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Differential regulation of the human progesterone receptor gene through an estrogen response element half site and Sp1 sites

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Abstract

The progesterone receptor (PR) gene is regulated by estrogen in normal reproductive tissues and in MCF-7 human breast cancer cells. Although it is generally thought that estrogen responsiveness is mediated by interaction of the ligand-occupied estrogen receptor (ER) with estrogen response elements (EREs) in target genes, the human progesterone receptor (PR) gene lacks a palindromic ERE. Promoter A of the PR gene does, however, contain an ERE half site upstream of two adjacent Sp1 sites from +571 to +595, the +571 ERE/Sp1 site. We have examined the individual contributions of the ERE half site and the two Sp1 sites in regulating estrogen responsiveness. Transient transfection assays demonstrated that both Sp1 sites were critical for estrogen-mediated activation of the PR gene. Interestingly, rather than decreasing transcription, mutations in the ERE half site increased transcription substantially suggesting that this site plays a role in limiting transcription. Chromatin immunoprecipitation assays demonstrated that Sp1 was associated with the +571 ERE/Sp1 site in the endogenous PR gene in the absence and in the presence of estrogen, but that ERα was only associated with this region of the PR gene after MCF-7 cells had been treated with estrogen. Our studies provide evidence that effective regulation of transcription through the +571 ERE/Sp1 site requires the binding of ERα and Sp1 to their respective *cis* elements and the appropriate interaction of ERα and Sp1 with other coregulatory proteins and transcription factors.

Keywords: Progesterone receptor; ERE; Sp1; Estrogen receptor; Gene expression

1. Introduction

The human progesterone receptor (PR) gene is controlled by 17β-estradiol (E_2) in normal reproductive tissues and in MCF-7 breast cancer cells. Treatment of MCF-7 cells with E_2 increases PR gene expression. PR mRNA and protein reach maximal levels after MCF-7 cells have been treated with E_2 for 72 h [1–3].

The human PR gene encodes two proteins, the 120 kDa PR-B and the 94 kDa PR-A, which lacks 164 amino-terminal amino acids that are present in PR-B [4]. It has been hypothesized that two promoters, Promoter A (+464 to +1105) and Promoter B (–711 to +31), are responsible for the production of PR-A and PR-B, respectively [4,5].

Estrogen's effects are mediated through its interaction with two intracellular estrogen receptors (ERs), ERα and ERβ. Classical models of estrogen action have proposed that the cellular response to estrogen is initiated by the interaction of these receptors with their cognate recognition sequence, the estrogen response element (ERE). Yet, in spite of the fact that both PR Promoters A and B are estrogen responsive, neither contains a palindromic ERE [4]. Thus, estrogen responsiveness of the PR gene must be mediated by *cis* elements other than an ERE.

Previously, we demonstrated that estrogen responsiveness of the human PR gene is derived in part from the interaction of ERα and Sp1 with a region in Promoter A from +571 to +595, which contains an ERE half site and two adjacent Sp1 sites [6] and will be referred to as the +571 ERE/Sp1 site [6]. Although we previously demonstrated that the +571 ERE/Sp1 site was able to function as a transcriptional enhancer in transient transfection assays, there was some question about the individual contributions of the ERE half site and two Sp1 sites in the overall responsiveness of the +571 ERE/Sp1 site to hormone.

In this study, we have assessed the contributions of the ERE half site and two adjacent Sp1 sites using a variety of

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in vivo and in vitro assays. Our studies suggest that each of the individual *cis* elements in the +571 ERE/Sp1 site is important in conferring estrogen responsiveness to the PR gene, but that the ERE half site and Sp1 sites have distinctly different effects on transcription.

2. Materials and methods

2.1. Oligonucleotides and plasmid construction

The sequences of oligos containing the wild-type and mutant +571 ERE/Sp1 sites have been published [6]. The oligo pairs, which had *Bgl*II ends, were annealed and inserted into the *Bgl*II-cut, dephosphorylated chloramphenicol acetyl transferase (CAT) reporter plasmid, TATA-CAT [7] to create wild-type or mutant +571 ERE/Sp1 TATA-CAT vectors. The plasmids were transformed into the DH5α strain of *Escherichia coli*, sequenced and purified on two cesium chloride gradients.

2.2. Cell culture and transient transfection assays

U2 osteosarcoma (U2-OS) cells were maintained in culture and transfected with Lipofectin (Gibco BRL, Grand Island, NY, USA) as described [8,9] with 3 μg of the indicated reporter plasmid and 150 ng of the β-galactosidase vector CMVβ-gal (Promega, Madison, WI, USA). Since we determined that expression of $ER\alpha_{EGA\rightarrow GSV}$ was twofold less than the expression of the wild-type ERα (data not shown), 25 ng of the wild-type human ERα expression vector CMV5hERα [10] or 50 ng of an expression vector encoding an ERE binding deficient human ERα [11] were included as indicated. A previous study also demonstrated that $ER\alpha_{EGA\rightarrow GSV}$ is expressed twofold less efficiently than the wild-type ERα [11]. Cells were exposed to ethanol vehicle or 10 nM E_2 for 24 h. β-Galactosidase activity was determined at room temperature as previously described [12] and used to normalize the amount of CAT activity in each sample. CAT assays were carried out as described [8,9].

2.3. Gel mobility shift assays

Gel mobility shift assays were carried out essentially as described [6]. ^{32}P-Labeled oligos (10,000 cpm) containing the +571 ERE/Sp1 with wild-type or mutant DNA sequence were incubated with the indicated amounts of purified Sp1 (Promega), baculovirus-expressed purified ERα [8,13], bacterially expressed purified ERα wild-type CD or the $CD_{EGA\rightarrow GSV}$ mutant for 15 min at room temperature as indicated. The purified ERα was prepared from Sf9 cells that had been treated with E_2 15 min prior to harvest. BSA, ovalbumin and/or KCl were added as needed to maintain constant protein (20 μg) and salt concentrations. Low ionic strength gels and buffers were prepared as described [14]. Radioactive bands were visualized by autoradiography.

2.4. Pull-down assay using in vitro translated proteins

Flag/His-tagged Sp1 was expressed in stably transfected SL-2 cells, kindly provided by Dr. Guntram Suske (Institut fur Molekularbiologie und Tumorforschung, Philipps-Universitat, Marburg, Germany) as described [15]. The expressed Sp1 was immobilized on M2-agarose (Sigma, St. Louis, MO, USA) or Ni-NTA (Qiagen Inc., Valencia, CA, USA), washed three times with Buffer A (20 mM Tris, 400 mM KCl, 20% glycerol, 0.2 mM EDTA), twice with Buffer B (20 mM Tris, 150 mM KCl, 20% glycerol, 0.2 mM EDTA), and then washed and resuspended in Buffer C (20 mM Tris, 50 mM NaCl, 0.2% NP-40, 1 mM DTT) containing protease inhibitors (50 μg/ml leupeptin, 5 μg/ml phenylmethylsulfonylfluoride, 1 μg/ml pepstatin, and 5 μg/ml aprotinin).

The ERα expression vectors pBSK-ERα [16], pBSK-ERα(1–530), pBSK-ERα(ABC), pBSK-ERα(AB), pBSK-ERα(DEF) [17], pET15b-ERα(304–554), and pET15b-ERα(304–595) [18] kindly provided by Dr. Benita Katzenellenbogen (University of Illinois, Urbana, IL, USA) and the pET21b(+):Flag:hERαDBD [19] and the $CD_{EGA\rightarrow GSV}$ mutant vector generously provided by Dr. David Shapiro (University of Illinois) were used to synthesize ^{35}S-labeled full-length ERα and the truncated ERα proteins 1–530, AB, ABC, DEF, E, EF, CD, and $CD_{EGA\rightarrow GSV}$ in vitro. The ^{35}S-labeled proteins were synthesized using the TNT T7 Quick Coupled Transcription/Translation System (Promega) and incubated at 4 °C for 1 h with the immobilized Flag/His-Sp1 in Buffer C with M2-agarose (ERα, 1–530, AB, ABC, DEF, E, and EF) or Ni-NTA (CD and $CD_{EGA\rightarrow GSV}$) in the absence or presence of E2. 200 μg Flag peptide and 400 μg BSA/ml (50% slurry) were included in reactions with the Ni-NTA resin. After one wash with Buffer A, two washes with Buffer C, and one wash with 50 mM Tris, pH 8.0, proteins were eluted with 2× sample buffer (250 mM Tris, 0.8% SDS, 40% glycerol, 2.88 M β-mercaptoethanol). Eluted proteins were separated by SDS–PAGE, and autoradiograms were exposed for 1–2 days.

2.5. Western blots

Nuclear extracts were prepared from MCF-7 cells that had been treated with ethanol vehicle or 10 nM E_2 for 24 h. 10 μg of nuclear proteins were separated by SDS–PAGE and transferred to a 0.45 μM nitrocellulose membrane (Osmonics, Westborough, MA, USA). The membrane was blocked with T_{50}BS solution (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5 mM thimerosal) containing 5% Carnation nonfat dry milk for 1 h at room temperature. The ERα-specific antibody sc-8002 and the Sp1-specific antibody sc-59G (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were diluted 1:10,000 and 1:1000, respectively, in T_{50}BS containing 5% milk. Antibodies were incubated with the membrane for 1 h at room temperature. Blots were washed with T_{50}BS with

0.05% Tween 20 and incubated with an appropriate horse radish peroxidase coupled secondary antibody (Zymed, San Francisco, CA, USA) at a 1:10,000 dilution in T_{50}BS with 5% milk for 1 h at room temperature. Blots were washed, incubated with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL, USA) for 5 min, and exposed to film.

2.6. Chromatin immunoprecipitation (ChIP) assays

ChIP assays were carried out essentially as described [9]. MCF-7 cells were exposed to ethanol vehicle or 10 nM E_2 for 24 h. The ERα-specific antibody, sc-8002 or Sp1-specific antibody, sc-59 (Santa Cruz Biotechnology) was used for immunoprecipitation of protein–DNA complexes. PCR primers flanking the +571 ERE/Sp1 site (5′GCTCCCCACTTGCCGCTCGCTG3′ and 5′TCGGGAATATAGGGGCAGAGGGAGGAGAA3′) produced 181 bp DNA fragments. As a negative control, primers that annealed from −711 to −693 and from −458 to −436 of the PR gene were used to produce a 275 bp fragment [9]. This region of the PR gene does not contain an identifiable ERE or Sp1 site.

3. Results

3.1. E_2 and ERα are required for activation of the +571 ERE/Sp1 site

To test the ability of the +571 ERE/Sp1 site to activate transcription, U2-OS cells, which do not express ERα, were transfected with a CAT reporter plasmid containing a TATA sequence alone (TATA-CAT) or in combination with the +571 ERE/Sp1 site (+571 ERE/Sp1 TATA-CAT) in the absence or in the presence of a human ERα expression vector. A β-galactosidase expression vector was also included to normalize for differences in transfection efficiency. When +571 ERE/Sp1 TATA-CAT and the ERα expression vector were included and cells were treated with E_2, there was a 5.3-fold increase in CAT activity compared to ethanol-treated cells (Fig. 1A, +571 ERE/Sp1 TATA-CAT, +ERα). There was also a slight increase in the basal expression of +571 ERE/Sp1 TATA-CAT in the presence of the ERα expression vector as we have noted previously with other estrogen-responsive promoters [6,20–22]. Hormone treatment had no effect on CAT activity when the reporter plasmid contained the TATA sequence alone (TATA-CAT). Furthermore, when the ERα expression vector was not included (−ERα), no increase in CAT activity was observed with TATA-CAT or with +571 ERE/Sp1 TATA-CAT regardless of hormone treatment. Combined with earlier studies in CHO cells [6], in which E_2 increased transcription of +571 ERE/Sp1 TATA-CAT, these studies support a role for the +571 ERE/Sp1 site in mediating estrogen responsiveness of the human PR gene.

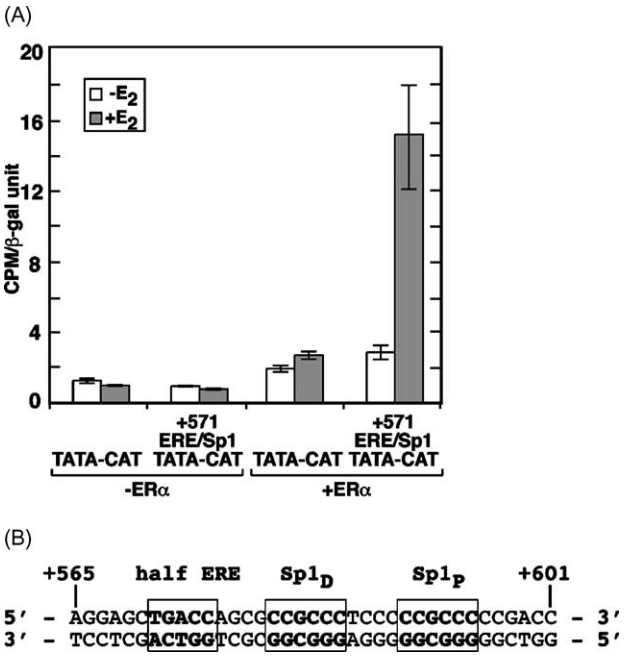

Fig. 1. Transient cotransfections with reporter plasmids containing the wild-type +571 ERE/Sp1 site. (A) U2-OS cells were transfected with a β-galactosidase expression plasmid and a reporter plasmid containing the +571 ERE/Sp1 site and a TATA box (+571 ERE/Sp1 TATA-CAT) or a TATA box alone (TATA-CAT). A human ERα expression plasmid was not (−ERα) or was (+ERα) included as indicated. Cells were treated with ethanol vehicle or 10 nM E_2 for 24 h and CAT activity was determined. Data from three independent experiments were combined and values are presented as the mean ± S.E.M. (B) The sequence of the +571 ERE/Sp1 site previously reported by Kastner et al. [4] is shown. The locations of the ERE half site and the proximal ($Sp1_P$) and distal ($Sp1_D$) are indicated.

3.2. EREα and Sp1 bind to the +571 ERE/Sp1 site

We next examined the ability of ERα and Sp1 to bind to the +571 ERE/Sp1 site in gel mobility shift assays. When ^{32}P-labeled oligos containing the +571 ERE/Sp1 site were combined with purified Sp1, a distinct gel-shifted band was formed that was supershifted by an Sp1-specific antibody, but not by an ERα-specific antibody (Fig. 2, Lanes 1–3). When ^{32}P-labeled oligos containing the +571 ERE/Sp1 site were combined with purified ERα, a more rapidly migrating protein–DNA complex was formed that was supershifted by an ERα-specific antibody, but not by an Sp1-specific antibody (Lanes 4–6). When Sp1 and ERα were combined with the ^{32}P-labeled oligos, both ERα–DNA and Sp1–DNA complexes were formed (Lane 7). The more slowly migrating complex was supershifted by the Sp1-specific antibody (Sp1→, Lane 8) and the more rapidly migrating complex was supershifted by the ERα-specific antibody (ERα→, Lane 9). Thus, the more slowly migrating complex contained Sp1 and the more rapidly migrating complex contained ERα. These assays demonstrate that both ERα and Sp1 bind directly to the +571 ERE/Sp1 site.

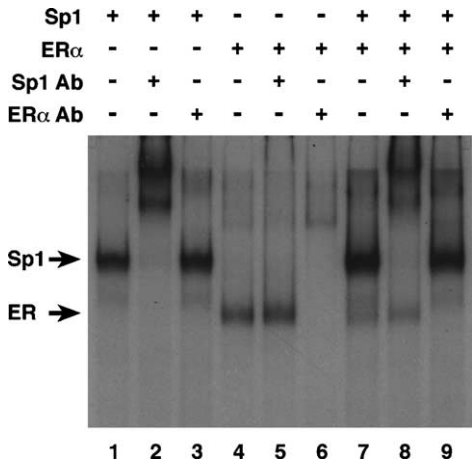

Fig. 2. Gel mobility shift assays with +571 ERE/Sp1 containing oligos and purified ERα and Sp1. ^{32}P-Labeled oligos containing the +571 ERE/Sp1 site were incubated with 3 ng purified Sp1 (Lanes 1–3), 50 fmol purified, E_2-occupied ERα (Lanes 4–6), or 3 ng purified Sp1 and 50 fmol purified, E_2-occupied ERα. The ^{32}P-labeled oligos were fractionated on a nondenaturing gel and visualized by autoradiography. The free probe, which is not visible, was run off the gel so that the protein–DNA complexes could be more effectively separated from the antibody–protein–DNA complexes.

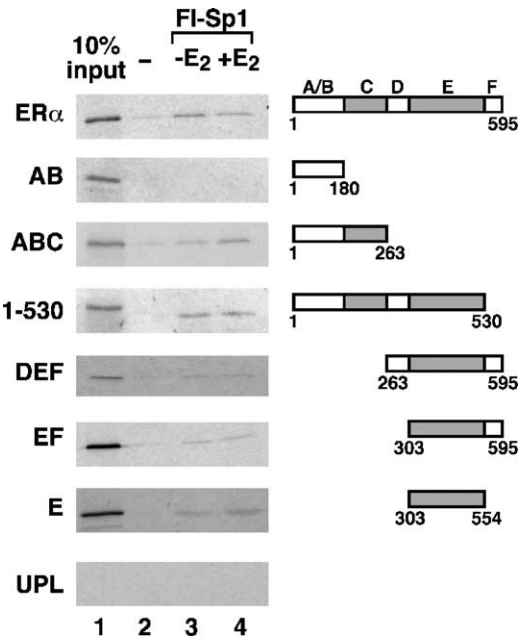

Fig. 3. Interaction of ERα and Sp1. Flag-affinity resin without (Lanes 2) or with purified Flag-tagged Sp1 (Fl-Sp1, Lanes 3 and 4) was combined with in vitro-translated ^{35}S-labeled full-length or truncated ERα or unprogrammed lysate (UPL), which was processed with no ERα DNA template. E_2 was added as indicated. After extensive washes, proteins were eluted and fractionated on a denaturing gel. The gel was dried and subjected to autoradiography. 10% of input was included for reference (Lanes 1). Assays were carried out at least three times and a representative experiment is shown.

3.3. Sp1 interacts directly with the DNA and ligand binding domains of ERα

In addition to binding to their cognate recognition sites, ERα and Sp1 can interact with each other [23], but the region(s) of the receptor required for this interaction are unclear. To identify the region(s) of the receptor required for interaction with Sp1, immobilized Flag-tagged Sp1 was incubated with in vitro-translated, ^{35}S-labeled full-length or truncated ERα. As seen in Fig. 3, the full-length receptor bound to Sp1 in the absence and in the presence of E_2, but the amino-terminal AB region of ERα was unable to bind to Sp1. However, when this amino-terminal region was combined with the DNA binding domain (DBD), the ^{35}S-labeled ABC was retained by Sp1. Likewise, the combined hinge, ligand binding domain (LBD) and carboxy terminus (DEF) bound to Sp1, as did deletion mutants containing only the LBD and carboxy terminus (EF) or the LBD alone (E). No binding was detected when no template was included to direct ERα synthesis (unprogrammed lysate, UPL). These studies demonstrate that the ERα DBD and LBD interact directly with Sp1.

3.4. E_2-occupied ERα differentially activates transcription of reporter plasmids containing wild-type and mutant +571 ERE/Sp1 sites

The abilities of ERα and Sp1 to interact with each other and with DNA and the close proximity of the ERE half site and Sp1 sites led us to speculate that the interaction of these DNA-bound proteins might be required for efficient estrogen responsiveness. To determine the individual contributions of the ERE half site, the proximal Sp1 site, and the distal Sp1 site in regulating transcription, transient transfection assays were carried out with a reporter plasmid that contained a +571 ERE/Sp1 site with wild-type DNA sequence or with mutations in the distal Sp1 site, the proximal Sp1 site, both Sp1 sites, or the ERE half site. U2-OS cells were transfected with ERα and β-galactosidase expression vectors and one of the CAT reporter plasmids. When these cells were transfected with an ERα expression vector, the endogenous PR gene was activated and PR protein levels were increased in response to hormone (data not shown). Similar to the results shown in Fig. 1, the wild-type +571 ERE/Sp1 site induced a 4.3-fold increase in transcription in the presence of E_2 (Fig. 4, +571 ERE/Sp1 TATA-CAT). In contrast, transcription was significantly decreased when the reporter plasmid contained mutations in the distal Sp1 site (+571 mut Sp1 D TATA-CAT) or the proximal Sp1 site (+571 mut Sp1 P TATA-CAT). A reporter plasmid containing mutations in both Sp1 sites (+571 mut Sp1 P/D TATA-CAT) was the least efficient in activating transcription. These data indicate that both Sp1 sites play critical roles in enhancing transcription through the +571 ERE/Sp1 site. Surprisingly, when a reporter plasmid containing mutations in the ERE half site (TGACC→TGATT) was utilized, a dramatic increase in transcription was observed in the absence and in the presence of E_2 (+571 mut ERE TATA-CAT). We

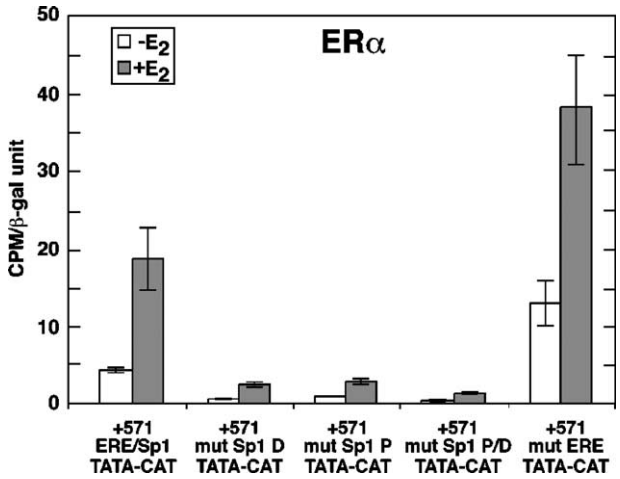

Fig. 4. Transient cotransfections with ERα and reporter plasmids containing a wild-type or mutant +571 ERE/Sp1 site. U2-OS cells were transfected with a β-galactosidase expression vector, a human ERα expression vector, and a reporter plasmid containing a TATA box and the +571 ERE/Sp1 site with wild-type DNA sequence (+571 ERE/Sp1 TATA-CAT) or with mutations in the distal Sp1 site (+571 mut Sp1 D TATA-CAT), the proximal Sp1 site (+571 mut Sp1 P TATA-CAT), both Sp1 sites (+571 mut Sp1 P/D TATA-CAT), or the ERE half site (+571 mut ERE TATA-CAT). Cells were treated with ethanol vehicle or 10 nM E_2 for 24 h and CAT activity was determined. Data from three independent experiments were combined and values are presented as the mean ± S.E.M.

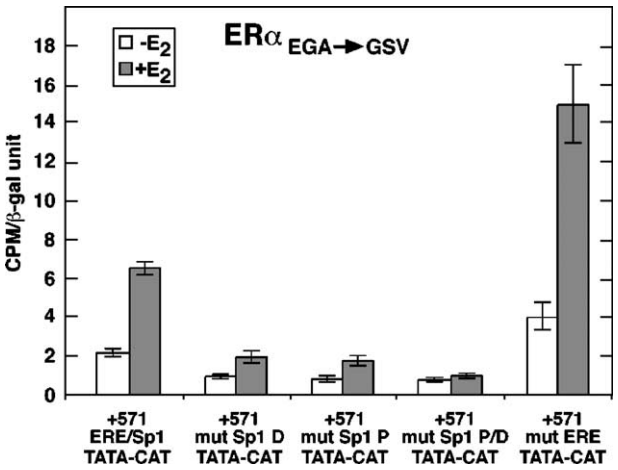

Fig. 5. Transient cotransfections with $ERα_{EGA→GSV}$ and reporter plasmids containing a wild-type or mutant +571 ERE/Sp1 site. U2-OS cells were transfected with a β-galactosidase expression vector, a human $ERα_{EGA→GSV}$ expression vector with 3-amino acid substitutions in the DBD, and a reporter plasmid containing a TATA box and the +571 ERE/Sp1 site with wild-type DNA sequence (+571 ERE/Sp1 TATA-CAT) or with mutations in the distal Sp1 site (+571 mut Sp1 D TATA-CAT), the proximal Sp1 site (+571 mut Sp1 P TATA-CAT), both Sp1 sites (+571 mut Sp1 P/D TATA-CAT), or the ERE half site (+571 mut ERE TATA-CAT). Cells were treated with ethanol vehicle or 10 nM E_2 for 24 h and CAT activity was determined. Data from three independent experiments were combined and values are presented as the mean ± S.E.M.

believe that this increase in transcription was not due to the introduction of a novel transcription factor binding site since a different mutation in the ERE half site (TAGAC) produced similar results (data not shown). The increase in transcription observed with reporter plasmids containing a mutant ERE half site suggests that binding of ERα to the ERE half site plays a role in limiting transcription of the PR gene in transient transfection assays.

3.5. An E_2-occupied $ERα_{EGA→GSV}$ mutant differentially activates transcription of reporter plasmids containing the wild-type or mutant +571 ERE/Sp1 site

While the decreased transcription observed with reporter plasmids containing mutated Sp1 sites was anticipated, the increased transcription observed with the mutated ERE was unexpected. To better understand the basis of this increased activity, transient transfection assays were carried out with an ERα expression vector containing three amino acid substitutions (EGA→GSV) in the DBD that abolishes the ability of the receptor to bind to an ERE [11]. Transcription induced by this mutant receptor ($ERα_{EGA→GSV}$) could not be due to direct binding of the receptor to DNA, but would instead reflect the ability of the receptor to interact effectively with Sp1 or other transcription factors involved in forming an active transcription complex. Transient transfection assays were carried out with the $ERα_{EGA→GSV}$ expression vector, a β-galactosidase expression vector, and a CAT reporter plasmid containing the +571 ERE/Sp1 site with wild-type sequence or with mutations in the distal Sp1 site, the proximal Sp1 site, both Sp1 sites, or the ERE half site. Inclusion of the +571 ERE/Sp1 site resulted in a 2.9-fold increase in transcription in the presence of E_2 compared to ethanol vehicle (Fig. 5, +571 ERE/Sp1 TATA-CAT). As seen with the wild-type receptor in Fig. 4, transcription was decreased when the reporter plasmid contained mutations in either the distal (+571 mut Sp1 D TATA-CAT) or proximal (+571 mut Sp1 P TATA-CAT) Sp1 site and was further decreased when both Sp1 sites were mutated (+571 mut Sp1 P/D TATA-CAT). Thus, both Sp1 sites are important in increasing estrogen-mediated transactivation. As seen with the wild-type receptor, when a reporter plasmid containing mutations in the ERE half site (+571 mut ERE TATA-CAT) was utilized, an increase in transcription was observed both in the absence and in the presence of E_2. Thus, the pattern of activation was quite similar with the wild-type ERα and the mutant receptor, but the magnitude of the response was significantly diminished with $ERα_{EGA→GSV}$ compared to ERα.

3.6. The wild-type CD and the $CD_{EGA→GSV}$ mutant interact with Sp1 and enhance Sp1 binding to the +571 ERE/Sp1 site

One potential explanation for the decreased transcription observed with $ERα_{EGA→GSV}$ was that the interaction of this receptor with Sp1 might be compromised. To determine whether this was the case, in vitro binding assays were carried out with in vitro transcribed ^{35}S-labeled CD, which

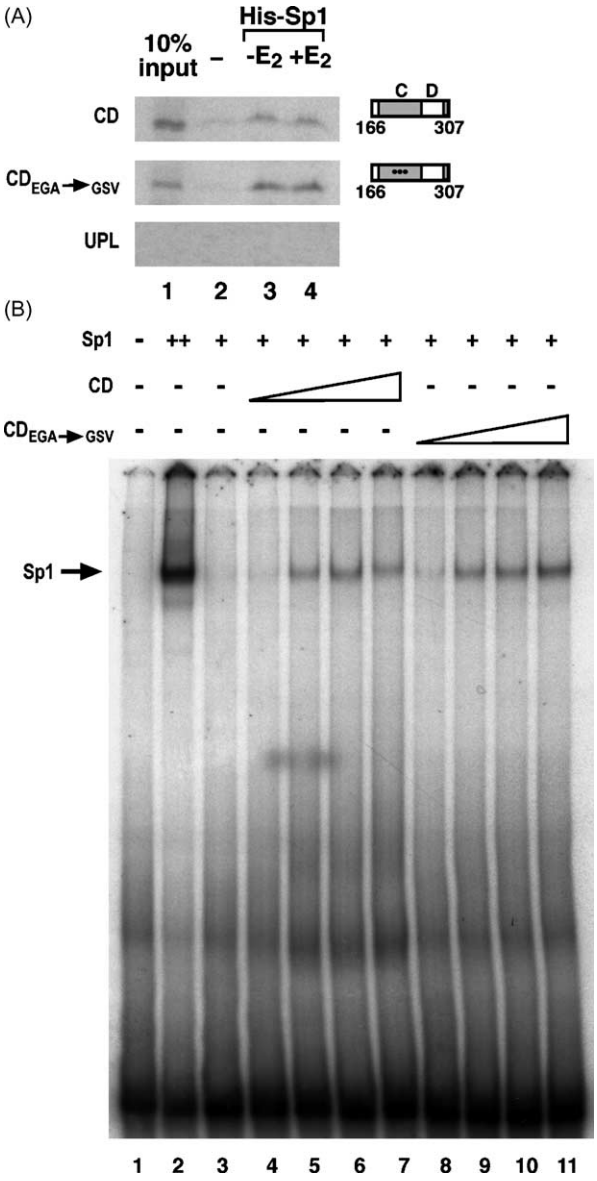

Fig. 6. Interaction of Sp1 with the wild-type CD and the $CD_{EGA\rightarrow GSV}$ mutant. (A) Nickel resin without (Lanes 2) or with purified His-tagged Sp1 (His-Sp1, Lanes 3 and 4) was combined with in vitro-translated ^{35}S-labeled wild-type CD or the $CD_{EGA\rightarrow GSV}$ mutant. E_2 was added as indicated and samples were processed as described in Fig. 3. 10% of input was included for reference (Lanes 1). (B) ^{32}P-Labeled oligos containing the +571 ERE/Sp1 site were incubated alone (Lane 1), with 3 ng purified Sp1 (Lane 2), or with 0.25 ng purified Sp1 (Lanes 3–11) and increasing amounts (5, 50, 100 or 250 ng, Lanes 4–7 and 8–11) of purified wild-type CD (Lanes 4–7) or the $CD_{EGA\rightarrow GSV}$ mutant (Lanes 8–11).

contained the DBD and hinge region of the receptor with wild-type amino acid sequence, or $CD_{EGA\rightarrow GSV}$, which contained the 3-amino acid substitution in the DBD. Both the wild-type CD and the $CD_{EGA\rightarrow GSV}$ mutant bound similarly to the His-tagged Sp1 resin (Fig. 6, Panel A, Lanes 3–4), but did not bind to the Ni-NTA resin alone (Lanes 2).

We previously demonstrated that ERα dramatically increased the binding of Sp1 to the two Sp1 sites present in the +571 ERE/Sp1 site [6]. To determine whether the ability of the receptor to enhance Sp1 binding to the +571 ERE/Sp1 site was compromised when the DBD contained the EGA→GSV mutation, gel mobility shift assays were carried out with purified ERα wild-type CD or the $CD_{EGA\rightarrow GSV}$ mutant. ^{32}P-Labeled oligos containing the +571 ERE/Sp1 sequence were incubated with 3 ng Sp1 (Fig. 6, Sp1→, Lane 2) or with 0.25 ng of Sp1 alone (Lane 3) or with increasing amounts of the wild-type CD or the $CD_{EGA\rightarrow GSV}$ mutant (Lanes 4–11). A dose-dependent increase in Sp1 binding was observed when purified wild-type CD was added to the reaction (Sp1→, Lanes 4–7). Likewise, when increasing concentrations of the purified $CD_{EGA\rightarrow GSV}$ mutant were added to the binding reactions, a dose-dependent increase in Sp1 binding was observed (Sp1→, Lanes 8–11). This enhanced binding observed was not due to differences in protein concentrations since protein and salt concentrations were kept constant in all binding reactions. While we cannot rule out the possibility that the interaction of the full-length ERα with Sp1 might be compromised by the mutation of the DBD, our gel mobility shift experiments support the idea that the $CD_{EGA\rightarrow GSV}$ mutant was as effective in fostering Sp1 binding as the wild-type CD.

3.7. ERα and Sp1 are associated with the endogenous +571 ERE/Sp1 site in native chromatin

Transient transfection assays combined with in vitro binding assays can be very useful in identifying *cis* elements and *trans* acting factors that modulate transcription. Our experiments had demonstrated that the +571 ERE/Sp1 site played an important role in regulating transcription of a reporter plasmid containing this DNA sequence. However, a question of far greater importance is whether ERα and Sp1 interact with this region of the PR gene as it exists in native chromatin. To determine whether these proteins are involved in regulating transcription of the endogenous PR gene, ChIP assays were carried out in MCF-7 cells that had been treated with ethanol vehicle or E_2 for 24 h. A discrete amplified product was obtained when MCF-7 cells had been treated with E_2 and the protein–DNA complexes were immunoprecipitated with an ERα- or Sp1-specific antibody (Fig. 7A, +571 ERE/Sp1, Lanes 6 and 8). Interestingly, an amplified product was also obtained when cells were treated with ethanol and the protein–DNA complexes were immunoprecipitated with an Sp1-specific antibody (+571 ERE/Sp1, Lane 7) indicating that Sp1 was associated with the +571 ERE/Sp1 site both in the absence and in the presence of E_2. Genomic DNA that had not been subjected to immunoprecipitation was also readily amplified (+571 ERE/Sp1, Input, Lanes 1 and 2). In contrast, no amplified product was obtained when cells were treated with ethanol vehicle and the protein–DNA complexes were immunoprecipitated with an ERα-specific antibody (+571 ERE/Sp1, Lane 5) or when antibody was omitted (Lanes 3 and 4) regardless of hormone exposure. To ensure that the ERα or Sp1 containing

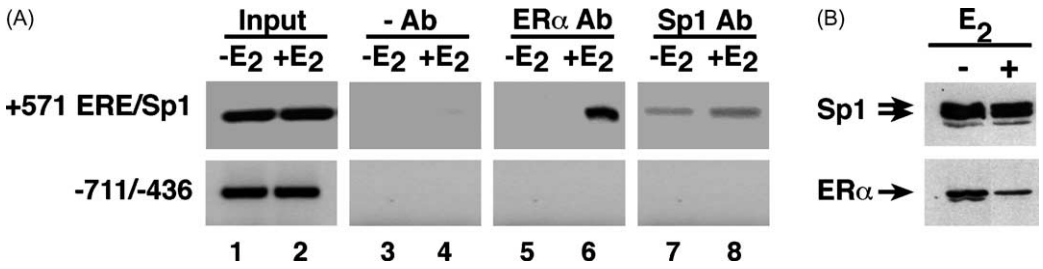

Fig. 7. Chromatin immunoprecipitation of ERα- and Sp1-associated DNA. (A) MCF-7 cells, which had been exposed to ethanol vehicle (−E_2) or 10 nM E_2 (+E_2) for 24 h, were treated with formaldehyde to form protein–protein and protein–DNA crosslinks, lysed and sonicated. Immunoprecipitation of the protein–DNA complexes was carried out in the absence (−Ab) or in the presence of ERα- (ERα Ab) or Sp1-specific (Sp1 Ab) antibody. Primers flanking the +571 ERE/Sp1 site (+571 ERE/Sp1) or a region in the PR gene that did not contain an Sp1 site (−711/−436) were used in PCR amplification. Amplified DNA was fractionated on an agarose gel and visualized by ethidium bromide staining. Genomic DNA that had not been subjected to immunoprecipitation was included to demonstrate that each DNA preparation could be used as a template in PCR reactions (Input). (B) Nuclear extracts (10 μg) from MCF-7 cells that had been treated with ethanol or E_2 for 24 h were fractionated on a denaturing gel, transferred to nitrocellulose membrane and incubated with either an Sp1- (Sp1→) or ERα-specific (ERα→) antibody. A chemiluminescent substrate was used for detection.

protein–DNA complexes were specifically immunoprecipitated, we determined whether a region of the PR gene that contains neither an ERE nor an Sp1 site (−711 to −436) could be amplified. No DNA product was observed when an ERα- or Sp1-specific antibody (−711/−436, Lanes 5–8) or no antibody (−711/−436, Lanes 3 and 4) was utilized for immunoprecipitation regardless of hormone exposure. In contrast, when genomic DNA that had not been subjected to immunoprecipitation was used as a control, an amplified product was obtained for the −711 to −436 region of the PR gene (−711/−436, Lanes 1 and 2). These data indicate that Sp1 is associated with the +571 ERE/Sp1 site in the endogenous PR gene both in the presence and absence of E_2 but that ERα is associated with this site only after E_2 treatment of MCF-7 cells.

To ensure that the differences we detected in binding of ERα and Sp1 were not due to differences in the expression of these proteins, MCF-7 cells were treated with ethanol vehicle or E_2 for 24 h. Nuclear extracts were prepared and subjected to Western analysis with either an Sp1- or ERα-specific antibody. The Sp1-specific antibody identified two proteins with apparent molecular weights of ∼100 kDa (Fig. 7B, Sp1→), which most likely represented different phosphorylation states of Sp1 [24]. No changes in Sp1 levels were detected after various times of E_2 treatment. However, the levels of ERα were significantly decreased after 24 h of hormone exposure (ERα→) as has been reported previously [25]. Thus, the differential association of ERα and Sp1 with the endogenous +571 ERE/Sp1 site in the absence and in the presence of E_2 is not due to differences in protein expression. The receptor was associated with the endogenous +571 ERE/Sp1 site after 24 h of E_2 treatment even though the levels of ERα were substantially decreased at this point in time.

4. Discussion

The PR gene contains a number of highly conserved transcription factor binding sites including an ERE half site, a CAAT box, and Sp1 and AP-1 sites [4,26–28]. The +571 ERE/Sp1 site, particularly the ERE half site and distal Sp1 site are highly conserved in the human [4], rat [27], and mouse [26] PR genes. We have now shown that both the proximal and distal Sp1 sites in the +571 ERE/Sp1 site are important in increasing PR gene expression and that the ERE half site may actually play an important role in limiting PR gene expression.

4.1. Role of ERα in estrogen-mediated transcription

It is clear that estrogen plays a critical role in regulating PR gene expression in MCF-7 breast cancer cells [1–3] and that estrogen's effects are mediated through ERα in these cells. ERα plays an important role in regulating transcription by interacting directly with the ERE half site. First, ERα binds directly to the +571 ERE/Sp1 site in gel mobility shift assays [Fig. 2 and Ref. [6]]. Second, the ERE half site is protected by ERα in in vitro DNase I footprinting experiments [6]. Third, the ERE half site in the endogenous PR gene is more effectively protected in in vivo footprinting experiments when MCF-7 cells are treated with E_2 than when cells are maintained in a hormone-free environment [6]. In addition to binding directly to DNA, ERα enhances Sp1 binding to the proximal and distal Sp1 sites in the +571 ERE/Sp1 site [6]. Furthermore, these tandem Sp1 sites in the endogenous PR gene are more protected when MCF-7 cells are treated with E_2 than when cells are maintained in a hormone-free environment [6]. The absolute requirement of an ERα expression vector to achieve estrogen responsiveness of a reporter plasmid containing the +571 ERE/Sp1 site in transient transfection assays confirms the importance of this protein in mediating hormone responsiveness. More importantly, E_2 treatment of MCF-7 cells, which is required for activation of the PR gene, promotes the recruitment of ERα to the +571 ERE/Sp1 site in the endogenous PR gene. Thus, our studies have documented the interaction of ERα with the ERE half site and with DNA-bound Sp1 using a variety of different techniques and support the idea that these

interactions are physiologically relevant and are involved in regulating hormone responsiveness of the PR gene.

The overall involvement of ERα in regulating PR gene expression through the +571 ERE/Sp1 site presents an intriguing paradox. When the ERE half site is mutated so that ERα can no longer bind directly to DNA, but can still enhance Sp1 binding, transcription is significantly increased. One would then anticipate that utilizing the $ER\alpha_{EGA\rightarrow GSV}$ mutant would produce similar results. Although the pattern of expression is similar with ERα and $ER\alpha_{EGA\rightarrow GSV}$, the magnitude of the response is significantly reduced when cells are transfected with the $ER\alpha_{EGA\rightarrow GSV}$ expression vector. This decreased responsiveness most likely results from conformational aberrations in the structure of the mutant receptor such that it fails to interact appropriately with other regulatory proteins and provide a functional platform upon which to establish an active transcription complex.

The fact that both ERα and $ER\alpha_{EGA\rightarrow GSV}$ significantly enhance transcription of reporter plasmids containing mutations in the ERE suggests that the ERE half site plays a role in regulating transcription. Interestingly, previous work from our laboratory showed that when the ERE half site is mutated, Sp1 binding is significantly increased [6] implying that the ERE half site may constrain the DNA and inhibit it from assuming the optimal conformation required for Sp1 binding. Our present work demonstrates that when the ERE half site is mutated, transcription is significantly enhanced. The enhanced binding of Sp1 to the proximal and distal Sp1 sites when the ERE half site has been mutated could explain the enhanced transcription we observe in the absence of hormone. In the presence of hormone, the association of the receptor with DNA-bound Sp1 at the +571 ERE/Sp1 site further augments transcription and confers estrogen responsiveness. The failure of the receptor to bind to the mutated ERE half site would almost certainly result in the formation of a distinctly different complex of proteins than is associated with the native gene sequence. This idea is supported by work from our laboratory and others, which demonstrates that the formation of transcription complexes is altered by DNA sequence and the selective exposure of protein surfaces for interaction with other transcription factors and coregulatory proteins [8,13,20,29–31]. It appears that the role of the ERE half site in this region of the PR gene may be to anchor the receptor to this region of the gene and assist in the ordered recruitment of proteins and the assembly of an active transcription complex.

4.2. Role of Sp1 sites in regulating PR gene expression

Estrogen responsiveness is conferred to a number of genes by the interaction of ERα with DNA-bound Sp1 [32]. We have demonstrated the importance of the proximal and distal Sp1 sites in the +571 ERE/Sp1 site in enhancing transcription and modulating estrogen responsiveness. Mutations in either the proximal or the distal Sp1 site reduces Sp1 binding [6] and reporter plasmids containing mutations in the proximal or the distal Sp1 site have significantly decreased transcription. Mutation of both Sp1 sites abrogates the ability of Sp1 to interact with DNA [6] and dramatically decreases transcription of a reporter plasmid containing mutations in these two Sp1 sites. These combined experiments demonstrate that both of the Sp1 sites in the +571 ERE/Sp1 site are required for effective activation of PR gene expression.

We have now identified two different regions in the PR gene that contain tandem Sp1 sites and play a role in regulating estrogen responsiveness of the PR gene. The −61 Sp1 site [22] and the +571 ERE/Sp1 site [6] each contain adjacent Sp1 sites with the proximal Sp1 sites containing near-consensus Sp1 sequences [33,34]. Thus, it was not surprising that there were similarities in the interaction of Sp1 with these two PR regions. The −61 Sp1 and +571 ERE/Sp1 sites both bind purified Sp1 and Sp1 present in MCF-7 nuclear extracts [6,22]. ERα enhances Sp1 binding to both Sp1 sites in in vitro binding assays and both estrogen and ERα are required to confer estrogen responsiveness to a simple heterologous promoter containing either of these two sites. However, it is also clear that there are distinct differences in the −61 Sp1 and +571 ERE/Sp1 sites. Unlike the +571 ERE/Sp1 site, the −61 Sp1 site lacks an ERE half site and is unable to bind directly to ERα. Interestingly, the −61 Sp1 site is a significantly more potent transcriptional enhancer than the +571 ERE/Sp1 site in the absence and in the presence of E_2 [22]. However, the +571 ERE/Sp1 site is significantly more active when the ERE half site has been mutated indicating that, in the absence of an ERE half site, the tandem Sp1 sites activate transcription to a greater extent and yet still maintain estrogen responsiveness. This hormone responsiveness in the absence of an ERE half site is most likely due to the ability of ERα to interact directly with Sp1 and enhance its binding to DNA.

4.3. Regulation of the endogenous PR gene

While the use of mutant receptors and mutated binding sites can help to provide clues about the regulation of gene expression, certainly a question of far greater importance is to define how an endogenous gene is regulated in target cells. MCF-7 cells are estrogen-responsive cells that express low levels of PR when maintained in a hormone-free environment [1]. Interestingly, we have demonstrated that when MCF-7 cells are maintained in a hormone-free environment, Sp1 is associated with the +571 ERE/Sp1 site in the endogenous PR gene and this site is protected from DNase I cleavage in in vivo footprinting experiments [6]. Thus, we propose that binding of Sp1 to the +571 ERE/Sp1 site helps to maintain basal PR levels in MCF-7 cells in the absence of hormone.

PR gene expression is significantly increased in MCF-7 cells in response to E_2 treatment. Our ChIP experiments suggest that ERα plays a critical role in regulating transcription through the +571 ERE/Sp1 site of the endogenous PR gene. ERα is only associated with the +571 ERE/Sp1 site

after cells have been exposed to hormone. Furthermore, this site is more extensively protected in in vivo footprinting experiments when MCF-7 cells have been treated with E_2 [6]. These combined experiments reflect the character of the Sp1 and ERα proteins. Sp1 is a general transcription factor and is involved in regulating PR gene expression in the absence and in the presence of hormone. In contrast, ERα is a ligand-induced transcription factor and associates with the +571 ERE/Sp1 site only after cells have been treated with hormone. The association of ERα with this site can be through interaction with the ERE half site and/or through association with DNA-bound Sp1. This very elegant combinatorial approach to regulating gene expression with multiple *cis* elements and coordinating *trans* acting factors affords a target cell considerable versatility in responding to hormonal cues and fluctuating levels of endogenously expressed regulatory proteins.

The abilities of ERα and Sp1 to interact with each other and with their cognate recognition sequences could help to form an interconnected network upon which a basal transcription complex is established. In fact, our in vivo footprinting experiments revealed an extended region of protection flanking the +571 ERE/Sp1 site after estrogen treatment of MCF-7 cells [6]. This may reflect the formation of a large protein–DNA complex containing ERα and Sp1 as well as other associated *trans* acting factors involved in regulating PR gene expression. In fact, both ERα and Sp1 interact directly with TFIID components. The association of ERα with TBP, TFIIB, and $TAF_{II}30$ [35–37] and the interaction of Sp1 interaction with TBP, $TAF_{II}130$, and $TAF_{II}55$ [38–40] may help to form the protein–protein and protein–DNA network required for activation of the human PR gene.

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