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Estrogen Receptor-Sp1 Complexes Mediate Estrogen-induced Cathepsin D Gene Expression in MCF-7 Human Breast Cancer Cells*

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Cathepsin D is an estrogen (17β-estradiol, E_2)-inducible lysosomal protease. A putative estrogen receptor (ER)-Sp1-like sequence (GGGCGG$(n)_{23}$ACGGG) has been identified in the non-coding strand of the cathepsin D promoter (-199 to -165), and electromobility shift assays of nuclear extracts from MCF-7 and HeLa cells confirm that both the ER and Sp1 protein bind to ^{32}P-labeled ER/Sp1 oligo. For example, nuclear extracts from MCF-7 cells bind to the ^{32}P-labeled ER/Sp1 oligo; however, ER/Sp1 binding can be decreased by selective competition with excess unlabeled estrogen responsive element and Sp1 oligos, immunodepletion with ER or Sp1 antibodies, and by treating cells with ICI 164,384, an antiestrogen which inhibits formation of ER homodimer. Moreover, E_2-induced chloramphenicol acetyltransferase (CAT) activity in MCF-7 cells cotransfected with a human estrogen receptor expression plasmid and a plasmid containing an ER/Sp1 sequence cloned upstream to a thymidine kinase promoter and a CAT reporter. In cotreatment studies, ICI 164,384 inhibited E_2-induced CAT activity. In contrast, E_2 did not induce CAT activity in MCF-7 cells transfected with plasmids containing mutations in the ER or Sp1 segments of the ER/Sp1 oligo, thus confirming that both cognate binding sites are required for estrogen responsiveness.

MCF-7 human breast cancer cells express the estrogen receptor (ER)[1] and represent a prototypical estrogen-responsive cell line which has been extensively utilized for mechanistic studies on estrogen- and antiestrogen-induced responses (1–3). Cathepsin D is an aspartyl protease, and the role of estrogens on the intracellular regulation of procathepsin D, a 52-kDa protein, and CATH-D (a 34-kDa protein) have been extensively investigated (1–12). 17β-Estradiol (E_2) significantly increases *CATH-D* gene transcription and intracellular protein formation, and within 24 h after hormone treatment, the extracellular levels of the 52- and 34-kDa proteins are also significantly increased. This induction response is not inhibited by the nonsteroidal partial antiestrogen, tamoxifen (7, 13), whereas ICI 164,384, a steroidal "pure" antiestrogen, inhibits *CATH-D* gene transcription (7, 13–15). CATH-D also exhibits mitogenic activity, and Briozzo and co-workers (16) reported that this protease degraded the extracellular matrix of endothelial bovine corneal cells (17). This observation led to the suggestion that CATH-D secreted from breast cancer cells facilitates tumor invasiveness and metastasis (17, 18). Subsequent studies reported that there was no correlation between CATH-D secretion and tumor invasiveness in the Boyden chamber assay of invasion (19). Although the precise role of this protein in the development of breast cancer has not been determined, CATH-D has been used clinically as a negative prognostic indicator for disease-free survival in women with breast cancer (20–24).

E_2 induces the expression of the *CATH-D* gene in ER-responsive breast cancer cells by interacting with the transcriptional machinery at the promoter level. In the absence of E_2, the *CATH-D* gene transcription is initiated at multiple transcription start sites I–V; however, E_2 exclusively initiates transcription at the TATA-dependent transcription start site I on the CATH-D promoter (12). The promoter region of cathepsin D does not contain a classical palindromic estrogen-responsive element (ERE) (25) but contains several GC-rich boxes which can bind to the transcription factor Sp1 (12, 26). Sp1-dependent activation of transcription is TATA box-dependent (27), and recent studies have identified important regulatory sequences in the promoter region of the E_2-induced creatine kinase B gene in which weak interactions between the ER and an ERE DNA half-site are stabilized by an adjacent transcription factor, such as Sp1 (28). The mechanism of estrogen activation of the c-*myc* oncogene also involves similar interactions between the ER half-site and the Sp1 element on the c-*myc* downstream promoter (29). Interestingly, these ER/Sp1 elements function in either orientation as enhancer sequences, *in vitro* (28).

We have identified a putative ER/Sp1-like sequence in the non-coding strand of the CATH-D promoter (*i.e.* GGGCGG$(n)_{23}$ACGGG) (-199 to -165), and this study reports ^{32}P-labeled ER/Sp1 binding of nuclear extracts from HeLa and MCF-7 cells using electromobility shift assays (EMSAs). These assays demonstrate ER and Sp1 bind to this oligo, and, utilizing an ER/Sp1-thymidine kinase-CAT plasmid in transient transfection assays, E_2 induces chloramphenicol acetyltransferase (*CAT*) activity in MCF-7 cells. The results indicate that the ER/Sp1 sites are functional enhancer elements and are responsible for E_2-induced TATA-dependent transcription of the *CATH-D* gene *in vivo*.

MATERIALS AND METHODS

Chemicals, Cells, Antibodies, and Oligonucleotides—ICI 164,384 was kindly provided by Dr. A. Wakeling; the MCF-7 cells and HeLa cells were obtained from ATCC. The ERE and xenobiotic responsive element (XRE) oligo for the EMSAs have previously been described (30, 31). The ER/Sp1, *ER*/Sp1 and ER/*Sp1* oligos (see below) were synthesized by and purchased from the DNA Technologies Laboratory, Texas A&M Univer-

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[1] The abbreviations used are: ER, estrogen receptor; CAT, chloramphenicol acetyltransferase; CATH-D, cathepsin D; EMSA, electromobility shift assay; ERE, estrogen responsive element; E_2, 17β-estradiol; hER, human estrogen receptor; XRE, xenobiotic responsive element; Ab, antibody; Me_2SO, dimethyl sulfoxide.

sity. The complementary strands were annealed and the 5′-overhangs were used for cloning into the thymidine kinase-CAT vector (25). MCF-7 and HeLa cells were cultured, and nuclear extracts were isolated as described elsewhere (32). The Sp1 oligo was purchased from Promega Biological Research Products; the human estrogen receptor (hER) was kindly provided by Dr. Ming Tsai (Baylor College of Medicine). Rat ER antisera (ER Ab) and rat ER peptide (ER blocking peptide) was kindly provided by Dr. Koji Yoshinaga (National Hormone and Pituitary Program, NIH). Sp1 monoclonal antibody (Sp1 Ab) was purchased from Santa Cruz Biotech (Santa Cruz, CA).

The oligonucleotides were as follows. ER/Sp1 oligo (antisense strand), 5′ GATCCTGGGCG**GG GCAA**CCTCGGGCA CGCACAGCGC-CCG**GGCGGG**GGGCGGGGA 3′; ER/Sp1 oligo (antisense strand), 5′ GATCCTGGGCG**GG GCAA**CCTCGGGCA CGCACAGCGCCCG**GA-CAGG**GGGCGGGGA 3′; ER/Sp1 oligo (antisense strand), 5′ GATCCT-GGGCG**GA ACAA**CCTCGGGCA CGCACAGCGCCCG**GGCGGG**GG-GCGGGGA 3′. ER, Sp1, and mutated bases are noted in bold type.

EMSA—The oligonucleotides were annealed and labeled at the 5′ end using T4-polynucleotide kinase and [γ-^{32}P]ATP (33). DNA binding was measured using EMSAs. Five to 10 μg of nuclear extracts from MCF-7 or HeLa cells treated with 10 nM E_2 for 24 h and different chemicals for the times as indicated were incubated with 1 μg of poly d(I-C) for 15 min at 20 °C to bind nonspecific DNA-binding proteins. Following addition of ^{32}P-labeled DNA (final concentration, 1 nM), the mixture was incubated for another 15 min at 20 °C. Excess unlabeled DNA (50–200-fold) was added 5 min before adding ^{32}P-labeled DNA to compete for specific DNA-protein binding. Reaction mixtures were loaded onto a 7% non-denaturing polyacrylamide gel (acrylamide: bisacrylamide, 30:0.8) and run at 110 V in TBE buffer (0.09 M Tris, 0.09 M borate and 2 mM EDTA, pH 8.3). Gels were dried and protein-DNA binding was visualized by autoradiography and quantitated with a Betagen Betascope 603 blot analyzer.

Immunodepletion and Super Shift Assays—MCF-7 cells were maintained in Dulbecco's modified Eagle's-F-12 medium as described earlier. The cells were treated with the appropriate chemicals and nuclear extracts were obtained. For immunodepletion studies, 5 μg of nuclear extract were incubated with 20 ng of Sp1 Ab, 40 ng of ER Ab, 100 ng of CATH-D Ab, and 40 ng of ER Ab (incubated for 18 h with blocking peptide) for 2 h. These extracts were then subjected to EMSAs as described earlier. The gels were dried and subjected to autoradiography, and the intensity of the specific bands was quantitated using a Betagen Betascope 603 blot analyzer. For the super shift studies the specific and the nonspecific Ab were incubated for 20 min after incubation with radiolabeled probe. Aliquots of 20, 40, 40, 100, and 100 ng of Ab were used for the Sp1, ER, ER + peptide, rabbit IgG, and mouse IgG, respectively. The extracts were subjected to EMSAs, and the gels were dried and visualized using autoradiography.

Cloning and CAT Assays—The oligonucleotides were cloned into the thymidine kinase-CAT vector at the *Bam*HI and *Hin*dIII sites as previously described (25). Ligation products were transformed into DH5α bacterial cells, and clones obtained were verified by restriction mapping. Cultured MCF-7 cells (32) were cotransfected with a Polybrene (200 μg/ml) solution containing 10 μg of appropriate plasmid DNA and the hER plasmid. After 6 h, the cells were shocked with 25% glycerol in Hank's solution and washed twice with the same solution and grown in Dulbecco's modified Eagle's/F-12 medium (without phenol red) and 5% stripped fetal bovine serum. Cells were dosed 12 h after shocking with appropriate chemicals dissolved in Me_2SO; control cells were treated with Me_2SO alone (<0.1%). Two days later the cells were removed by manual scraping, cell extracts were obtained, and 100 μg of protein extract were used to determine CAT activity as described elsewhere (33, 34). The percent protein conversion into acetylated chloramphenicol was quantitated using the counts/min obtained from the Betascope 603 blot analyzer. The TLC plates were subjected to autoradiography using a Kodak X-Omat film for at least 12 h.

RESULTS

Identification of ER/Sp1 Elements on the Cathepsin D Promoter—The estrogen-responsive region of the CATH-D promoter is a 240-base pair fragment located at −123 to −364 of the initiation codon (12). Examination of the non-coding strand revealed the following putative ER/Sp1 sites within this region: 3′ −199 . . . GGGCGG $(n)_{23}$ ACGGG . . . −165 5′. An oligonucleotide (49-mer), which included the ER/Sp1 site and adjacent elements, was prepared (ER/Sp1 oligo) and used in the following analysis of the role of ER/Sp1 in regulation of E_2-induced

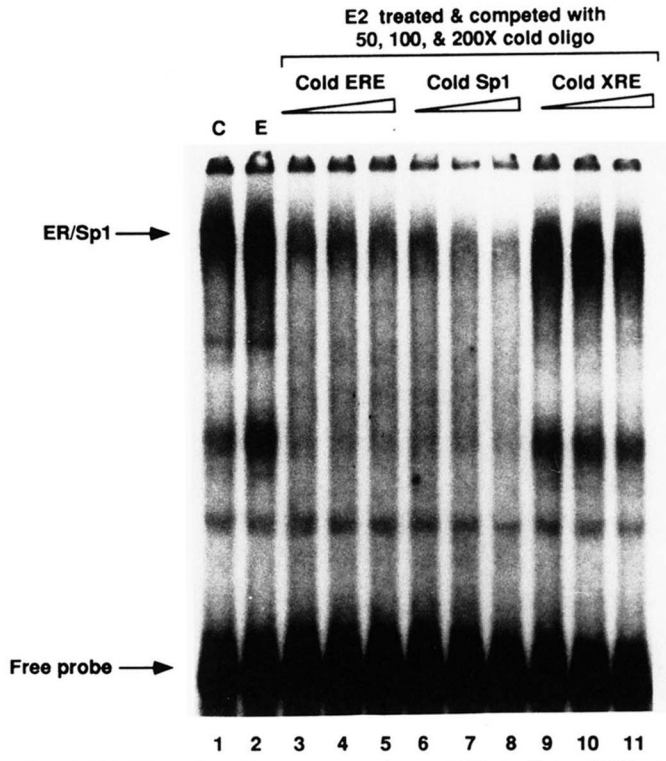

FIG. 1. **Binding of nuclear extracts from MCF-7 cells to ER/Sp1 oligo.** The cells were treated with Me_2SO (*lane 1*) or 10 nM E_2 (*lanes 2–11*); the nuclear extracts were isolated and analyzed by electromobility shift assays as described under "Materials and Methods." The retarded ER/Sp1 bands (see *arrow*) were visualized by autoradiography and quantitated using a Betagen 604 Betascope blot analyzer. The intensity values in *lanes 2–11* relative to the control band (*lane 1*, 100 ± 20%) were 150 ± 11, 40 ± 8, 42 ± 7, 38 ± 5, 42 ± 6, 30 ± 3, 10 ± 2, 143 ± 14, 150 ± 15, and 90 ± 12% (*lanes 2–11*, respectively; means ± S.D. for three determinations. The intensities in *lanes 3–8* were all significantly lower ($p < 0.05$) than observed in the E_2-treated cells (*lane 2*).

CATH-D gene expression.

EMSA Using Nuclear Extracts from MCF-7 Human Breast Cancer Cells—Fig. 1 illustrates the characteristic EMSA pattern using nuclear extracts from MCF-7 cells and ^{32}P-labeled ER/Sp1 oligo. The results show that nuclear extracts from control (Me_2SO-treated) cells bound to the ER/Sp1 oligo (*lane 1*); binding was increased with extracts from E_2-treated cells and decreased after preincubation with 50-, 100-, and 200-fold excess of unlabeled ERE (*lanes 3–5*) or unlabeled Sp1 (*lanes 6–8*) oligos. In contrast, competition with a 50-, 100-, and 200-fold excess of a nonspecific XRE oligo (31, 32) (*lanes 8–11*) did not affect binding to the ^{32}P-labeled ER/Sp1 oligo. An additional estrogen-inducible band with higher mobility was also observed but not further investigated. The results in Fig. 2 demonstrate that incubation of nuclear extracts with the ER/Sp1 (*wt*) oligo results in formation of a retarded ER·Sp1 complex (*lane 3*) which is competitively displaced by 100-fold unlabeled oligo (*lane 2*). In contrast, these extracts do not form a retarded band with oligos mutated in the ERE half-site (*lane 1*) or the Sp1 binding site (*lane 4*).

Effect of ER and Sp1 Specific Antibodies on ER/Sp1 Binding in EMSAs—Fig. 3 shows that when antibodies specifically raised against ER (*lane 5*) or Sp1 (*lane 2*) proteins are incubated with MCF-7 cell nuclear extracts for 20 min following incubation with radiolabeled ER/Sp1 oligo, a band of decreased mobility is formed due to super shifting of the ER/Sp1 band by the specific antibodies. Incubation with nonspecific IgG does not affect ER·Sp1 complex formation (Fig. 3, *lanes 3* and *4*); preincubation of the ER antibody with a peptide which blocks antibody function removes the specific antibody band (Fig. 3,

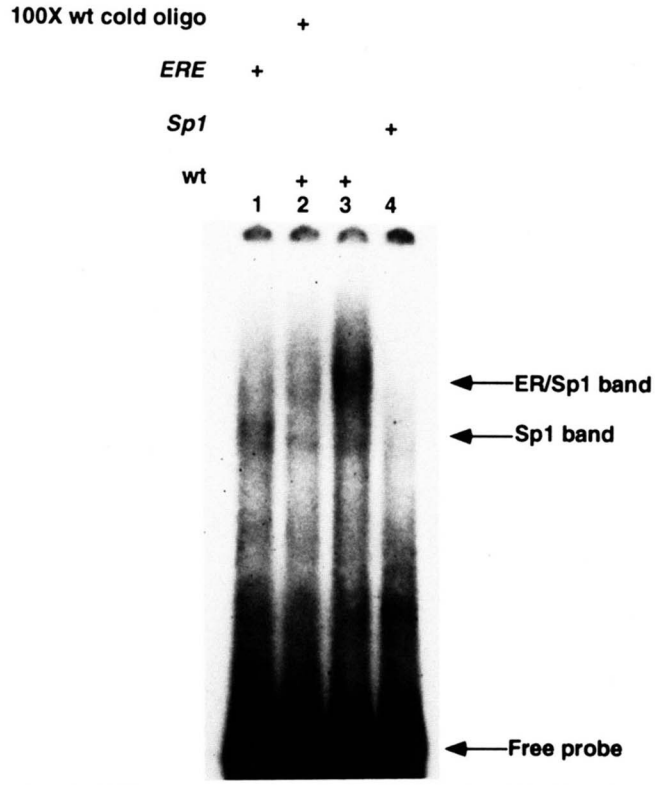

FIG. 2. **EMSAs performed with the ERE/Sp1 and ERE/Sp1 mutant oligos.** Nuclear extracts from MCF-7 cells were otained as described under "Materials and Methods" and subjected to EMSAs using radiolabeled oligos mutated at the ERE half-site (*ERE*) or at the Sp1 site (*Sp1*). *Lane 1* is an EMSA performed using the mutated ERE site and it illustrates an Sp1 band with increased mobility when compared to lane 3 which is the wild-type (*wt*) ER/Sp1 band (see *arrow*). *Lane 4* is an EMSA performed using an oligo mutated at the Sp1 site and illustrates no significant ER/Sp1 or Sp1 bands. *Lane 2* is an EMSA performed using radiolabeled wt ER/Sp1 oligo which is preincubated with a 100-fold excess of unlabeled wt ER/Sp1 oligo.

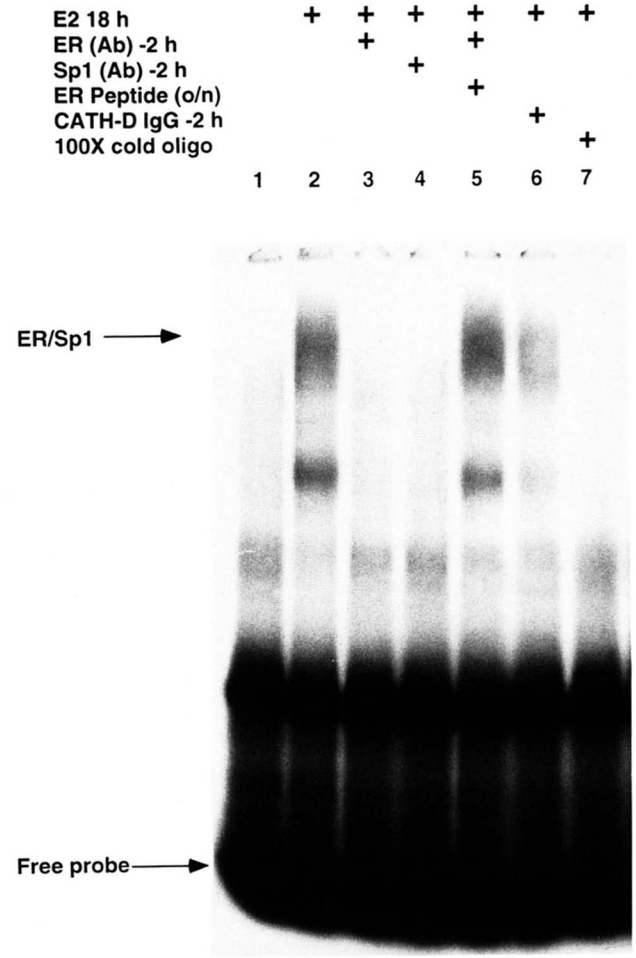

FIG. 3. **Effect of preincubation of MCF-7 nuclear extracts with ER and Sp1 antibodies on ER/Sp1 binding.** Cells were treated with Me_2SO (*lane 1*) or 10 nM E_2 (*lanes 2–7*). The nuclear extracts were isolated and incubated with 40 ng of ER Ab (*lane 3*) or 20 ng of Sp1 Ab (*lane 4*) for 2 h. *Lanes 5* and *6* were nuclear extracts preincubated with nonspecific CATH-D IgG (100 ng) and ER Ab incubated overnight at 4 °C with 10 μg/ml blocking peptide. The preincubated extracts were analyzed by electromobility shift assays as described under "Materials and Methods." The retarded ER/Sp1 and Sp1 bands (see *arrows*) were visualized by autoradiography and quantitated using the Betagen 603 Betascope blot analyzer. The intensity values in *lanes 2–7* relative to the control lane (*lane 1*, 100 ± 20%) were 190 ± 30, 84 ± 17, 110 ± 15, 200 ± 25, 195 ± 14 and 85 ± 10% (*lanes 2–7*, respectively; means ± S.D. for three determinations). The intensities in *lanes 3*, *4* and *7* were all significantly lower ($p < 0.05$) than observed in the E_2-treated cells (*lane 2*). Specific preincubation with 100 × cold ER/Sp1 oligo resulted in decreased ER/Sp1 binding (*lane 7*).

lane 6). Preincubation of MCF-7 cell nuclear extracts with the ER or Sp1 antibodies for 2 h followed by EMSAs resulted in a significant decrease in the ER/Sp1 binding (Fig. 4, *lanes 3* and *4*). Preincubation with nonspecific IgG (*lane 6*) or peptide-blocked ER antibody (*lane 5*) did not decrease ER/Sp1 binding. These results indicate that immunodepletion of the nuclear extracts with ER or Sp1 specific antibody can result in decreased formation of ER·Sp1 complexes *in vitro*. The above results support our hypothesis that ER and Sp1 proteins are directly involved in formation of these enhancer complexes.

EMSA of Nuclear Extracts from MCF-7 Cells Treated with E_2, ICI 164,384, and E_2 + ICI 164,384—^{32}P-Labeled ER/Sp1 binding of nuclear extracts from MCF-7 cells cotreated with E_2 and 1 μM ICI 164,384 for 8, 6, 4, and 2 h (Fig. 5, *lanes 3–6*, respectively) exhibited a time-dependent decrease in formation of the retarded band compared to Me_2SO (control) or E_2-treated cells (Fig. 5, *lanes 1* and *2*, respectively), and this is consistent with the reported effects of ICI 164,384 on destabilization of the nuclear ER homodimer (35).

EMSA Pattern of Nuclear Extracts from HeLa Cells after Transfection with an hER Expression Plasmid—Nuclear extracts from HeLa cells transfected with an hER expression plasmid gave an ER/Sp1 retarded band (Fig. 6, *lane 1*) which was increased in intensity after treatment with E_2 (*lane 2*); in contrast, minimal ER/Sp1 binding was observed in nuclear extracts from untreated HeLa cells (*lane 3*), although a lower molecular weight retarded band (labeled *Sp1*) was observed. The effect of various unlabeled DNA sequences as competitors for ^{32}P-labeled ER/Sp1 binding of nuclear extracts from HeLa cells transfected with hER was also determined using EMSAs. Competition with a 100-fold excess of the following unlabeled oligos, ER/Sp1 (*lane 6*), Sp1 (*lane 5*), and ERE (*lane 4*), all decreased the intensity of the ER/Sp1 band. Preincubation with unlabeled ERE shifts the ER/Sp1 band to an Sp1-retarded complex (*lanes 3* and *4*).

Northern Blot Analysis of Cathepsin D mRNA in MCF-7 Cells in the Presence of 1 μM ICI 164,384—The time-dependent effects of 1 μM ICI 164,384 on E_2-induced CATH-D mRNA levels were determined in MCF-7 cells (Fig. 7). Compared to control (Me_2SO) cells (*lane 1*), E_2 induces CATH-D mRNA levels. In cotreatment studies, ICI 164,384 significantly inhibited E_2-induced CATH-D mRNA levels after exposure of the cells to the antiestrogen for 8 or 6 h (*lanes 4* and *5*) and the inhibitory effect decreased with decreasing time of exposure to ICI 164,384. The effect of ICI 164,384 on CATH-D mRNA levels corresponded to a parallel decrease in ER/Sp1 binding in the EMSA (Fig. 5).

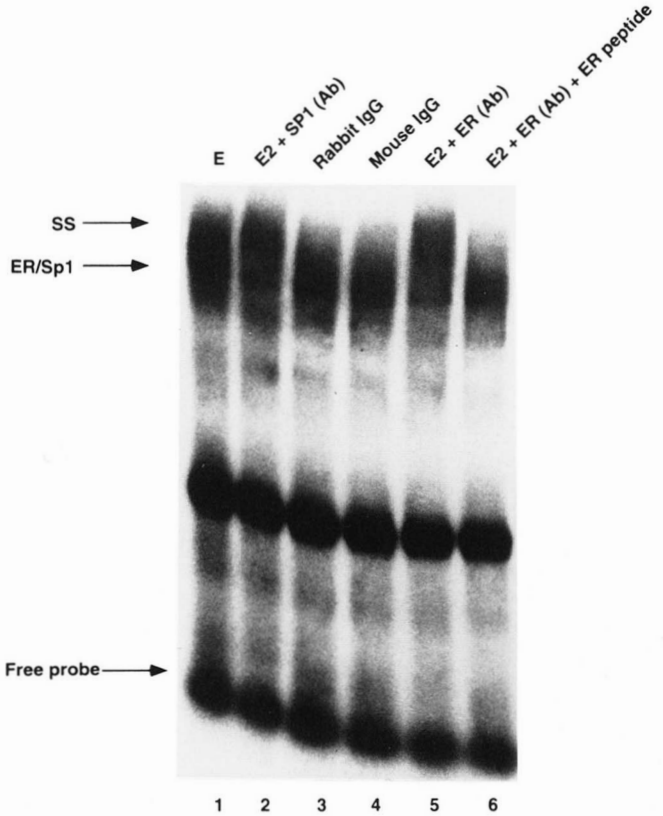

FIG. 4. **Supershifting of ER/Sp1 band with ER and Sp1 antibodies in EMSAs using MCF-7 cell nuclear extracts.** Cells were treated with 10 nM E_2 (*lanes 1–6*) and nuclear extracts were incubated with radiolabeled ER/Sp1 oligo followed by incubation with 20 ng of Sp1 Ab, 100 ng of rabbit IgG, 100 ng of mouse IgG, 40 ng ER Ab, and ER Ab (incubated for 18 h at 4 °C with ER peptide blocker; 40 ng). These nuclear extracts were analyzed by electromobility shift assays as described under "Materials and Methods." The retarded ER/Sp1 and super shift (*SS*) bands (see *arrows*) were visualized by autoradiography. The bands in *lanes 2* and *5* indicate the formation of a retarded band in addition to the ER/Sp1 band resulting from specific supershifting of the ER/Sp1 band by Sp1 and ER Ab, respectively.

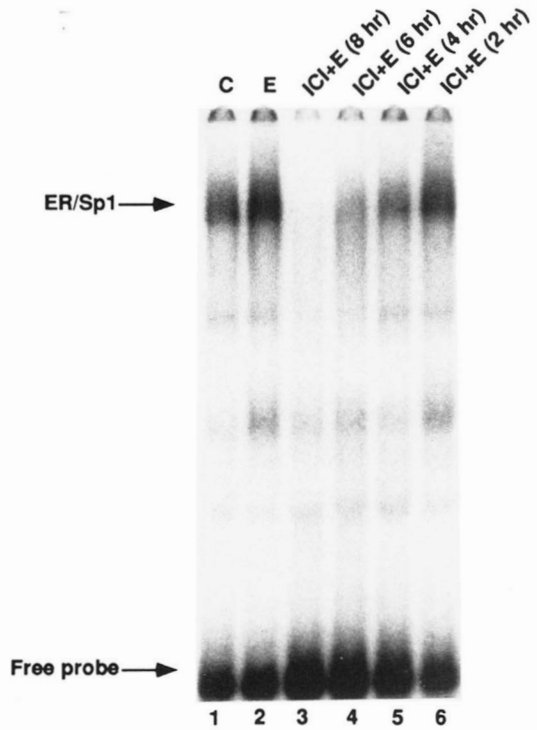

FIG. 5. **Binding of nuclear extracts from MCF-7 cells treated with 1 μM ICI 164,384 for 8, 6, 4, and 2 h (*lanes 3–6*, respectively).** Nuclear extracts from these cells were isolated and analyzed by electromobility shift assays as described under "Materials and Methods." The retarded ER/Sp1 bands (see *arrow*) were visualized by autoradiography and quantitated using a Betagen 603 Betascope blot analyzer. The intensity values in *lanes 2–6* relative to the control band (*lane 1*, 100 ± 15%) were 230 ± 28, 10 ± 5, 25 ± 7, 64 ± 11, and 145 ± 14% (*lanes 2–6*, respectively; means ± S.D. for three determinations). The intensities in *lanes 3–5* were all significantly lower ($p < 0.05$) than observed in E_2-treated cells (*lane 2*).

CAT Assays—The results in Fig. 8 summarize the effects of 10 nM E_2 on the induction of CAT activity in MCF-7 cells transiently transfected with plasmids containing the ER/Sp1 oligo and oligos with mutations in the ERE (*'ERE'*) or Sp1 (*'Sp1'*) genomic sequences. The results demonstrated that E_2 induced a 5.9-fold increase in CAT activity in cells transfected with the plasmid containing the ER/Sp1 oligo (*lane 2*) compared to cells treated with Me_2SO (control, *lane 1*). In contrast, CAT activities in Me_2SO and E_2-treated cells transiently transfected with the plasmids containing oligos mutated in the ERE (*lanes 6* and *7*, respectively) or Sp1 (*lanes 8* and *9*, respectively) sequence were not significantly different. Relatively high constitutive CAT activity was observed in extracts from cells transiently transfected with the plasmid containing the mutated ERE (*lanes 6* and *7*). In MCF-7 cells transfected with the estrogen-responsive ER/Sp1-thymidine kinase-CAT plasmid, ICI 164,384 caused a concentration-dependent decrease in E_2-induced CAT activity (*lanes 3* through *5*). In separate experiments (data not shown), incubation of MCF-7 cell nuclear extracts with the mutant oligos, *'ERE'*/Sp1 and ERE/*'Sp1*, did not result in formation of an ER·Sp1 complex as determined by EMSAs.

DISCUSSION

CATH-D is an estrogen-regulated gene which requires a functional ER for transactivation. Previous studies have demonstrated that ICI 164,384, a "pure" antiestrogen, which inhibits formation of the nuclear ER homodimer (35) also inhibited E_2-induced CATH-D mRNA levels and secretion of this protein. The results summarized in Fig. 4 illustrate the time-dependent inhibition of E_2-induced CATH-D mRNA levels and this resembled the reported parallel decrease in ER homodimer levels. Analyses of the 5′-promoter region of the *CATH-D* gene have identified several Sp1 binding sites in the sense and antisense strands as well as GC boxes which are typical of "housekeeping" genes (36). Redecker and co-workers (36) did not identify a functional TATA box in the 5-region, whereas estrogen induces TATA-dependent initiation of *CATH-D* gene transcription almost exclusively from transcription start site I (12). It was suggested that the differences observed in these studies may be due to differential regulation of gene transcription by estrogen (from transcription start site I) and calcitriol (possibly from transcription start site V) (12). Examination of the noncoding strand of the CATH-D promoter shows that it contains a $GGGCG(n)_{23}ACGGG$ (−199/−165) ER/Sp1 sequence similar to that described for the E_2-regulated *myc* protooncogene (29). This study demonstrates that the ER/Sp1 sequence plays a role in the regulation of *CATH-D* gene transcription by estrogens.

The characteristic EMSA pattern in Fig. 1 shows that ER/Sp1 complexes are formed *in vitro* using nuclear extracts from MCF-7 cells and a ^{32}P-labeled ER/Sp1 oligonucleotide which corresponds to the sequences identified on the CATH-D promoter. In addition, preincubation with several unlabeled oligonucleotides (Fig. 1, *lanes 3–12*) confirms the specific involvement of the ER and Sp1 proteins in this complex. The results illustrated in Figs. 2–4 further support our hypothesis that ER and Sp1 proteins are directly involved in formation of the ER·Sp1 complex. EMSAs of nuclear extracts from MCF-7 cells in-

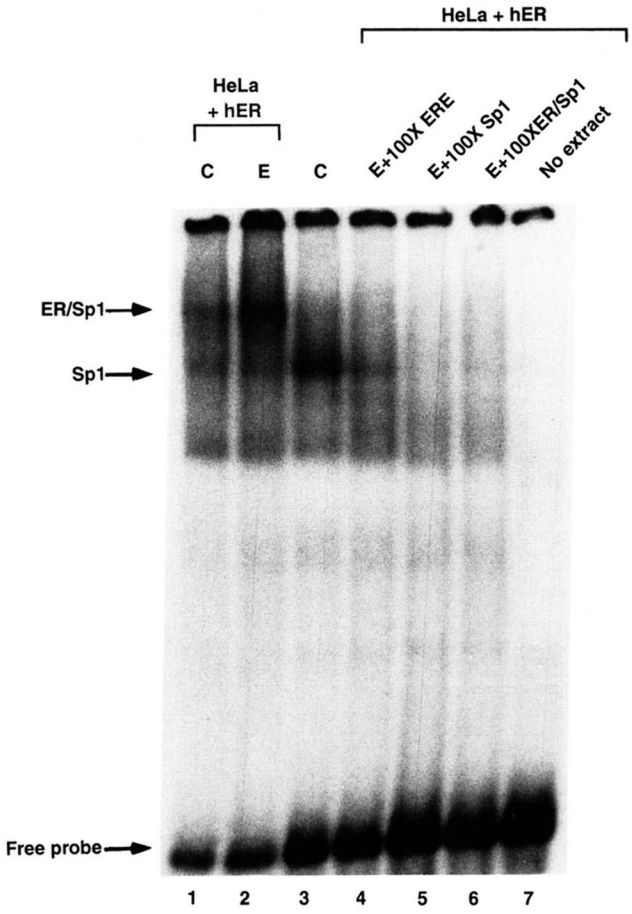

FIG. 6. **Binding of nuclear extracts from HeLa cells treated with Me_2SO (*lane 3*) and nuclear extracts from HeLa cells co-transfected with hER and treated with Me_2SO (*lane 1*) and 10 nM E_2 (*lanes 2, 4, 5,* and *6*).** The nuclear extracts were isolated and analyzed by electromobility shift assays as described under "Materials and Methods." The retarded ER/Sp1 and Sp1 bands (see *arrows*) were visualized by autoradiography and quantitated using the Betagen 603 Betascope blot analyzer. The intensity value for the ER/Sp1 banding E_2-treated cells (*lane 2*) was 270 ± 20% relative to the control band (*lane 1*, 100 ± 17%). No significant binding for the ER/Sp1 band was observed from *lanes 3* to *7*. The intensity values for the Sp1 band in *lane 4* is 22 ± 3% when compared to Me_2SO-treated HeLa cells in *lane 3* (100 ± 14%). The specific preincubation with an 100-fold excess of cold Sp1 oligo prevents any significant ER/Sp1 and Sp1 binding.

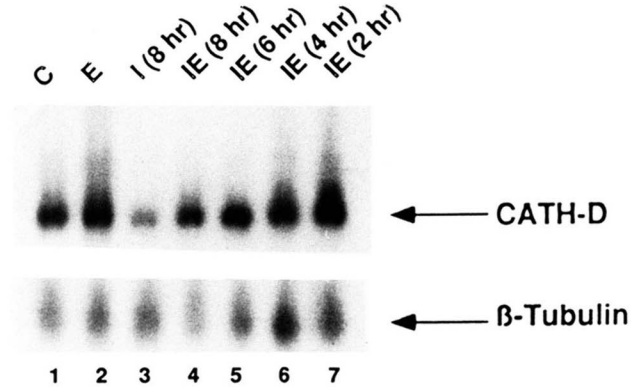

FIG. 7. **Northern analysis of mRNA from MCF-7 cells treated with ICI 164,384.** The cells were treated with Me_2SO (*lane 1*), 10 nM E_2 for 24 h (*lane 2*) and 1 μM ICI 164,384 for 8 h (*lane 3*). Cells were cotreated with 10 nM E_2 for 24 h and 1 μM ICI 164,384 for 8, 6, 4, and 2 h (*lanes 4–7*, respectively). Cell extracts were obtained and total RNA was isolated and subjected to Northern analysis as described under "Materials and Methods." The blots were visualized by autoradiography and quantitated using a Betagen 603 Betascope blot analyzer. The intensity values in *lanes 2–7* relative to the control lane (*lane 1*, 100 ± 6%) were 176 ± 8, 55 ± 8, 80 ± 5, 105 ± 4, 123 ± 6, and 163 ± 12% (*lanes 2–7*, respectively; means ± S.D. for three determinations). The intensities in *lanes 3* through *5* were all significantly lower ($p < 0.05$) than observed in the E_2-treated cells (*lane 2*); the intensity values for CATH-D were standardized to β-tubulin which was used as an internal control.

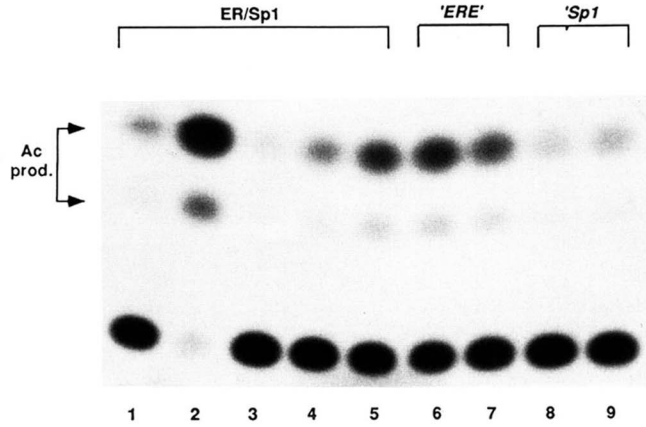

FIG. 8. **Effect of ICI 164,384 on E_2-induced ER/Sp1-thymidine kinase-CAT activity in MCF-7 cells.** The cells were co-transfected with hER and ER/Sp1-thymidine kinase-CAT (*lanes 1–5*) or *'ERE'*/Sp1-thymidine kinase-CAT (mutated ERE half-site) (*lanes 6* and *7*) or ER/*'Sp1*-thymidine kinase-CAT (mutated Sp1 site) (*lanes 8* and *9*) plasmids. The transient transfection assay was performed as described under "Materials and Methods." Cells were treated with Me_2SO (*lane 1*), 10 nM E_2 for 48 h (*lane 2*), 1 μM ICI 164,384 for 24 h (*lane 3*), E_2 + 1 μM ICI 164,384 (*lane 4*), and E_2 + 0.1 μM ICI 164,384 (*lane 5*), Me_2SO (*lane 6*), 10 nM E_2 (*lane 7*), Me_2SO (*lane 8*), and 10 nM E_2 (*lane 9*). The relative intensity values for *lanes 2–5* when compared to control cells (*lane 1*, 100 ± 20%) were 590 ± 100, 80 ± 113, 250 ± 70, and 340 ± 50% (*lanes 2–5*, respectively; means ± S.D. for three determinations). The relative intensity values for E_2-treated cells (*lane 7*) when compared to control cells (*lane 6*, 100 ± 11%) was 75 ± 17%. The relative intensity values for E_2-treated cells (*lane 9*) when compared to control cells (*lane 8*, 100 ± 13%) was 98 ± 20%. The intensity values for lanes through *5* are significantly lower ($p < 0.05$) than E_2-treated cells (*lane 2*); the results expressed above for the two mutated plasmids (*'ERE'* and *'Sp1*) are from two separate experiments and are expressed as means ± S.D. for three determinations.

cubated with ^{32}P-labeled oligos mutated in the Sp1 or ERE half-site did not form an ER·Sp1 complex (Fig. 2, *lanes 4* and *1*, respectively). Immunodepletion of ER or Sp1 from nuclear extracts (Fig. 3, *lanes 3* and *4*) decreases formation of the ER/Sp1 complex *in vitro*; moreover, the ER·Sp1 complex is supershifted after incubation with ER or Sp1 antibodies (Fig. 4, *lanes 5* and *2*, respectively). The requirement for the ER in formation of the ER·Sp1 complex was confirmed by measuring ER/Sp1 binding in nuclear extracts from HeLa cells in the presence or absence of cotransfected hER (Fig. 5) and in MCF-7 cells treated with the antiestrogen ICI 164,384 (Fig. 5). EMSA analysis of extracts from HeLa cells (Fig. 1) demonstrated that formation of the ER·Sp1 complex was observed only after transfection with hER in the absence (*lane 1*) or presence (*lane 2*) of E_2 and, in MCF-7 cell nuclear extracts, the band intensity was decreased by competition with excess unlabeled ERE, Sp1, and ER/Sp1 oligos. The antiestrogen ICI 164,384 interferes with formation of the ER homodimer resulting in decreased nuclear estrogen receptor levels (35). Treatment of MCF-7 cells with ICI 164,384 resulted in a time-dependent decrease in CATH-D mRNA levels (Fig. 7), and this corresponded with the reported time-dependent decrease in nuclear ER levels in these cells (35) and paralleled the time-dependent decrease in ER/Sp1 binding in MCF-7 cells (Fig. 5). These data demonstrate that the ER/Sp1 sequence located in the promoter region of the *CATH-D* gene binds both ER and Sp1.

The functional activity of the CATH-D ER/Sp1 sequence was investigated by cloning the sequence upstream to a thymidine

kinase promoter with a CAT reporter gene as previously described (25). The results (Fig. 8) demonstrate that the ER/Sp1 sequence is estrogen-responsive (*lane 2*) and ICI 164,384 inhibits the E_2-induced response (*lanes 3–5*). The requirement of the ER/Sp1 sequence for estrogen-responsiveness was further investigated using plasmids which contain mutations in the ERE-half site (*'ERE'*) and in the Sp1 sequence (*'Sp1'*). The constitutive CAT activity in extracts from cells transiently transfected with these plasmids was highly variable (Fig. 8, *lanes 6* and *8*), and it was evident that a higher overall induction response was observed for the plasmid containing a mutated ERE-half site but an intact Sp1 sequence. These data demonstrate that Sp1-mediated transcriptional activation from the mutated *'ERE'*-promoter is not dependent on a functional ERE half-site whereas mutations in the Sp1 oligo resulted in only minimal induction of CAT activity by E_2. However, despite the differences in the constitutive CAT activities of extracts from cells transiently transfected with plasmids containing ERE·half site or Sp1 mutations, E_2 did not induce CAT activity in these assays. In addition, the mutant oligos did not form an ER·Sp1 complex as determined by EMSAs (Fig. 2). Thus, these data confirm the requirement for an intact ER/Sp1 sequence for estrogen-responsiveness.

The molecular mechanism for Sp1-mediated transcriptional activation has not been unequivocally determined; however, there is evidence that TATA binding factors including TFIID and other coactivators may be required (27, 37). Similar mechanisms may also govern ER/Sp1-mediated transactivation in which coactivators may play a role. However, at present, the nature of the specific protein-DNA and protein-protein interactions required for the induction response have not been determined and are currently being investigated in this laboratory.

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