reduction in the ERα mRNA levels regardless of the presence of 17β-estradiol (Fig. 8). As a negative control, the expression of the β-actin gene was not significantly altered under the same conditions (Fig. 8). Collectively, our data suggest that the 2 AP-1 sites in the UGT2B15 promoter are insufficient to facilitate a classic inductive response to PMA.
The \textit{UGT2B17} promoter responds to 17β-estradiol via a mechanism similar to that of the \textit{UGT2B15} promoter

The proximal promoter region from nucleotides -1 to -1662 of \textit{UGT2B15} is 91% identical with that of \textit{UGT2B17}. Due to this sequence homology, all five \textit{cis}-activating elements that are involved in 17β-estradiol-mediated \textit{UGT2B15} gene expression, are totally conserved in the \textit{UGT2B17} promoter (Fig. 9A). As we already demonstrated that the endogenous UGT2B17 mRNA levels were increased by 78-fold in MCF-7 cells treated with 10 nM 17β-estradiol over that of the vehicle-treated cells (Fig. 1), it is reasonable to assume that the \textit{UGT2B17} promoter may also be up-regulated by 17β-estradiol in a manner similar to the \textit{UGT2B15} promoter.

To test this hypothesis, we prepared a reporter construct of 2B17-650/+40Luc by cloning the \textit{UGT2B17} promoter region from nucleotides +42 to -650 into the pGL3-basic vector and tested its capability to drive transcription upon 17β-estradiol stimulation in MCF-7 cells in transient transfections. As shown in Fig. 9B, compared to that of the controls treated with vehicle alone, 17β-estradiol treatment combined with transfected ERα resulted in a nearly 19-fold increase in promoter activity; however, this up-regulation was almost abolished by simply mutating the proximal AP1 site.

We further quantified endogenous UGT2B17 mRNA levels from MCF-7 cells treated with siRNAs directed against ERα, c-Jun, and Fra-2 by quantitative real-time RT-PCR. Our results showed that a significant decrease in the mRNA levels of ERα, c-Jun, and Fra-2 by siRNAs (Fig. 6) respectively reduced UGT2B17 mRNA levels to 3%, 36%, and 10% of that in cells treated with scramble siRNAs (Fig. 6A, B, and C).
Taken together, these results suggest that the same five \textit{cis}-activating elements confer estrogen-responsiveness to both \textit{UGT2B15} and \textit{UGT2B17} promoters in MCF-7 cells.
DISCUSSION

In this study, we show that both UGT2B15 and UGT2B17 expression are up-regulated by 17β-estradiol in ER-positive breast cancer MCF-7 cells. Furthermore, this effect is mediated by the recruitment of ERα, c-Jun and Fra-2 to a 278-bp ERU composed of 2 AP-1 sites, one imperfect ERE site, and two consensus ERE half-sites in the proximal promoters of these UGT genes. The induction of UGT2B15 expression by 17β-estradiol and the contribution of an AP-1 site in this process has been reported previously (Harrington et al., 2006; Kininis et al., 2007). Our work extends these findings by demonstrating the importance of an additional four cis-activating elements and the cooperative action of ERα, c-Jun and Fra-2 in estrogen inducibility of both UGT genes.

Most ERUs in other genes are composed of 2 or more ERE half sites or imperfect EREs. The ERU of UGT2B15 and UGT2B17 has three such sites as determined by mutation analyses. Of note, these sites in other genes bind ERα poorly in EMSAs (Kraus et al., 1994; Murdoch et al., 1995), but can be identified in ChIP analyses, a phenomenon observed in our study. However, the UGT2B15 and UGT2B17 ERU also contains AP-sites, as mutation of these sites strongly reduces 17β-estradiol inducibility. Results from our EMSAs showed that AP-1 factors bound the distal AP-1 site mainly as heterodimers c-Jun/Fra-2 (Fig. 4A). These observations were in agreement with our observed 17β-estradiol-induced in vivo recruitment of c-Jun and Fra-2 to the region containing the UGT2B15 ERU (Fig. 7CD). Of note, our results indicate that the two AP-1 sites do not function as classic TREs. This is in contrast to other genes where, for example, the binding of c-Jun and Fra-2 to an AP-1 site in the Fra-1 proximal promoter was found to mediate PMA-inducibility of this promoter in human lung epithelial A549 cells (Adiseshaiah et al., 2008). A genome-wide screen for in vivo ERα
binding sites by ChIP-on-ChIP assays showed that ERα was recruited to the region harbouring the UGT2B15 ERU in MCF-7 cells upon 17β-estradiol-stimulation (Laganiere et al., 2005). These findings were verified by our ChIP assays, which clearly demonstrated 17β-estradiol-mediated *in vivo* recruitment of ERα to the UGT2B15 ERU (Fig. 7). The significance of ERα, c-Jun, and Fra-2 in the 17β-estradiol-mediated *UGT2B15* induction was further established by our siRNA silencing experiments (Fig. 6).

Taken together, these data indicate that probably both classic (ERα/ERE) and non-classical (ERα/AP-1) genomic pathways are involved in 17β-estradiol-stimulated *UGT2B15* expression in MCF-7 cells. Given the proximity of the AP-1 and imperfect ERE and ERE-half sites, we propose that transcriptional co-activator complexes associate with these sites after 17β-estradiol treatment and interact to form a super complex that is now permissive for transcriptional activation. A similar mechanism has been proposed to explain the 17β-estradiol-mediated transcriptional synergism between an AP-1 site and an ERE site (separated by 52 bp) on the *pS2* promoter in HepG2 cells (Barkhem et al., 2002) or the insulin-like growth factor-1-induced transcriptional activation of the same *pS2* promoter in MCF-7 cells (Baron et al., 2007). Very recently, studies have shown that 17β-estradiol-modulated *GREB1* gene transcription in MCF-7 cells is mediated by coordinated ERα binding to three consensus EREs, which are located at -21.2, -9.5, and -1.6 kb upstream of the closest *GREB1a* transcription start site (Deschenes et al., 2007). The authors detected the formation of 17β-estradiol-induced chromatin loops between each ERE and the transcription start site by chromatin capture assays. Hence, they proposed that these EREs can physically associate with each other through multiple chromatin loops, either in a super complex with the transcription start site or in a transient binary interaction in response to estrogen (Deschenes et al., 2007). In this sense, we can not rule out the possibility that additional functional imperfect
ERE half-sites in that portion of the promoter (data not shown).

Since all five cis-activating elements within the UGT2B15 ERU are completely conserved in the UGT2B17 promoter at similar positions relative to the translation start site (Fig. 9A), we propose that these same elements most likely account for our observed 17β-estradiol-activated UGT2B17 gene transcription in MCF-7 cells (Fig. 1). This assumption was supported by the reported 17β-estradiol-induced ERα recruitment to the promoter region containing the UGT2B17 ARU (Laganiere et al., 2005). To further support our hypothesis, we demonstrated by siRNA silencing that 17β-estradiol-stimulated UGT2B17 gene expression was significantly reduced after repression of ERα, c-jun, and Fra-2 expression by their respective siRNAs (Fig. 6). Furthermore, mutation of the proximal AP-1 site alone almost completely abolished promoter activity (Fig. 9B). However, the functionalities of the remaining four cis-activating elements remain to be verified by mutagenesis.

Estrogens can negatively regulate their own signalling by multiple mechanisms as discussed in detail elsewhere (Harrington et al., 2006). One mechanism involves the inactivation and elimination of estrogen by glucuronidation. Whereas UGT1A1, 1A3, 1A8, and 1A10 are active in forming the 3-glucuronide, only UGT2B7 and 2B17 have the capacity to form the 17β-glucuronide from 17β-estradiol. Under basal conditions, the levels of all these UGTs are very low (Fig. 1) and presumably would have little impact on inactivating estrogen. However, the dramatic increase in the levels of UGT2B17 after 17β-estradiol treatment would increase the capacity of the cell to eliminate 17β-estradiol as the 17β-glucuronide, and potentially provide a negative feedback loop controlling the magnitude of the response to
active estrogen. In this context, the regulation of UGT2B17 by 17β-estradiol, which has not been reported previously, would be of significant importance in maintaining estrogen homeostasis. An alternate mechanism for controlling estrogen signalling, would be to regulate the removal of precursors required for estrogen synthesis.

It has been reported that 75% of estrogen synthesis before menopause and nearly 100% after menopause in women, occurs in peripheral target tissues from precursor steroids of adrenal origin (Labrie, 1991). The breast is capable of in situ synthesis of both 17β-estradiol and dihydrotestosterone from the common precursor, testosterone, by aromatase and 5α-reductase, respectively, especially after menopause (Sasano et al., 2008). One study even demonstrated that in situ synthesis of 17β-estradiol in breast tumors predominated over uptake from plasma after menopause (Yue et al., 1998). Given that UGT2B15 and UGT2B17 are the only UGTs capable of glucuronidating, both testosterone and dihydrotestosterone (Bowalgaha et al., 2007; Turgeon et al., 2001), their induction by 17β-estradiol would lead to removal of these estrogen precursors and hence to a subsequent decrease in estrogen synthesis and estrogen signalling. Thus up-regulation of UGT2B15 and UGT2B17 levels by estrogens might play a crucial role in the local homeostasis of estrogens, androgens and their precursors within the breast, especially after menopause. The breast and MCF-7 cells contain androgen receptors, in addition to estrogen receptors. As androgens acting through the androgen receptor are antagonistic to estrogen signalling, the capacity of UGT2B15 and 2B17 to inactivate dihydrotestosterone may also impact on estrogen signalling effects by reducing the antagonism of the androgen receptor signalling pathway in MCF-7 cells and the breast.

In addition to their glucuronidation of steroid hormones, UGT2B15 is also active on the metabolites of these hormones. It has been shown to conjugate endogenous catechol
estrogens, such as 4-hydroxy estrone and 4-hydroxy estradiol (Turgeon et al., 2001). 4-Hydroxy estrone and 4-hydeoxy estradiol are present in breast tumors (Rogan et al., 2003), and are believed to be involved in carcinogenesis of breast cancer and other human cancers, partly because their quinone metabolites may react with DNA at purine bases to form depurinating adducts generating highly mutagenic apurinic sites (Cavalieri et al., 2006). Thus, their clearance from the breast through 17β-estradiol-enhanced UGT2B15 and UGT2B17 activity could minimize the exposure of breast tissues to these carcinogens.

In summary, this study identifies c-Jun, Fra-2, and ERα as important factors mediating the response of UGT2B15 to 17β-estradiol in MCF-7 cells. In the presence of 17β-estradiol, these transcription factors are recruited to a 278 bp ERU in the UGT2B15 proximal promoter to enhance transcription of this UGT gene. This study also identifies UGT2B17 as a novel estrogen-inducible gene in MCF-7 cells, and our preliminary results suggest that it is up-regulated by 17β-estradiol in a manner similar to that of UGT2B15. These studies provide an insight into the mechanisms controlling expression of these major androgen-conjugating UGTs in breast cancer cells and suggest a role for UGT2B15 and UGT2B17 in maintenance of the local homeostasis of sex steroids within the breast.
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REFERENCES


**FOOTNOTES**

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FIGURE LEGENDS

Fig. 1. 17-β Estradiol elevates UGT2B15 and UGT2B17 mRNA levels in MCF-7 cells. MCF-7 cells preincubated for 6 days in phenol red-free RPMI containing 5% CCD-stripped FBS were treated with either 17β-estradiol (E2) at three different concentrations (0.1 nM, 1 nM, or 10 nM) or 0.1% ethanol (vehicle) for 24 h. Total RNA was extracted and reverse-transcribed to generate cDNA, followed by quantitative real-time PCR to calculate the copy numbers of target transcripts as described under ‘Materials and Methods’. Relative expression levels were calculated as copies of each target transcript per $10^9$ copies (for all UGTs) or $10^3$ copies (for β-Actin) of 18S rRNA transcripts. Data shown are from a representative experiment performed in triplicate with the error bars indicating one standard deviation. Inset, data for β-Actin.

Fig. 2. Deletion analysis of the UGT2B15 promoter in MCF-7 cells indicates the presence of cis-activating elements responsive to 17β-estradiol. A series of luciferase reporter constructs carrying varying lengths of the UGT2B15 proximal promoter are shown on the left. Transient transfections with MCF-7 cells were carried out using 100 ng of each promoter construct with or without 2.5 ng of ERα expression plasmid. 5 h post-transfection, cells were treated with either 10 nM 17β-estradiol (E2) or 0.1% ethanol (vehicle). 40 h post-treatment, cells were harvested and assayed for firefly luciferase activity as described under ‘Materials and Methods’. The relative luciferase activities of UGT2B15 promoter constructs are expressed as fold induction over that of the promoterless pGL3-basic vector (set at a value of 1) for the respective conditions, as indicated by “0.1% ethanol”, “10 nM E2”, and “10 nM E2/ERα”, respectively. Data shown are from a representative experiment performed in triplicate with error bars representing one standard deviation.
Fig. 3. Mutational analysis of the UGT2B15 proximal promoter identifies five functional cis-activating elements responsible for estrogen-inducibility. A), a schematic depiction of the five putative cis-activating elements in the UGT2B15 proximal promoter from nucleotides -3 to – 485, including two AP-1 sites beginning at nucleotides -454 (5’-AP-1) and -431 (3’-AP-1), two ERE half-sites at -250 bp (5’-ERE half-site) and -176 bp (3’-ERE half-site), and an imperfectly palindromic ERE site -346 bp. The sequences at these sites (WT) and their mutations (MT) are shown. B), Shown at the left are sixteen mutated constructs of the UGT2B15 proximal promoter with mutations at one or more of the aforementioned five putative sites indicated by black boxes. Transient transfections with MCF-7 cells were carried out using 100 ng of each promoter construct with 2.5 ng of ERα expression vector. 5 h post-transfection, cells were treated with 10 nM 17β-estradiol in phenol red-free RPMI with 5% CCD-stripped FBS. 40 h post-treatment, cells were harvested and assayed for firefly luciferase activities as described under ‘Materials and Methods’. Data shown are representative experiments performed in triplicate with error bars representing one standard deviation. After normalizing to the promoterless pGL3-basic vector, the relative luciferase activities of the mutants are expressed as a percentage of that obtained from the relevant wild-type constructs (set at a value of 100%).

Fig. 4. c-Jun and Fra-2 bind to the distal AP-1 site in the UGT2B15 promoter. EMSAs were performed with 32P-end-labeled double-stranded oligonucleotide probes encompassing either the distal AP-1 site (5’-AP-1) (A) or the proximal AP-1 site (3’-AP-1) (B) of the UGT2B15 promoter using 5 µg of MCF-7 nuclear extracts as described in ‘Materials and Methods’. In competition assays, unlabelled probes were added at a 10- (lanes 2 and 4) and 100-fold (lanes 3 and 5) molar excess of labelled probe before the addition of labelled probe. In supershift assays, 2 µg of antibodies (lanes 8-16 as indicated) were added immediately after
the addition of labelled probe and incubated for 30 min at room temperature. Arrows indicate the major DNA/protein complexes pertinent to the 5′-AP-1 site (a, b and c) and the 3′-AP-1 site (d), as well as the supershifted band (SS). Lane 17 is a replicate of lane 1 (A), to indicate the relative positions of these DNA/protein complexes. hWT, labelled probe; hMT, labelled mutated probe; cWT, unlabelled probe; cMT, unlabelled mutated probe.

**Fig. 5. A),** Recombinant human ERα does not bind to the two ERE half-sites and the imperfect ERE of the UGT2B15 promoter. EMSAs were performed with 1 µl of in-vitro transcribed /translated recombinant human ERα and 50,000 cpm (~1ng) of 32P-end-labeled double-stranded oligonucleotide probes encompassing either the consensus vitERE site of the Xenopus Vitellogenin A2 gene or one of the three UGT2B15 imperfect ERE and ERE half-sites (as indicated) as described in ‘Materials and Methods’. For supershift assays, 2 µg of anti-ERα antibody (lanes 3, 6, 9, and 12) were added immediately after the addition of labelled probe and incubated for 30 min at room temperature. The specifically bound ERα-DNA retarded complexes (A), the ERα antibody supershifted complexes (SS), and the non-specifically retarded complexes (NS) are indicated with brackets. **B),** Probes encompassing the ERE half-sites of the UT2B15 proximal promoter significantly inhibit the formation of ERα/DNA complexes on the vitERE site of the Xenopus Vitellogenin A2 gene promoter. EMSAs (lanes 2-10) with the vitERE probe were performed as above. For competition assays, unlabelled wild-type (WT, lanes 3, 5, 7, and 9) or mutated (MT, lanes 6, 8, and 10) probes were added at 100-fold molar excess before the addition of labelled probes.

**Fig. 6.** siRNAs against ERα, c-Jun and Fra-2 reduce UGT2B15 and UGT2B17 mRNA levels in 17β-estradiol-treated MCF-7 cells. MCF-7 cells were transfected with the indicated siRNAs for 5 h, and then cultured in fresh phenol red-free RPMI with 6% CCD-
stripped FBS. 48 h Post-transfection, cells were treated with 10 nM 17β-estradiol for 24 h, harvested and target mRNAs quantified by real-time RT-PCR as described in ‘Materials and Methods’. After normalizing to 18S rRNA, the relative expression levels of target genes in MCF-7 cells transfected with ERα, c-Jun, or Fra-2 siRNAs were expressed as a percentage of that (set at a value of 100%) in control cells treated with scramble siRNA. Data shown are representative experiments performed in triplicate with error bars representing one standard deviation.

**Fig. 7. ERα, c-Jun, and Fra-2 are recruited to the UGT2B15 proximal promoter in 17β-estradiol-treated MCF-7 cells.** Cells pre-cultured in phenol red-free RPMI supplemented with 6% CCD-stripped FBS for 6 days were treated with either 10 nM 17β-estradiol (A, C) or 0.1% ethanol (vehicle) (B, D) for 2 h and subjected to chromatin immunoprecipitation assays, followed by real-time PCR to quantify the precipitated DNA of target sequences as described under ‘Materials and methods’. After normalizing to the reference gene, HCG3, data were expressed as the-fold enrichment in DNA samples precipitated with 10 µg of each of the antibodies as indicated compared to that (set a value of 1) in the control DNA samples precipitated from equivalent amounts of pre-immune IgG serum (IgG). Data shown are the average of duplicates from one representative experiment. Regions analysed by PCR in the pS2/TFF1 promoter (A and B) and the UGT2B15 promoter (C and D) are indicated with black boxes.

**Fig. 8. The phorbol ester, PMA does not elevate UGT2B15 mRNA levels in MCF-7 cells.** Cells cultured in phenol red-free RPMI with 6% CCD-stripped FBS for 6 days were treated respectively with 0.1% ethanol (vehicle), 10 nM 17β-estradiol for 24 h, 100 nM PMA for 6 h, and a combination of 10 nM 17β-estradiol for 24 h and 100 nM PMA for 6 h. Cells were
harvested and total RNA was extracted, followed by quantification of target mRNAs by real-time RT-PCR as described in ‘Materials and Methods’. After normalizing to 18S rRNA, the relative mRNA levels of target genes (UGT2B15, MMP-1, ERα, and β-actin) were expressed as a fold change over that (set at a value of 1) of the vehicle-treated cells. MMP-1, a TPA-inducible gene, acts as a positive control for these experiments. Data shown are a representative experiment performed in triplicate with error bars representing one standard deviation.

Fig. 9. The UGT2B17 proximal promoter contains an ERU and is upregulated by 17β-estradiol in MCF-7 cells. A), The proximal promoters of the UGT2B15 (top) and UGT2B17 (bottom) genes are aligned using GeneBank records AF179881 and AF179874, respectively. The sequences of the promoter regions are numbered on the left relative to the adenine base of the initiator codon (bold), which is assigned as +1. Conserved nucleotides are indicated by an asterix under the sequences and deletions and insertions are represented by hyphens. The five putative cis-activating elements conferring estrogen-inducibility to the UGT2B15 proximal promoter are boxed and totally conserved in the UGT2B17 promoter. B), Luciferase constructs of the UGT2B17 promoter carrying the region from nucleotides +42 to 650 relative to the translation start site were transfected into MCF-7 cells with or without cotransfection of ERα expression plasmid. 5 h post-transfection, cells were treated with either 10 nM 17β-estradiol or 0.1% ethanol (vehicle). 40 h post-treatment, cells were harvested and assayed for firefly luciferase activities as described under ‘Materials and methods’. Data shown are the results from experiments with the wild type construct (2B17-650/+40Luc) and the construct (2B17-650/+40LucMut) with a mutation in the 3’-AP-1 site. The data are the means of triplicate samples from one representative experiment, expressed as fold induction.
over the promoterless pGL3-basic vector (set at a value of 1). Error bars represent one standard deviation.
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3'AP-1m\textsuperscript{b} & TATGAATTACAGGCAACAAAAGTTT \\
3'EREhwt & AAATATAATGAGGTCATCAATCTTTTGTTG \\
3'EREhm & AAATAAATATGAAAATCAATCTTTTGTTG \\
5'EREhwt & CTTACATATTCTAGGTCATAAAAAATTATTG \\
5'EREhm & CTTACATATTCTATTTTTAAAAATTATTG \\
EREfw & TATGAATTGCAACAAAAGTTT \\
EREfm & TATGAATTACAGGCAACAAAAGTTT \\
Xenopus \textit{VitellogeninA2} \textit{vitERE} & AAAGTCAGGTCACAGTGACCTGACCAAGCTGAATCAAAAG \\

\textbf{Quantitative real-time PCR} \\
c-Fos-For & TGATGACCTGGGCTTCCCAG \\
c-Fos-Rev & CAAAGGCTCGGTCTTCAGC \\
c-Jun-For & CTCTCTCTCTCTCTCTCTGAGGAGGTCAGCG \\
c-Jun-Rev & GTCTGAGGCTCCTCCTGAGGAGGTCAGCG \\
ER\textalpha{}-For & TTCGGCTCCAACGGCCTGGGGTTT \\
ER\textalpha{}-Rev & GGTACTGGCCAATCTTTCTCTGAGGAGGTCAGCG \\
Fra-2-For & TAGATATGCTAGGTCAGGAGGTCAGCG \\
Fra-2-Rev & TCTTGATCACGCCAGGTCTTGGGA \\
18S rRNA-For & CGATGCTCTTAGCTGAGTGT \\
18S rRNA-Rev & GGTCCAAGAATTTCACCTCT \\
1A3-For & ATGGCAATGTTGAACAATATG \\
1A3-Rev & GGTCTGAATTGGCTGTTAGTAATC \\
1A7-For & TGGCTCGTGCAGGGTGGACTG \\
1A7-Rev & TTCGCAATGGTGCCGTCCAGC \\
1A8-For & CTGCTGACCTGTGCTTGGGTTT
MOL 57380

1A8-Rev        CCATTGAGCATCGGCGAAAT
2B28-For        TCTTTTTGATCCCAATGACGCATT
ß-Actin-For     CTGGCGGCACCACCATGTACCCT
ß-Actin-Rev     GGAGGGGCCGACTCGTCATACT

**ChIP assay**

2B15-ChipF1     TGCCGTTTGAGTTGTATAATTACTTCTTC
2B15-ChipR1     GAATAGGTGCATGCCAAGGAGACC
2B15-ChipF2     GGGGGGTAGAGGCTATTGTATAG
2B15-ChipR2     GAATATGTAAGTAACCTGTCTTATGT
2B15-ChipR3     TTAATATCGTGAGTAAGTAATGTCTTC
2B15-ChipF3     GAGTGGGGGGTTAGAGGCTATTGTATAG
HCG3-ChipF      ATGGTGACTACTACGAGGT
HCG3-ChipR      CACCTGCTTGAATCTCCTCT
TFF1/pS2-For    GGCCATCTCTCACAATGACTACTTCTTC
TFF1/ps2-Rev    GCCAGGCTCTGTGCGTAAAGGACCG

* Primers are numbered relative to the adenine base of the initiator codon (ATG), which is assigned as +1. Incorporated restriction sites of both Mul I (ACGCGT) and Kpn I (GGTACC) are in italics.

* Oligos are the same for both mutagenesis and EMSAs except that those used for the proximal AP-1 site in mutagenesis and EMSAs are labelled with a and b, respectively. Mutated sequences are highlighted in bold.
Figure 1

[Graph showing the copies of UGT mRNA per 10^9 copies of 18S rRNA transcripts for different concentrations of E2 (0 nM, 0.1 nM, 1 nM, 10 nM).]
Figure 2

The figure shows a bar graph comparing the relative luciferase activity of various promoter constructs. The constructs are labeled as follows:

- 2B15-2716/-3Luc
- 2B15-747/-3Luc
- 2B15-705/-3Luc
- 2B15-595/-3Luc
- 2B15-556/-3Luc
- 2B15-458/-3Luc
- 2B15-412/-3Luc
- 2B15-253/-3Luc
- 2B15-202/-3Luc
- 2B15-154/-3Luc
- 2B15-54/-3Luc
- pGL3-basic (Luc)

The x-axis represents the promoter constructs, while the y-axis represents the relative luciferase activity. Different conditions are indicated by different colors:

- 10 nM E2 and ERα
- 10 nM E2
- 0.1% Ethanol

The bars show the activity levels under these conditions for each construct.
Figure 4

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A

B

a
b
c

SS

d

a
b
c

A

B
Figure 5A

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ERα + Ab
ERα
Free probe

SS
A
NS
Figure 5B

[Diagram showing different treatments and their effects on viERE, 3' ERE half-site, 5' ERE half-site, and ERE full-site.]
Figure 6

Effects on mRNA levels by siRNAs

A

ERα siRNA

B

c-Jun siRNA

C

Fra-2 siRNA

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Figure 8

![Bar chart showing the relative mRNA levels of MMP-1 under different conditions.](molpharm.aspetjournals.org)

Legend:
- □ No E2/no PMA
- □ 100 nM PMA
- □ 10 nM E2
- □ 100 nM PMA/10 nM E2