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Transcriptional Activation of Rat Creatine Kinase B by 17β-Estradiol in MCF-7 Cells Involves an Estrogen Responsive Element and GC-Rich Sites

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Abstract The rat creatine kinase B (CKB) gene is induced by estrogen in the uterus, and constructs containing rat CKB gene promoter inserts are highly estrogen-responsive in cell culture. Analysis of the upstream −568 to −523 region of the promoter in HeLa cells has identified an imperfect palindromic estrogen response element (ERE) that is required for hormone inducibility. Analysis of the CKB gene promoter in MCF-7 breast cancer cells confirmed that pCKB7 (containing the −568 to −523 promoter insert) was estrogen-responsive in transient transfection studies. However, mutation and deletion analysis of this region of the promoter showed that two GC-rich sites and the concensus ERE were functional cis-elements that bound estrogen receptor α (ERα)/Sp1 and ERα proteins, respectively. The role of these elements was confirmed in gel mobility shift and chromatin immunoprecipitation assays and transfection studies in MDA-MB-231 and Schneider *Drosophila* SL-2 cells. These results show that transcriptional activation of CKB by estrogen is dependent, in part, on ERα/Sp1 action which is cell context-dependent. J. Cell. Biochem. 84: 156–172, 2002.

Key words: CKB; estrogen; ERα/Sp1; promoter analysis

Creatine kinase B (CKB) catalyzes phosphorylation of creatine, and this enzyme is highly inducible by estrogens in the female rat reproductive tract and mammary tumors, as well as in human breast tumors and tissue [Notides and Gorski, 1966; Reiss and Kaye, 1981; Walker and Kaye, 1981; Kaye et al., 1986; Sömjen et al., 1989, 1991; Pentecost et al., 1990; Crombie et al., 1994]. CKB is one of the most sensitive markers of estrogen exposure in the rat uterus, and constructs containing CKB gene promoter inserts are also estrogen-responsive in transient transfection experiments in multiple cell types [Spatz et al., 1992; Wu-Peng et al., 1992; Sukovich et al., 1994; Castro-Rivera et al., 1999]. Analysis of the CKB gene promoter has identified multiple binding sites for diverse nuclear transcription factors types [Benfield et al., 1988; Hobson et al., 1988, 1990; Horlick and Benfield, 1989; Horlick et al., 1990; Mitchell and Benfield, 1990; Spatz et al., 1992; Wu-Peng et al., 1992; Sukovich et al., 1994] and at least two regions of the promoter have been characterized as estrogen-responsive [Wu-Peng et al., 1992; Sukovich et al., 1994]. One study reported that constructs containing downstream sequences between −195 to −37 were induced by 17β-estradiol (E2) in HeLa cells. Subsequent linker-scanning mutations of this region of the promoter showed that the −75 to −45 was the minimal sequence required for hormone activation [Sukovich et al., 1994]. Binding sites for the estrogen receptor (ER) were not present in this region of the CKB gene promoter, and both CCAAT and TA rich motifs appear to be important for ER action.

Another study characterized an upstream −627 to −404 region of the CKB gene promoter

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as E2-responsive in HeLa cells and subsequent deletion analysis showed that the active sequence (−568 to −525) contained a nonconsensus estrogen response element (ERE) (GGTCA GAA CACCC) flanked by two GC-rich Sp1 binding sites [Wu-Peng et al., 1992]. Extracts containing ERα protein bound to the nonconsensus ERE and constructs containing mutations in the perfect (GGTCA) ERE half-site (ERE½) were inactive in transient transfection assays in HeLa cells. Previous studies in this laboratory have demonstrated that transcriptional activation of several genes by E2 was due to interactions of ERα/Sp1 with Sp1(GC-rich) $(N)_x$ERE½ motifs in which both ERα and Sp1 proteins bound promoter DNA [Krishnan et al., 1994, 1995; Porter et al., 1996; Vyhlidal et al., 2000]. ERα/Sp1 also activates GC-rich promoters that bind only Sp1 protein, and ligand activation does not require interactions of ERα with DNA [Porter et al., 1997; Duan et al., 1998; Sun et al., 1998; Wang et al., 1998, 1999; Xie et al., 1999, 2000; Qin et al., 1999; Dong et al., 1999; Samudio et al., 2001]. ERα/Sp1 action is highly cell context dependent and is observed in both ER-negative MDA-MB-231 and ER-positive MCF-7 breast cancer cell lines, whereas minimal E2-responsiveness was obtained in HeLa cells [Saville et al., 2000]. Studies in both breast and non-breast cancer cell lines show that constructs derived from the CKB gene promoter are highly inducible by E2 [Spatz et al., 1992; Wu-Peng et al., 1992; Sukovich et al., 1994; Castro-Rivera et al., 1999]. Previous studies on the c-*fos* gene identified the E2-responsive sequence in the proximal region of the promoter (−1300 to −1060); deletion analysis showed that an imperfect palindromic ERE at −1212 was required for hormone activation in transient transfection studies in HeLa cells [Weisz and Rosales, 1990]. In contrast, analysis of this region of the promoter in breast cancer cells showed that a downstream GC-rich motif at −1168 that bound ERα/Sp1 was required for hormone-induced transactivation of constructs (e.g., −1220 to −1155) containing both ERE and GC-rich sequences [Duan et al., 1998]. The ERE/GC-rich motifs in the c-*fos* gene promoter are similar to those described in the −568 to −525 region of the CKB gene promoter [Wu-Peng et al., 1992] and therefore, we have further investigated the mechanism of hormonal regulation of this region of the promoter in breast cancer cells. Our results confirm the high inducibility of constructs containing the upstream −568 to −523 region of the CKB gene promoter in breast cancer cells. In contrast to studies of this promoter in HeLa cells [Wu-Peng et al., 1992], our results show that both the nonconsensus ERE and GC-rich sites are independently inducible by E2 in transient transfection studies.

MATERIALS AND METHODS

Cell Culture Conditions

MCF-7, MDA-MB-231, and Schneider's *Drosophila* line 2 (SL2) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). MCF-7 cells were maintained in MEM with phenol red and supplemented with 10% fetal calf serum (FCS) plus 0.2 × antibiotic/antimycotic solution, 0.035% sodium bicarbonate, 0.011% sodium pyruvate, 0.1% glucose, 0.238% HEPES, and 6×10^{-7}% insulin. MDA-MB-231 cells were maintained in Dulbecco's modified eagle's medium nutrient mixture F-12 Ham (DME F-12) medium supplemented with 10% FCS (Intergen, Purchase, NY) plus 0.2 × antibiotic/antimycotic solution and 0.22% sodium bicarbonate. Cells were incubated in an air−carbondioxide (95:5) atmosphere at 37°C and passaged every 3–5 days without becoming confluent. Cells were grown in DME F-12 medium without phenol red and 2.5% charcoal-stripped FSC for 2–3 days before dosing. SL2 cells were grown at room temperature in T-150 flasks in Schneider's medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% FCS (heat inactivated at 56°C for 30 min) and 0.5 × antibiotic/antimycotic solution.

Chemicals, Biochemicals, and Oligonucleotides

DME F-12 without phenol red, phosphate buffered saline (PBS), acetyl CoA, E2, 100 × antibiotic/antimycotic solution were purchased from Sigma Chemical Company (St. Louis, MO). Minimum Essential Medium (MEM) was purchased from Life Technologies (Grand Island, NY). [γ-^{32}P]ATP (3,000 Ci/mmol) and [^{14}C]chloramphenicol (53 mCi/mmol) were purchased from NEN Research Products (Boston, MA). Poly d(I-C), restriction enzymes (Hind III and Bam HI), and T4-polynucleotide kinase were purchased from Boehinger Mannheim (Indianapolis, IN) or Promega (Madison, WI). Oligonucleotides were synthesized by the Gene Technologies Laboratory (Texas A&M

University), Genosys Biotechnologies, Inc. (Woodlands, TX), or Life Technologies (Grand Island, MD). Human ER monoclonal antibody H222 was purchased from Abbott Laboratories (Abbott Park, IL). Recombinant human ERα protein was purchased from PanVera (Madison, WI). The pCKB1 and pCKB6 constructs, which contain 2.9 and 0.2 kb CKB promoter sequences, respectively, were provided by Dr. Benfield (DuPont Merck Pharmaceutical Company, Wilmington, DE). The human estrogen receptor α (ERα ≡ HEG0) expression plasmid was provided by Dr. Mingjer Tsai (Baylor College of Medicine, Houston, TX). The ERα-DNA binding domain (DBD) deletion mutant HE11 and pPacSp1 expression plasmids were supplied by Dr. Pierre Chambon (Institut de Genetique et de Biologie Moleculaire et Cellulaire, Illkirch, France) and Dr. Robert Tjian (UC Berkeley, CA), respectively. Recombinant human Sp1 protein was purchased from Promega (Madison, WI). Plasmid preparation kit was purchased from Qiagen (Valencia, CA) or Bio-Rad Laboratories (Hercules, CA). Polyacrylamide (40%) was obtained from National Diagnostics (Atlanta, GA). All other chemicals and biochemicals were of the highest quality available from commercial sources.

The oligonucleotide structures (sense strand) and their descriptors are given below and used throughout the manuscript. The Sp1, consensus and nonconsensus ERE sequences are *underlined*, the mutated bases are indicated with an *asterisk*, and the Hind III and Bam HI linker sequences are *italicized*. The numbers in parenthesis indicate the positions within the 5′-promoter region of CKB gene.

Consensus ERE: 5′-GTCCAAAGTCAGGTCACAGTGACCTGATCAAAGTT-3′

Consensus Sp1 oligo: 5′-*AGCTT*ATTCGATCGGGGCGGGGCGAGC*G*-3′

CKB7 (−568 to −523) oligo: 5′-*AGCTT*GGGCCCGCCCAAGGTCAGAACACCCTGGGTGCTTCCGGGGCGGGACC*G*-3′

CKB7m1 (−568 to −523) oligo: 5′-*AGCTT*GGGCCCGCCCAAC*A*TCAGAACACCCTGGGTGCTTCCGGGGCGGGACC*G*-3′

CKB7m2 (−568 to −523) oligo: 5′-*AGCT*TGGGCA*A*T*A*A*A*AAGGTCAGAACACCCTGGGTGCTTCCGGGGCGGGACC*G*-3′

CKB7m3 (−568 to −523) oligo: 5′-*AGCTT*GGGCCCGCCCAAGGTCAGAACACCCTGGGTGCTTCCT*T*T*T*A*T*T*GACC*G*-3′

CKB7m4 (−568 to −523) oligo: 5′-*AGCTT*GGGCA*A*T*A*A*A*AAGGTCAGAACACCCTGGGTGCTTCCT*T*T*T* A*T*T*GACC*G*-3′

CKB7m5 (−568 to −523) oligo: 5′-*AGCTT*GGGCA*A*T*A*A*A*AAC*A*TCAGAACACCCTGGGTGCTTCCT*T*T*T*A*T*T*GACC*G*-3′

CKB8 (−568 to −523) oligo: 5′-*AGCTT*GGGCCCCGCCCAATGGGTGCTTCCGGGCGGGACC*G*-3′

CKB8m1 (−568 to −523) oligo: 5′-*AGCTT*GGGCA*A*T*A*A*A*AATGGGTGCTTCCT*T*T*A*T*T*GACC*G*-3′

CKB9 (−568 to −523) oligo: 5′-*AGCTT*GGGCAAGGTCAGAACACCCTGGGTGCTTCCGACC*G*-3′

CKB9m1 (−568 to −523) oligo: 5′-*AGCTT*GGGCAAC*A*TCAGAACACCCTGGGTGCTTCCGACC*G*-3′

Cloning

The pBLTATA-CAT plasmid was made by digesting the pBLCAT2 vector with *Bam HI* and *Xho I* to remove the thymidine kinase promoter, and the double-stranded E1B-TATA oligonucleotide containing complementary 5′-overhangs was then inserted into the corresponding sites [Porter et al., 1996]. The CKB7, CKB8, CKB9, and their mutants CKB7m1, CKB7m2, CKB7m3, CKB7m4, CKB7m5, CKB8m1, and CKB9m1 were cloned into the pBLTATA-CAT at the *Hind III* and *Bam HI* sites to give pCKB7, pCKB8, pCKB9, pCKB7m1, pCKB7m2, pCKB7m3, pCKB7m4, pCKB7m5, pCKB8m1, and pCKB9m1 plasmids, respectively. Construction of the pPac constructs for studies in SL2 cells have previously been described [Samudio et al., 2001]. All the ligation products were transformed into DH 5α competent *E. coli* cells, plasmids were isolated, and correct clonings were confirmed by restriction enzyme mapping and DNA sequencing using Sequitherm Cycle Sequencing Kit from Epicentre Technologies (Madison, WI). Plasmid preparation for transfection was carried out by alkaline lysis followed by two cesium chloride gradient centrifugations or by the Qiagen Plasmid Mega Kit or Bio-Rad Maxiprep Kit for plasmid preparation.

Construction of CKB Promoter Deletion Constructs

pCKB1 was digested with Bgl II to release a fragment containing CKB promoter sequence −1461 to +5. After gel electrophoresis and

purification (Qiagen, Valencia, CA), the Bgl II fragment was ligated to the promoterless vector, pBLCAT3 (ATCC, Rockville, MD), to give pCKB2. For construction of pCKB3, pCKB4, and pCKB5, unidirectional nested deletions into CKB promoter insert in pCKB2 were performed using Exo III/Mung Bean Nuclease Deletion Kit (Stratagene, La Jolla, CA). In brief, 30 μg of pCKB2 was digested with Pst I to create 3′-overhang at the multiple cloning sites (MCS) of pCKB2, which is resistant to exonuclease III digestion. After heat-inactivation (75°C for 15 min) and ethanol precipitation, the Pst I-digested pCKB2 was further digested with Bam HI whose cutting site is also located at MCS of pCKB2 but closer to 5′-end of CKB insert than the Pst I site described above. Twenty micrograms of the double-digested DNA was mixed with 50 μl of 2X Exonuclease III buffer, 10 μl of fresh 100 mM β-mercaptoethanol, and 400 U of exonuclease III in a total volume 100 μl. The reaction mixture was incubated at 34°C and, at each time point (1 min), 25 μl aliquots from the reaction mixture were removed and added to 175 μl aliquots of diluted mung bean nuclease buffer and placed on dry ice. After heat-inactivation at 68°C for 15 min, 15 U of mung bean nuclease was added to each time point tube and the reaction mixture was incubated for 30 min at 30°C. The resulting plasmids containing blunt-ended CKB promoter inserts were re-circularized by blunt-end ligation. Following DH5α cell transformation, bacterial colonies were isolated, and plasmid size was determined by Bgl II digestion and eleterophoresis. The CKB promoter deletions in pCKB3, pCKB4, and pCKB5 were further confirmed by DNA sequencing.

Transient Transfection and CAT Assays

Cultured MCF-7 and MDA-MB-231 cells were transiently transfected by the calcium phosphate method with 10 μg reporter plasmids and 5 μg of ERα, HE11 expression plasmid or empty construct (pcDNA3.1, Invitrogen, Inc., Carlsbad, CA) as a control. Three hours prior to transfection, the medium was replaced with 5 ml of the charcoal stripped DME F-12 medium; cells were transfected, and after 14 to 16 h, media was changed and cells were treated with 10 nM E2 in charcoal-stripped DME F-12 medium. After 48 h, cells were washed once with 5 ml of PBS, harvested by scraping, and CAT activity in cell lysates was determined as previously described [Wang et al., 1998, 1999]. For SL2 cell transfection, 2 ml of cells per well were pipetted into 6-well plates, and after incubation for 24 h at room temperature, cells in each well were transfected with 0.5 ml of transfection cocktail containing 1 μg of reporter plasmid, different amount of pPacERα, pPacSp1, or pPacDBD, 250 ml of 2X HBS, and 15 μl of 2.5 M $CaCl_2$. The empty vector, pPac, was used to maintain 3 μg of plasmid for each experiment. After incubation for 20 h at room temperature, cells were treated with 10 nM E2 or solvent carrier (ethanol) for about 48 h, harvested by scraping, and CAT activity determined as previously described [Wang et al., 1998, 1999]. Intensities of radiolabeled acetylated bands were quantitated using a Packard Instant Imager (Meriden, CT). CAT activity was calculated as the percentage of that observed in cells treated with DMSO (arbitrarily set at 100%).

Electrophoretic Mobility Shift Assays

Oligonucleotides were annealed and labeled at the 5′-end using T4-polynucleotide kinase and [γ-^{32}P]ATP. Gel mobility shift assays were performed by incubating different concentrations of recombinant Sp1, ERα or Sp1 plus ERα proteins with ^{32}P-labeled oligonucleotides (50,000 cpm) in the presence or absence of excess unlabeled oligonucleotides as previously described [Wang et al., 1998, 1999]. For ERα antibody supershift studies, specific and nonspecific antibodies were added for 20 min after initial incubation with radiolabeled oligonucleotides.

Chromatin Immunoprecipitation (ChIP) Assay

Breast cancer cells were grown in 60 mm tissue culture plates to 80–95% confluency, transfected with pCKB1 or $pSp1_3$ (5 μg) as described above, and treated with DMSO or 10 nM E2 for various times. Formaldehyde was added to the medium to give a 1% solution and incubated with shaking for 10 min at 20°C. Glycine was then added (0.125 M) and, after further incubation for 10 min, media was removed, cells were washed with PBS and 1 mM PMSF, scraped and collected by centrifugation. Cells were then resuspended in swell buffer (85 mM KCl, 0.5% NP-40, 1 mM PMSF, 5 μg/ml leupeptin and aprotinin at pH 8.0), homogenized, and nuclei were isolated by centrifugation at 1,500*g* for 30 s. Nuclei were then

resuspended in sonication buffer (1% SDS, 10 mM EDTA, 50 mM Tris at pH 8.1), and sonicated for 45–60 s to obtain chromatin with appropriate fragment lengths (500–1,000 bp). The sonicated extract was then centrifuged at 15,000*g* for 10 min at 0°C, aliquoted and stored at −70°C until used. The crosslinked chromatin preparations were diluted in buffer (1% Triton X, 100 mM NaCl, 0.5% SDS, 5 mM EDTA and Tris at pH 8.1), and 20 μl of Ultralink protein A or G or A/G beads (Pierce) was added per 100 μl chromatin and incubated for 4 h at 4°C. A 100 μl aliquot was saved and used as the 100% input control. This aliquot was treated at 65°C to reverse crosslinks and DNA was purified as described below. The crosslinked mixture was then centrifuged; salmon sperm DNA, specific antibodies, and 20 μl Ultralink beads were added and the mixture incubated for 6 h at 4°C. Samples were then centrifuged; beads were resuspended in dialysis buffer, vortexed for 5 min at 20°C, and centrifuged at 15,000*g* for 10 s. Beads were then resuspended in immunoprecipitation buffer (11 mM Tris, 500 mM LiCl, 1% NP-40, 1% deoxycholic acid at pH 8.0) and vortexed for 5 min at 20°C. The procedures with the dialysis and immunoprecipitation buffers were repeated (3–4 times), and beads were then resuspended in elution buffer (50 nM $NaHCO_3$, 1% SDS, 1.5 μg/m sonicated salmon sperm DNA), vortexed, incubated at 65°C for 15 min. Supernatants were then isolated by centrifugation and incubated at 65°C for 6 h to reverse protein–DNA crosslinks. Wizard PCR kits (Promega) were used for additional DNA cleanup and PCR was used to detect the presence of promoter regions immunoprecipitated commercially available ERα or Sp1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) with selected antibodies. The following primers were used for PCR analysis of immunoprecipitated promoter regions.

Cathepsin D	Fw (−294)	5′-TCCAGACATCCTCTCTGGAA-3′
	Rv (−54)	5′-GGAGCGGAGGGTCCATTC-3′
Cathepsin D (exon 2)	Fw (+2469)	5′-TGCACAAGTTCACGTCCATC-3′
	Rv (+2615)	5′-TGTAGTTCTTGAGCACCTCG-3′
$pSp1_3$	Fw (6128)	5′-GTTTGTCCAAACTCATCAATG-3′
	Rv (105)	5′-CTTTATGTTTTTGGCGTCTTC-3′
pCKB1	Fw (−678)	5′-GGAAAGAACCTGGGGATTTG-3′
	Rv (−319)	5′-GCCTTCCCTGCTTCCTG-3′

Statistical Analysis

Statistical significance was determined by ANOVA and Scheffe's test, and the levels of probability are noted. Results are expressed as means ± standard error (SE) for at least three separate experiments.

RESULTS

Deletion Analysis of the CKB Gene Promoter (−2900 to +5)

Results in Figure 1A illustrate the high inducibility (22 to 37-fold) of pCKB1 (−2900 to +5), pCKB2 (−1461 to +5), and pCKB3 (−1206 to +5) constructs in MCF-7 cells; pCKB4 (−533 to +5), which does not contain the nonconsensus ERE at −550, was significantly less hormone-inducible. Subsequent deletion analysis showed that constructs pCKB5 and pCKB6 containing downstream −233 to +5 and −195 to +5 CKB gene promoter inserts were also E2-responsive as previously reported in HeLa cells [Sukovich et al., 1994]. E2-responsiveness of these constructs in ER-positive MCF-7 cells was only observed after cotransfection with ERα expression plasmid. Previous studies on constructs containing inserts from the E2-responsive proximal promoter region of the cathepsin D gene show that induction of reporter gene activity by E2 in MCF-7 cells also required cotransfection with ERα expression plasmid [Cavailles et al., 1989, 1993; Savouret et al., 1991; Krishnan et al., 1994, 1995]. This has been attributed to expression of high copy numbers of the plasmid and limiting levels of endogenous ERα in the transfected cells [Savouret et al., 1991]. Moreover, transient transfection studies in MCF-7 or T47D cells using constructs containing E2-responsive regions from the pS2, c-*myc*, c-*fos*, and progesterone receptor gene promoters also required cotransfection with ERα to observe hormone inducibility [Dubik and Shiu, 1988; Berry et al., 1989; Weisz and Rosales, 1990; Zacharewski et al., 1994]. Cotransfection with ERα expression plasmid in ER-positive breast cancer cells is also required to observe hormone-induced transactivation of GC-rich and AP1 constructs regulated by ERα/Sp1 and ERα/AP1, respectively [Webb et al., 1995; Paech et al.,

1997; Porter et al., 1997; Duan et al., 1998; Sun et al., 1998; Wang et al., 1998, 1999; Dong et al., 1999; Qin et al., 1999; Xie et al., 1999, 2000; Saville et al., 2000; Samudio et al., 2001]. In MCF-7 cells transfected with plasmids containing 1, 2, 3, or 4 tandem consensus EREs, induction (> 1.5-fold) by E2 in the absence of cotransfected ERα was observed only with the latter two constructs [Sathya et al., 1997], and similar results were observed for MCF-7 cells in this laboratory (data not shown). Not surprisingly, ERα cotransfection is required for hormone-induced transactivation of most E2-responsive gene promoters in ER-positive and ER-negative cells, and thus, important cell context-dependent effects in these cell lines can be determined in transient transfection studies.

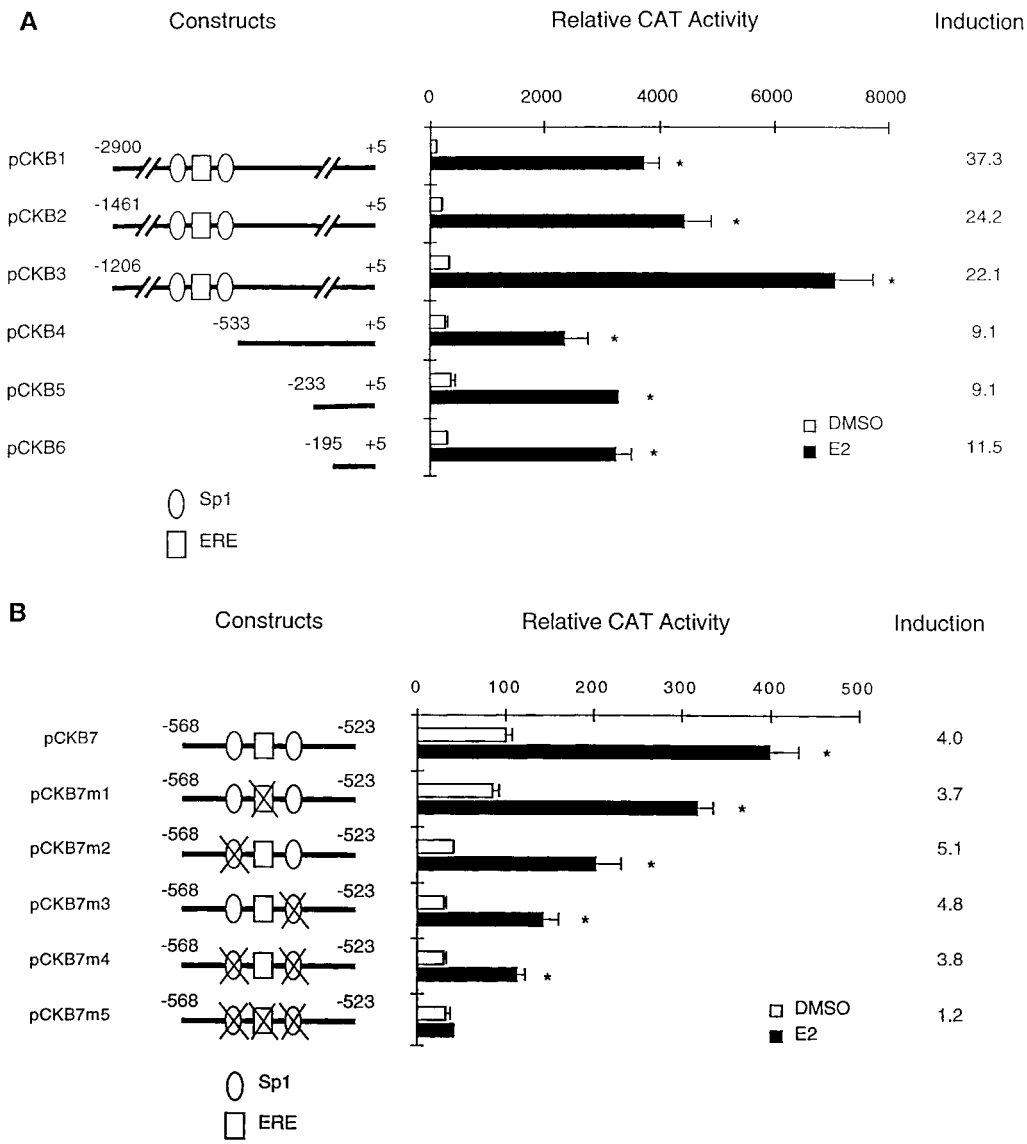

Fig. 1. Transcriptional activation of pCKB constructs in MCF-7 cells. **A**: 5′-Deletion analysis. Cells were transiently transfected with ERα expression plasmid and pCKB construct, treated with 10 nM E2, and CAT activities in the different treatment groups were determined as described in the Materials and Methods. Significant ($P < 0.05$) induction by E2 is indicated by an asterisk. **B**: Mutation and (**C**) deletion variants of pCKB7. The wild-type and mutant pCKB7 constructs, and plasmids containing ERE or Sp1 deletions of CKB7 were transfected and treated as described in (A) and reporter gene activity was determined as described in the Materials and Methods. Significant ($P < 0.05$) induction by E2 is indicated with an asterisk. Results are expressed as means ± SE for at least three replicate experiments for each treatment group.

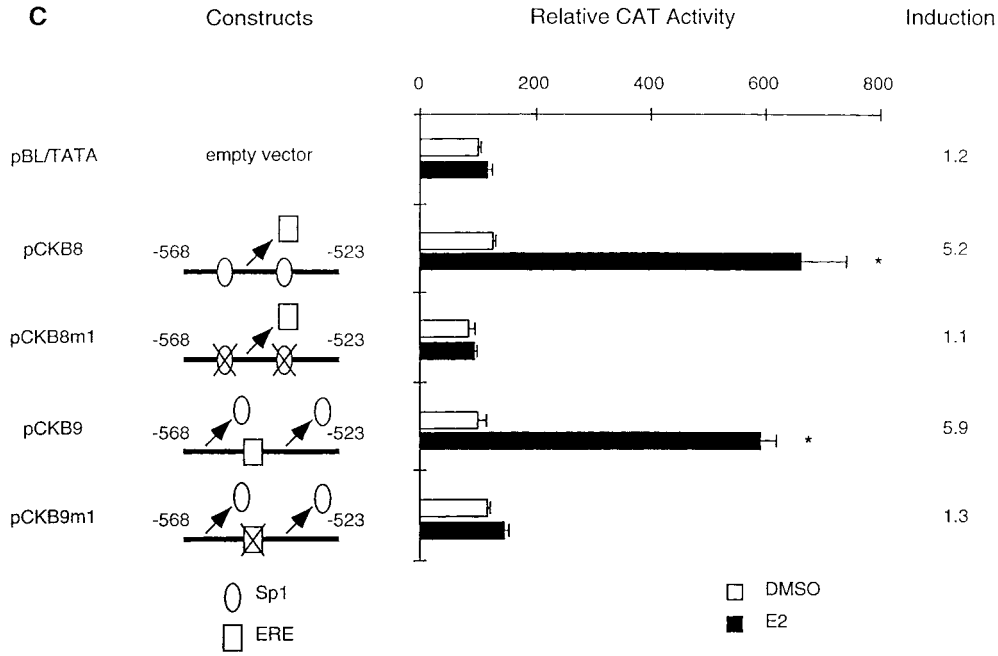

Fig. 1. *(Continued)*

Mutation and Deletion Analysis of CKB Gene Promoter (−568 to −523)

Previous studies have demonstrated that the upstream region (−568 to −523) of the CKB gene promoter was E2-responsive in HeLa cells [Sukovich et al., 1994], and similar results were obtained in MCF-7 cells transiently transfected with pCKB7 (Fig. 1B). In HeLa cells, it was suggested that the GC-rich sites may play a role in enhancing activity of the imperfect palindromic ERE at −550 [Sukovich et al., 1994] and therefore we analyzed this region of the promoter by mutation and deletion analysis. The role of the ERE and two GC-rich sites were investigated by successive mutations of the ERE (pCKB7m1), the upstream and downstream GC-rich sites (pCKB7m2 and pCKB7m3, respectively), both GC-rich sites alone (pCKB7m4). pCKB7m5 is mutated in both GC-rich sites and the ERE. The results show that the GC-rich sites alone (pCKB7m1) and the ERE alone (pCKB7m4) are sufficient for transactivation by E2 and mutation of all three sites resulted in loss of hormone-responsiveness. These results suggest that both ERα/Sp1 and ERα interactions with GC-rich sites and the nonconsensus ERE are required for activation of pCKB7 by E2. Mutation of the consensus ERE half-site (GGTCA → CATCA) to give pCKB7m1 leaves a nonconsensus CACCC sequence that may weakly interact with ERα; moreover, previous studies in the uteroglobin gene promoter show interactions of Sp proteins with CACCC motifs [Scholz et al., 1998]. Therefore, hormone-responsiveness of both GC-rich Sp1 binding sites was further confirmed in transfection studies with pCKB8 in which the ERE was deleted (Fig. 1C). E2-induced a 5.2-fold increase in CAT activity in cells transfected with pCKB8, whereas mutation of both GC-rich sites (pCKB8m1) abrogated the induction response. Similarly, E2 induced a 5.9-fold increase in CAT activity in MCF-7 cells transfected with a construct (pCKB9) in which the two GC-rich sites were deleted and subsequent mutation of the ERE (pCKB8m1) resulted in loss of hormone inducibility.

Activation of pCKB7 was also determined in ER-negative MDA-MB-231 cells and E2 induced reporter gene activity in these cells after transfection with wild-type ERα and HE11 (DBD-deletion mutant) (Fig. 2). These results suggest that the GC-rich sites alone were sufficient for ERα/Sp1 activation. Both ERα (HEG0 or HE0) and HE11 mediated induction by E2 in cells transfected with pCKB8 (containing the GC-rich sites), confirming that the GC-rich sites were functional cis-elements for ERα/Sp1 action. In contrast, only ERα (but not HE11)

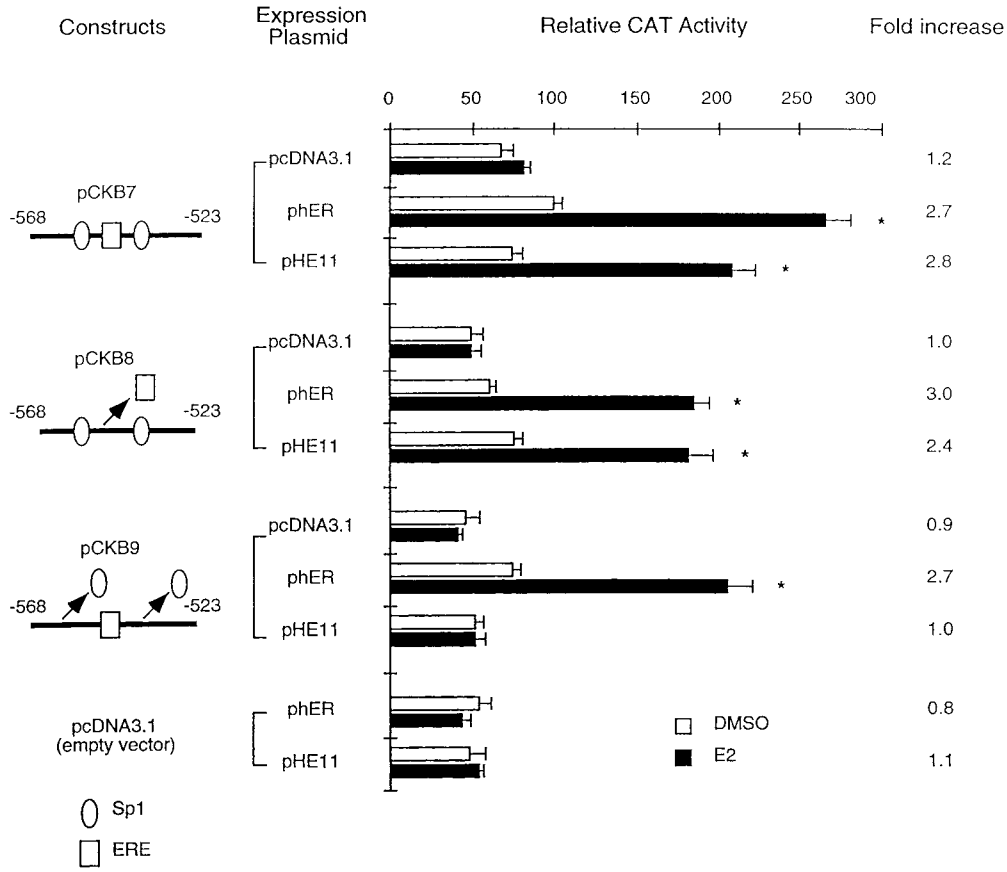

Fig. 2. Comparative activation of pCKB constructs by ERα and HE11 in MDA-MB-231 cells. Cells were transiently transfected with ERα or HE11 expression plasmids and pCKB7, pCKB8, or pCKB9, treated with 10 nM E2, and CAT activity was determined as described in the Materials and Methods. Significant ($P < 0.05$) induction by E2 is indicated with an asterisk. Results are expressed as means ± SE for three replicate determinations for each treatment group.

mediated induction of pCKB9 which contained the nonconcensus ERE. Similar results were observed using the DBD deletion mutant of HEG0 (data not shown).

Activation of Wild-Type and Mutant pCBK7 in SL-2 Cells

Drosophila SL-2 cells do not express endogenous ERα or Sp1 and were used as a model for studying the roles of both transcription factors in activation of pCKB7. The results in Figure 3A show that in cells transfected with pCKB7, cotransfection with ERα expression plasmid enhanced CAT activity by up to 2.7-fold, whereas no effect was observed with pCKBm1 containing a mutated ERE. Activation of the GC-rich sites was also observed in Schneider cells transfected with pCKBm1 and a > 40-fold enhancement of CAT activity was observed using 200 or 2,000 ng of Sp1 expression plasmid (Fig. 3A). In contrast, cotransfection with a plasmid expressing an Sp1 variant that does not contain a DBD did not increase reporter gene activity, and similar results were obtained after cotransfection of Sp1 expression plasmid with the triple mutant pCKB7m5 construct. Interactions of ERα and Sp1 were investigated in SL-2 cells cotransfected with pCKB7m1, Sp1 expression plasmid (50 ng) plus different concentrations (2–200 ng) of ERα expression plasmid (Fig. 3C). There was a maximal 2.8-fold activation of pCKB7m1 in SL-2 cells cotransfected with Sp1 (50 ng) plus ERα (20 ng) expression plasmids compared to activity observed after transfection with Sp1 alone (1.0-fold activity). In contrast, ERα did not enhance or affect activity in SL-2 cells transfected with pCKB7m1 and 50 ng of the DBD-variant form of Sp1.

Protein Interactions With the −568 to −523 Region of the CKB Gene Promoter

Previous studies showed that both ER and Sp1 proteins bind to the −568 to −523 region of the CKB gene promoter [Wu-Peng et al., 1992; Sukovich et al., 1994], and nuclear extracts from MCF-7 cells bound [^{32}P]CKB7 to give a complex pattern of bands (data not shown); however, there was some concern regarding Sp1 protein interactions with this region of the CKB gene promoter [Wu-Peng et al., 1992]. The results in

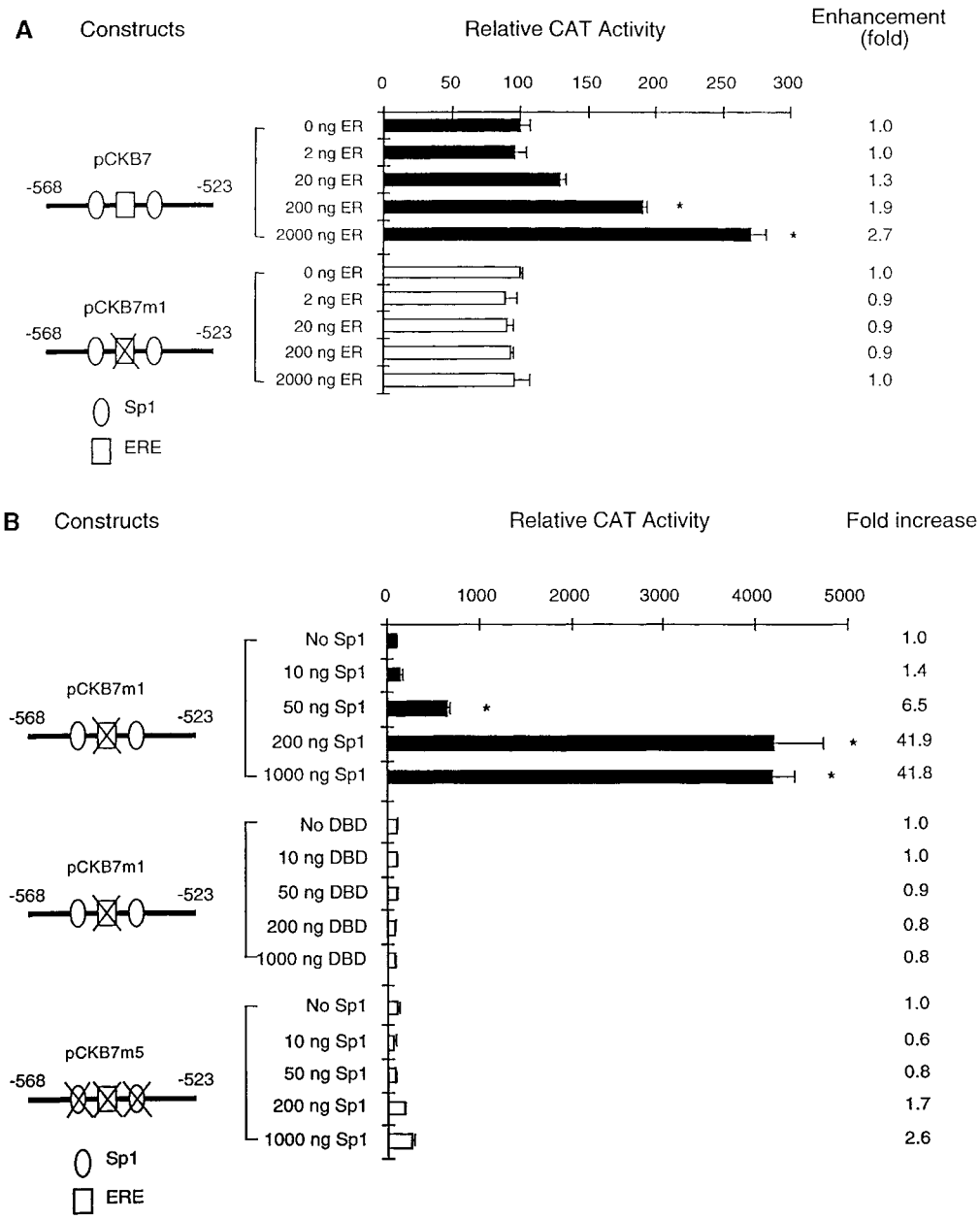

Fig. 3. Estrogen inducibility of pCKB constructs in Schneider SL-2 cells. **A**: Induction by ERα. SL-2 cells were cotransfected with ERα expression plasmid and pCKB7 or pCBK7m1, treated with 10 nM E2, and CAT activity was determined as described in the Materials and Methods. Significant ($P < 0.05$) induction by E2 is indicated with an asterisk. **B**: Induction by Sp1. Transfection experiments were carried out as described above in (A) using pCKB7m1 or pCKB7m5 and different amounts of wild-type or DBD-mutant Sp1 expression plasmid (50, 200, and 1,000 ng). Significant ($P < 0.05$) induction is indicated with an asterisk. **C**: Sp1 and ERα interactions in SL-2 cells. Cells were transfected with ERα, wild-type or DBD-mutant Sp1 expression plasmids and pCKB7m1 as described in (A). Significantly ($P < 0.05$) enhanced activity resulting from cotransfection with Sp1 (50 ng) and different amounts of ERα are indicated with an asterisk. Results are expressed as means ± SE for three separate determinations for each treatment group.

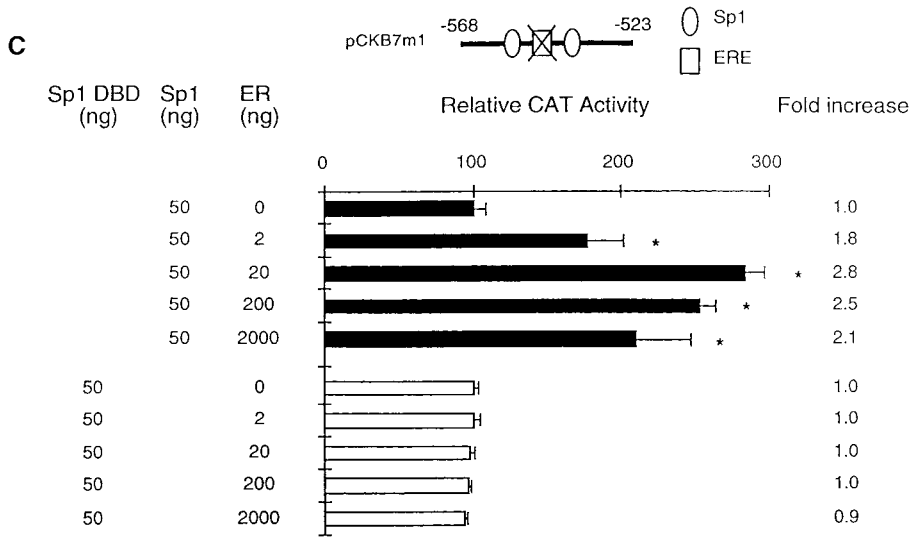

Fig. 3. *(Continued)*

Figure 4A show that [^{32}P]CKB7 bound Sp1 protein (lanes 2–4), and intensity of the retarded band was decreased after competition with unlabeled concensus Sp1 and CKB7 oligonucleotides (lanes 5 and 7), but not by mutant Sp1 and CKB7m4 oligonucleotides (lanes 6 and 8). It was suggested that interactions of ERα and Sp1 in this region of the CKB gene promoter may be dependent, in part, on cooperative ERα and Sp1 binding and therefore, the effects of Sp1 interactions with CKB7 were also investigated using [^{32}P]CKB7m1 (ERE mutation). The results (Fig. 4B) show that both [^{32}P]CKB7m1 (lane 3) and [^{32}P]Sp1 (lane 2) oligonucleotides bind Sp1 protein, and intensity of the Sp1(protein)-[^{32}P]CBK7m1 complex is decreased after competition with concensus Sp1 (lane 4) and CKB7 (lane 6) oligonucleotides (lanes 5 and 7). Previous studies showed that Sp1 protein bound GC-rich sequences, and coincubation with ERα enhanced retarded band intensity but did not form a ternary (ERα/Sp1-DNA) supershifted complex [Porter et al., 1997; Duan et al., 1998; Sun et al., 1998; Wang et al., 1998, 1999; Qin et al., 1999; Xie et al., 1999, 2000; Dong et al., 1999; Saville et al., 2000; Samudio et al., 2001]. Kinetic studies showed that ERα enhanced the "on-rate" of the Sp1-DNA complex [Porter et al., 1997]. Incubation of [^{32}P]CKBm1 (ERE mutant) with Sp1 protein gave a retarded band (lane 2) (Fig. 5A) and intensity of this band significantly increased (2 to 3-fold) after coincubation with recombinant human ERα (lanes 3–5). ERα alone did not bind [^{32}P]CKBm1 (lane 6) but formed a retarded band with [^{32}P]ERE (lane 7) that was supershifted with ERα antibodies (lane 8) but not by non-specific IgG (lane 9). Similar results were obtained using [^{32}P]CKB8 (ERE deleted) (Fig. 5B) which bound Sp1 protein to form a retarded band (lane 2) that was enhanced after coincubation with ERα (lanes 3–5). These results show that Sp1 protein binds the −568 to −523 region of the CKB gene promoter in the absence of the functional ERE motif and intensity of the Sp1-DNA complex is enhanced by coincubation with ERα.

ChIP Assay

The ChIP assay was also used to investigate protein interactions with promoter regions of an endogenous gene (cathepsin D) and transfected pCKB1 or pSp1$_3$ (Fig. 6). Preliminary studies showed that for several genes, maximal immunoprecipitation of hormone-responsive promoter regions by ERα antibodies was observed 15–45 min after treatment with hormone (data not shown). The results illustrated in Figure 6 show that in MCF-7 cells transfected with pSp1$_3$ (panel B) or pCKB1 (panel A), antibodies to ERα and Sp1 proteins immunoprecipitated the GC-rich promoter regions. Interestingly, both ERα and Sp1 antibodies also immunoprecipitated the GC-rich region of pSp1$_3$ in untreated MCF-7 cells, whereas this was not observed for pCKB1, and this may be due, in part, to the higher GC-content of pSp1$_3$ and the known ligand-independent physical interactions of ERα with Sp1 protein [Saville et al., 2000]. Previous studies reported that ERα antibodies immunoprecipitated the E2-responsive −54 to

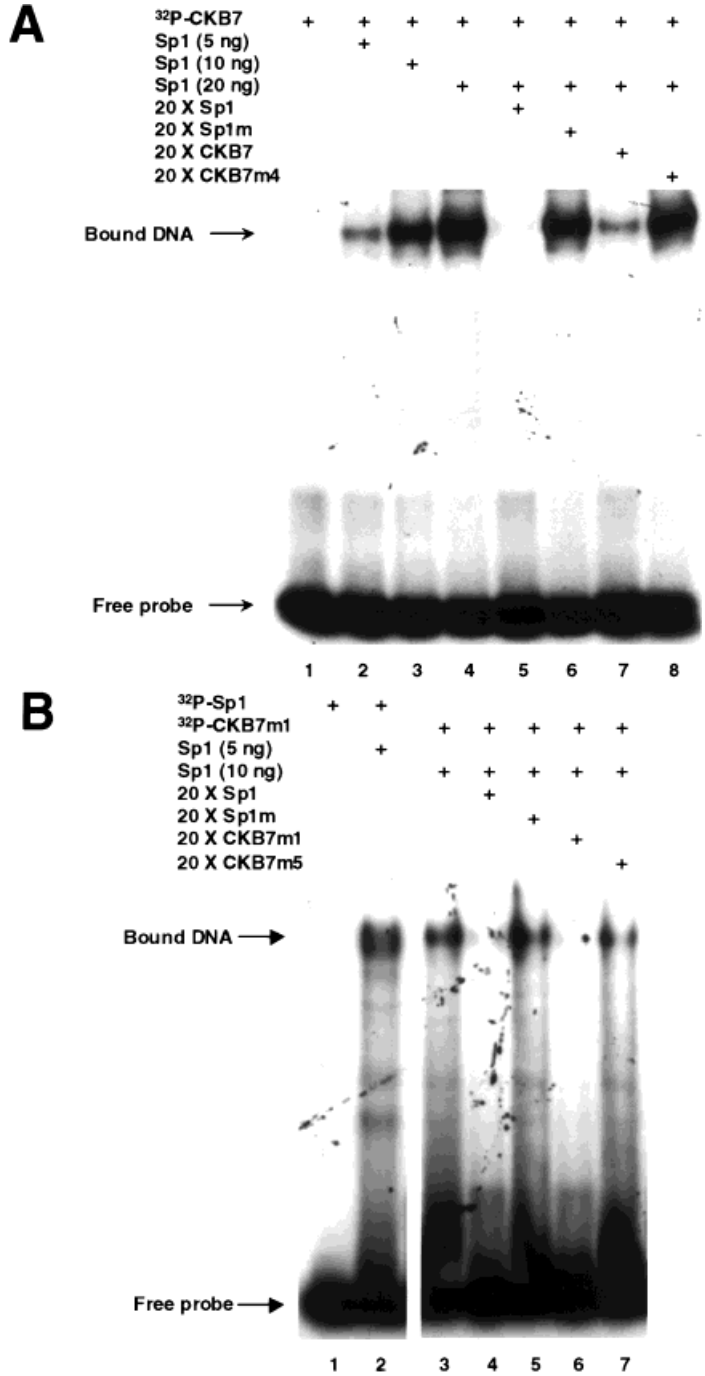

Fig. 4. Binding of Sp1 protein to the CKB gene promoter. **A**: Sp1 binding to [^{32}P]CKB7. Sp1 protein (5–20 ng) was incubated with [^{32}P]CKB7 in the presence or absence of 20-fold excess of wild-type (Sp1 or CKB7) or mutant (Sp1m or CKB7m4) oligonucleotides and analyzed in gel mobility shift assays as described in the Materials and Methods. **B**: Sp1 binding to [^{32}P]CKB7m1. [^{32}P]CKB7m1 or [^{32}P] Sp1 were incubated with Sp1 protein (5 or 10 ng) in the presence or absence of 20-fold excess of different unlabeled oligonucleotides (Sp1, Sp1m, CKB7m1, or CKB7m5) and analyzed in gel mobility shift assays as described in the Materials and Methods. Results illustrated in (A) and (B) were typical of those observed in duplicate experiments. Specifically-bound retarded bands are indicated (Bound DNA →) and this band is supershifted by Sp1 antibodies (data not shown).

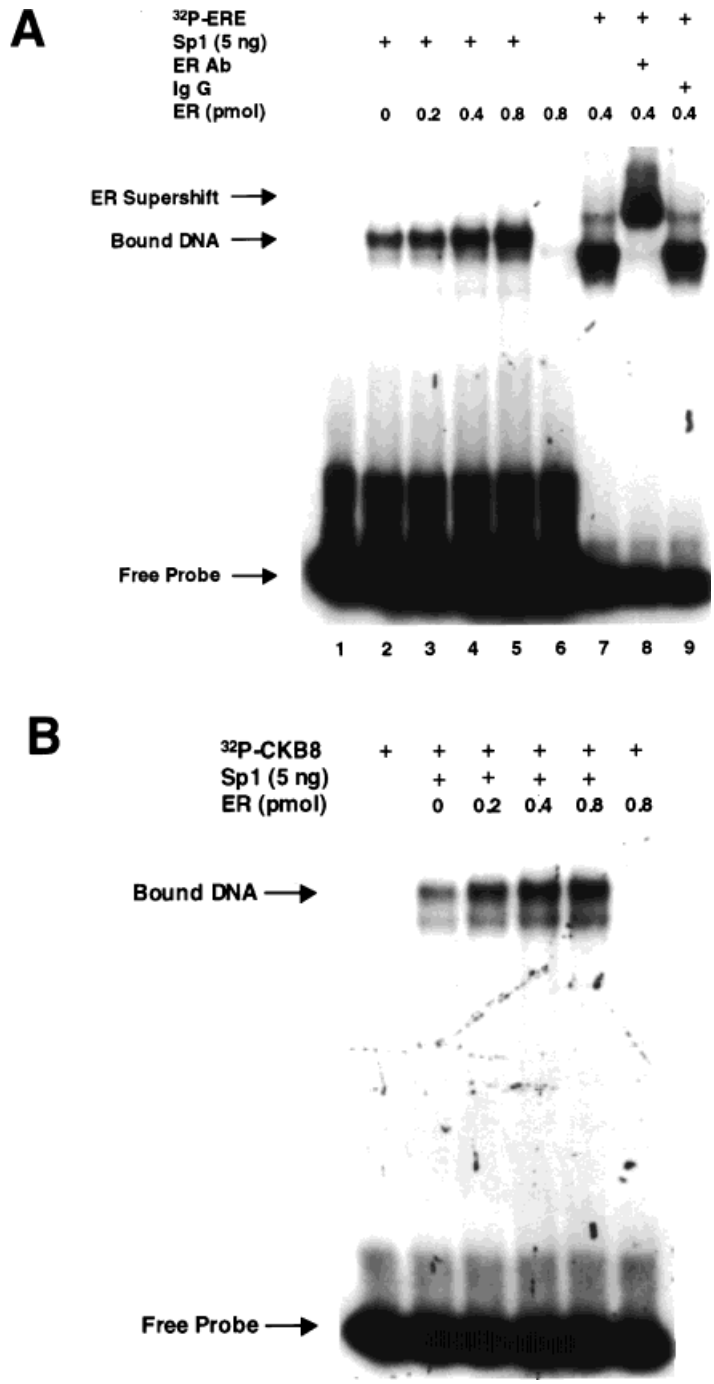

Fig. 5. ERα protein enhances Sp1-DNA binding to the CKB gene promoter. **A**: ERα/Sp1 binding to [^{32}P]CKB7m1 and ERα binding to [^{32}P]ERE. [^{32}P]Oligonucleotides (CKB7m1 or ERE) were incubated with Sp1, ERα, or Sp1 plus ERα (combined) proteins and analyzed in gel mobility shift assays as described in the Materials and Methods. Bound DNA (→) represents the Sp1-DNA complex and a band supershifted with ERα antibodies is also indicated. ERα antibodies did not affect the Sp1-DNA retarded band. **B**: ERα/Sp1 binding to [^{32}P]CKB8. Recombinant Sp1 (5 ng) and ERα (0–0.8 pmol) protein were incubated with [^{32}P]CKB8 and analyzed in a gel mobility shift assay as described in the Materials and Methods. ERα enhanced Sp1-DNA complex formation by 2 to 3-fold using [^{32}P]CKB7m1 or [^{32}P]CKB8, and similar results were observed in duplicate experiments. E2 (10 nM) was used in experiments with ERα, however, ERα/Sp1 interactions in this assay are observed in the presence or absence of E2 [Porter et al., 1997].

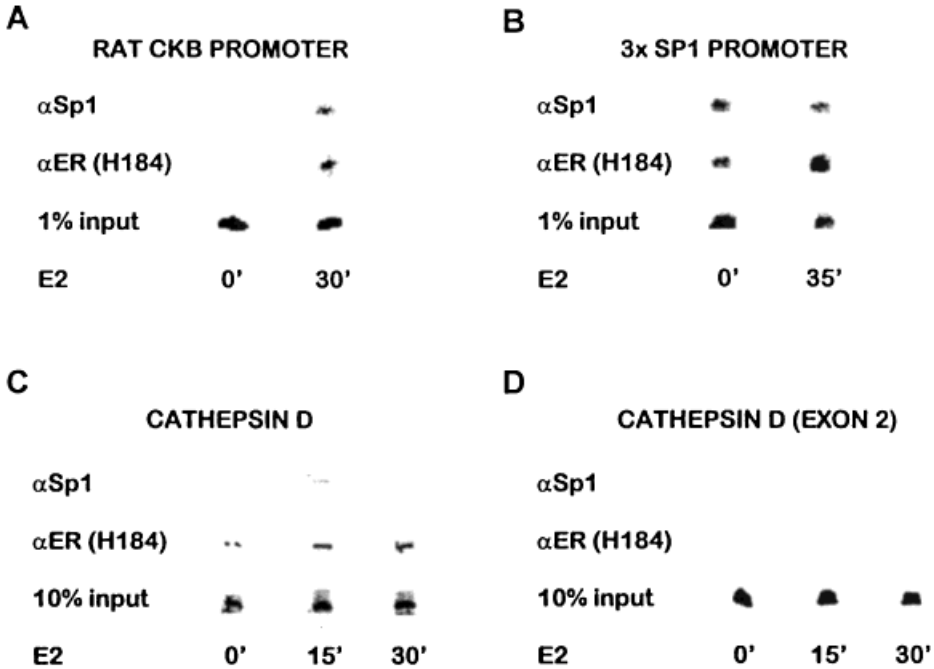

Fig. 6. ChIP assay of ERα/Sp1 interactions with CKB and other GC-rich promoters. Immunoprecipitation of GC-rich regions of the CKB and consensus GC-rich promoter were determined in transient transfection assays with pCKB1 and $pSp1_3$ followed by PCR analysis of immunoprecipitated chromatin fragments as described in the Materials and Methods. Experiments with pCKB and $pSp1_3$ (panels A and B) were carried out separately, and comparable results were observed in at least two separate experiments. ChIP assay on the cathepsin D gene promoter (panels C and D) used the endogenous promoter, and the analysis was carried out in cells treated with DMSO or 10 nM E2 (but no transfection).

−294 region of the cathepsin D gene promoter after treatment with E2 for 45 min [DiRenzo et al., 2000; Shang et al., 2000], and a weak band was also detected in solvent-treated cells. Similar results were observed in this study after incubation with E2 for 15 or 30 min, and Sp1 antibody also immunoprecipitated the E2-responsive region of the cathepsin D gene promoter after 15 min; a weaker band was observed after 30 min (panel C). These results are consistent with the reported ERα/Sp1 action on the cathepsin D gene promoter in MCF-7 cells [Krishnan et al., 1994, 1995]. As an additional negative control for this experiment, it was shown that neither ERα or Sp1 antibodies immunoprecipitated a region in exon 2 of the cathepsin D gene (panel D). These results show that the ChIP assay can detect ERα/Sp1 interactions with transiently transfected pCKB1 (panel A) as well as endogenous gene promoters (cathepsin D) (panel C).

DISCUSSION

CKB gene expression is highly inducible in the rodent uterus [Notides and Gorski, 1966; Reiss and Kaye, 1981; Crombie et al., 1994], and constructs containing CKB gene promoter inserts are highly inducible in both ER-positive and ER-negative transformed cell lines [Benfield et al., 1988; Hobson et al., 1988, 1990; Horlick and Benfield, 1989; Horlick et al., 1990; Mitchell and Benfield, 1990; Spatz et al., 1992; Wu-Peng et al., 1992; Sukovich et al., 1994; Castro-Rivera et al., 1999]. Deletion analysis of the CKB gene promoter in HeLa cells has identified at least two E2-responsive regions, namely an upstream region (−568 to −523) that binds ER and other transcription factors and a more proximal region (−195 to −37) that does not directly bind ER [Wu-Peng et al., 1992; Sukovich et al., 1994]. A similar pattern of E2-responsiveness was also observed in MCF-7 cells using a series of CKB-derived constructs (Fig. 1). Sukovich et al. [1994] identified TA-rich and a CCAAT sequence in the proximal −75 to −45 region of the promoter that was E2-responsive in HeLa cells cotransfected with ERα, HE19 (AF1 deletion mutant) or HE74, an ERα variant that has six mutations in the DBD that prevent direct binding to promoter DNA. Their studies did not identify transacting

factors required for activation of this region of the CKB gene promoter and this is currently being investigated in our laboratory.

Wu-Peng et al. [1992] extensively investigated hormonal regulation of the upstream CKB promoter region and showed the transcriptional activation by E2 required cotransfection with ERα expression plasmid and HE11, a DBD deletion mutant was inactive. The upstream sequence contains an imperfect palindromic ERE with consensus (GGTCA) and nonconsensus (CACCC) half-sites flanked by two GC-rich Sp1 binding sites. In transient transfection studies in HeLa cells, only the nonconsensus ERE was required for E2-responsiveness since a GGTCA → CATCA mutation abrogated the hormone-induced response in HeLa cells [Wu-Peng et al., 1992]. The high E2-inducibility of constructs containing the −582 to −523 region of the CKB gene was surprising for a promoter containing an imperfect palindromic ERE and it was previously suggested that the flanking GC-rich sites may also play a role in E2-responsiveness [Wu-Peng et al., 1992]. Another possible explanation for these results could be the formation of DNA-bound ERα/Sp1 complexes through GC-rich $Sp1(N)_xERE$ half-site motifs which have previously been identified as E2-responsive elements in the cathepsin D ($N = 23$), heat shock protein 27 ($N = 10$) and transforming growth factor α ($N = 30$) gene promoters [Krishnan et al., 1994, 1995; Porter et al., 1996; Vyhlidal et al., 2000]. Initial studies with [^{32}P]CKB-7 showed that nuclear extracts from MCF-7 cells did not give the typical broad ERα/Sp1-DNA complex associated with binding to an $Sp1(N)_xERE$ half-site (data not shown), and this region of the CKB promoter independently bound ERα [Wu-Peng et al., 1992] and human recombinant Sp1 protein (Figs. 4 and 5).

Deletion and mutation analysis of the −568 to −523 (i.e., CKB7) region of the CKB gene promoter (Figs. 1–3) showed that E2-responsiveness in ER-positive MCF-7 and ER-negative MDA-MB-231 breast cancer cells, and in Schneider (insect) SL-2 cells was due to independent contributions of the nonconsensus ERE (e.g., pCKB9) and GC-rich sites (e.g., pCKB8) that bound ERα/Sp1. The characteristics of these E2-responsive GC-rich elements in the CKB gene promoter were similar to those observed for several other genes regulated by ERα/Sp1 through GC-rich motifs including DNA polymerase α, c-*fos*, E2F1, retinoic acid receptor α1, thymidylate synthase, *bcl*-2, adenosine deaminase, and insulin growth factor binding protein 4 [Porter et al., 1997; Duan et al., 1998; Sun et al., 1998; Wang et al., 1998, 1999; Dong et al., 1999; Qin et al., 1999; Xie et al., 1999, 2000; Samudio et al., 2001]. For example, constructs containing E2-responsive promoter inserts from these genes were activated in cells cotransfected with ERα or HE11 (DBD deletion mutant), and similar results were observed for pCKB7 or pCKB8 (Fig. 2C). Previous studies showed that the GC-rich sites in CKB gene promoters were not functional in transfection studies [Wu-Peng et al., 1992]; however, this is not unexpected since hormone-responsiveness of GC-rich sites is cell context-dependent and has been observed in ER-positive breast/endometrial and LnCAP prostate cancer cells [Saville et al., 2000], but not in HeLa cells [Weisz and Rosales, 1990; Wu-Peng et al., 1992]. The gel mobility shift data on binding of Sp1 and Sp1 plus ERα proteins to [^{32}P]CKB7 were also typical of interactions of these proteins with other GC-rich oligonucleotides derived from E2-responsive gene promoters [Porter et al., 1997; Duan et al., 1998; Sun et al., 1998; Wang et al., 1998, 1999; Dong et al., 1999; Qin et al., 1999; Xie et al., 1999, 2000; Petz and Nardulli, 2000; Saville et al., 2000; Salvatori et al., 2000; Li et al., 2001; Samudio et al., 2001]. Sp1 protein binds GC-rich sequences and coincubation with ERα increased Sp1-DNA binding by enhancing the "on-rate" of complex formation [Porter et al., 1997]. Even though ERα and Sp1 physically interact [Porter et al., 1997], the failure to observe a ternary complex in gel mobility shift assays is not unprecedented. For example, sterol regulatory element-binding protein, cyclin D1, and the human T cell lymphotropic virus type 1 Tax protein enhance DNA binding of Sp1, ER and other DNA-bound nuclear proteins without forming a supershifted complex [Matthews et al., 1992; Zhao and Giam, 1992; Armstrong et al., 1993; Franklin et al., 1993; Anderson and Dynan, 1994; Sanchez et al., 1995; Zwijsen et al., 1997]. We have also used the ChIP assay to confirm ERα/Sp1 interactions within the −678 to −319 region of the CKB gene promoter (Fig. 6, panel A), and both ERα and Sp1 antibodies also immunoprecipitated the GC-rich regions in the transfected $pSp1_3$ construct and in the cathepsin D gene (panels B and C, respectively). This

ChIP data supports results of previous transactivation, immunoprecipitation and gel mobility shift assays [Porter et al., 1997; Duan et al., 1998; Sun et al., 1998; Wang et al., 1998, 1999; Dong et al., 1999; Qin et al., 1999; Xie et al., 1999, 2000; Petz and Nardulli, 2000; Saville et al., 2000; Salvatori et al., 2000; Li et al., 2001; Samudio et al., 2001] showing that ERα and Sp1 are associated with GC-rich motifs in E2-responsive genes/constructs.

In summary, results of this study demonstrate that E2-responsiveness of the upstream −568 to −523 region of the CKB gene promoter is regulated independently through an imperfect palindromic ERE and two GC-rich motifs in breast cancer cells. ERα/Sp1 action through GC-rich sites has been reported for several other genes in breast cancer cells [Porter et al., 1997; Duan et al., 1998; Sun et al., 1998; Wang et al., 1998, 1999; Dong et al., 1999; Qin et al., 1999; Xie et al., 1999, 2000; Samudio et al., 2001], and recent studies have characterized similar mechanisms for hormone activation of low density lipoprotein receptor, telomerase, progesterone receptor, epidermal growth factor receptor, and the receptor for advanced glycation end products in other cell lines [Kyo et al., 1999; Petz and Nardulli, 2000; Salvatori et al., 2000; Tanaka et al., 2000; Li et al., 2001]. Functional Sp1 interactions with other members of the nuclear receptor superfamily including retinoic acid/X receptors, progesterone receptor, chick ovalbumin upstream promoter, transcription factor, and the androgen receptor have also been reported [Rohr et al., 1997; Owen et al., 1998; Pipaón et al., 1999; Suzuki et al., 1999; Lu et al., 2000] suggesting an expanding role for nuclear receptor/Sp1 complexes in regulating gene expression. This study also illustrates the importance of cell context in ERα/Sp1 action since the GC-rich sites in the CKB promoter were E2-responsive in breast but not HeLa cells [Wu-Peng et al., 1992]. We are currently using chromatin immunoprecipitation and transient transfection assays to investigate the timing of coactivator and ERα/Sp1 assembly on GC-rich sites and the role of other nuclear proteins in mediating cell context-dependent activation of E2-regulated genes.

ACKNOWLEDGMENTS

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