A Novel Estradiol/Estrogen Receptor α-dependent Transcriptional Mechanism Controls Expression of the Human Prolactin Receptor

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Prolactin (PRL), a hormone of the lactogen/cytokine family, is produced in the anterior pituitary gland and exerts diverse cellular actions through its transmembrane receptors (PRLR) in several target tissues. Prolactin is a major factor in the proliferation and differentiation of breast epithelium and is the primary hormone in the stimulation and maintenance of lactation. Prolactin is a tumor pro-

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Prolactin exerts diverse functions in target tissues through its membrane receptors, and is a potent mitogen in normal and neoplastic breast cells. Estradiol (E2) induces human prolactin receptor (hPRLR) gene expression through stimulation of its generic promoter (PIII). This study identifies a novel E2-regulated non-estrogen responsive element-dependent transcriptional mechanism that mediates E2-induced hPRLR expression. E2 stimulated transcriptional activity in MCF7A2 cells transfected with PIII lacking an estrogen responsive element, and increased hPRLR mRNA and protein. The abolition of the E2 effect by mutation of Sp1 or C/EBP elements that bind Sp1/Sp3 and C/EBPβ within PIII indicated the cooperation of these transfactors in E2-induced transcription of the hPRLR. DNA affinity protein assay showed that E2 induced estrogen receptor α (ERα) binding to Sp1/Sp3 and C/EBPβ DNA-protein complexes. The ligand-binding domain of ERα was essential for its physical interaction with C/EBPβ, and E2 promoted this association, and its DNA binding domain was required for transactivation of PIII. Co-immunoprecipitation studies revealed tethering of C/EBPβ to Sp1 by E2-activated ERα. Chromatin immunoprecipitation analysis showed that E2 induced recruitment of C/EBPβ, ERα, SRC1, p300, pCAF, TFIIB, and Pol II, with no change in Sp1/Sp3. E2 also induced promoter-associated acetylation of H3 and H4. These findings demonstrate that an E2/ERα/Sp1, and C/EBPβ complex with recruitment of coactivators and TFIIB and Pol II are required for E2-activated transcriptional expression of the hPRLR through PIII. Estradiol produced in breast stroma and adipose tissue, which are major sources of estrogen in post-menopausal women, could up-regulate hPRLR gene expression and stimulate breast tumor growth.

Proinflammatory effects of estradiol on the proliferation of these cells (8). There is local production of PRL in mammary epithelial cells, and increased expression of the PRLR long form occurs in a significant number of human mammary tumors (9–12). A lower ratio of short (inhibitory forms)/long (activating form) receptors reported in breast tumor tissues could cause unopposed prolactin-mediated stimulatory actions of the long form and may contribute to breast tumor development and progression (7). Moreover, the PRL antagonist G129R was reported to cause apoptosis in breast cancer cells (8, 13). These findings, and the correlation between serum PRL and the incidence and progression of breast tumors (11, 12, 14), indicate that PRL has a role in human breast cancer. Stromal and adipose tissue are the major sources of estrogen in post-menopausal women, and could exert paracrine control of prolactin and prolactin receptor expression in adjacent mammary epithelial cells.

Our previous studies on the hPRLR gene revealed its complex 5′ genomic structure, with multiple (six) alternative non-coding exons 1 and promoter utilization (15, 16). These include the preferentially utilized, generic promoter 1/exon-1 (PIII/hE1), which is also present in rat and mouse, and five human specific exon-1 promoters (hE1hE1–hE1hE1) (15–17). These forms were found to be expressed in breast cancer cells, and variably in other tissues (16). Quantitative competitive reverse transcriptase-PCR analysis showed that E2 induced increases of PRLR non-coding exon-1 hE1 (generic) mRNA transcripts directed by promoter III (hPIII) in breast cancer cells. Also, in transfection studies E2 activated the hPIII promoter (18). This promoter contains functional Sp1 and C/EBP sites that bind Sp1/Sp3 and C/EBPβ, respectively (16, 19). The lack of a formal CRE in the hPIII promoter suggested that the effect of estradiol is mediated through association of the activated CRE with relevant DNA binding transactors. Thus, although there is not a classical CRE within the hPRLR promoter/5′-flanking region of hPIII, our initial studies demonstrated an estrogen regulatory role in hPRLR expression. In this study we have investigated control mechanism(s) underlying human PRLR gene expression, and characterized a novel non-classical CRE-independent mechanism by which estrogen regulates hPRLR gene expression.

EXPERIMENTAL PROCEDURES

Reporter Gene Constructs and Expression Vectors, Expression, and Purification of Glutathione S-Transferase (GST) C/EBPβ Fusion Protein—All plasmids were constructed by standard recombinant DNA techniques. The hPRLR PIII reporter pG12 gene constructs have been previously
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described (16). These include constructs of the pGL2 reporter gene plasmid (Promega, Madison, WI) with insertion of DNA fragment (−931/−112 bp) containing the 5′-flanking region/promoter/exon 1 of hPRLR hPIII or the hPIII promoter/exon 1 (−480/−112 bp), either wild-type or harboring mutations in Sp1 and/or C/EBP (within the hPIII) or the ERE half-site (upstream to the promoter) (Fig. 1A). The constructs were numbered relative to the translation initiation codon (ATG +1) in exon 3 (E3) (Fig. 1A). All plasmid constructs were restriction mapped and sequenced.

The hERα/pcDNA 3.1 expression constructs containing specific deletions, and C-terminal fusion of a V5 tag (Fig. 6A) with inclusion of Kozak sequence were generated by conventional PCR. ERα and its truncated forms were synthesized in vitro using the TNT T7 Quick-coupled transcription/translation system (Promega). One-tenth of the individual reactions (5 µl) were assessed as input of the pulldown analysis (45 µl). The GST C/EBPβ fusion expression construct was prepared by inserting full-length C/EBPβ cDNA into the pET41a vector (Novagen, Madison WI) at EcoRI and Xhol sites in-frame with 5′ GST.

Bacterial BL21a (DE3) pLysS strains transformed with pET41a or the pET41a/C/EBPβ-GST fusion construct were cultured at 37 °C for 16 h (A$_{600}$ of 0.6 to 0.7). Cells were then incubated with 0.2 mM isopropyl-β-D-thiogalactopyranoside (Invitrogen) for 1 h at 37 °C. Cells harvested and lysed by sonication in B-PER bacterial lysis buffer were subjected to pulldown assays (45 µl) by beads suspended in 20 µl of washing buffer (50 mM Tris, pH 8.0, 0.14 M NaCl, 0.008 M sodium phosphate, 0.002 M potassium thiocyanate) (Promega, Madison WI) at EcoRI and XhoI sites in-frame with 5′ GST.

The GST pulldown reaction mixture (45 µl of reaction mixture) were subjected to pulldown assays by incubation with GST (control) or GST-C/EBPβ (attached to glutathione-Sepharose beads) for 16 h at 4 °C in the presence or absence of 100 nM E$_2$ in a total volume of 500 µl of the binding buffer (50 mM NaCl, 50 mM Tris, pH 8.0, 0.5 mM dithiothreitol, 0.05% Nonidet P-40 and proteases inhibitors mixture). Beads were collected by centrifugation and washed five times in 500 µl of washing buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 0.1% Nonidet P-40 and protease inhibitors mixture). Washed beads suspended in 20 µl of sample buffer (Tris glycine, 2× SDS, Invitrogen) were denatured at 100 °C for 5 min. The soluble fraction was recovered by centrifugation, and resolved in 4–20% Tris glycine gels (Invitrogen). Interactions were evaluated by Western blot analysis using antibodies to Sp1, Sp3, C/EBPα, and γH2AX.

Preparation of Nuclear Extracts and Western Blot—Nuclear extracts from MCF-7 cells were cultured with 17β-estradiol (0–100 nM) at the times indicated, using the RNA isolation kit (Stratagene). Prior to reverse transcription reaction, total RNA was treated with DiNase 1 to remove any possible copurified DNA. 2 µg of DNA was reversed transcribed using a SuperScript II kit (Invitrogen) containing a mixture of oligo(dT)$_{20}$ and random hexamer primers. The first-strand DNA from 100 ng of RNA was used as a template in a real-time PCR with SYBR Green Master Mix and an ABI 7500 sequence detection system (Applied Biosystems). The cycling program was set as follows: denature at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The primers utilized for PCR to detect hPRLR mRNA transcribed from promoter hPIII were a specific forward primer derived from non-coding exon 1 (hE31) (−135/−114) and reverse primer 5′-CATTCTCAGAAAGCCAGGTGAG-3′ from sequences located in common hE3 (−49/+71). The primers for detecting β-actin were primers 5′-TATG-GTGGCGACACTTTCGCTCCC-3′ and 3′-AGTCGACCACTTTCGCTCCC-3′. The specificity of the PCR products was verified by melting curve analyses at the end of the PCR. The standard curves were created by a 10-fold serial dilution of the pcDNA/PRLR vector. Results presented are from two individual experiments, each sample was assayed in triplicate, and normalized to the level of β-actin mRNA and expressed as fold change from controls.

Preparation of Nuclear Extracts Immunodepleted of ERα—Antibody-conjugated agarose beads were used to deplete ERα from nuclear extracts of MCF-7 cells as previously described (22) with modifications. Briefly, 40 µg of ERα polyclonal antibody (Santa Cruz) was incubated with 200 µl of protein A-agarose beads in 1 ml of binding buffer (0.14 M NaCl, 0.008 mM sodium phosphate, 0.002 mM potassium phosphate, and 0.01 M KCl) for 2 h, and subsequently washed three
times with PBST (PBS buffer, 0.02% Tween 20) buffer and twice with PBS buffer. The beads were then incubated with 100 μg of nuclear protein for 2 h at 4°C with rotation. The suspension was centrifuged, and the supernatant was subjected to one more round of depletion by re-incubation for 1 h with 200 μl of 50% slurry protein A-agarose bead antibody. The ERα-depleted nuclear extracts were analyzed by Western blots to confirm the removal of ERα from the nuclear protein extracts.

**DNA Affinity Protein Assay (DAPA)—** DAPA were performed essentially as previously described (23). 5′-Biotin end-labeled sense and anti-sense oligonucleotides corresponding to the wild-type Sp1 binding site (−373CATGACTCTCTCTCTGATAG−355) and its mutant (5′-CAGTGaTaTCTaCTTGATCA-3′) to the C/EBP wild-type binding site (−386ATAATTGTGGCAACTGACT−368) and its mutant (5′-ATAAAATACCAtATAGCTGACT-3′) of the hPRLR promoter III were custom made by GeneProbe Technology, Inc. (Gaithersburg, MD). The oligomers were annealed and gel purified by 12% polyacrylamide gel electrophoresis. 50 μg of nuclear protein extracts were preincubated with 40 μl of streptavidin-agarose (50% slurry from Invitrogen) for 1 h at 4°C with gentle agitation. The suspension was centrifuged, and the supernatant collected by centrifugation was incubated with 1% SDS, 0.1% NaHCO3. After reversal of cross-linking by heating at 80°C for 10 min and diluted with buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl at pH 8.1) followed by re-immunoprecipitation with a different relevant antibody. Subsequent steps of ChIP re-immunoprecipitation were as for the initial immunoprecipitations.

**Co-immunoprecipitation (Co-IP)—** 100 μg of nuclear protein prepared from MCF7 cells were initially subjected to preincubation with 40 μl of protein A-agarose (50% slurry) and 2 μg of normal rabbit or mouse immunoglobulin G (IgG) in the immunoprecipitation assay buffer (1X PBS, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) for 30 min at 4°C with gentle agitation. The recovered supernatant was incubated with 2 μg of an antibody to a member of the complex for 2 h at 4°C in the presence of 1X protease inhibitor mixture. Then, 50 μl of protein A-agarose in 50% slurry was added, and the incubation was continued for overnight. Protein A-precipitated protein complex was recovered by brief centrifugation, followed by three times washes with immunoprecipitation assay buffer. The harvested beads resuspended in 25 μl of 2X protein sample buffer containing 5% β-mercaptoethanol were boiled for 5 min to release the bound protein. The samples were then analyzed by Western blot with a specific antibody to another member of the complex. For Co-IP of ERα with C/EBPβ we utilized ERα antibody cross-linked to protein G-agarose beads to immunoprecipitate ERα employing the Size X Protein G Kit (Pierce). This avoided masking of the C/EBPβ band (45 kDa) by the immunoglobulin heavy chain dissociated from the immunoprecipitates. The samples normalized by input were expressed as -fold increase over control untreated cells in basal conditions (C) designated as 1.

**RESULTS**

**Identification of Sites Critical for E2-induced Transcriptional Activation of the hPRLR Gene—** Previous studies demonstrated that the hPRLR promoter (−480/−112) contains C/EBP and Sp1 functional elements that bind C/EBPβ and Sp1/Sp3 and that both contribute to basal transcriptional activity (16). Furthermore, 5′-flanking regions to the promoter did not influence basal transcriptional activity in T-47D and MCF-7 cells (16). In addition E2 was found to activate PIII in T-47 D cells transfected with the hPRLR-luciferase construct (18). In this study we initially explored the functional domains within the hPRLI promoter and 5′-flanking region involved in the E2 activation of transcriptional activity in MCF-7 cells (Fig. 1). E2 stimulated the hPRLR −480/−112 hPRLR promoter/luciferase construct 6–8-fold, and neither the addition of 5′-flanking sequences (−481/−931) nor the mutation of ER one-half element at −240G/GCTA−798 had effect in the E2 activation of transcriptional activity (Fig. 1B). The E2 activation was inhibited to basic construct levels by the addition of the E2 receptor antagonist ICI 182,780 to the cultures (Fig. 1C). Mutation of either Sp1 or C/EBP sites inhibited E2 activation to near basal control values in both the −931/−112 construct or in the −480/−112 promoter construct (Fig. 1, B and C). This indicated a required effect of cooperative effects of transactors C/EBPβ and Sp1/Sp3 in E2 activation of hPRLR transcription through hPRLI.

**Estrogen Activated the Transcription of hPRLR and Caused Increases in mRNA and Receptor Protein Expression—** The hPRLR promoter activity was dose dependently increased by E2 treatment of MCF-7 cells for 24 h (Fig. 2A). Induction of promoter activity was observed with 1 nM E2 (2–3-fold), and a 6–8 fold increase was observed with 100 nM E2 (Fig. 2A). In temporal studies, 100 nM E2-induced increases in promoter activity were initially observed at 6 h (1-fold), and continue to increase at
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12 (3–4-fold) and 24 h (6–8-fold) (Fig. 2B). In parallel studies, we evaluated whether the endogenous expression of the hPRL gene governed by its natural promoter could be regulated by E₂. Real-time PCR analysis of RNA from E₂-treated and -untreated MCF-7 cells demonstrated a dose-dependent increase of mRNA levels (Fig. 2C). These results paralleled those obtained for the activation of promoter activity (Fig. 1A). A significant activation of endogenous hPRLR exon 1 (E13) gene transcripts directed by the hPIII promoter was observed following 6 h incubation of the cells with 100 nM E₂ (2-fold), and further increases were observed at 12 and 24 h (Fig. 2D). Furthermore, PRLR protein dose-related increases were induced by E₂ (Fig. 2E) and these were observed at 12 and 24 h of treatment with 100 nM estradiol (Fig. 2D). Our results showed clear correlation between the transcriptional responses of the hPRLR gene to E₂ and demonstrated that hPRLR transcription markedly induced by E₂ caused significant activation of hPRLR gene expression in MCF-7 cells.

Interaction of ERα with C/EBPβ and Sp1/Sp3 in the Activation of hPRLR Gene Transcription—Our results from transient transfection studies indicated that DNA-bound Sp1 and C/EBPβ have a central role in E₂-induced hPRLR gene expression through the hPIII promoter by interacting with ER protein (Fig. 1). To determine the nature of their participation we conducted DAPA using biotin-labeled C/EBP and Sp1 wild-type and mutant double-stranded sequences of hPIII PRLR as probe and nuclear extracts from control and cells treated with 100 nM E₂, in the presence or absence of 5 µM ICI or ICI alone. Relative luciferase activities were normalized by the activity of co-transfected β-galactosidase and expressed as fold over control (untreated cells) for the constructs indicated. Mutated sequences are indicated below the sequences.

FIGURE 1. Estrogen activates hPIII promoter activity. Identification of regions and cis-elements involved in E₂ activation of hPRLR transcription through hPIII. A, schematic representation of hPRLR with generic hPIII promoter, non-coding alternative exon-1 (E13), common non-coding exons 2, and part of 3 and coding exons 3–10 of hPRLR (16). Exons 3–10 are utilized for translation of the long and intermediate form of PRLR; in addition, exon 11 is utilized for translation of the short forms of the receptor S1a and S1b. Exon 10 sequences are not present in the S1b short form (4). B and C, effect of E₂ on transcription of the hPRLR gene directed through the hPIII promoter. Shown are representations of the DNA fragments (−931/−112 bp) and (−480/−112 bp) used in the preparation of expression constructs. These include the wild-type construct with sequences of promoter hPIII (−480/−358 bp) and relevant cis-elements, indicated by symbols, and most of non-coding exon-1, hE13 (−357/−112 bp). 5'-Flanking sequences (−931/−481 bp) to the promoter are included in the constructs in B. Constructs of wild-type hPRLR in pG52 vector and mutants (mutated elements indicated by X symbols) were transiently transfected/expressed in MCF-7 cells. Also, cells were transfected with promoterless vector pG52 (Basic). After transfection MCF-7 cells were treated with and without 100 nM E₂ for 24 h in the presence or absence of 5 µM ICI, or ICI alone. Relative luciferase activities were normalized by the activity of co-transfected β-galactosidase and expressed as fold over control (untreated cells) for the constructs indicated. Mutated sequences are indicated below the sequences.
DAPA utilizing these nuclear protein preparations were used to determine whether bound Sp1/Sp3 and C/EBPβ targeted ERα to the complex (Fig. 3A, middle and right). The expected binding of Sp1, Sp3, and C/EBPβ to the wild-type but not the mutated hPRLR promoter sequences was revealed by their respective antibodies and their absence when their specific DNA sequences were mutated. This is consistent with findings from electrophoretic mobility shift analysis that showed Sp1/Sp3 and C/EBPβ binding to their cognate sequences of hPRLR transfected in T47D and MCF-7 cells (16). Moreover, nuclear protein from cells treated with E2 revealed association of ERα with its binding element (Fig. 3A, middle and right). No differences from control were, however, observed when nuclear extracts of cells treated with ICI (not shown) or ICI/E2 were employed. These results demonstrated that association of ERα with Sp1/Sp3 and C/EBPβ bound to their respective binding elements was observed (Fig. 3B, right). Such interactions were specific because they were only observed when utilizing nuclear preparations from cells treated with the ICI, which contain only trace amounts of ERα. These findings indicate that in addition of the mutual recruitment of ERα through the Sp1 or C/EBPβ bound to Sp1 and C/EBPβ.

Also, the possible inclusion in the complex of coactivators known to associate with ERα was investigated. Western blots (Fig. 3B, middle) demonstrated that E2 did not affect the protein levels of coactivators (SRC1, p300/CBP, and pCAF). However, an increased association of coactivators presumably to ERα recruited through the Sp1 or C/EBPβ bound to their respective elements was observed (Fig. 3B, right). Such interactions were specific because they were only observed when utilizing nuclear preparations of cells treated with E2 that contain high concentration of ERα (middle and right), similarly Sp1/Sp3 and ERα were co-precipitated by C/EBPβ antibody (middle), and Sp1/Sp3 and C/EBPβ by the ERα antibody (right). In all
cases, interactions were observed when using nuclear extracts of cells treated with E2, whereas minor or no interaction was present when utilizing control or nuclei preparations of cells treated with ICI. This confirms our initial proposal about the nature of the core interacting members of the complex and the requirement of ER/H9251 in complex formation.

Subsequent studies were directed to determine whether ER/H9251 per se or E2-activated ER/H9251 was required for the formation of the complex. For these studies we performed DAPA analysis and co-IP using MCF-7 nuclear cell extracts immunodepleted of endogenous ER/H9251 and incubated with a constant amount of exogenously added recombinant ER/H9251 in the presence or absence of E2. DAPA demonstrated that association of ER/H9251 to SP1 and C/EBP/H9252 bound to their respective elements was highly dependent on its activation by E2 (Fig. 5A). This interaction was also demonstrated by co-IP where ERα in the presence of E2 was coprecipitated by the Sp1 or C/EBP/H9252 antibody, whereas only a minor band was observed in the absence of the hormone (Fig. 5B). These results demonstrated that an activated ERα complex was required for its interaction with Sp1 and C/EBPβ.

A requirement of ERα to link Sp1 and C/EBPβ within the complex was indicated in DAPA analyses utilizing nuclear extracts from cells treated with E2 (Fig. 3A, middle and right). Furthermore, studies showed that hor-
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C-terminal fusion of a V5 tag were generated to analyze the protein-protein interaction by pulldown assay with GST-C/EBPβ (Fig. 6). One construct contained the N-terminal region of ERα that harbors the AF-1 domain but lacks the DNA-binding domain (DBD), hinge region (H), and C-terminal ligand-binding (LBD)/AF-2 domains (M1). Another deletion construct included most of the sequence of ERα (DNA-BD, H, and LBD/AF-2) with a truncation of the AF-1 domain (M2). An additional construct excluded the AF-1 and DBD and included the H-LBD/AF2 domain (M3), another contained the LBD/AF2 domain (M4) and one contained only the LBD (M5) (Fig. 6A). Two additional constructs comprising the AFI, DNA-BD, H (M6) or AF1, DNA-BD (M7) domains were also employed. The AFI construct (M1), and constructs containing DNA-BD, H (M6), or DNA-BD domains (M7), showed no interaction (Fig. 6C, right). In contrast, other constructs lacking either the AF1 domain, alone (M2) or with the DBD (M3), as well as the hinge region (M4) or all of these and also the AF2 domain, only containing the LBD domain (M5), showed basal interaction with C/EBPβ that was markedly increased by E2 (Fig. 6C, left). Furthermore, the observed differences of the ERα/C/EBPβ interaction or E2 effect on the interaction were not related to variations in protein expression of the construct (Fig. 6B). Thus, these studies indicated that the LBD of ERα was responsible for its interaction with C/EBPβ.

Definition of the functional region(s) of ERα necessary for hPIII transcriptional activation through the complex C/EBPβ/Sp1 was subsequently pursued in cotransfection studies with ERα deletion constructs and the hPRLR hPRLR promoter/reporter gene construct in HCC 1806 breast cancer cells, which lack ERα expression. All constructs were found to have total cellular and also nuclear expression (Fig. 6, D and E). The nuclear expression of ERα constructs (M2–M5), which contained the LBD, was increased by E2 treatment of cells. In contrast, the expression of constructs lacking the LBD (M1, M6, and M7) were unchanged by E2 treatment. All constructs with the exception of M1 (AF1 domain) contained the nuclear localization sequence(s) (24). These competently entered the nucleus. The relatively small size of M1 permitted its entry to the nucleus, probably by diffusion. In cells cotransfected with wild-type ERα, E2 caused a 3–4-fold increase in transcriptional activity. Expression of a construct lacking the DBD but bearing the AF1, LBD/AF2, LBD, and hinge (H) regions did not transactivate the promoter. Only the M2 ERα deletion construct containing the DBD, the LBD interacting domain, as well as inactive functional regions (AF2 and H), caused E2-induced transcriptional activation of hPIII that was equivalent to the wild-type construct (Fig. 6F). Based on these findings, we conclude that sequences within the DNA-binding domain are also required for functional transactivation of the hPIII promoter by E2.

Endogenous Recruitment of Individual Components of the Complex on the PIII hPRL Promoter—Our previous studies demonstrated that the hPRLR hPRLR promoter binds Sp1 and C/EBPβ through their elements (16). In this study, we provide evidence for the existence of a complex anchored by these two elements with activated ERα acting as a connector between these transfactors. Subsequently, the impact of E2/ERα on the endogenous recruitment of core transfactors to the complex and other factors/cofactors associated were investigated (Fig. 7). ChIP assays showed no apparent differences in the recruitment of Sp1/Sp3 to the hPRL hPRLR promoter in the presence or absence of E2 with or without addition of the Erα-specific antagonist MPP (1 μM) or MPP alone. However, recruitment of C/EBPβ and ERα was highly induced by E2 but was prevented by MPP (Fig. 7, A and C). The association of these transfactors in the complex was further shown in re-ChIP assays by the subsequent use of C/EBPβ and ERα antibodies and in reverse order (Fig. 7D). No association of ERβ was observed (not shown). The association

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**FIGURE S.** A. Effect of E2 on the recruitment of ERα by Sp1 and C/EBPβ. B. Effect of E2 on ERα induced complex formation. C. Linking of C/EBPβ and Sp1 to the complex by E2-activated ERα. A and C, ChIPs were carried out with incubation of MCF-7 nuclear extracts, depleted of ERα with 5′ biotin-labeled wild-type Sp1 or C/EBPβ in the presence or absence of added recombinant ERα and E2. The avidin-precipitated complexes were subjected to Western blot analyses for immunodetection of ERα (A) C/EBPβ or Sp1 (C). Nuclear extracts depleted of ERα, in the presence or absence of added ERα with or without E2 were subjected to a co-immunoprecipitation assay with C/EBPβ or Sp1 antibody followed by immunoblotting using ERα immunosignal and IgG (heavy chain).
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**FIGURE 6. Analyses of interaction of ERα with C/EBPβ.** A, schematic diagram of the wild-type (WT) ERα/V5 fusion protein and various deletion protein fragments of ERα/V5. Arrows indicate the position of nuclear localization signal sequences (24) (NSL). B, Western blot of expressed ERα/V5 fusion protein and fragments used for pulldown assay. C, pulldown assay of immobilized GST-C/EBPβ with ERα/V5 and deletion protein fragments in the presence and absence of 100 nM estradiol. Pulldown assays of GST with ERα and its mutant proteins were used as negative controls (not shown). The complexes precipitated by immobilized glutathione resin were extracted and subjected to Western blot analyses for immunodetection with V5 antibody to reveal ERα and fragments associated with C/EBPβ. Estrogen effect on the interaction between C/EBPβ and ERα wild-type and deletion constructs. –Fold increase induced by estradiol derived from bound values (percent of input). B and C illustrate one of three independent experiments. D–F, co-transfection studies of the wild-type hPRLR gene promoter/reporter gene construct (~480/~112 bp) with the wild-type and ERα mutant constructs in HCC 1806 breast cancer cells. D, Western blot analyses of total cell expression of co-transfected ERα constructs. E, Western blot analyses of the nuclear expression of cotransfected ERα constructs. F, luciferase activities were normalized by β-galactosidase activity and expressed as mean ± S.E. of three independent experiments, each performed in triplicate.
of E2-activated ERα with the complex within the promoter region (−497/−321 bp) was clearly established. In contrast, no association of ERα, transactors, AcH3/H4, TFIIB, and Pol II with the −881/−773-bp DNA fragment containing the ERα half-site non-functional element was observed (Fig. 7B). This element and sequences 5' to the promoter were shown (Fig. 1) not to participate in E2-induced hPRLR transcription (Fig. 1B). SRC-1, p300, and pCAF coactivators were recruited to the complex by E2/ERα. E2 treatment likely caused the marked increase of
both acetylated H3 and H4 within the promoter domain, and such association was specific for this region (Fig. 7, A and C). The epigenetic changes induced by E2/ERα localization could provide a more accessible promoter environment for recruitment of components of general transcriptional machinery. Specific recruitment of TFIIIB and RNA polymerase II to the hPRLR gene promoter hPIII was observed upon E2 treatment.

**DISCUSSION**

These studies on the molecular basis of transcriptional regulation of the prolactin receptor by estradiol have demonstrated that estradiol increases reporter activity up to 8-fold in MCF-7 cells transfected with the PRLR PIII promoter containing Sp1 and C/EBPβ elements that bind transcription factors Sp1/Sp3 and C/EBPβ, respectively. Also, that both of these functional elements are essential for E2 action. Consistent with these findings, estradiol increased PRLR transcription directed by its PIII promoter in a dose-dependent manner. In the absence of an ERE, indirect effects of E2/ERα through interaction with Sp1 and C/EBPβ bound to their cognate elements caused transcriptional activation of the hPRLR hPIII promoter in breast cancer cells. The assembly of an E2/ERα, Sp1, and C/EBPβ complex was required for transcriptional expression of the hPRLR following the PIII promoter in MCF-7 cells. E2/ERα increased recruitment of C/EBPβ to the hPRLR promoter-linked C/EBPβ with Sp1 within the complex. Estradiol induced the association of ERα with C/EBPβ through the LBD of ERα, as well as recruitment of coactivators p300, SRC-1, and pCAF to the complex, with consequent region-specific changes in histone acetylation. These hormone/receptor-induced associations and chromatin changes favored TFIIIB and RNA polymerase recruitment and the activation of hPIII-directed hPRLR transcription.

The actions of estradiol that regulate transcription of the PRLR gene through its PIII promoter do not involve direct binding of the agonist-activated ER to an ERE. This promoter is devoid of a classical ERE, and through its PIII promoter do not involve direct binding of the agonist-activated ER to an ERE. This promoter is devoid of a classical ERE, and through its PIII promoter do not involve direct binding of the agonist-activated ER to an ERE. This promoter is devoid of a classical ERE, and through its PIII promoter do not involve direct binding of the agonist-activated ER to an ERE. This promoter is devoid of a classical ERE, and through its PIII promoter do not involve direct binding of the agonist-activated ER to an ERE. This promoter is devoid of a classical ERE, and through its PIII promoter do not involve direct binding of the agonist-activated ER to an ERE. This promoter is devoid of a classical ERE, and through its PIII promoter do not involve direct binding of the agonist-activated ER to an ERE. This promoter is devoid of a classical ERE, and through its PIII promoter do not involve direct binding of the agonist-activated ER to an ERE. This promoter is devoid of a classical ERE, and through its PIII promoter do not involve direct binding of the agonist-activated ER to an ERE. This promoter is devoid of a classical ERE, and through its PIII promoter do not involve direct binding of the agonist-activated ER to an ERE. This promoter is devoid of a classical ERE, and through its PIII promoter do not involve direct binding of the agonist-activated ER to an ERE. This promoter is devoid of a classical ERE, and through its PIII promoter do not involve direct binding of the agonist-activated ER to an ERE. This promoter is devoid of a classical ERE, and through its PIII promoter do not involve direct binding of the agonist-activated ER to an ERE. This promoter is devoid of a classical ERE, and through its PIII promoter do not involve direct binding of the agonist-activated ER to an ERE. This promoter is devoid of a classical ERE, and through its PIII promoter do not involve direct binding of the agonist-activated ER to an ERE. This promoter is devoid of a classical ERE, and through its PIII promoter do not involve direct binding of the agonist-activated ER to an ERE. This promoter is devoid of a classical ERE, and through its PIII promoter do not involve direct binding of the agonist-activated ER to an ERE. This promoter is devoid of a classical ERE, and through its PIII promoter do not involve direct binding of the agonist-activated ER to an ERE. This promoter is devoid of a classical ERE, and through its PIII promoter do not involve direct binding of the agonist-activated ER to an ERE. This promoter is devoid of a classical ERE, and through its PIII promoter do not involve direct binding of the agonist-activated ER to an ERE. This promoter is devoid of a classical ERE, and through its PIII promoter do not involve direct binding of the agonist-activated ER to an ERE. This promoter is devoid of a classical ERE, and through its PIII promoter do not involve direct binding of the agonist-activated ER to an ERE. This promoter is devoid of a classical ERE, and through its PIII promoter do not involve direct binding of the agonist-activated ER to an ERE. This promoter is devoid of a classical ERE, and through its PIII promoter do not involve direct binding of the agonist-activated ER to an ERE. This promoter is devoid of a classical ERE, and through its PIII promoter do not involve direct binding of the agonist-activated ER to an ERE. This promoter is devoid of a classical ERE, and through its PIII promoter do not involve direct binding of the agonist-activated ER to an ERE.
C/EBPβ and Sp1 Mediate E2/ERα Regulation of hPRL Expression

Studies have demonstrated that Sp1/Sp3 and CEBPβ are essential participants in the transcriptional activation of the PRLR by E2, revealing novel functions for these transcription factors, and that E2/ERα is the key effector in integrating the core activation mechanism in PRLR transcription.

REFERENCES

1. Wennho, N., Gebre-Medhin, M., Grilli-Linde, A., Ohlsson, C., Isaksson, O. G., and Tornell, J. (1997) J. Clin. Invest. 100, 2744–2751
2. Harvey, P. W. (2005) J. Appl. Toxicol. 25, 179–183
3. Hu, Z. Z., Zhuang, L., and Dufau, M. L. (1998) Trends Endocrinol. Metab. 9, 94–100
4. Hu, Z. Z., Meng, J., and Dufau, M. L. (2001) J. Biol. Chem. 276, 41086–41094
5. Bhatavdekar, J. M., Patel, D. D., Shah, N. G., Vora, H. H., Suthar, T. P., Ghosh, N., Chikhlikar, P. R., and Trivedi, T. I. (2000) Eur. J. Endocrinol. 26, 540–547
6. Peire, S. C., and Chen, W. Y. (2001) J. Endocrinol. 171, R1–R4
7. Meng, J., Tsai-Morris, C. H., and Dufau, M. L. (2004) Cancer Res. 64, 5677–5682
8. Chen, W. Y., Ramamoorthy, P., Chen, N., Sticca, R., and Wagner, T. E. (1999) Clin. Cancer Res. 5, 3583–3593
9. Abu-Rediar, F. A., El-Gamal, B. A., Ibrahim, N. A., and El-Aaser, A. A. (2000) Tumori 86, 24–29
10. Ingram, D. M., Nottage, E. M., and Roberts, A. N. (1990) Med. J. Aust. 153, 469–473
11. Bartsch, C., Bartsch, H., Fuchs, U., Lippert, T. H., Bellmann, O., and Dufau, M. L. (1989) Cancer 64, 426–433
12. Dowsett, M., McGarrick, G. E., Harris, A. L., Coombes, R. C., Smith, I. E., and Jeffcoat, S. A. (1983) Br. J. Cancer 47, 763–769
13. Peire, S. C., and Chen, W. Y. (2004) Oncogene 23, 1248–1255
14. Willis, K. I., London, D. R., Ward, H. W., Butt, W. R., Lynch, S. S., and Rudd, B. T. (1977) Br. Med. J. 1, 425–428
15. Hu, Z. Z., Zhuang, L., Meng, J., and Dufau, M. L. (1999) J. Endocrinol. Metab. 84, 1153–1156
16. Hu, Z. Z., Zhuang, L., Meng, J., and Dufau, M. L. (2002) Endocrinology 143, 2139–2142
17. Hu, Y. L., Lei, Z. M., and Rao, C. V. (1996) Endocrinology 137, 3897–3905
18. Leondires, M. P., Hu, Z. Z., Dong, J., Tsai-Morris, C. H., and Dufau, M. L. (2000) J. Steroid Biochem. Mol. Biol. 82, 263–268
19. Hu, Z. Z., Zhuang, L., Meng, J., and Dufau, M. L. (1998) J. Biol. Chem. 273, 26225–26235
20. Ujhazy, P., Klobusicka, M., Babusikova, O., Strausbauch, P., Mihich, E., and Ehrke, M. J. (1994) Int. J. Cancer 59, 83–89
21. Zhang, Y., and Dufau, M. L. (2003) Mol. Cell. Biol. 23, 6958–6972
22. Zhang, Y., and Dufau, M. L. (2002) J. Biol. Chem. 277, 33431–33438
23. Ylikomi, T., Boocquel, M. T., Berry, M., Gromemeyer, H., and Chambon, P. (1992) EMBO J. 11, 3681–3694
24. Parce, K., Webb, P., Kuiper, G. G., Nilsson, S., Gustafsson, J., Kucherer, P. J., and Scanlan, T. S. (1997) Science 277, 1508–1510
25. Webb, P., Nguyen, P., Valentine, C., Lopez, G. N., Kwok, G. R., McNerney, E., Katzellenbogen, B. S., Enmark, E., Gustafsson, J. A., Nilsson, S., and Kucherer, P. J. (1999) Mol. Endocrinol. 13, 1672–1685
26. Kucherer, P. J., Agard, A. D., Greene, D. L., Scanlan, T. S., Shiu, A. K., Uhet, R. M., and Webb, P. (2000) J. Steroid Biochem. Mol. Biol. 74, 311–317
27. Saville, B., Worrnke, M., Wang, F., Nguyen, T., Enmark, E., Kuiper, G., Gustafsson, J. A., and Salee, S. (2000) J. Biol. Chem. 275, 5379–5387
28. Potratz, S. T., Bellido, T., Mocha1, H., Grabb, D., and Manolagas, S. C. (1994) J. Clin. Investig. 93, 944–950
29. Ray, A., Prefontaine, K. E., and Ray, P. (1994) J. Biol. Chem. 269, 12940–12946
30. Ray, P., Ghosh, S. K., Zhang, D. H., and Ray, A. (1997) FEBS Lett. 409, 79–85
31. Stein, B., and Yang, M. X. (1995) Mol. Cell. Biol. 15, 4971–4979
32. Galien, R., Evans, H. F., and Garcia, T. (1996) Mol. Endocrinol. 10, 713–722
33. Galien, R., and Garcia, T. (1997) Nucleic Acids Res. 25, 4242–4249
34. Harrington, W. R., Sheng, S., Barnett, D. H., Petz, L. N., Katzellenbogen, J. A., and Katzellenbogen, B. S. (2003) Mol. Cell. Endocrinol. 206, 13–22
35. Khanha-Gupta, A., Zibello, T., Simkevich, C., Rossmann, A. G., and Berliner, N. (2000) Blood 95, 3734–3741
36. Lopez-Rodriguez, C., Botella, L., and Corbi, A. L. (1997) J. Biol. Chem. 272, 29120–29126
37. Lee, Y. H., Yano, M., Liu, S. Y., Matsuura, E., Johnson, P. F., and Gonzalez, F. J. (1994) Mol. Cell. Biol. 14, 1383–1394
38. Foti, D., Iuliano, R., Chieffari, E., and Brunetti, A. (2003) Mol. Cell. Biol. 23, 2720–2732
39. Lee, Y. H., Williams, S. C., Baer, M., Sterneck, E., Gonzalez, F. J., and Johnson, P. F. (1997) Mol. Cell. Biol. 17, 2038–2047
40. Huisman, M., Draganeva, Y., Romahn, E., and Jehnichen, P. (2000) Biochem. J. 352,
C/EBPβ and Sp1 Mediate E2/ERα Regulation of hPRLR Expression

Ing, N. H., Beekman, J. M., Tsai, S. Y., Tsai, M. J., and O’Malley, B. W. (1992) J. Biol. Chem. 267, 17617–17623

Baniahmad, A., Ha, I., Reinberg, D., Tsai, S., Tsai, M. J., and O’Malley, B. W. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 8832–8836

Fondell, J. D., Roy, A. L., and Roeder, R. G. (1993) Genes Dev. 7, 1400–1410

Blanco, J. C., Wang, I. M., Tsai, S. Y., Tsai, M. J., O’Malley, B. W., Jurutka, P. W., Haussler, M. R., and Ozato, K. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 1535–1539

MacDonald, P. N., Sherman, D. R., Dowd, D. R., Jefcoat, S. C., Jr., and DeLisle, R. K. (1995) J. Biol. Chem. 270, 4748–4752

Chen, H. W., and Privalsky, M. L. (1997) Mol. Endocrinol. 129, 55–61

Masuyama, H., Jefcoat, S. C., Jr., and MacDonald, P. N. (1997) Mol. Endocrinol. 11, 218–228

Jacq, X., Brou, C., Lutz, Y., Davidson, I., Chambon, P., and Tora, L. (1994) Cell 79, 107–117

Sabbah, M., Kang, K. I., Tora, L., and Redeuilh, G. (1998) Biochem. J. 336, 639–646

Felzien, L. K., Farrell, S., Betts, J. C., Mosavin, R., and Nabel, G. J. (1999) Mol. Cell. Biol. 19, 4241–4246