Identification of a Functional Imperfect Estrogen-Responsive Element in the 5′-Promoter Region of the Human Cathepsin D Gene†

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ABSTRACT: 17β-Estradiol (E2) induces cathepsin D gene expression in MCF-7 human breast cancer cells. Previous studies have identified an Sp1-imperfect estrogen-responsive element (ERE) half-site [GGGCGG-$(N)_{23}$ACGGG] (−199 to −165) in the promoter region which forms an Sp1−estrogen receptor (ER) complex and confers E2 responsiveness on the corresponding Sp1−ERE−chloramphenicol acetyl transferase (CAT) construct. Further analysis of downstream regions of the promoter identified a CGCCC-$(N)_3$TGACC sequence (−119 to −107) which is homologous to the adenovirus major late promoter element (MLPE) and binds the ER to form a retarded band in a gel electrophoretic mobility shift assay. The corresponding promoter−CAT construct is also E2−inducible. The MLPE resembles an imperfect palindromic ERE containing imperfect (5′) and perfect (3′) ERE half-sites; analysis of oligonucleotides with mutations in these half-sites shows that only the perfect ERE half-site is required for binding the ER, whereas both sites are required for transactivation. *In vivo* exonuclease III footprinting showed that treatment with E2 also enhanced binding at the MLPE site. Identification of this second functional enhancer sequence in the 5′-promoter region of cathepsin D is consistent with the increasingly complex cell-specific regulation of hormone-responsive genes.

Cathepsin D is a lysosomal protease which has been identified in numerous breast cancer cell lines and in mammary tumors (Westley & Rochefort, 1980; Westley et al., 1984, 1989; Morisset et al., 1986; May & Westley, 1987; Westley & May, 1987; Cavailles et al., 1988, 1989, 1991, 1993; May et al., 1993; Augereau et al., 1994; Krishnan et al., 1994, 1995). Several studies have demonstrated that high levels of cathepsin D in primary mammary tumors are associated with poor prognosis for disease free survival of these patients (Spyratos et al., 1989; Thorpe et al., 1989; Tandon et al., 1990; Kute et al., 1992; Rochefort, 1992; Krishnan et al., 1995). Garcia and co-workers (1990) have also reported that tumor cells which overexpress cathepsin exhibit increased metastatic potential; however, the precise role of cathepsin D in tumor development and growth is unknown (Briozzo et al., 1988; Montcourrier et al., 1990; Rochefort, 1990; Johnson et al., 1993). Regulation of cathepsin D gene expression has been extensively investigated in human breast cancer cells and other cancer cell lines derived from hormone-responsive tissues. 17β-Estradiol (E2)[1] induces cathepsin D gene expression in MCF-7 human breast cancer cells, and this is accompanied by increased intra- and extracellular levels of both cathepsin D and procathepsin D (52 kDa protein). Five major transcription start sites have been identified for cathepsin D (Cavailles et al., 1993; May et al., 1993), and E2 is induced from transcription start site I and is dependent on an intact TATA box (−48 to −44) (Cavailles et al., 1993).

Analysis of the proximal promoter region of the cathepsin D gene revealed multiple estrogen-responsive element (ERE) half-sites, AP1 and GC-rich Sp1 binding sites (Redecker et al., 1991; Cavailles et al., 1993; May et al., 1993; Augereau et al., 1994). Augereau and co-workers (1994) identified a nonconsensus palindromic ERE at residue −261 (5′-GGGC-CGGGCTGACCCCGCGGG-3′). However, in transient transfection studies utilizing a promoter-tk−CAT construct containing this sequence, estrogen responsiveness was not observed; however, constructs containing this imperfect ERE linked to other downstream sequences could be induced by E2. The −252 to −124 region of the cathepsin D promoter was also estrogen-responsive, and various sequences were protected in a DNase I footprinting experiment (Augereau et al., 1994). Research in this laboratory (Krishnan et al., 1994, 1995) has identified an E2-responsive Sp1−ERE half-site [GGGCGG$(N)_{23}$ACGGG] in the noncoding strand of the cathepsin D promoter (−199 to −165). Gel electrophoretic mobility shift assays confirmed formation of an estrogen receptor (ER)−Sp1 complex which required both the imperfect ERE half-site and the Sp1 site. Moreover, plasmids containing the Sp1−ERE sequence linked to a chloramphenicol acetyltransferase (CAT) reporter gene were E2-responsive (Krishnan et al., 1994). In a subsequent study,

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[1] Abbreviations: E2, 17β-estradiol; ERE, estrogen receptor element; ER, estrogen receptor; CAT, chloramphenicol acetyltransferase; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; AhR, aryl hydrocarbon receptor; DRE, dioxin-responsive element; iDRE, inhibitory dioxin-responsive element; hER, human estrogen receptor; MLPE, major late promoter element; AF, activation function.

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Table 1: Oligonucleotides from the Human Cathepsin D Gene Used for the Transient Transfection and Eletrophoretic Mobility Shift Assays[a]

oligo-nucleotides	locations	sequences (sense strand)
CD/2	−208 to −101	5′-*AGC TT*C CCC GCC CCC CGC CCG GGC GCT GTG CGT GCC CGA GGT TGC CCC GCC CAG GCC AGG CCC CGC TCC GCC CCG CCC CGC GCA CGC CGG CCG **CGC CC**A CG**T GAC C**GG TCC G*G*-3′
CD/2_{M4}	−208 to −101	5′-*AGC TT*C CCC GCC CCC ATA ACG GGC GCT GTG CGT GCC CGA GGT TGC CCC GCC CAG GCC AGG CCC CGC TCC GCC CCG CCC CGC GCA CGC CGG CCG **CGC CC**A CG**T GAC C**GG TCC G*G*-3′
CD/2_{M2}	−208 to −101	5′-*AGC TT*C CCC GCC CCC CGC CCG GGC GCT GTG CGT GCC CGA GGT TGC CCC GCC CAG GCC AGG CCC CGC TCC GCC CCG CCC CGC GCA CGC CGG CCG **CGC CC**A CG**T AAA A**GG TCC G*G*-3′
CD	−145 to −101	5′-*AGC TT*C CGC CCC GCC CCG CGC ACG CCG GCC G**CG CCC** ACG **TGA CC**G GTC CC*G*-3′
CD/L	−145 to −119	5′-*AGC TT*C CGC CCC GCC CCG CGC ACG CCG GCC CC*G*-3′
CD/R	−120 to −101	5′-*AGC TT*G **CGC CC**A CG**T GAC C**GG TCC G*G*-3′
CD/R_{M1}	−120 to −101	5′-*AGC TT*G AAC ACA CGT GAC CGG TCC G*G*-3′
CD/R_{M2}	−120 to −101	5′-*AGC TT*G CGC CCA CGT AAA AGG TCC G*G*-3′
CD/R_{M3}	−120 to −101	5′-*AGC TT*G AAC ACA CGT AAA AGG TCC G*G*-3′

[a] All oligonucleotides contain a *Hin*dIII site at the 5′-end and a *Bam*HI site at the 3′-end for cloning into the pBL/TATA CAT vector. The *Hin*dIII and *Bam*HI linker sequences are italicized. The numbering is based on a system with 1 as the first nucleotide of the translation codon as described (Cavailles et al., 1993; Augereau et al., 1994). The bold letters indicate the imperfect palindromic ERE sequence. The mutated bases in the $CD/2_{M4}$, $CD/2_{M2}$, CD/R_{M1}, CD/R_{M2}, and CD/R_{M3} oligonucleotides are underlined.

it was also shown that inhibition of E2-induced cathepsin D gene expression and CAT activity by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was accompanied by disruption of the ER−Sp1−DNA complex (Krishnan et al., 1995). The apparent mechanism of this inhibitory response involved interaction of the nuclear aryl hydrocarbon receptor (AhR) complex with a dioxin-responsive element (DRE) (−181 to −175) located between the Sp1 and imperfect ERE half-sites. The 5′-promoter region of the cathepsin D gene contains several other potential downstream inhibitory DRE (iDRE) sequences at −130 to −126 (overlapping), −88 to −92, and −38 to −42. Results of preliminary studies using constructs containing the −208 to −101 and −145 to −101 regions of the cathepsin D promoter suggested that other *cis*-acting genomic elements within the latter sequence (−145 to −101) may also play a role in E2-induced transactivation. An imperfect palindromic ERE (CGCCCACGTGACC) has been identified in this study; this DNA sequence binds the ER to form a retarded band in a gel electrophoretic mobility shift assay and confers E2 responsiveness on the derived promoter−reporter construct. Augereau and co-workers (1994) previously identified this nonconsensus ERE as a viral major late promoter element (MLPE) and also showed that this site was protected against DNase I digestion using nuclear extracts from MCF-7 cells treated with E2. These observations are consistent with results of the present study which characterizes the MLPE as a second E2-responsive region in the proximal promoter region of the cathepsin D gene.

EXPERIMENTAL PROCEDURES

Chemicals and Biochemicals. Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham (DME F-12) without phenol red, phosphate-buffered saline (PBS), acetyl-CoA, E2, and 100× antibiotic/antimycotic solution were purchased from Sigma Chemical Co. (St. Louis, MO). Fetal calf serum (FCS) was obtained from Intergen (Purchase, NY). Minimum Essential Medium (MEM) was purchased from Life Technologies (Grand Island, NY). [γ-^{32}P]ATP (3000 Ci/mmol) and [^{14}C]chloramphenicol (53 mCi/mmol) were purchased from NEN Research Products (Boston, MA). Poly[d(I-C)], restriction enzymes *Hin*dIII and *Bam*HI, and T4-polynucleotide kinase were purchased from Boehringer Mannheim (Indianopolis, IN). DNA oligonucleotides (Table 1) were synthesized and purchased from Genosys (Woodlands, TX). The estrogen response element (ERE) and mutant ERE (Kumar & Chambon, 1988) were synthesized by the Gene Technologies Laboratory at Texas A&M University. The polymerase chain reaction (PCR) reagents were purchased from Perkin−Elmer (Branchburg, NJ) and Stratagene (La Jolla, CA). ER antibody H222 was purchased from Abbott Laboratories, and ER antibodies for blocking ER binding to EREs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) (rabbit polyclonal IgG against the C terminus of the ER). All other chemicals and biochemicals were the highest quality available from commercial sources.

Cell Culture Maintenance and Growth. MCF-7 human breast cancer cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD) and maintained in MEM with phenol red and supplemented with 10% FCS plus antibiotic/antimycotic solution, 0.035% sodium bicarbonate, 0.011% sodium pyruvate, 0.1% glucose, 0.238% Hepes, and 6×10^{-7}% insulin. Hela cells were also purchased from ATCC and maintained in DME F-12 supplemented with 5% FCS plus antibiotic/antimycotic solution and 0.22% sodium bicarbonate.

Plasmids and Plasmid Construction. The pBL/CAT2 and pBluescript (BS) plasmids were purchased from ATCC and Strategene. The human estrogen receptor (hER) expression plasmid was provided by Ming-jer Tsai (Baylor College of Medicine, Houston, TX). HE15 and HE19 are expression plasmids coding for mutant hERs. In HE15, the amino acids from 282 to 595 are deleted, whereas HE19 is truncated from amino acid 1 to 178 (Kumar et al., 1987). pBL/TATA/CAT plasmid was prepared in this laboratory by removing the *Bam*HI/*Xho*I fragment (i.e. the thymidine kinase promoter) from pBL/CAT2 and ligating E1B TATA DNA oligo into the *Bam*HI/*Xho*I sites. For construction of the pCD/355 plasmid containing the cathepsin D promoter insert, −365 to −10 (Redecker et al., 1991), two primers were designed: A (sense strand), 5′-CCGAAGCTTGGCCGGGACAGGGGT-CAC-3′; and B (antisense strand), 5′-GTGGGATCCGGC-CGGGTCGGAGAGGG-3′. The PCR condition was 95 °C for 4 min, 95 °C for 1 min, and 75 °C for 1 min for 30 cycles and 75 °C for 5 min. The resultant PCR product was cut with *Hin*dIII and *Bam*HI at 37 °C overnight and ligated into the pBL/TATA/CAT vector. pCD2, $pCD/2_{M4}$, $pCD/2_{M2}$, pCD, pCD/L, pCD/R, pCD/R_{M1}, pCD/R_{M2}, and pCD/

R_{M3} were generated by ligation of appropriate double-stranded oligonucleotides (Table 1) into the pBL/TATA/CAT (or pBS for pCD/BS) vector and ligation products transformed into DH 5α competent *Escherichia coli* cells. Identification of the cloned sequences was confirmed by restriction enzyme mapping and DNA sequencing using the Sequitherm cycle sequencing kit from Epicentre Technologies (Madison, WI). Plasmid preparations for transfection utilized alkaline lysis followed by cesium chloride gradient centrifugations (two times) or the Plasmid Mega Kit from Qiagen (Chatsworth, CA).

Transient Transfection. Cells from maintenance were trypsinized, seeded in 100 mm petri dishes with 10 mL of phenol red free DME F-12 medium plus 5% charcoal-stripped FCS, and grown until they were 50−60% confluent. About 3 h prior to transfection, old medium was replaced with 5 mL of the charcoal-stripped DME F-12 medium. Cells in each petri dish were transfected with 1 mL of transfection cocktail containing 5 μg of test plasmid, 4 μg of hER, 50 μL of 2.5 M $CaCl_2$, and 500 μL of HBS (pH 7.05). Cotransfection of the hER expression plasmid was required due to overexpression of the promoter−reporter constructs; this requirement has also been observed using constructs derived from cathepsin D and other E2-responsive gene promoters (Cavailles et al., 1991; Augereau et al., 1994; Krishnan et al., 1994, 1995). After incubation for 14−16 h at 37 °C, cells were washed once with 5 mL of PBS and treated with 10^{-9} M E2 in 10 mL of dextran-coated charcoal-stripped DME F-12 medium. After 48 h, cells were washed once with 5 mL of PBS and harvested by scraping. Cells were lysed in 200 μL of 0.25 M Tris-HCl (pH 7.6) by three cycles of freezing in liquid nitrogen for 2 min, thawing at 37 °C for 2 min, sonication for 3 min, and vortexing for 1 min. Cell debris was pelleted, and the protein concentration in the supernatant was measured by the method of Bradford (1976) using bovine serum albumin as the standard. An aliquot of cell lysate equal to 80 μg of protein was diluted to 120 μL with 0.25 M Tris-HCl (pH 7.6) and incubated with 1 μL of [^{14}C]chloramphenicol (53 mCi/mmol) and 42 μL of 4 mM acetyl-CoA for 6 h at 37 °C. The reaction was stopped by vortexing with 700 μL of ethyl acetate. The extract (600 μL) was dried and redissolved in 20 μL of ethyl acetate, and the acetylated products were resolved by thin-layer chromatography (TLC) (Whatman Lab Sales, NY) using a 95:5 chloroform/methanol solvent mixture. The percent protein conversion into acetylated chloramphenicol was quantitated using the counts per min obtained from the Betagen Betascope 603 blot analyzer. The TLC plates were subjected to autoradiography using a Kodak X-Omat film for about 20 h.

Gel Electrophoretic Mobility Shift Assay (GMSA). The complementary oligonucleotides were annealed and labeled at the 5′-end using T4 polynucleotide kinase and [^{32}P]ATP (Sambrook et al., 1989). Nuclear extracts (5 μg) from MCF-7 or Hela cells treated with 10^{-9} M E2 for 12 h were incubated in HEGD [25 mM Hepes, 1.5 mM EDTA, 10% glycerol (v/v), and 1.0 mM dithiothreitol at pH 7.5] buffer with 1 μg of poly[d(I-C)] and 0.1 mM KCl for 15 min at 20 °C to bind nonspecific DNA-binding proteins. Following addition of ^{32}P-labeled DNA (final concentration, 10^{-9} M), the mixture was incubated for another 15 min at 20 °C. Excess unlabeled DNA (50−2000-fold) was added 5 min before adding ^{32}P-labeled DNA to compete for specific DNA binding. The double-stranded oligonucleotides for unlabeled competition assays were Sp1 (sense strand 5′-ATTCGATCGGGGCGGGGCGAGC-3′), wild-type ERE, and those summarized in Table 1. For the immunodepletion study, 5 μg of nuclear extract was incubated in HEGD buffer and 0.1 M KCl with 2 μg of ER antibody overnight on ice and 20 °C for 1 h before ^{32}P-labeled probe was added. Reaction mixtures were loaded onto a 5% nondenaturing polyacrylamide gel and run at 110 V in 1× TBE buffer (0.09 M Tris, 0.09 M boric acid, and 2 mM EDTA, at pH 8.3). A similar protocol was used for the supershift experiments except that the antibody (H222) was added after the [^{32}P]oligonucleotide. Gels were dried, and protein−DNA complexes were visualized by autoradiography and quantitated with a Betagen Betascope 603 blot analyzer.

Table 2: Induction of CAT Activity by E2 in MCF-7 Cells Transiently Transfected with the pBL/TATA, pCD/355, pCD2, and pCD Plasmids[a]

	relative CAT activity (% of DMSO)			
treatment	pBL/TATA	pCD/355	pCD2	pCD
DMSO	100 ± 9	100 ± 3	100 ± 9	100 ± 16
E2 (1 nM)	109 ± 7	709 ± 22[b]	365 ± 10[b]	420 ± 36[b]

[a] Cells were cotransfected with 3 μg of hER and 5 μg of pBL/TATA (control), pCD/355, pCD2, or pCD and treated with 1 nM E2 or DMSO 2 days before determination of CAT activity. pBL/TATA is a vector without a cathepsin promoter insert; pCD/355, pCD2, and pCD contain cathepsin promoter sequences −365 to −10, −208 to −101, and −145 to −101, respectively. Results are expressed as means ± SE for at least three determinations for each treatment group. [b] Significantly higher ($p > 0.05$) than observed after treatment with DMSO.

In Vivo Footprinting. MCF-7 cells were grown to 50% confluence, then transfected with 10 μg of the cathepsin D promoter plasmid (pCD/355) for 24 h, and then maintained with or without estradiol (10^{-7} M) for 12 h. For *in vivo* footprinting of transcription factors, the cells were harvested and nuclei were isolated and digested by exonuclease III digestion (5000 units/mL), in conjunction with *Hin*dIII (1000 units/mL), as described previously (Archer et al., 1992). After purification of the genomic DNA, 10 μg of each sample was analyzed using Taq polymerase (100 units/mL) amplification with a ^{32}P-labeled oligonucleotide specific for the CAT reporter gene as described (Archer et al., 1992). Purified extended products were analyzed on 5 or 7% polyacrylamide denaturing gels and exposed to Kodak X-MAT AR film at −80 °C.

Statistical Analysis. Statistical significance was determined by ANOVA and Scheffe's test. Results are expressed as means ± standard error for three separate determinations for each treatment group.

RESULTS

The results in Table 2 summarize the induction of CAT activity by 10^{-9} M E2 in MCF-7 cells transiently cotransfected with 4 μg of the human estrogen receptor (hER) plasmid and 5 μg of the pBL/TATA, pCD/355, pCD2, or pCD constructs. pBL/TATA is a control vector which does not contain an insert, whereas pCD/355, pCD2, and pCD contain cathepsin D promoter sequences −365 to −10, −208 to −101, and −145 to −101, respectively, cloned into pBL/TATA. The results show that the full length (pCD/355) and deletion (pCD2 and pCD) constructs are all E2-inducible (3.65−7.09-fold). pCD2 contains the E2-responsive GGG-

Table 3: Induction of CAT Activity by E2 in MCF-7 Cells Transiently Transfected with the pCD Plasmid, a Requirement for Cotransfection with hER[a]

	relative CAT activity (% DMSO)					
treatment	0 μg of hER	1 μg of hER	3 μg of hER	5 μg of hER	HE19	HE15
DMSO	100 ± 14	100 ± 7	100 ± 6	100 ± 5	100 ± 7	100 ± 7
E2 (1 nM)	155 ± 5[b]	233 ± 7[b]	323 ± 18[b]	416 ± 63[b]	299 ± 17[b]	74 ± 6

[a] MCF-7 cells were transiently transfected with pCD and different amounts of hER or hER variants and treated with E2. CAT activity was determined as described in Experimental Procedures and results are expressed as means ± SE for three separate determinations for each treatment group. [b] Significantly higher ($p < 0.05$) than activity in cells treated with DMSO alone.

Table 4: Induction of CAT Activity by E2 in MCF-7 Cells Transiently Transfected with the CD/2, CD/2_{M4}, and CD/2_{M2} Plasmids[a]

treatment	pCD/2	pCD/2_{M4}	pCD/2_{M2}
DMSO	100 ± 6.0	100 ± 12	100 ± 6
E2 (1 nM)	498 ± 37[b]	293 ± 24[b]	273 ± 37[b]

[a] Cells were cotransfected with 2 μg of hER and 5 μg of pCD/2, pCD/2_{M4}, or pCD/2_{M2} and treated with 1 nM E2 or DMSO 2 days before determination of CAT activity as described in Experimental Procedures. Results are expressed as means ± SE for at least three determinations for each treatment group. [b] Significantly higher ($p < 0.05$) than observed after treatment with DMSO.

$CGG(N)_{23}ACGGG$ Sp1−ERE (half-site) (Krishnan et al., 1994, 1995), whereas this sequence is not contained in pCD. E2 significantly induced CAT activity in MCF-7 cells transiently transfected with pCD at concentrations of 10^{-10}, 10^{-9}, and 10^{-8} M [233 ± 15, 391 ± 18, and 280 ± 14% of control (100%), respectively] and maximal induction was observed 24 h after treatment (data not shown). E2-induced activity was dependent on cotransfection with hER in which 5 μg of this expression plasmid gave a maximal response (Table 3); in cotransfection with variant ER expression plasmids containing N-terminal (HE19) and C-terminal (HE15) deletions, CAT activity was induced by E2 only with HE19 which contains the ligand-dependent transactivation function 2 (AF2) (Table 3). These results were consistent with previous studies showing that cotransfection with hER was required for induction of cathepsin D promoter−reporter constructs (Augereau et al., 1994; Krishnan et al., 1994, 1995).

The induction of CAT activity by E2 has been determined in MCF-7 cells transiently transfected with pCD and different amounts of pBS/CD where the CD oligonucleotide has been inserted into the Bluescript in which the reporter gene is expressed only in prokaryotes. Thus, the CD insert in pBS/CD serves as a competitor with pCD for nuclear factors which bind the −145 to −101 sequence from cathepsin D. The results show that, as the concentration of pBS/CD was increased from 0, 10, 20, and 40 μg, there was a significant decrease in E2-induced CAT activity (4.31 ± 0.21, 3.33 ± 0.34, 2.60 ± 0.07, and 2.18 ± 0.08, respectively, compared to untreated cells; the last two values were significantly decreased), suggesting that induction was due to *trans*-acting factors binding to the CD region of the promoter (results are expressed as means ± SE for three separate determinations; the total amount of DNA was kept constant for each experiment). The results of this study were similar to those previously reported for competition for C/EBP binding sites in Hep G2 cells (Pimental et al., 1993).

Gel electrophoretic mobility shift assays were utilized to investigate nuclear protein interactions with the ^{32}P-labeled CD promoter. Incubation of nuclear extracts from MCF-7 cells with the [^{32}P]CD oligonucleotide gave a single major retarded band (Figure 1, lane 1); the intensity of the retarded complex was decreased after competition with a 50- or 200-fold excess of unlabeled ERE (lanes 2 and 3, respectively), whereas no significant decrease was observed after competition with a 50- and 200-fold excess of unlabeled Sp1 oligonucleotide (lanes 4 and 5, respectively) or mutant ERE (lanes 6 and 7, respectively). These data suggested that the retarded band was associated with an ER−CD complex, and this was further investigated using nuclear extracts from Hela cells (Figure 2). Incubation of nuclear extracts from Hela cells with [^{32}P]CD gave two relatively weak bands (lane 3) compared to the band using nuclear extracts from MCF-7 cells (lane 2). Incubation of nuclear extracts from Hela cells transiently transfected with hER resulted in formation of an ER−[^{32}P]CD complex (lane 4), and the intensity of the retarded band was decreased by competition with a 50-fold excess of unlabeled ERE (lane 5) or CD oligonucleotides

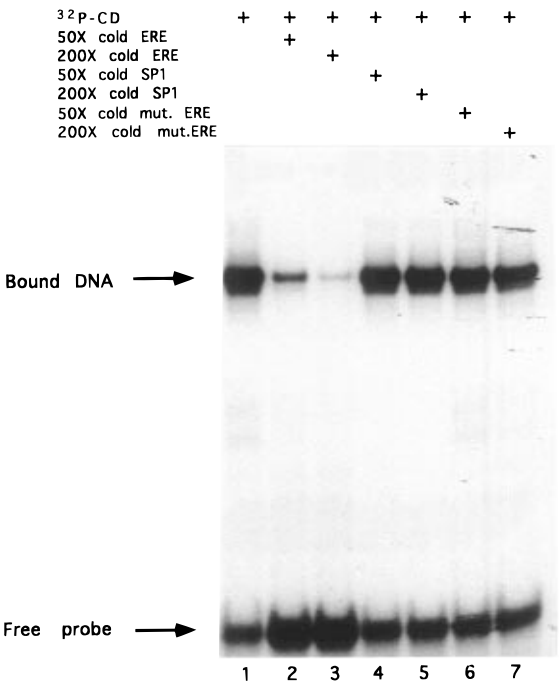

FIGURE 1: Binding of nuclear extracts from MCF-7 cells to the CD oligonucleotide. The ^{32}P-labeled CD oligonucleotide was incubated with nuclear extracts from cells treated with 10^{-9} M E2 and analyzed by gel electrophoretic mobility shift assay. The retarded band was visualized by autoradiography and quantitated using a Betagen 503 Betascope blot analyzer. The intensity values in lanes 2−7 relative to that of the control band (lane 1, 100 ± 20%) were as follows: lane 2, 18.8 ± 0.9; lane 3, 5.7 ± 0.06; lane 4, 99.0 ± 2.1; lane 5, 96.4 ± 0.7; lane 6, 95.8 ± 0.08; and lane 7, 90.0 ± 1.2% (means ± standard error from three determinations). Unlabeled ERE (lanes 2 and 3) significantly decreases the intensity of the bound DNA complex ($p < 0.05$), whereas competition with unlabeled Sp1 or ERE oligonucleotides did not decrease the intensity of the retarded band.

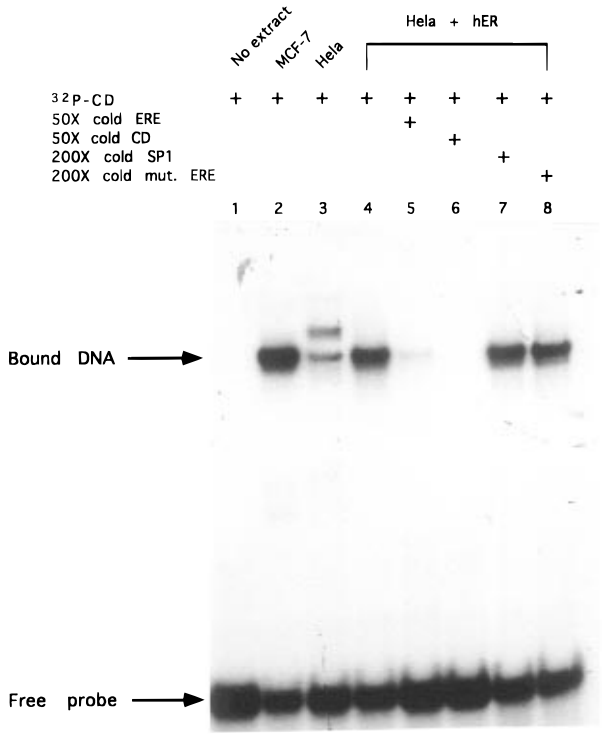

FIGURE 2: Binding of nuclear extracts from Hela cells cotransfected with hER to the CD oligonucleotide. The formation of the retarded band with [^{32}P]CD was determined using nuclear extracts from MCF-7 cells treated with E2, Hela cells and Hela cells, cotransfected with hER and treated with 10^{-9} M E2. The retarded ER band was visualized by autoradiography and quantitated using the Betagen 603 Betascope blot analyzer as described in Figure 1. The intensity values in lanes 3−8 relative to that of the band from MCF-7 cells (lane 2, 100 ± 4.5%) were: lane 3, 22.02 ± 1.52; lane 4, 65.47 ± 1.23; lane 5, 6.42 ± 0.79; lane 6, 0.14 ± 0.03; lane 7, 62.10 ± 2.88; and lane 8, 38.09 ± 0.81%. The intensity of the retarded ER band using Hela cells extracts was significantly increased after hER transfection, and E2 treatment (lane 4 versus lane 3, $p < 0.0001$) significantly decreased competition with 50× cold ERE ($p < 0.0001$) or CD ($p < 0.0001$) but was not affected by 200× cold Sp1 or mutated ERE oligonucleotides ($p > 0.05$). Lane 1 is a free probe in the absence of nuclear extract.

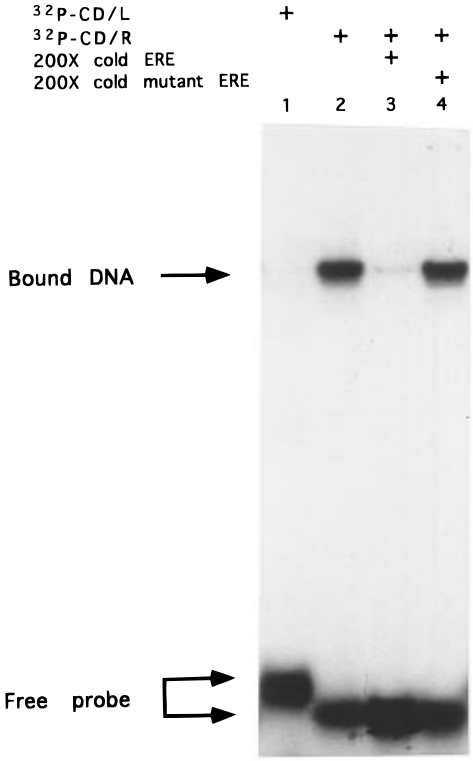

FIGURE 3: Binding of nuclear extracts from E2-treated MCF-7 cells to [^{32}P]CD/R and [^{32}P]CD/L. Binding of the ^{32}P-labeled CD/L and CD/R oligonucleotides was determined as described in Experimental Procedures. The relative intensity values (±) were as follows: lane 1, 50 ± 9; lane 2, 844 ± 48; lane 3, 60 ± 4; and lane 4, 738 ± 58% (means ± SE from three determinations). The nuclear extracts from E2-treated MCF-7 cells bound to [^{32}P]CD/R (lane 2), whereas minimal binding to [^{32}P]CD/L (lane 1) was observed. The intensity of the [^{32}P]CD/R−ER band was significantly decreased by competition with unlabeled ERE (lane 3) but not mutant ERE (lane 4). The retarded band intensity in lane 2 was significantly higher in lanes 1 and 3 ($p < 0.01$), but similar to that observed in lane 4.

(lane 6) but not by competition with a 200-fold excess of unlabeled Sp1 or mutant ERE oligonucleotides (lanes 7 and 8, respectively). These results also suggest that the retarded band was due to interactions between the ER and CD oligonucleotide.

The CD oligonucleotide contains a GC-rich region at the 5′-end and potential imperfect palindromic ERE sequence CGCCCACGTGACC at the 3′-end containing perfect (3′) and imperfect (5′) half-sites. The results in Figure 3 demonstrate that formation of the ER−[^{32}P]CD retarded band (Figures 1 and 2) was associated with the region of the oligonucleotide containing the imperfect palindromic ERE (CD/R) and not the Sp1 binding site (CD/L). Incubation of nuclear extracts from MCF-7 cells with [^{32}P]CD/L or [^{32}P]-CD/R (Figure 3) showed that a retarded band was formed primarily with the latter oligonucleotide (lane 2), and the intensity of the retarded band was decreased after competition with a 200-fold excess of ERE (lane 3) but not mutant ERE (lane 4); in contrast, [^{32}P]CD/L only formed weak retarded bands (lane 1). Incubation of [^{32}P]ERE, [^{32}P]CD/R, or [^{32}P]-CD/R_{M1} with nuclear extracts from E2-treated MCF-7 cells gave a single major retarded band (lanes 1, 4, and 7) which was supershifted with H222 ER antibodies (lanes 2, 5, and 8). The mobilities of the ER−DNA complexes and the supershifted bands were similar for the three oligonucleotides (Figure 4). Preincubation of nuclear extracts with preimmune serum had no effects on formation of the retarded bands (lanes 3, 6, and 9). Further confirmation that the CD/R oligonucleotide binds the ER was obtained in competitive binding studies with [^{32}P]ERE (data not shown). The results showed that a 10−200-fold excess of unlabeled ERE or CD/R oligonucleotides competitively decreased formation of the bound [^{32}P]ERE−ER band, whereas mutant ERE did not decrease formation of the retarded band.

The functional activity of CD/R was investigated using a pCD/R construct containing the −120 to −101 region of the cathepsin D promoter in a pBL/TATA/CAT reporter plasmid. The results shown in Figure 5 summarize the induction of CAT activity by E2 in MCF-7 cells transiently transfected with hER plus pCD/R or plasmids mutated in the imperfect and perfect ERE half-site and both half-sites (pCD/R_{M1}, pCD/R_{M2}, and pCD/R_{M3}, respectively). There was a 3.25-fold induction of CAT activity by E2 using the wild-type construct, whereas no significant induction was observed with the mutant plasmids. The role of the MLPE imperfect palindromic ERE (−119 to −107) and the Sp1−ERE half-site (−199 to −165) (Krishnan et al., 1994) as E2-responsive enhancer sequences was investigated by determining the induction of CAT activity by E2 in cells transiently transfected with pCD/2, pCD/2_{M4}, or pCD/2_{M2}. PCD/2_{M4} and pCD/2_{M2} were mutated in the Sp1 and perfect ERE half-

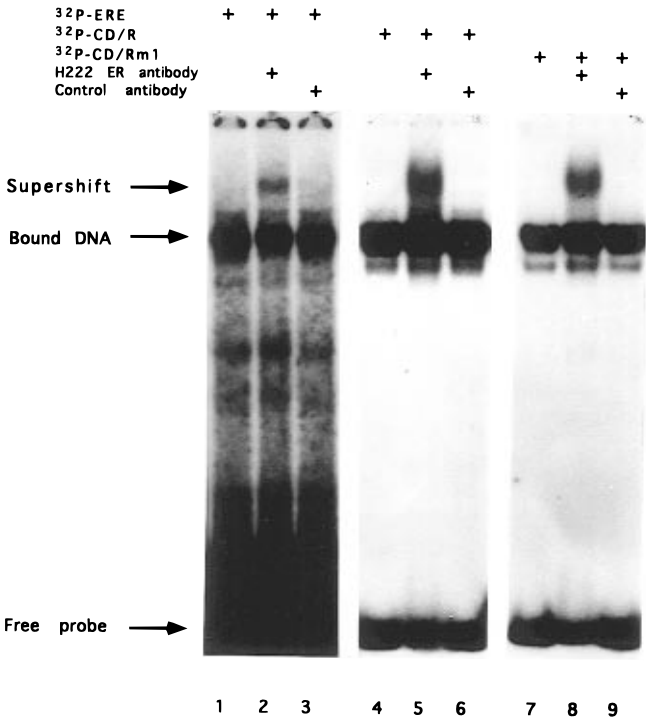

FIGURE 4: Supershift of [^{32}P]ERE, [^{32}P]CD/R, and [^{32}P]CD/R_{M1}−ER complexes with ER antibodies. Nuclear extracts from MCF-7 cells were treated with E2 and were incubated with the radiolabeled oligonucleotides alone (lanes 1, 4, and 7) and analyzed by gel electrophoretic mobility shift assay as described in Experimental Procedures. In parallel experiments, the specifically bound DNA−protein complexes were incubated with 2 μg of H222 ER antibody (lanes 2, 5, and 8) or a control antibody (lanes 3, 6, and 9) and analyzed by gel electrophoretic mobility shift assay as described in Experimental Procedures; the gels were visualized by autoradiography. The specifically bound and supershifted bands are indicated with arrows. ER blocking studies with [^{32}P]CD/R and ER antibodies decreased formation of the specifically bound retarded band (data not shown).

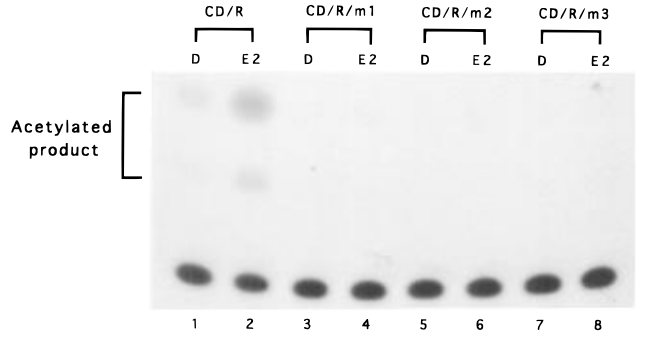

FIGURE 5: Effect of E2 on pCD/R and mutated analogs on CAT activities in MCF-7 cells with the requirement for the wild-type promoter sequence. MCF-7 cells were transiently cotransfected with hER and pCD/R, pCD/R_{M1}, pCD/R_{M2}, or pCD/R_{M3} and treated with 10^{-9} M E2 for 48 h before harvest. CAT activity in MCF-7 cells transfected with pCD/R plus hER plasmids and treated with DMSO was used as a control (lane 1, 100 ± 6.3%). The intensity values relative to that of lane 1 were as follows: lane 2, 325.1 ± 40.7; lane 3, 40.0 ± 4.3; lane 4, 50.7 ± 2.3; lane *5*, 30.4 ± 2.2; lane *6*, 30.9 ± 0.7; lane 7, 25.0 ± 2.2; and lane 8, 35.9 ± 2.1% (means ± SE from three determinations). E2 significantly increased CAT activity in MCF-7 cells cotransfected with hER and the wild-type pCD/R plasmids ($p < 0.0001$), whereas no induction was observed using the mutated plasmid constructs ($p < 0.05$).

site, respectively. Both mutants were E2-responsive (Table 4), suggesting that the imperfect ERE and Sp1−ERE half-site were active as enhancer sequences.

The direct and competitive binding of the wild-type and mutant CD/R oligonucleotides with nuclear extracts was also determined (Figure 6A,B). The results show that both wild-type [^{32}P]CD/R and [^{32}P]CD/R_{M1} form a complex with nuclear extracts (Figure 6A, lanes 1 and 2), whereas [^{32}P]-CD/R_{M2} and [^{32}P]CD/R_{M3} did not form retarded bands. Similar results were obtained in competitive binding studies using nuclear extracts from MCF-7 cells and wild-type [^{32}P]-CD/R (Figure 6B); a 10−200-fold excess of unlabeled CD/R (lanes 2 and 3) and CD/R_{M1} (lanes 4−6) competitively decreased formation of the ER−[^{32}P]CD/R complex whereas no competition was observed with a 10−200-fold excess of unlabeled pCD/R_{M2} (lanes 7−9) or pCD/R_{M3} (lanes 10−12). Thus, despite formation of a retarded ER−CD/R_{M1} complex, the corresponding plasmid (pCD/R_{M1}) was not E2-responsive.

In vivo footprinting was determined in MCF-7 cells transfected with pCD/355 and then subjected to an *in vivo* exonuclease III footprinting analysis. The results of this experiment are shown in Figure 7. Binding of proteins to the MPLE site is indicated by the exonuclease III-dependent stops seen (compare lanes 2 and 6). Consistent with the hormone-induced increase in activity observed in cells treated with E2, an increase in the binding at the MPLE and Sp1 sites is observed (compare lanes 6 and 7) in response to E2 addition. The other major enhanced band (located above the MLPE band) is associated with increased binding to the Sp1 site located within the CD/l sequence.

DISCUSSION

Steroid hormone receptors regulate diverse biological and physiological processes by modulating tissue-specific expression of target genes in response to specific hormones and their agonists (Evans, 1988; Beato, 1989; O'Malley, 1990; Mangelsdorf et al., 1995). The classical model for E2-induced responses involves interaction of the homodimeric ER with a palindromic ERE typified by the GGTCA(N)$_3$TGACC sequence identified in the 5′-promoter region of the chicken and *Xenopus* vitellogenin genes (Burch et al., 1988; Klein-Hitpass et al., 1988; Kumar & Chambon, 1988). Subsequent studies indicate that modulation of gene expression by the ER and other steroid hormone receptors is complex and involves different domains within the ER and interaction of the ER with multiple *cis* genomic responsive elements and other nuclear proteins (Kumar et al., 1987; Maurer & Notides, 1987; Tora et al., 1988; Berry et al., 1989, 1990; Richard & Zingg, 1990; Slater et al., 1990; Weisz & Rosales, 1990; Darwish et al., 1991; Doucas et al., 1991; Savouret et al., 1991; Shupnik & Rosenzweig, 1991; Hyder et al., 1992, 1995a,b; Pham et al., 1992; Wu-Peng et al., 1992; Cavailles et al., 1994; Dana et al., 1994; Halachmi et al., 1994; Hyder & Stancel, 1994; Landel et al., 1994; Tzukerman et al., 1994; Astruc et al., 1995; Montano et al., 1995; Webb et al., 1995; Blobel & Orkin, 1996). This complexity is responsible, in part, for the tissue/organ-specific expression of genes in the presence of hormones.

For many estrogen-responsive genes, functional *cis* genomic sequences required for estrogen responsiveness have been localized to one specific region within their respective 5′- or 3′-flanking regions. Augereau and co-workers (1994) have extensively investigated the proximal promoter region of the cathepsin D gene and have identified several transcription factor binding sites, including three nonconsensus ERE

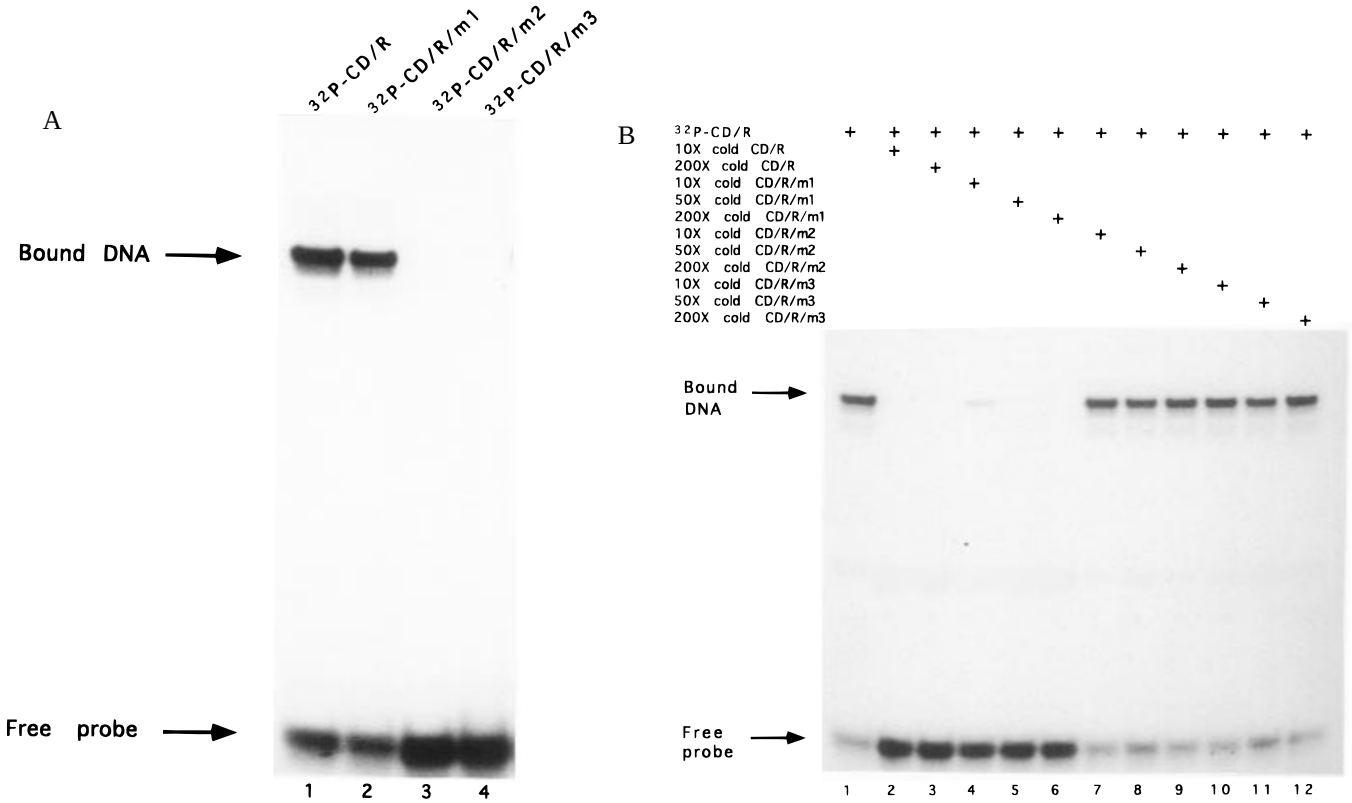

FIGURE 6: Binding of nuclear extracts from MCF-7 cells to wild-type and mutant [^{32}P]CD/R oligonucleotides: direct binding (A) and competition studies (B). (A) The ^{32}P-labeled CD/R, CD/R_{M1}, CD/R_{M2}, and CD/R_{M3} oligonucleotides were incubated with nuclear extracts from E2-treated MCF-7 cells and analyzed by gel electrophoretic mobility shift assay. Binding of nuclear extracts to wild-type [^{32}P]CD/R was used as a control (lane 1, 100.0 ± 2.8%). The intensity values relative to that of the control were as follows: lane 2, 65.8 ± 4.5; lane 3, 2.4 ± 0.3; and lane 4, 1.8 ± 0.3% (mean ± SE from three determinations). ER binding was significantly reduced when the less conserved side (underlined) of the imperfect palindromic ERE of CD/R, CGCCCACGTGACC, was mutated to AACACAGCTGACC (lane 2, [^{32}P]CD/R_{M1}) ($p < 0.0001$). Mutation of the perfect ERE half-site or both half-sites resulted in almost complete loss of ER binding ($p < 0.0001$) (lane 3 for [^{32}P]CD/R_{M2} and lane 4 for [^{32}P]CD/R_{M3}). (B) [^{32}P]CD/R was incubated with E2-treated MCF-7 nuclear extracts and analyzed as described above. The intensity of the [^{32}P]CD/R-ER retarded band (lane 1) was set at 100 ± 5% and relative intensities were: lane 2, 6.0 ± 0.8; lane 3, 1.2 ± 0.1; lane 4, 12.5 ± 1.9; lane 5, 4.7 ± 0.7; lane 6, 2.9 ± 0.3; lane 7, 95.5 ± 0.6; lane 8, 101.9 ± 8.3; lane 9, 108.2 ± 5.4; lane 10, 109.2 ± 8.9; lane 11, 105.5 ± 7.9; and lane 12, 103.3 ± 2.8%. ER binding to [^{32}P]CD/R was significantly decreased in the presence of a 10−200-fold excess of unlabeled CD/R or CD/R_{M1} (lanes 2−6, $p < 0.0001$). The competitive binding EC_{50} values for CD/R and CD/R_{M1} were determined, and the results indicate that the ER binding affinity of CD/R was 14% higher than observed for CD/R_{M1}. However, ER binding to [^{32}P]CD/R was not reduced in the presence of unlabeled CD/R_{M2} or CD/R_{M3} oligonucleotides ($p > 0.05$).

sequences (E1, E2, and E3). One of these sequences at −261 (E2) bound the ER in a gel mobility shift assay; however, in transient transfection assays, the E2 element alone did not confer estrogen responsiveness on a tk−CAT construct but interacted with other downstream or upstream elements. In contrast, studies in this laboratory initially identified a GGGCGG(N)$_{23}$ACGGG (−199 to −165) sequence in the noncoding strand of the cathepsin D gene, and results of transient transfection and gel mobility shift assays indicate that an Sp1−ER complex is directly involved in E2 responsiveness (Krishnan et al., 1994, 1995). Moreover, in transient transfection studies with constructs containing the upstream Sp1−ERE (half-site) sequence (pCD/355 and pCD2) and a downstream region (pCD) which did not contain this sequence, CAT activity was induced by E2 using all three constructs (Tables 2 and 3) and the AF-2 domain of the ER was required for the induction response observed for pCD. The functional activity of the CD oligonucleotide (−141 to −101) was further confirmed by competition assays which utilized a pBS/CD plasmid containing the CD insert ligated into pBluescript which is an expression vector only in prokaryotic cells. Increasing concentrations of pBS/CD decreased induction of CAT activity in cells transiently transfected with pCD, thus confirming that the CD oligonucleotide insert (in pBS/CD) competes for nuclear transcription factors required for E2 responsiveness of pCD. Results of previous studies showed that the −120 to −103 region of the cathepsin D promoter is protected in *in vitro* DNase I protection assays using nuclear extracts from estrogen-treated MCF-7 cells (Augereau et al., 1994). However, Augereau and co-workers (Augereau et al., 1994) reported that, in transient transfection studies, constructs containing this sequence (−124 to −13) were not estrogen-responsive. In contrast, results obtained with pCD in this study show that the −145 to −101 region of the cathepsin D promoter confers E2 responsiveness on the corresponding plasmid construct. The reason for the differences observed in this study and the research reported by Augereau and co-workers (1994) may be related to the thymidine kinase promoter used in their constructs which can give high background activity.

The CD oligonucleotide contains two overlapping Sp1 binding sites (−145 to −135) and at least one perfect ERE half-site, TGACC (−111 to −107), which potentially could form an Sp1−ER complex. However, results of gel electrophoretic mobility shift assays using [^{32}P]CD plus nuclear

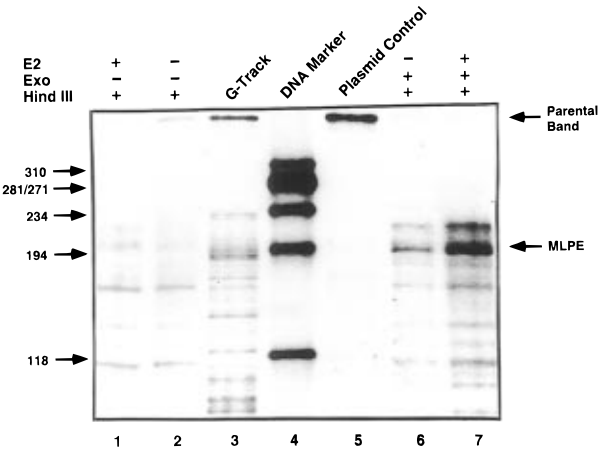

FIGURE 7: *In vivo* exonuclease III footprinting: hormone-dependent and -independent DNA−protein interactions on the human cathepsin D gene promoter. MCF-7 cells were transiently transfected with pCD/355 plasmid and treated with (lane 7) and without (lane 6) 10^{-7} M E2 for 12 h prior to harvesting. Isolated nuclei were digested with *Hin*dIII and exonuclease III, as previously described (Archer et al., 1992). Following linear *Taq* polymerase amplification with a ^{32}P-labeled oligonucleotide specific for the CAT reporter gene (Archer et al., 1992), purified extended products were analyzed on 5 or 7% polyacrylamide denaturing gels prior to autoradiography at −8 °C. The arrow indicates the exonuclease stop corresponding to the binding site for MLPE: lanes 1 and 2, sample controls without Exo III; lane 3, G sequencing track; lane 4, ϕX 174 size marker; and lane 5, pure pCD/355 cut with *Hin*dIII followed by linear amplification.

extracts from MCF-7 (Figure 1) or Hela cells (Figure 2) indicated that an intense specifically bound retarded band was formed, and the intensity of this band was decreased only by competition with excess wild-type ERE but not Sp1 or mutant ERE oligonucleotides. Formation of this retarded band was further localized to the −120 to −101 region of the promoter (CD/R, Figures 3 and 4), and retarded bands associated with the consensus [^{32}P]ERE and [^{32}P]CD/R oligonucleotides exhibited comparable mobilities and were supershifted by ER antibody H222 (Figure 4). The consensus palindromic [^{32}P]ERE forms a complex with the ER homodimer, and therefore, the results in Figure 4 suggest that [^{32}P]CD/R forms a similar complex with the ER homodimer.

The adenovirus MPLE was previously identified within the CD/R region (Augereau et al., 1994), and closer inspection indicates that the CGCCC(N)$_3$TGACC sequence is a nonconsensus ERE containing imperfect (5′) and perfect (3′) half-sites. The interaction of the ER with the MLPE was confirmed by *in vivo* exonuclease III footprinting analysis (Archer et al., 1992; Mymryk & Archer, 1995). Treatment of the cells with E2 resulted in increased binding at the MLPE site (Figure 7, lane 7), and this corresponded to hormone-induced transactivation using pCD/355 in transient transfection studies (Table 2). A second enhanced band (above the MLPE) corresponded to binding to the Sp1 motif within pCD/L, and the functional significance of this interaction is currently being investigated. The E2-dependent increase of a prebound transcription factor observed for the MLPE (and Sp1) sites has also previously been observed with the transcription factor NF1 on the MMTV promoter (Mymryk & Archer, 1995)

Since the [^{32}P]CD/R oligonucleotide forms a specifically bound complex with the ER, the relative importance of the two ERE half-sites in the formation of this complex was determined in both direct and competitive binding studies (Figure 6A,B). The data show that mutations in both ERE half-sites (CD/R$_{M3}$) and perfect half-site CD/R$_{M2}$ result in a loss of both direct and competitive binding activity. In contrast, [^{32}P]CD/R$_{M1}$ which is mutated only in the imperfect ERE half-site forms a complex with ER (Figure 6A, lane 2), and unlabeled CD/R$_{M1}$ competitively decreased formation of the retarded ER−[^{32}P]CD/R complex (Figure 6B, lanes 4−6). Probit analysis of the EC$_{50}$ values obtained in the competition studies (Figure 6B) indicated that the ER binding of wild-type CD/R was only 14% higher than that observed for CD/R$_{M1}$. In contrast, E2 did not induce CAT activity in MCF-7 cells transfected with promoter−reporter constructs containing the three mutant CD/R$_{M1}$, CD/R$_{M2}$, or CD/R$_{M3}$ constructs, whereas a 3.2-fold induction was observed with a plasmid containing the wild-type CD/R oligonucleotide (Figure 5). The lack of correspondence between ER binding to [^{32}P]CD/R$_{M1}$ and the estrogen insensitivity of the corresponding CD/R$_{M1}$ construct is consistent with results of other studies. For example, the nonconsensus E2 element previously identified in the cathepsin D gene promoter (−269 to −257) binds ER in a gel mobility shift assay, whereas the corresponding E2-tk−CAT construct alone was not estrogen-responsive (Augereau et al., 1994). Hyder and co-workers (1995a) previously identified an imperfect ERE [GCAGA-(N)$_3$TGACC] within the coding sequence of the protooncogene c-*jun*. The imperfect c-*jun* ERE binds ER in a gel mobility shift assay and confers E2 inducibility on reporter plasmids in mammalian cells which resembles the properties reported in this paper for the CGCCC(N)$_3$TGACC sequence in the cathepsin D gene promoter. Moreover, for E2-induced transcriptional activation, both the imperfect and perfect ERE half-sites are required using the ERE derived from the c-*jun* coding sequence (Hyder et al., 1995a) or the cathepsin D gene promoter (Figure 5). The formation of a retarded band using [^{32}P]CD/R$_{M1}$ which contained mutations only in the imperfect ERE half-site (Figure 6) paralled results reported for the ERE from c-*jun* in which mutations of the imperfect half-site also did not abolish formation of a retarded band (Hyder et al., 1995a).

Previous studies have also identified an Sp1−ERE half-site (−199 to −165) as a functional E2-responsive enhancer sequence (Krishnan et al., 1994). The results in Table 4 compare induction of CAT activity by E2 in MCF-7 cells transiently transfected with pCD/2, which contains the −208 to −101 region of the cathepsin D gene promoter, and constructs mutated in the Sp1 site within the Sp1−ERE motif (CD/2$_{M4}$) and in the perfect ERE half-site within the MLPE sequence (CD/2$_{M2}$). A 5-fold induction of CAT activity was observed using pCD/2 containing the wild-type −208 to −101 promoter sequence (Table 4). However, both mutant plasmid constructs were also E2-responsive (2.7−2.9-fold induction of CAT activity), suggesting that the Sp1−ERE half-site (−199 to −105) and the imperfect palindromic ERE (−119 to −107) are functional E2-responsive enhancer sequences in the cathepsin D gene promoter.

In summary, this study has identified a functional estrogen-dependent enhancer sequence [CGCCC(N)$_3$TGACC] in the 5′-promoter region of the cathepsin D gene which was previously identified as an adenovirus MLPE (Augereau et al., 1994). However, this site also resembles a nonconsensus ERE, forms a complex with the ER in a gel electrophoretic

mobility shift assay, and confers E2 inducibility on promoter−reporter constructs in MCF-7 cells. These results are consistent with the reported E2-induced *in vitro* protection of the MLPE against DNase I digestion (Augereau et al., 1994) and the *in vivo* exonuclease III footprinting analysis reported in this study (Figure 7). Identification of this second functional estrogen-responsive enhancer sequence in the 5′-promoter region of the cathepsin D gene is consistent with the increasingly complex regulation of hormone-dependent genes which are expressed and induced in a cell-specific manner (Katzenellenbogen et al., 1996). For example, estrogen induces cathepsin D gene expression in ER-positive MCF-7 cells but not in the Ishikawa endometrial cancer cell line (Miralles et al., 1994; Hua et al., 1995). The relative importance and function of the Sp1−ERE (Krishnan et al., 1994, 1995), MLPE, and other estrogen-responsive enhancer sequences (Augereau et al., 1994) in the cathepsin D gene promoter in various cell lines is currently being investigated as a model for understanding cell-specific gene expression.

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