

Mechanism of transcriptional regulation of LRP16 gene expression by 17- β estradiol in MCF-7 human breast cancer cells

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Abstract

LRP16 gene expression is induced by 17- β estradiol (E2) via estrogen receptor alpha (ER α) in MCF-7 human breast cancer cells. A previous study also demonstrated that ectopic expression of LRP16 gene promoted MCF-7 cell proliferation. To explore the mechanism of hormone-induced LRP16 gene expression, the LRP16 gene promoter region (-2600 to -24 bp upstream of the LRP16 gene translation starting site) was analyzed in the present study by using different 5'-truncated constructs, and a luciferase reporter. The 5'-flanking sequence of -676 to -24 bp (pGL3-S5) was found to be E2-responsive. After exchange of the fragment from -213 to -24 bp with the TK gene proximal promoter region in pGL3-S5, E2 still induced reporter gene activity in MCF-7 and HeLa cells. Sequence analysis showed that the pGL3-S6 (-676 to -214) sequence contains two motifs that may contribute to E2-induced transactivation; namely, an estrogen-responsive element (ERE) half-site/Sp1 at -246 to -227 bp and an E-box site at -225 to -219 bp. Further deletion and mutation analysis of these two motifs indicated that both the 1/2 ERE and Sp1 binding sites were required for E2 action, while E-box deletion did not affect the luciferase activity in MCF-7 and HeLa cells. The results of gel mobility shift and chromatin immunoprecipitation assays confirmed that both ER α and Sp1 were required for hormone-induced transactivation, which involved both ER α and Sp1 directly binding to DNA. Taken together, these findings suggest that ER α and Sp1 play a role in activation of the human LRP16 gene promoter.

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Introduction

Estrogen and estrogen receptor (ER) play important roles in several physiologic processes, including development of the female and male reproductive systems as well as bone, vascular and neuronal functions (Chow *et al.* 1992, Turner *et al.* 1994, Farhat *et al.* 1996). They are also important pathologic factors in the genesis and malignant progression of breast cancer (Dong *et al.* 1999, Khan *et al.* 2003). ER α regulates the transcription of various genes as a transcription factor that binds to estrogen response elements or interacts with other transcription factors upstream of the target genes (Klinge 2001, Safe 2001, Petz *et al.* 2002). There are many reports concerning target genes that are transcriptionally activated by ER α , but the entire mechanism of the pathway from ER α leading to the proliferation and progression of mammary tumors is far from clarified (Finlin *et al.* 2001, van 't Veer *et al.* 2002). Breast cancer cell lines have been extensively used as models for understanding the mechanisms associated with 17 β -

estradiol (E2)-induced cell growth and for development of antiestrogenic and anticarcinogenic agents for treatment of this disease (Dickson & Lippman 1995, Levenson & Jordan 1997, Briand & Lykkesfeldt 2001). MCF-7 cells were among the first ER-positive human breast cancer cell lines characterized as responsive to the mitogenic effects of estrogens in cell culture and in athymic nude mice bearing MCF-7 cell xenografts (Brooks *et al.* 1973, Soule *et al.* 1973, Lippman *et al.* 1975, Levenson & Jordan 1997).

LRP16 is a novel gene that was cloned from human lymphocyte cells by our group in 2000, using restriction length genomic scanning (RLGS); the cDNA was then isolated by the rapid amplification of cDNA end (RACE) technique (GenBank accession no. AF202922) (Yu *et al.* 2000). LRP16 contains an open reading frame for a protein of 325 amino acids. Although the physiologic roles of the LRP16 gene have not been delineated, the fact that ectopic expression of the LRP16 gene stimulated MCF-7 cell proliferation suggests that LRP16 may play an important role in carcinogenesis and/or

progression of hormone-dependent breast cancer (Han *et al.* 2003a,b).

Our previous studies have shown that E2 induces LRP16 gene expression and reporter gene activity in MCF-7 human breast cancer cells transiently transfected with constructs containing LRP16 gene promoter inserts linked to a reporter gene (Han *et al.* 2003a,b). A 2.6 kb fragment (upstream of the translation starting site of human LRP16 gene) that has E2 responsiveness was identified (Han *et al.* 2003a,b). In our previous study, the reporter gene activity was observed not only in the ER-positive MCF-7 cells, but also in the ER-negative COS-7 cells. These data suggest that the effect of E2-induced transactivation is a direct interaction between ER α and the promoter region of the LRP16 gene. The main purpose of the experiments reported here is to illustrate the mechanism of transcriptional regulation of LRP16 gene expression by E2, and to determine *cis*-elements present in the 2.6 kb fragment of human LRP16 gene promoter that confers the E2 transactivation effect. A proximal region of -676 to -24 bp was first found to be important for E2 action by deletion mutants. Further analysis of deletion and mutation constructs showed that either the estrogen-responsive element (ERE) half-site (1/2) at -246 to -242 bp or the GC-rich Sp1 binding site at the -236 to -227 bp site was critical for E2 responsiveness in both MCF-7 and HeLa cells. Subsequently, gel mobility shift assays and chromatin immunoprecipitation assays confirmed that both ER α and Sp1 directly bind to DNA.

Materials and methods

Chemicals and biochemicals

E2, antibiotic solution, and polydeoxyinosine-deoxycytidine (poly-dI●dC) were purchased from Sigma. Fetal calf serum (FCS) and Dulbecco's Modified Eagle's Medium (DMEM) were obtained from Gibco-BRL (Grand Island, NY, USA). Transfection reagent SuperFect, PCR product and plasmid extraction kits were purchased from Qiagen. [γ ³²P] ATP was purchased from YaHui Chemical Co. (Beijing, China). The luciferase enzyme assay system, T4 DNA ligase and T4-polynucleotide kinase were purchased from Promega. LA Taq DNA polymerase and various restriction enzymes were purchased from TaKaRa (Otsu, Shiga, Japan). Sp1 and ER α antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Chromatin immunoprecipitation (ChIPs) assay kits were purchased from Upstate Co. (Lake Placid, NY, USA). FCS was stripped twice with a 1:2 ratio of dextran-coated charcoal (0.01 M Tris-HCL, 0.25% Nort A charcoal and 0.025% dextran, pH 8.0) at 45 °C for 40 min to remove endogenous hormones. Purified ER α protein was obtained from PanVera Corp.

(Madison, WI, USA), and the purified Sp1 protein was obtained from Promega. The synthetic oligonucleotides derived from the LRP16 gene promoter, consensus Sp1 and consensus ERE oligonucleotide were synthesized by ShengGong Biotechnology (Shanghai, China). The sequences for these nucleotides (sense strands) are summarized below. The GC-rich sequences and ERE1/2 sites are underlined, E-box sites are noted by italic type, mutations are underlined and substituted bases are indicated by boldface type:

- pu1, 5'-GAGCTC (SacI) GGT GAG AGC TGA GGA TAT AAC G-3'
- pu2, 5'-GAGCTC (SacI) CCT CGT ACG GCC ATC CAT GG-3'
- pu3, 5'-GAGCTC (SacI) TAT ATT GCC CAG GCT GGT CTT G-3'
- pu4, 5'-GAGCTC (SacI) GCT CAC CGC AAC CTC CGC CTT C-3'
- pu5, 5'-GAGCTC (SacI) AGA TTC TGC TCC AGC TGA GCC -3'
- pd, 5'-AAGCTT (HindIII) CCG CCC ACT TGG ACT CTA TTT-3'
- pd6, 5'-AGATCT (BglII) ATA *CAC ACG TGC* CGC CCC GCC-3'
- pd7, 5'-AGATCT (BglII) CGG ATG GAG CCC CGC CTG A-3'
- pd8, 5'-AGATCT (BglII) GCG CCC GCC CGG GCT CCC AGT GTC GC-3'
- pd8 m1, 5'-AGATCT (BglII) GCG CCC G **TT** CGG GCT CCC AGT GTC GC-3'
- pd8 m2, 5'-AGATCT (BglII) GCG CCC GCC CGG GCT CAA AGT GTC GC-3'
- pd8 m3, 5'-AGATCT (BglII) GCG CCC **GTT** CGG GCT CAA AGT GTC GC-3'
- S5p (-254 to -224), 5'-TCC GCG ACA CTG GGA GCC CGG GCG GGC GCG-3'
- consensus Sp1, 5'- AGC TTA TTC GAT CGG GGC GGG GCG AGC G-3'
- Sp1 (mutant), 5'- AGC TTA TTC GAT CGA AGC GGG GCG AGC G-3'
- consensus ERE, 5'-GTC CAA AGT CAG GTC ACA GTG ACC TGA TCA AAG A-3'
- ERE (mutant), 5-GTC CAA AGT CAG GAC ACA GTG TCC TGA TCA AAG A-3.

Plasmids

The vectors, including pGEM-T-easy, pGL3-Basic, pRL-SV40, pGL3-CMV and pRL-TK, were purchased from Promega. A human ER α expression vector, ps5ER α , was kindly provided by Dr Hajime Namata of Kyushu University, Japan. Constructs of pGL3-S₁ (-2223 to -24 bp), pGL3-S₂ (-1775 to -24 bp), pGL3-S₃ (-1354 to -24 bp), pGL3-S₄ (-1064 to -24 bp) and pGL3-S₅ (-676 to -24 bp) were made by PCR amplification, using pGL3-S₀ (-2623 to -24 bp) as the

template, and inserted into the pGL3-basic vector at SacI and HindIII cloning sites. The forward primers with SacI restriction linker used for pGL3-S₁ to pGL3-S₅ constructs were pu1, pu2, pu3, pu4 and pu5 respectively, and the reverse primer with HindIII restriction linker was pd. The pGL3-TK construct was made by inserting a TK promoter, which was obtained by BglII and HindIII enzyme digestion from a pRL-TK plasmid, into the pGL3-basic vector at the same cloning sites. pGL3-S₆ (−676 to −214 bp), pGL3-S₇ (−676 to −251 bp), pGL3-S₈ (−676 to −225 bp), pGL3-S_{8 m1} (−676 to −225 bp with GC-rich region mutation), pGL3-S_{8 m2} (−676 to −225 bp with ERE1/2 mutation) and pGL3-S_{8 m3} (−676 to −225 bp with both ERE1/2 and GC-rich region mutations) were made by ligation of appropriate restriction fragments into the pGL3-TK plasmid. The mutation sites were introduced through PCR primers. pu5 was used as the forward primer for pGL3-S₆, pGL3-S₇, pGL3-S₈, pGL3-S_{8 m1}, pGL3-S_{8 m2} and pGL3-S_{8 m3} constructs, and pd6, pd7, pd8, pd8 m1, pd8 m2 and pd8 m3 were used as reverse primers for these constructs respectively. The LRP16 promoter fragments amplified by PCR were first cloned into the pGEM-T easy TA vector. All ligation products were transformed into DH5 α -competent cells. Constructions were confirmed by restriction enzyme mapping and DNA sequencing. High-quality plasmids for transfection were prepared with the Qiagen Megaprep plasmid kit.

Preparation of MCF-7 nuclear extracts

MCF-7 cells were plated into 100 mm dishes and exposed to 100 nM E2 for 72 h, and then the cells were harvested and pelleted. Pelleted cells were resuspended in 1 ml buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 1.0 mM EDTA, 10 mM KCl, 1.0 mM DTT, 0.5 mM PMSF, 100 μ g/ml pepstain A and 100 μ g/ml aprotinin), incubated on ice for 45 min, homogenized and then centrifuged at 20 000 *g* for 30 min. The nuclei were resuspended in buffer B (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 1.0 mM EDTA, 0.5 M KCl, 1.0 mM DTT, 0.5 mM PMSF, 100 μ g/ml pepstain A and 100 μ g/ml aprotinin) and incubated on ice for 30 min. The nuclear extract was centrifuged at 20 000 *g* for 30 min, aliquoted, assayed for protein concentration and stored at −80 °C.

Cell lines and culture conditions

The HeLa and MCF-7 cells were purchased from ATCC (Rockville, MD, USA). HeLa cells were maintained in DMEM supplemented with 10% FCS and 100 U/ml penicillin-streptomycin, while MCF-7 cells were maintained in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 10 mg/ml bovine insulin

and 100 U/ml penicillin-streptomycin in a humid atmosphere with 5% CO₂ at 37 °C.

Transient transfection and luciferase assay

For transfection experiments, MCF-7 and HeLa cells were seeded in 35 mm culture dishes in DMEM supplemented with 1% dextran-coated charcoal FCS and grown until they were 50% confluent. Reporter plasmids (1 μ g) were transiently cotransfected with the ER α expression vector (1 μ g) with the SuperFect reagent, according to the manufacturer's instructions. An amount of 5 ng *Renilla* luciferase control vector, pRL-SV40 (as an internal standard), was added to each dish to assess the transfection efficiency. Cells were incubated for 12 h and then treated with E2 (100 nM) or DMSO for 34–40 h. The cells were lysed and harvested by the Dual-Luciferase reporter assay system. The luciferase activity in the lysates was analyzed with a Lumat LB 9507 (Promega). The experiments for each treatment group were carried out at least in triplicate.

Gel mobility shift assays with nuclear extracts and purified proteins

Gel mobility shift assays were carried out essentially as previously described (Xie *et al.* 2000). The nucleotides were synthesized, purified and annealed, and 1.75 pmol specific oligonucleotides were ³²P-labeled at the 5'-end with T4 polynucleotide kinase and [³²P]ATP. Nuclear extracts were incubated in HEPES with ZnCl₂ and 1 μ g polydeoxyinosine-deoxycytidine for 10 min on ice to remove nonspecific DNA-binding proteins. Subsequently, a 100-fold excess of unlabeled wild-type or mutant oligonucleotide competitors for the competition experiments was incubated with the nuclear extracts for 5 min on ice. The mixture was then incubated for 15 min at room temperature with the appropriate ³²P-labeled DNA probe, and then antibodies were added for 20 min on ice. When purified proteins were used, ³²P-labeled S5p probe and 200 fmol purified ER α protein or 1 ng purified Sp1 protein were added to the reaction mixture in the presence of 1 μ g polydeoxyinosine-deoxycytidine and incubated for 15 min at 25 °C. E2 was added to the binding reactions containing purified ER α protein at a final concentration of 20 nM. The reaction mixture was then loaded onto a 6% polyacrylamide gel and electrophoresed at 200 V for 1.5 h in 0.5 \times TBE buffer. The gel was dried at 60 °C, and protein–DNA complexes were visualized by autoradiography.

Chromatin immunoprecipitation (ChIP) assays

MCF-7 cells (1 \times 10⁶) were grown in 10 cm tissue culture plates and treated with 100 nM E2 for various times. Formaldehyde was then directly added to the medium to

a final concentration of 1%, and it was then incubated for 10 min at 37 °C. The medium was then completely removed, and the cells were washed twice in ice-cold PBS containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin and 1 µg/ml pepstatin A). Cells were then scraped, collected by centrifugation for 4 min at 2000 *g* at 4 °C, resuspended in SDS lysis buffer (1% SDS, 10 mM EDTA and 50 mM Tris, pH 8.1) and incubated for 10 min on ice, and then the lysate was sonicated for 45 s to shear DNA to lengths of 200–1000 bp. This extract was then centrifuged at 15 000 *g* for 10 min at 4 °C, aliquoted, and stored at –80 °C until used. An aliquot (4 µl) of salmon sperm DNA/protein A agarose-50% slurry (Upstate Co.) was then added per 100 µl chromatin and incubated for 30 min at 4 °C with agitation. A 100 µl aliquot was saved and used as the 100% input control. The agarose was pelleted by brief centrifugation and the supernatant fraction was collected. Specific antibodies or nonspecific IgG was added, and the mixture was incubated overnight at 4 °C. Subsequently, 4 µl salmon sperm DNA/protein A agarose-50% slurry were added per 100 µl chromatin and incubated for an additional 1 h at 4 °C with rotation. Samples were then briefly centrifuged, and the supernatant that contained unbound, non-specific DNA was carefully removed. The protein A agarose/antibody/protein complex was incubated for 3–5 min with 1 ml of a series of buffers provided by Upstate (ChIP Assay Kit, catalog no. 17–295). Subsequently, 250 µl freshly prepared elution buffer (1% SDS and 0.1 M NaHCO₃) were added to the pelleted protein A agarose/antibody/protein complex, the mixture was vortexed briefly to mix it thoroughly, and it was then incubated at room temperature for 15 min with rotation. The agarose was removed by centrifugation, and the supernatant fraction was transferred to another tube. The elution from the agarose was repeated, and the supernatant fraction was combined. An aliquot of 5 M NaCl (1 µl) was added per 20 µl combined eluates and incubated at 65 °C for 4 h to reverse protein–DNA cross-links. Subsequently, 10 µl 0.5 M EDTA, 20 µl 1 M Tris-HCL (pH 6.5) and 2 µl 10 mg/ml proteinase K were added to the eluates, and the mixture was incubated for 1 h at 45 °C. DNA was recovered by phenol/chloroform extraction and ethanol precipitation, and was resuspended in TE buffer for PCR analysis. PCR was used to detect the presence of promoter regions immunoprecipitated with ER α or Sp1 antibodies (Santa Cruz Biotechnology). The primers –356, 5'-GCCGACGCTCAGCCAACCAG-3' and –210, 5'-TCGTATACACGTGCGCGC-3' (LRP16 gene promoter); and 5'-GGGGTGGCTGTGAAGG TGGA-3' and 5'-CGTTGACGATGGCGTCCACC-3' (third exon in LRP16 gene, 115 bp) were synthesized and used for PCR analysis of the immunoprecipitated DNA.

Statistical analysis

Experiments were performed in triplicate, and the results were expressed as means \pm s.d. Statistical significance was determined by Statview and Student's *t*-test, and the levels of probability are noted for each experiment.

Results

Hormonal induced transactivation study of LRP16 gene promoter region in MCF-7 cells

Previous studies have demonstrated that E2 induces LRP16 gene expression in MCF-7 cells, and this leads to an 11-fold increase in luciferase activity in COS-7 cells transiently cotransfected with an ER α and pGL3-S₀ containing a –2623 to –24 bp LRP16 gene 5'-flanking sequence region insert (Han *et al.* 2003a). Interestingly, the initial transient transfection studies with a pGL3-S₀ vector construct showed that treatment with 10 nM, 100 nM and 1 µM E2 resulted in a 2.5-, 1.5- and 2-fold increase in reporter gene activity respectively (Fig. 1). However, after cotransfection with ER α , there was an enhanced (10–12-fold) induction of reporter gene activity after treatment with 10 and 100 nM E2. These results are consistent with several reported transfection studies in ER-positive breast cancer cells with E2-responsive constructs, which showed that enhanced hormone induction of gene expression is observed only after cotransfection with an ER α expression plasmid (Castro-Rivera *et al.* 2001, Samudio *et al.* 2001, Qin *et al.* 2002). This has been attributed to the high copy number of construct required in transfected cells where endogenous ER α becomes limiting, and the E2 responsiveness is enhanced by cotransfection with an ER α expression plasmid (Augereau *et al.* 1994). Therefore, the ER α expression vector was cotransfected with the constructs of interest in all of the subsequent experiments.

To identify the essential motifs contributing to E2 action in the promoter region of pGL3-S₀, we constructed a set of deletion mutants. The structures of the deletion mutants are indicated in Fig. 2A. As shown in Fig. 2B, E2-induced transactivation of the reporter gene in all six constructs, including pGL3-S₀, pGL3-S₁, pGL3-S₂, pGL3-S₃, pGL3-S₄ and pGL3-S₅. Among the fragments, the promoter regions of –2623 to –24 bp exhibited relatively lower fold increase in luciferase activities than did the other five fragments. This observation probably indicates that the region of –2.6 kb to –2.2 kb decreased E2-induced promoter activities. Furthermore, the results suggest that the promoter region of the LRP16 gene contains a possible negative regulatory sequence that influences E2 responsiveness.

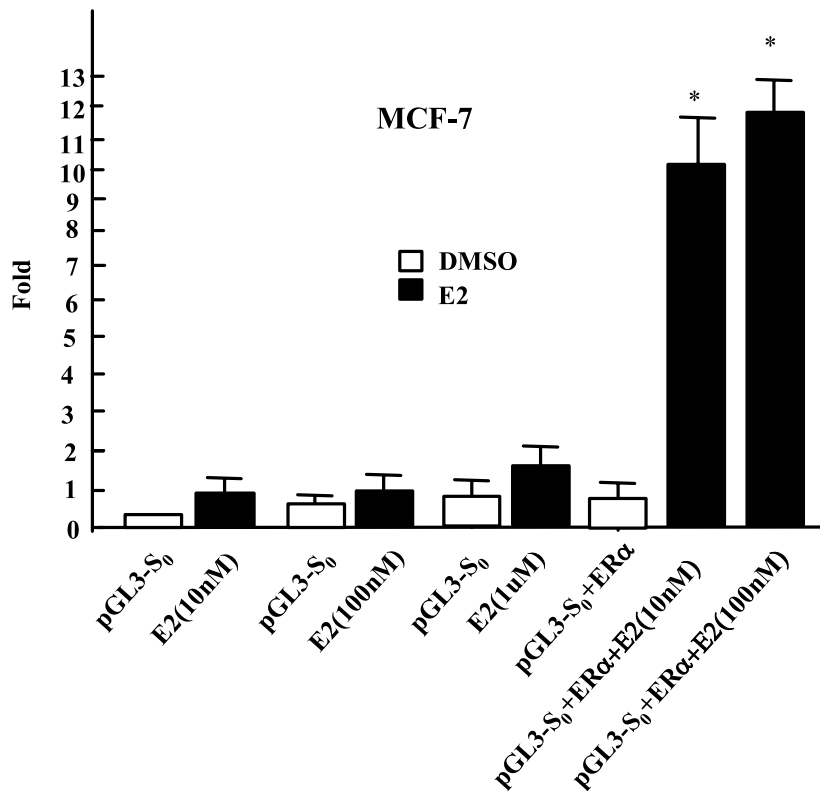


Figure 1 Hormone-induced activation of PGL3-S₀ in MCF-7 cells. Cells were transfected with PGL3-S₀ and 1 μ g ER α expression plasmid, and the effects of E2 on luciferase activity were determined, as described in Materials and methods. Results are expressed as mean \pm s.d. for at least three triplicate determinations for each treatment group. Statistically significant ($P < 0.05$) induction is indicated by an asterisk.

Although we cannot exclude the possibility that positive elements for E2 action exist within the fragment of -2.6 kb to -677 bp, deletion of this fragment did not affect the E2-induced transactivation activities. Therefore, we focused on the region of -676 to -24 bp to identify the *cis*-elements that may interact with ER α . Subsequently, two 3'-deletion mutants, pGL3-S₆ (-676 to -214 bp) and pGL3-S₇ (-676 to -251 bp), were constructed, and the structures are shown in Fig. 3A. In these two constructs, the TK promoter from a pRL-TK plasmid was inserted into the 3'-terminals of these two fragments because it can provide *cis*-elements for general transcription factors to bind. As can be seen in Fig. 3B, E2 stimulated a fourfold increase in luciferase activity by MCF-7 cells cotransfected with pGL3-S₆ compared to the control, but not in cells cotransfected with pGL3-S₇. These data indicate that a minimal 38 bp sequence of -214 to -251 bp is indispensable for hormone responsiveness of the LRP16 gene promoter located within the -676 to -214 bp region. Furthermore, this was also demonstrated in the ER-negative HeLa cells (Fig. 3C).

Estrogen enhances transcription of a reporter plasmid containing the ERE1/2/Sp1 binding site

The minimal 38 bp sequence of -214 to -251 bp of the human LRP16 gene promoter which contributed to E2 responsiveness, as illustrated above, contains an ERE1/2 site at -246 to -242 bp, a GC-rich Sp1 binding region at -236 to -227 bp and an E-box at -225 to -219 bp (Fig. 4A). A number of studies have suggested that a Sp1 site alone, or in combination with imperfect ERE sites or an ERE1/2 site, may be involved in conferring estrogen responsiveness (Krishnan *et al.* 1994, Porter *et al.* 1996, Scholz *et al.* 1998, Sun *et al.* 1998, Petz *et al.* 2000). Xing and Archer (1998) also have demonstrated that upstream stimulatory factors may mediate estrogen receptor activation by binding to E-box sites within the proximal promoter region of E2-induced genes. To determine whether the E-box is essential for estrogen responsiveness of the LRP16 gene promoter, we deleted it in the pGL3-S₈ (-676 to -225 bp) construct (Fig. 4B). By transient cotransfection assay, the E2-induced transactivation activities conferred by pGL3-S₆ (-676 to

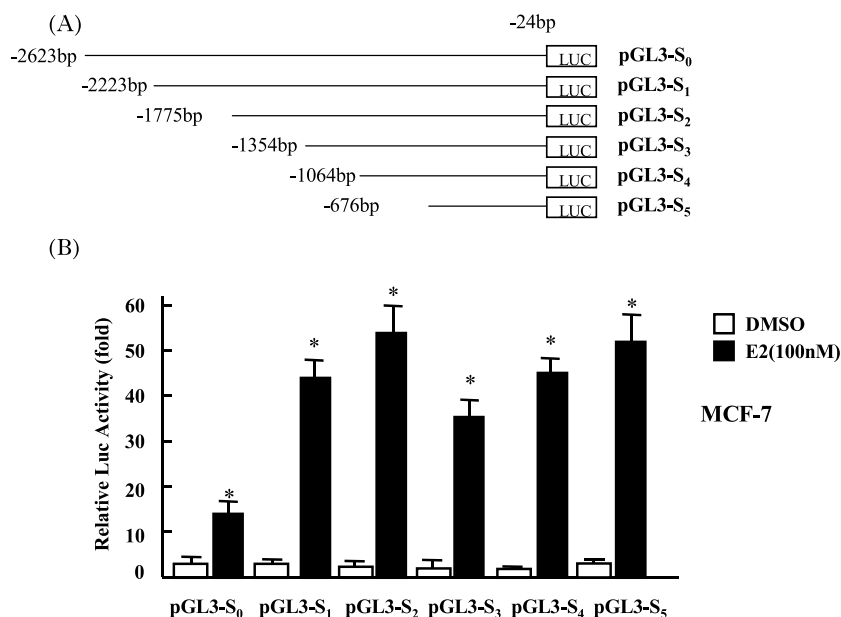


Figure 2 Luciferase assay with deletion mutants prepared from 5'-terminal region of the human LRP16 gene in MCF-7 cells. The structure of DNA fragments with various deletions is shown on the top panel (A). The numbers indicate nucleotide positions of the 5'-end from the first nucleotide of the codon for initiation of translation. Each fragment was linked to the luciferase gene on the pGL3-basic vector and used for transfection experiments. The names of the luciferase plasmids are shown on the right. The results of the luciferase assays with the deletion mutants are shown on the bottom panel (B). MCF-7 cells were transiently transfected with 1 μ g various deletion mutants and 1 μ g human ER α expression plasmid in an individual 35 mm Petri dish, and treated with DMSO or 100 nM E2. Luciferase activity was determined as described in Materials and methods. Results are expressed as means \pm S.D. for at least three triplicate determinations for each treatment group, and statistically significant ($P < 0.05$) induction is indicated with an asterisk.

-214 bp) and pGL3-S₈ (-676 to -225 bp) were compared. As shown in Fig. 4C and D, exposure to E2 led to significant increases in luciferase activity in both MCF-7 and HeLa cells cotransfected with either the pGL3-S₆ or pGL3-S₈ constructs. It was also observed that E-box deletion did not affect the reporter gene activity induced by E2. These observations indicate that the E-box is not indispensable in conferring the action of E2. However, it was not observed that the presence of the E-box inhibited the basal level of reporter gene in MCF-7 cells, as reported by Khan *et al.* (2003) in the CAD gene promoter system, but in HeLa cells the basal activities were only slightly increased in the absence of the E-box.

As shown above, the LRP16 promoter fragment of -251 to -225 bp of the human gene is significantly associated with E2-induced transactivation activity. To determine whether the functional enhancer element for conferring the E2 action is the GC-rich Sp1 binding site alone, or whether it also requires combination with the ERE1/2 site (ERE1/2/Sp1), we constructed a series of mutants, including pGL3-S_{8m1} (-676 to -225 bp with

GC-rich region mutation alone), pGL3-S_{8m2} (-676 to -225 bp with ERE1/2 mutation alone) and pGL3-S_{8m3} (-676 to -225 bp with both ERE1/2 and GC-rich region mutations). These structures are shown in Fig. 4B. The results of cotransfection demonstrated that mutation of both sites or either one markedly decreased the reporter gene activities induced by E2 (Fig. 4E and F). In the pGL3-S_{8m2} cotransfection groups, treatment with E2 resulted in only 2- and 1.5-fold increases in luciferase activity in MCF-7 and HeLa cells respectively. In contrast, in the pGL3-S_{8m1} and pGL3-S_{8m3} groups, the E2-induced transactivation activities maintained their basal levels in both cell lines. These findings suggest that both the ERE1/2 site and Sp1 binding sites are involved in estrogen-mediated activation of the LRP16 gene promoter.

Interactions of ER α and Sp1 proteins with the ERE1/2/Sp1 binding site

The transient transfection experiments provided evidence for the involvement of the ERE1/2/Sp1 binding

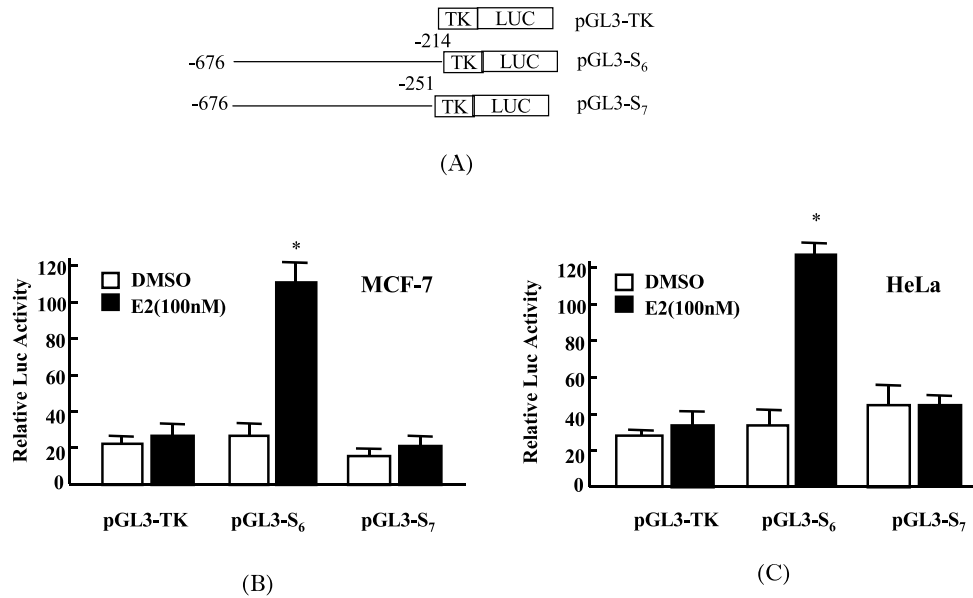


Figure 3 Effects of E2 on reporter gene activities with deletion mutants prepared from pGL3-S₅ in MCF-7 and HeLa cells. The structures of the deletion mutants with 3'-end deletion of pGL3-S₅ are shown in panel A. The names of plasmids are shown on the right. The minimal promoter region of the TK gene was from the pRL-TK plasmid, and was linked to the luciferase gene on the pGL3-basic vector. The fragments prepared from pGL3-S₅ were linked to the pGL3-TK plasmid. pGL3-TK was used as a negative control. Luciferase activities in MCF-7 and HeLa cells transfected with various constructs and human ER α expression plasmid are shown in panels B and C respectively. Results are expressed as mean \pm s.d. for at least three triplicate determinations for each treatment group, and statistically significant ($P < 0.05$) induction is indicated with an asterisk.

site in mediating E2 action of the LRP16 gene promoter. However, none of these studies allowed us to identify proteins that interact with this DNA sequence. Therefore, gel mobility shift assays were used to investigate the interactions of nuclear extracts from E2-treated MCF-7 cells with the corresponding ³²P-labeled oligonucleotides. Since we anticipated that ER α and Sp1 might bind to the S5p fragment, the consensus ³²P-ERE and ³²P-Sp1 probes were used as positive controls and antibodies against ER α , and Sp1 proteins were included in separate binding reactions. As shown in Fig. 5A, nuclear extracts from the MCF-7 cell line bound ³²P-ERE or ³²P-S5p to yield a major low-mobility DNA/protein complex, as indicated by the arrow (lanes 2 and 7). Competition with a 100-fold excess unlabeled ERE oligonucleotide markedly decreased the intensity of this band (lanes 3 and 8), but the intensity of this band was unaffected by a nonspecific immunoglobulin (Ig) antibody (lanes 5 and 10). Unexpectedly, the supershifted band was not clearly detected in either lane 4 or 9 after coinubation of nuclear extracts and ³²P-labeled probe with an ER α -specific antibody. Figure 5B shows the results of a gel mobility shift assay to identify Sp1 protein. Nuclear extracts from E2-treated MCF-7 cells bound ³²P-Sp1 oligonucleotides to give several bands (lane 2), and a major low-mobility Sp1-³²P-Sp1 complex

(indicated by an arrow) was observed (lanes 2, 4, 5 and 6). This latter band was significantly decreased after competition with 100-fold excess, unlabeled, consensus Sp1 oligonucleotide (lane 3), but was unaffected by adding mutant, unlabeled Sp1 oligonucleotide (lane 4) and after coinubation with nonspecific IgG antibody (lane 6). However, Sp1 antibody supershifted the complex to give a retarded band (lane 5). Lane 8 showed that only the major low-mobility band formed by nuclear extract-³²P-S5p interaction was similar to that observed with ³²P-Sp1. The specifically bound Sp1-³²P-S5p complex was decreased by nonlabeled, 100-fold excess Sp1 and S5p oligonucleotides (lanes 9 and 10). Although the Sp1-³²P-S5p complex in lane 12 appeared to be 'fainter' than that in lane 8, an effect possibly caused by the loading amount, it was not shifted after coinubation with nonspecific IgG antibody, while Sp1 antibody supershifted the mobility to give a smear-like complex (lane 11), and this site was similar to that observed in lane 5.

Although our gel mobility shift experiments using MCF-7 nuclear extracts suggested that Sp1 was involved in regulation of the LRP16 gene expression, they did not provide convincing evidence that the ER α was involved in formation of a protein-DNA complex. However, to our knowledge, few studies have provided evidence that

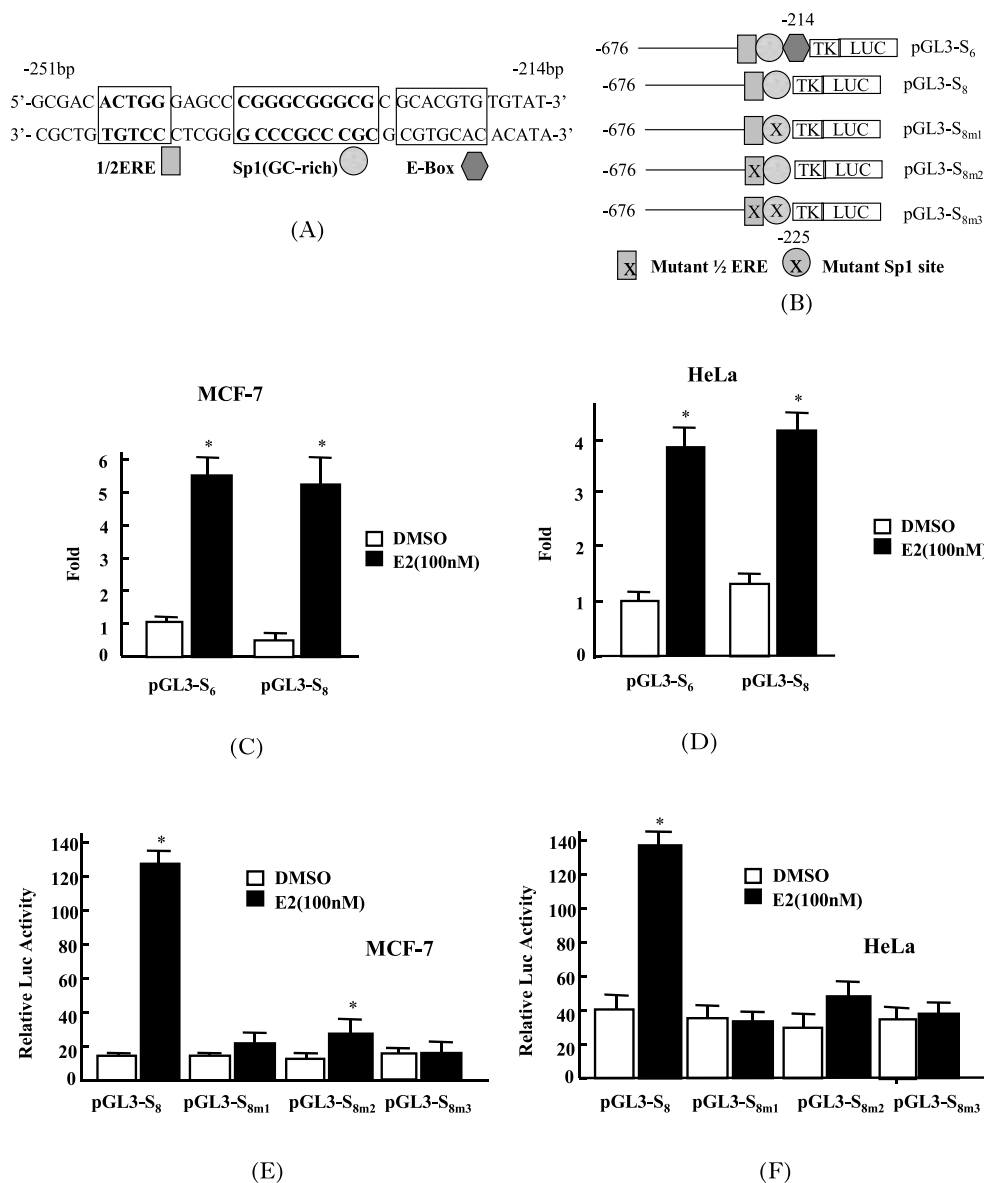


Figure 4 Luciferase assays with deletion and mutations of the minimal 38 bp fragment for E2-induced transactivation of pGL3-S₆ in MCF-7 and HeLa cells. Sequences of the ERE1/2 site, GC-rich Sp1 binding site and E-box site contained within the 38 bp fragment of -251 to -214 are shown in panel A. The rectangle, circle and hexagon represent the ERE1/2, GC-rich region and E-box respectively. The structures of a set of deletion and mutation constructs are shown in panel B, MCF-7 (panels C and E) and HeLa cells (panels D and F). Cells were transiently transfected with various constructs and 1 μ g human ER α expression plasmid, as described in Fig. 2. Results were expressed as mean \pm S.D. for at least three triplicate determinations for each treatment group, and statistically significant ($P < 0.05$) induction is indicated with an asterisk.

ER from MCF-7 nuclear extracts other than purified or *in vitro* translated ER α directly bind to DNA in gel mobility shift assays due to the formation of fragile protein-DNA complexes (Porter *et al.* 1996, Sun *et al.* 1998, Petz & Nardulli 2000). Therefore, gel mobility shift assays were performed with purified ER α and Sp1

proteins. The results, as indicated in Fig. 5C and D, showed that the major protein-DNA complex was formed by ER α -³²P-S5p or Sp1-³²P-S5p (lane 2), and the band was supershifted by the ER α - or Sp1-specific antibody (lane 3), but was not affected by nonspecific IgG (lane 4). Thus, these results suggest that the 1/2

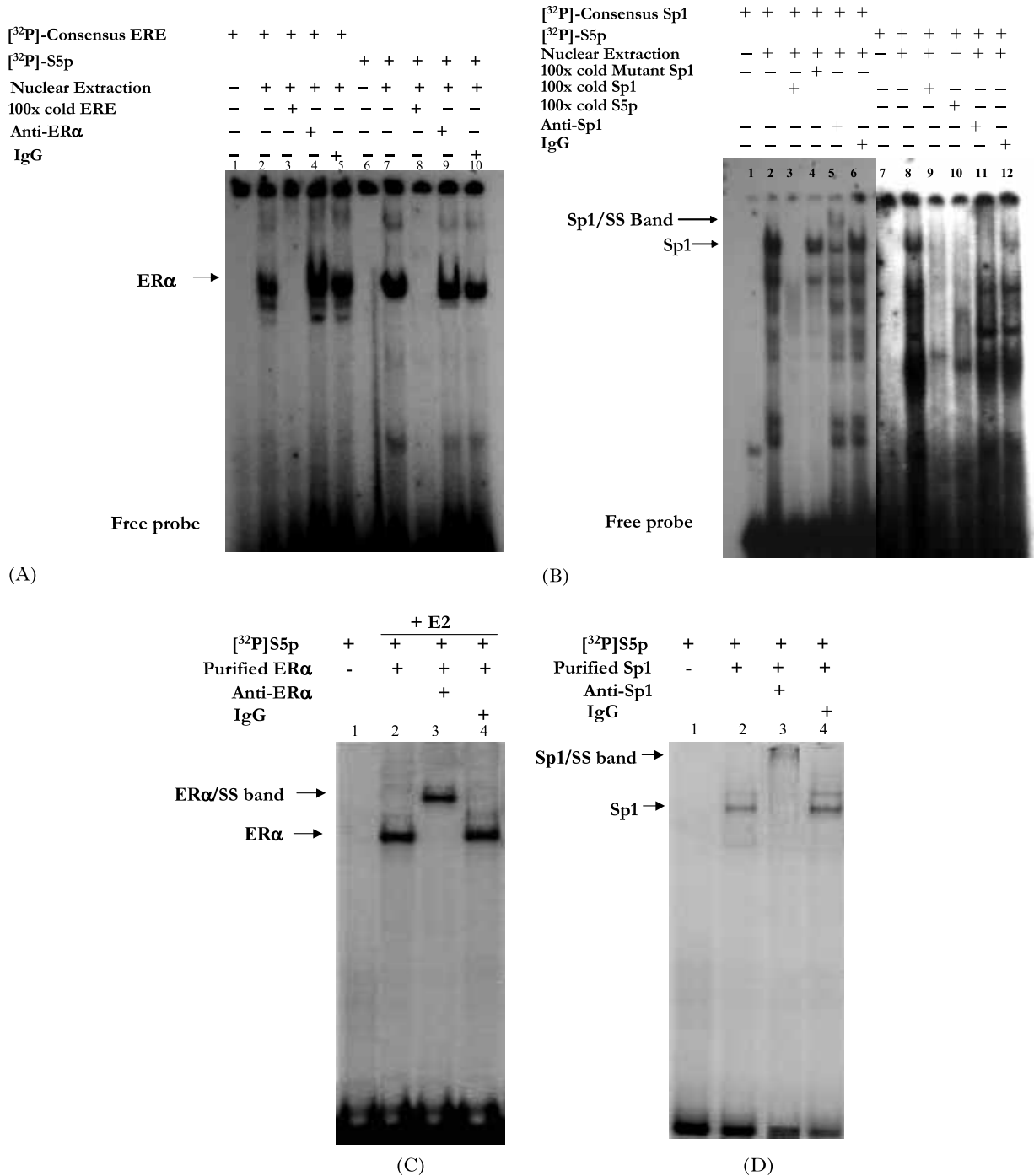


Figure 5 Gel mobility shift assays of S5p and ER α or Sp1 protein. (A) Binding to ER α protein. Nuclear extracts from MCF-7 cells treated with 100 nM E2 were incubated with ³²P-labeled consensus ERE or ³²P-labeled S5p (containing the ERE1/2/Sp1 motif (-254 to -224) region of the LRP16 gene promoter) and unlabeled wide-type consensus ERE or unlabeled S5p oligonucleotides, ER α antibody or nonspecific IgG, as described in Materials and methods. (B) Binding of Sp1 to ³²P-labeled consensus Sp1 or ³²P-labeled S5p in MCF-7 cells. Nuclear extracts from MCF-7 treated with 100 nM E2 were incubated with wild-type or mutant ³²P-labeled consensus Sp1 and unlabeled wide-type or mutant ³²P-labeled consensus Sp1, and ³²P-labeled S5p or unlabeled S5p or wild-type consensus Sp1, Sp1 antibody or nonspecific IgG, as described in Materials and methods. (C) ³²P-labeled S5p oligos binding to purified ER α protein. We incubated 200 fmol purified ER α protein treated with 20 nM with ³²P-labeled S5p and ER α antibody or nonspecific IgG. (D) ³²P-labeled S5p oligos binding to purified Sp1 protein.

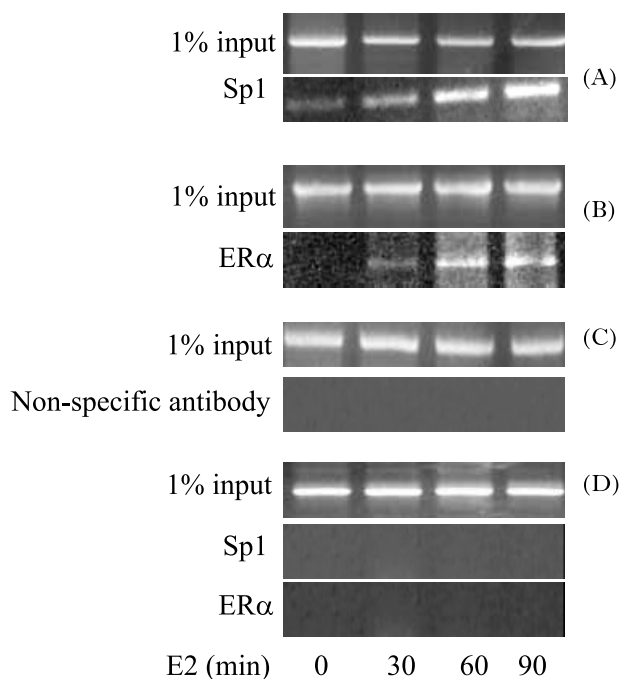


Figure 6 Analysis by ChIP of ER α and Sp1 interactions with the 5'-flanking sequence of the LRP16 gene. (A–C) The –329 to –210 region of the LRP16 gene promoter. MCF-7 cells were treated with 100 nM E2 for different time points; after immunoprecipitation of cross-linked complexes by Sp1 (A), ER α (B) antibody or nonspecific IgG (C), the chromatin was analyzed by PCR, as described in Materials and methods. (D) The third exon region of the LRP16 gene. After immunoprecipitation by Sp1 or ER α antibody, the third exon region of the LRP16 gene was detected by PCR, as described in Materials and methods.

ERE–Sp1 site (–246 to –227 bp) in the LRP16 gene promoter directly binds to ER α and Sp1 proteins.

Interactions of ER α and Sp1 proteins with the LRP16 gene promoter were also investigated in MCF-7 cells treated with 100 nM E2, using chromatin immunoprecipitation (ChIP) assays (Fig. 6). Cells were treated with E2 for different time points and then cross-linked with formaldehyde. Nuclear extracts were sonicated and immunoprecipitated with ER α and Sp1 antibodies. The interaction of ER α and Sp1 proteins with the proximal region of the LRP16 gene promoter (–356 to –210 bp) was determined by PCR. Sp1 antibodies immunoprecipitated this region of promoter in untreated MCF-7 cells and in cells treated with 100 nM E2 for 30, 60 and 90 min (Fig. 6A). ER α antibody did not immunoprecipitate this region of the promoter at 0 min; however, ER α antibodies gave bands at the 30-, 60- and 90-min time points (Fig. 6B). These data show that interactions of ER α and Sp1 with the human LRP16 gene promoter occur at 30-, 60- and 90-min time points after treatment with E2, and indicate a hormone-induced increase in ER α interactions with the LRP16 gene promoter. As a

control experiment, the fragment of –356 to –210 bp was not detected in the nonspecific IgG group (Fig. 6C). In other negative control experiments, ER α and Sp1 antibodies did not immunoprecipitate the third exon region of LRP16 gene (Fig. 6D). These data confirm the site of interactions of ER α and Sp1 with the human LRP16 gene promoter in E2-treated MCF-7 cells.

Discussion

A number of studies have demonstrated that breast cancer is a hormone-dependent multistep process. Although estrogens have been shown to play a central role in breast cancer development, their carcinogenicity in human breast epithelial cells has not yet been clearly demonstrated. An important pathway for the action of estrogen on the target cells is perhaps mediated via ER α and ER β , which function as ligand-activated transcription factors regulating gene expression as specific EREs in the gene promoter region (Katzenellenbogen & Katzenellenbogen 2000). Originally, the human LRP16 gene was isolated from lymphocytes in order to identify a leukemia relapse-related gene, but there was no difference between patients primarily diagnosed with acute myeloid leukemia (AML) and patients relapsed, with regard to AML, to LRP16 mRNA expression levels as determined by semiquantitative RT-PCR (Han *et al.* 2002). By computer-aided SAGE (serial analysis of gene expression) pattern analysis of LRP16 gene in MCF-7 cells, we have demonstrated that E2 induced five- to eightfold increases in LRP16 mRNA levels in MCF-7 cells, and this effect was observed as early as 3 h after addition of E2. Furthermore, we found that overexpression of the human LRP16 gene markedly increased the protein level of cyclin E in MCF-7 cells; more significantly, it stimulated MCF-7 cell proliferation (Han *et al.* 2003a). Some recent results also demonstrated that inhibition of LRP16 gene by small interfering RNA (siRNA) specific to LRP16 restrained proliferation in MCF-7 cells (not published). All these data not only demonstrated that LRP16 is a novel E2-regulated gene, but also implied that the LRP16 gene may play an important role in the carcinogenesis and/or progression of hormone-dependent breast cancer.

E2 leads to increases in LRP16 mRNA levels in MCF-7 cells and also in reporter gene activities in MCF-7 and COS-7 cells transfected with pGL3-S₀ construct, which contains a 2.6 kb insert upstream of the translation starting site of LRP16 gene, as previously reported (Han *et al.* 2003a,b). These data indicate that regulation of LRP16 gene expression by E2 occurs at the level of transcription.

In the present study, we attempted to identify the E2-responsive regions and functional enhancer elements within the 2.6 kb promoter region of the LRP16 gene

that are essential for E2-induced transactivation. Results of computer-aided analysis demonstrated that the fragment of -2623 to -24 bp does not contain a perfect palindrome ERE, but does contain 10 ERE1/2 sites densely distributed within the region of -2.5 to -1.8 kb (data not shown). Interestingly, deletion of the region of -2.6 to -1.8 kb did not decrease reporter gene activity. In contrast, its deletion increased E2 responsiveness (pGL3-S₀ vs pGL3-S₂). Therefore, it is unlikely that these ERE1/2 sites strongly contribute to E2 action, although E2-liganded ER α might interact with dense ERE1/2 sites, as deduced by Driscoll *et al.* (1998). Deletion analysis of -2623 to -24 bp identified the potential negative regulatory sequence within the -2.6 kb to -2.2 kb region. From further deletion and mutation analyses of the LRP16 gene promoter, we characterized a proximal E2-responsive region of -676 to -214 bp, and a 38 bp (-251 to -214 bp) fragment was found to be indispensable for E2-induced action conferred by pGL3-S₆ (-676 to -214 bp). Two positively acting elements were found in this 38 bp fragment: an ERE1/2 site in the region of -246 to -242 bp and an Sp1-binding motif in the region of -236 to -227 bp.

In the 38 bp fragment mentioned above, a conserved E-box motif (GCACGTG), which matches the consensus sequence CANNTG, was found in the region of -225 to -219 bp. Such motifs have been reported to be potential binding sites for basic helix-loop-helix (bHLH) transcription factors, *c-myc*, Myo D, and ubiquitous transcription factors USF-1 and USF-2 proteins (Maekawa *et al.* 1991, Hoffman & Chernak 1995, Elnitski *et al.* 1997, Gao *et al.* 1997, Gupta *et al.* 1997, Scholtz *et al.* 1997). Previous studies have demonstrated that E2 transiently induces *c-myc* gene expression in MCF-7 cells, and a synthetic antisense *c-myc* oligonucleotide partially inhibited E2-induced growth of MCF-7 cells (Dubik *et al.* 1987, Watson *et al.* 1991). Xing and coworkers also demonstrated that ER mediates recruitment of USF1/2 to the major late promoter element in the proximal -210 to -101 bp region of the cathepsin D gene, and that hormones stimulate E-box-dependent transactivation in human breast cancer cells (Xing & Archer 1998). Although USF1/2 was found to be recruited to the +54 to +78 bp E-box in the trifunctional carbamylphosphate synthetase/aspartate carbamyltransferase/dihydroorotase (CAD) gene promoter, E2-stimulated transcription was not affected by deletion of this motif (Khan *et al.* 2003). In our study, deletion of the E-box did not result in diminution of the E2-stimulated reporter gene activity driven by the LRP16 gene promoter region of -676 to -251 bp either in MCF-7 cells or in HeLa cells (Fig. 4C and D).

Sp1 was originally described as a *trans*-acting factor that binds to a GC-box (5'-GGGCGG) (Dyran & Tjian 1983, Gidoni *et al.* 1984). Subsequent comparison of

numerous Sp1 binding sites led to identification of a higher affinity, consensus Sp1 site, 5'-KYGGCGKRRY-3' (Briggs *et al.* 1986), and the discovery that sequences varying from this consensus sequence displayed decreased affinity for Sp1. The GC-rich Sp1 binding motif found in the promoter region of the LRP16 gene is CGGGCGGGCG (-236 to -227 bp) (Fig. 4A). It includes a typical GC-box (GGGCGG). Indeed, in the present case, substitution of two bases (-234 to -235 bp, GG to AA) within the Sp1-binding motif significantly reduced the effectiveness of E2 to levels approaching background in both MCF-7 and HeLa cells (Fig. 4E and F). Several reports have demonstrated that there is functional synergy between transcription factor Sp1 and the estrogen receptors, and that estrogen can modulate target gene expression through ER-enhanced Sp1 binding (Cavaillès *et al.* 1993, Augereau *et al.* 1994, Paech *et al.* 1997, Porter *et al.* 1997, Scholz *et al.* 1998, Petz & Nardulli 2000, Samudio *et al.* 2001). Substitutive mutation of the ERE1/2 site flanking the Sp1-binding region also markedly decreased the E2-induced responsiveness to 2- and 1.5-fold that of the basal levels in MCF-7 and HeLa cells respectively. These data indicate that the Sp1 site cannot effectively confer E2 action without its upstream ERE1/2 site, and suggest that synergic interaction of ER α and Sp1 requires direct binding of E2-liganded ER α to the ERE1/2 site.

The classic mechanism of ER α action is associated with estrogen-induced formation of a nuclear homodimer, binding to the ERE of a perfect or imperfect palindrome in target gene promoters, and interaction with other nuclear proteins and general transcription factors to activate gene expression (Driscoll *et al.* 1998, Klinge 2001). E2-dependent transactivation via ER α -Sp1 interactions has been characterized in the promoters of several E2-responsive genes, including *bcl-2*, *c-myc*, cathepsin D, transforming growth factor α (TGF α), progesterone receptor, retinoic acid receptor α 1, heat-shock protein 27, and others (Cavaillès *et al.* 1989, Krishnan *et al.* 1994, Porter *et al.* 1996, Sun *et al.* 1998, Dong *et al.* 1999, Petz & Nardulli 2000). Transactivation of target genes through interaction of ER α and Sp1 is mediated by at least two pathways: 1. interactions of the ER α -Sp1 complex with GC-rich sites in which only Sp1 protein binds DNA; 2. interaction of ER α -Sp1 proteins with 1/2 ERE(N)xSp1 or Sp1(N)xERE (1/2) motif DNA elements, where both ER α and Sp1 bind DNA elements. In this study, interactions of ER α -Sp1 with 1/2 ERE(N)xSp1 have been analyzed by gel mobility shift assays to give a broad, retarded band that binds both proteins, and to exhibit the expected supershifted band by using purified proteins. The results of gel mobility shift assays indicate that the ER α and Sp1 proteins directly bind well to the DNA elements (Fig. 5).

Previous studies have demonstrated that ER α and Sp1 physically interact, and ER α preferentially binds to the C-terminal DNA binding domain of Sp1 protein (Porter *et al.* 1997), the region that is also required for other transcription factors (Karseder *et al.* 1996, Lin *et al.* 1996). Our study investigated the interactions of ER α and Sp1 transcription factors with the LRP16 gene promoter, using a ChIP assay in MCF-7 cells at different time points after E2 treatment (Fig. 6). ER α and Sp1 antibodies immunoprecipitate the E2-responsive region (-329 to -210 bp) of the LRP16 promoter after treatment with E2 at 30, 60 and 90 min (Fig. 6A and B). The above-mentioned fragment was also immunoprecipitated at 0 min when Sp1 antibody was used, but not when ER α antibody was used. The results shown in Fig. 6 indicate that Sp1 association with the LRP16 gene promoter is ligand-independent, whereas ER α interaction is ligand-dependent. It is not surprising to observe that the functional interaction between Sp1 and ER α in transcriptional regulation of the LRP16 gene expression is ligand-dependent. Several reporters have well established the mechanisms of hormonal regulation of such genes as cad, progesterone receptor, transforming growth factor α and low-density lipoprotein receptor, in which DNA-dependent or -independent interactions of ER α and Sp1 proteins are hormone-dependent (Vyhldal *et al.* 2000, Li *et al.* 2001, Khan *et al.* 2003, Petz *et al.* 2004).

In summary, the results presented demonstrate that hormonal regulation of LRP16 gene expression is linked to liganded ER α and Sp1 interactions with an ERE1/2 and GC-rich site in the LRP16 promoter. Many of the genes, such as cyclin D1, E2F1, c-fos and bcl-2, which are regulated by ER α -Sp1 in ER-positive breast cancer cells, play a role in cell proliferation. Our previous study found that the LRP16 gene is also involved in this cellular process (Han *et al.* 2003a). These observations are consistent with a recent report showing that siRNA for Sp1 inhibits hormone-induced cell cycle progression in MCF-7 cells (Abdelrahim *et al.* 2002), and indicate that the LRP16 gene may play an important regulation role in breast cancer development and progression.

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