Cyclin D1 gene expression is induced by 17\(\beta\)-estradiol (E2) in human breast cancer cells and is important for progression of cells through the G\(_1\) phase of the cell cycle. The mechanism of activation of cyclin D1 is mitogen- and cell context-dependent, and this study describes the role of multiple promoter elements required for induction of cyclin D1 by E2 in estrogen receptor (ER)-positive ZR-75 breast cancer cells. Transcriptional activation of cyclin D1 by E2 was dependent, in part, on a proximal cAMP-response element at \(-66\), and this was linked to induction of protein kinase A-dependent pathways. These results contrasted to a recent report showing that induction of cyclin D1 by E2 in ER-positive MCF-7 and HeLa cells was due to up-regulation of c-jun and subsequent interaction of c-Jun-ATF-2 with the CRE. Moreover, further examination of the proximal region of the cyclin D1 promoter showed that three GC-rich Sp1-binding sites at \(-143\) to \(-110\) were also E2-responsive, and interaction of EREs and Sp1 proteins at these sites was confirmed by electromobility shift and chromatin immunoprecipitation assays. Thus, induction of cyclin D1 by E2 in ZR-75 cells is regulated through nuclear EREs/Sp1 and epigenetic protein kinase A activation pathways, and our results suggest that this mechanism may be cell context-dependent even among ER-positive breast cancer cell lines.

Mitogen stimulation of cell growth is accompanied by the coordinate expression of multiple genes and pathways including those required for different phases of cell cycle progression (1–7). Cyclin D1 is induced early in the G\(_1\) phase of the cell cycle, and cyclin D1-cyclin-dependent kinase complexes are important for phosphorylation of several key substrates involved in cell proliferation including retinoblastoma protein and other pocket proteins. The critical role for cyclin D1 in the rate of progression of cells through G\(_1\) has been studied in factors that regulate cyclin D1 gene expression in various cell types. Transcriptional activation of cyclin D1 depends in part on interaction of trans-acting factors with elements in the cyclin D1 gene promoter; it is clear from promoter analysis studies that the assembly of transcription factors is highly variable and dependent on multiple factors including the mi

togen and cell context (8–20). For example, p21\(^{\text{CIP1}}\) and p300 expression activated constructs containing cyclin D1 gene promoter inserts in JEG-3 human trophoblasts through interactions of proteins at a distal AP-1-like sequence at \(-954\) in the promoter (13). Overexpression of p60\(^{\text{erbB}}\) in MCF-7 breast cancer cells also activates cyclin D1 and involves activation of a cAMP-response element-binding protein (CREB)\(^1\) and activating transcription factor-2 (ATF-2) which interacts with a CRE at \(-66\) in the cyclin D1 promoter (16).

Cyclin D1 protein is overexpressed in \(-50\%\) of mammary carcinomas (21–23), and 17\(\beta\)-estradiol (E2) induces cyclin D1 gene expression in estrogen receptor (ER)-positive human breast cancer cell lines (24–29). Cyclin D1 also directly binds EREs and stimulates ligand-independent transactivation (30–33), and interaction of cyclin D1 with p300/CRE-binding protein-associated factor (PCAF) further stimulates ER/cyclin D1 action (34). Sabbah and co-workers (34) showed the E2-induced reporter gene activity in MCF-7 cells transfected with a construct containing the \(-944\) to \(+139\) region of the cyclin D1 reporter, and deletion analysis of this promoter in ER-negative HeLa cells identified a CRE at \(-66\) as the E2-responsive region. They identified a cAMP-dependent protein kinase A (PKA)-independent pathway for activation of this CRE, and transactivation was linked to induction of c-jun and interaction of c-Jun-ATF-2 heterodimers at the CRE. This study reports that E2 also induces cyclin D1 gene expression in ER-positive ZR-75 breast cancer cells, and deletion analysis of the promoter confirmed that the downstream CRE was E2-inducible through activation of PKA. Moreover, further examination of the promoter shows that three GC-rich Sp1-binding sites at \(-142\) to \(-110\) were also E2-responsive indicating that transcriptional activation of cyclin D1 by E2 involves multiple proximal cis-elements including GC-rich sites that bind hER\(\alpha\)-Sp1 complexes.

MATERIALS AND METHODS

Chemicals and Biochemicals—RPMI 1620, phosphate-buffered saline, acetyl coenzyme A, E2, 100\(\mu\)g/ml antibiotic/antimycotic solution, cyclin D1 antibody, cholera toxin plus 3-isobutyl-1-methylxanthine (CT), DME/F-12, and chloroquine were purchased from Sigma. Luciferase and \(\beta\)-galactosidase enzyme assay systems were obtained from Promega Corp. (Madison, WI). Fetal bovine serum (FBS) was obtained from Intergen (Purchase, NY) and JRH Biosciences (Lenexa, KS). \(^{33}\)P/ATP (3000 Ci/mmol), \(^{32}\)P[CTP], and \(^{3}JC\)lauralamphenicol (53 mCi/mmol)

\(^1\) The abbreviations used are: CREB, cAMP-response element-binding factor; AF, activation function; ATF, activating transcription factor; CD1, cyclin D1; ChiP, chromatin immunoprecipitation; CRE, cAMP-response element; CSS, charcoal-stripped serum; E2, 17\(\beta\)-estradiol; ER, estrogen receptor; ERE, human ER; ERE, estrogen-response element; PCAF, p300/CRE-binding protein associated factor; PKA, protein kinase A; DME, Dulbecco’s modified Eagle’s; FBS, fetal bovine serum; PCR, polymerase chain reaction; CT, cholera toxin; EMSA, electrophoretic mobility shift assay.
were purchased from PerkinElmer Life Sciences. Restriction enzymes (Xhol and KpnI) and T4-polymerase kinase were purchased from Promega Corp. All other chemicals and biochemicals were the highest quality available from commercial sources. CREB1, CREB, ATF3, and c-Jun rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). FBS was stripped two times with 20% ratio of dextran-coated charcoal (0.01 Mr Tris-HCl, 0.25% Nort A charcoal, 0.025% dextrose, pH 8.0) at 45 °C for 45 min.

**Cell Culture**—ZR-75A cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 medium with phenol red and supplemented with 10% FBS plus 0.2% antibiotic antimycotic solution, 0.22% sodium bicarbonate, dexamethasone, 0.45% dextrose, and 0.24% HEPES. Cells were grown in 150-cm² culture flasks in a air:carbon dioxide (95:5) atmosphere at 37 °C. For transfection studies with CREB-Gal4 chimera protein and constructs containing cyclin D1 promoter (wild type and mutant) inserts, cells were seeded in 6-well Falcon plates (70% confluent) in DME/F-12 media containing 2.5% charcoal-stripped serum (CSS) for 16–24 h prior to transfection. Nuclear extracts for gel mobility shift assays were also obtained from ZR-75 cells grown in DME/F-12 and 2.5% CSS for 16–24 h prior to treatment with 10 mM E2 for 1 h as described previously (28, 35). Cells for chromatin immunoprecipitation (ChIP) and Northern and Western blot assays were grown in 100- or 150-mm culture plates in serum-free DME/F-12 for 3 days to arrest cells in G0/G1. Serum-free DME/F-12 was added to cells, and cells were then transfected with pCD1 or pCD4 and treated with 10 nM E2 for use in gel mobility shift assays or Western blot analysis were essentially obtained as described previously (28, 35), and the amount/concentrations of nuclear extracts or proteins are indicated directly in the figures or legends.

**Northern and Western Blot Analysis**—Cells were cultured in DME/F-12 (serum-free) for 3 days and then treated with Me2SO or E2 (for 30 min, 1, 2, 6, 12, and 24 h). RNA was extracted using an RNA extraction kit from Tel-Test (Friendswood, TX), and Northern blot analysis was performed as described previously (35). The 874-base pair cyclin D1 cDNA used for Northern blot analysis was obtained using the following primers and conditions: AGGAAACCGCCCCACCAGGAA; antisense primer, TGTCGCAACCGCGCCACCT, β-Tubulin mRNA was used as an internal control to standardize cyclin D1 mRNA levels.

Aliquots of whole cell extracts (100 µg) for Western blot analysis were separated on 10% SDS-polyacrylamide gel (35) using cyclin D1 rabbit IgG obtained from Sigma. Protein concentrations were determined by the method of Bradford (36).

**Cell Transfection**—ZR-75 cells were transiently transfected for 6–18 h by calcium phosphate coprecipitation with 2–4 µg of reporter plasmid and 2 µg of pcDNA3.I/His/LacZβ-galactosidase as a control vector. The reporter plasmids were cotransfected 1:0.33 to 1:0.5 with ERα or ERβ variants expression vectors. Luciferase activities in the various treatment groups were performed on 30 µl of cell extract using the luciferase assay system, and results were normalized to β-galactosidase enzyme activity as described previously (28, 35).

**Chromatin Immunoprecipitation (ChIP) Assay—**ZR-75 or MCF-7 breast cancer cells were grown in 150-mm tissue culture plates to >70% confluency and treated with 10 mM E2 for various times. Formaldehyde was then added to the medium to give a 1% solution and incubated with shaking for 10 min at 20 °C. After addition of glycine (0.125 mM) and incubation for 10 min, the media were removed; cells were washed with phosphate-buffered saline and 1 mM phenylmethylsulfonyl fluoride, scraped, and collected by centrifugation. Cells were then resuspended in swell buffer (85 mM KCl, 0.5% Nonedet P-40, 1 mM phenylmethylsulfonyl fluoride, 5 µg/ml leupeptin and aprotinin at pH 8.0) and homogenized. Nuclei were isolated by centrifugation at 1500 x g for 30 s, then resuspended in sonication buffer (5% SDS, 0.1 M EDTA, 50 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 10 mM EDTA for 45–60 s) with appropriate fragment lengths (500–1000 base pair). This extract was then centrifuged at 15,000 x g for 10 min at 0 °C, aliquoted, and stored at −70 °C until used. The cross-linked chromatin preparations were diluted in buffer (1% Triton X-100, 100 mM NaCl, 0.5% SDS, 5 mM EDTA, and Tris-HCl, pH 8.1), and 20 µl of Ultralink protein A or G or A/G beads (Pierce) were added per 100 µl of chromatin and incubated

**Nuclear and Whole Cell Extracts**—Cells were treated with 10 mM E2 or Me2SO for 1 h prior to harvesting by trypsinization. Cells were then extracted in high salt (0.5 M potassium chloride), and nuclear extracts for use in gel mobility shift assays were obtained and stored in small aliquots at −80 °C as described previously (28, 35). Whole cell extracts were obtained from cells cultured in serum-free DME/F-12 for 3 days, as described above and then treated with Me2SO or 10 nM E2 (in Me2SO) for 2, 6, 12, 18, and 24 h, respectively. Whole cell lysates used in Western blot analysis were essentially obtained as described previously (28, 35) and stored at −80 °C until required.

**Plasmids and Oligonucleotides**—The cyclin D1 (pA3-Luc-CYCD) promoter plasmid constructs that contain the cyclin D1 regulatory regions (−145 to +130) fused to a luciferase reporter gene were described previously and then treated with Me2SO or 10 nM E2 (in Me2SO) for 2, 6, 12, 18, and 24 h, respectively. Whole cell lysates used in Western blot analysis were essentially obtained as described previously (28, 35) and stored at −80 °C until required.

**Glucocorticoid Receptor (GR)**—The GR-expressing cell line (GR) was a kind gift from Dr. Richard Goodman (Oregon Health Science Center). The following oligonucleotides were synthesized, purified, and annealed, and 5 pmol of specific oligonucleotides were 32P-labeled at the 5′-end using T4 polynucleotide kinase and [γ-32P]ATP. Gel mobility shift and supershift assays were performed as described previously (28, 35), and the amount/concentrations of nuclear extracts or proteins are indicated directly in the figures or legends.

**Northern and Western Blot Analysis**—Cells were cultured in DME/F-12 (serum-free) for 3 days and then treated with MeSO or E2 (for 30 min, 1, 2, 6, 12, and 24 h). RNA was extracted using an RNA extraction kit from Tel-Test (Friendswood, TX), and Northern blot analysis was performed as described previously (35). The 874-base pair cyclin D1 cDNA used for Northern blot analysis was obtained using the following primers and conditions: AGGAAACCGCCCCACCAGGAA; antisense primer, TGTCGCAACCGCGCCACCT, β-Tubulin mRNA was used as an internal control to standardize cyclin D1 mRNA levels.

**Aliquots of whole cell extracts (100 µg)** for Western blot analysis were separated on 10% SDS-polyacrylamide gel (35) using cyclin D1 rabbit IgG obtained from Sigma. Protein concentrations were determined by the method of Bradford (36).

**Chromatin Immunoprecipitation (ChIP) Assay**—ZR-75 or MCF-7 breast cancer cells were grown in 150-mm tissue culture plates to >70% confluency and treated with 10 mM E2 for various times. Formaldehyde was then added to the medium to give a 1% solution and incubated with shaking for 10 min at 20 °C. After addition of glycine (0.125 mM) and incubation for 10 min, the media were removed; cells were washed with phosphate-buffered saline and 1 mM phenylmethylsulfonyl fluoride, scraped, and collected by centrifugation. Cells were then resuspended in swell buffer (85 mM KCl, 0.5% Nonedet P-40, 1 mM phenylmethylsulfonyl fluoride, 5 µg/ml leupeptin and aprotinin at pH 8.0) and homogenized. Nuclei were isolated by centrifugation at 1500 x g for 30 s, then resuspended in sonication buffer (5% SDS, 0.1 M EDTA, 50 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 10 mM EDTA for 45–60 s) with appropriate fragment lengths (500–1000 base pair). This extract was then centrifuged at 15,000 x g for 10 min at 0 °C, aliquoted, and stored at −70 °C until used. The cross-linked chromatin preparations were diluted in buffer (1% Triton X-100, 100 mM NaCl, 0.5% SDS, 5 mM EDTA, and Tris-HCl, pH 8.1), and 20 µl of Ultralink protein A or G or A/G beads (Pierce) were added per 100 µl of chromatin and incubated
for 4 h at 4 °C. A 100-μl aliquot was saved and used as the 100% input control. Salmon sperm DNA, specific antibodies, and 20 μl of Ultralink beads were added, and the mixture was 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-HCl, 500 mM LiCl, 1% Nonidet P-40, 1% deoxycholic acid, 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 mM NaHCO₃, 1% SDS, 1.5 μg/ml sonicated salmon sperm DNA), vortexed, and 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 cross-links. Wizard PCR kits (Promega) were used for additional DNA clean up, and PCR was used to detect the presence of promoter regions immunoprecipitated with commercially available ERα or Sp1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). The following primers were used for PCR analysis of immunoprecipitated promoter regions: cathepsin D Fw (5'-294), 5'-TCC AGA CAT CCT CTC TGG AA-3', and Rv (5'-54), 5'-GGG GCC GAG GGT CCT TCA TAT-3'; cathepsin D (exon 2) Fw (5'-2468), 5'-TGG ACA AGT TCA CGT CCA TC-3', and Rv (5'-2615) 5'-TGT AGT TCT TGA GCA CCT CG-3'; cyclin D1 Fw (5'-204), 5'-GGG GAC GTG CAT TTC TAT GA-3', and Rv (5'-32) 5'-CAA AAC TCC CCT GTA GTC CGT-3'.

**Schneider Cell Maintenance and Transfection**—Cells were grown at room temperature in T-150 flasks in Schneider’s medium (Life Technologies, Inc.) supplemented with 5% FBS (heat-inactivated at 55 °C for 30 min) and 0.5% antibiotic/antimycotic solution. Cells were grown in 12-well plates, and luciferase activities in various treatment groups were determined and normalized to β-galactosidase activity (internal control) as described previously (28, 35). Statistical Analysis—Results of transient transfection studies are presented as means ± S.D. for at least three separate experiments for each treatment group. All other experiments were carried out at least two times to confirm a consistent pattern of responses. Statistical differences between treatment groups were determined by analysis of variance and Scheffe’s test.

**RESULTS**

**Hormonal Activation of Cyclin D1 Gene/Gene Promoter Constructs in ZR-75 Cells**—Treatment of ZR-75 cells with 10 nM E2 resulted in the induction of cyclin D1 mRNA levels within 30 min, and elevated expression subsequently decreased with time (Fig. 1A). These results are comparable to those reported previously in other ER-positive breast cancer cell lines (24–29). In addition, E2 also induces cyclin D1 protein (Fig. 1B). Initial transient transfection studies with pCD1 (−1745 to +130) in ZR-75 cells showed that treatment with 50 nM E2 alone resulted in a 2-fold increase in reporter gene activity (Fig. 2A). Transfection studies in ER-positive breast cancer cells with many E2-responsive constructs containing consensus and non-consensus ERs, GC-rich, or AP1 sites show that hormone-induced transactivation is observed only after cotransfection with an ER expression plasmid (37–60). This is due to the high copy numbers of plasmids in transfected cells and limiting levels of endogenous ER (37). The results summarized in Fig. 2A show that E2 induces luciferase activity in ZR-75 cells transfected with pCD1 and cotransfected with ERα, but not ERβ, expression plasmid. Deletion analysis of the cyclin D1 gene promoter showed that E2-induced reporter gene activity in ZR-75 cells transfected with pCD1, pCD2, pCD3, and pCD4 (Fig. 2B), and it was evident that deletion of the −1745 to −163 region of the promoter did not affect E2 responsiveness. Sabbah and coworkers reported previously (34) a time-dependent induction of reporter gene activity by E2 in growth-arrested MCF-7 cells transfected with constructs containing a cyclin D1 promoter insert (−944 to +139). Their cells were arrested in G1/S by using ICI 182,780 in the culture medium for 48 h. In this study, ZR-75 cells were grown in DME/F-12 media containing 0.1% CSS for 3 days to growth-arrest cells prior to hormone treatment. In ZR-75 cells transfected with pCD1 or pCD4, E2 significantly induced luciferase activity 6 or 12 h after hormonal treatment (Fig. 2, C and D), thus confirming time-dependent hormonal activation of cyclin D-derived constructs in ZR-75 cells as observed previously (34) in MCF-7 cells. Other transfection studies in breast cancer cells used 2% CSS, and time-dependent variations in E2 responsiveness were not observed.

**Deletion and Mutation Analysis of the Proximal Region of the Promoter**—The effects of cotransfection of hERα and mutants containing deletion of the DNA-binding domain (HE11), activation function 2 (AF2) (HE15), or AF1 (HE19) on transactivation in cells transfected with pCD1 were also investigated (Fig. 3A). The results showed that induction of reporter gene activity by E2 was observed only in cells transfected with hERα or HE11, and previous studies (35, 49–60) have shown that E2 activates HE11/Sp1 and HE11/AF1 in cells transfected with constructs containing E2-responsive GC-rich and AP1 promoters, respectively. A series of 5'- and 3'-deletion constructs containing the −163 to −100 (pCD5), −163 to −130 (pCD6), −130 to −100 (pCD7), and −107 to +100 (pCD8) regions of the cyclin D1 gene promoter were used in transient transfection studies to identify specific E2-responsive elements within this region of the promoter (Fig. 3B). pCD8 contains a CRE-binding site, and E2 responsiveness has been linked previously to activation of ATF-2 and c-Jun and their subsequent interaction as a heterodimer with the CRE (34). In addition, E2 responsiveness of pCD5, pCD6, and pCD7, and CRE suggests that GC-rich motifs that bind Sp1 family proteins are also important for E2 action. Mutation analyses of the GC-rich region of the cyclin D1 promoter was carried out to define further their role for functional interactions with ERα/Sp1 (Fig. 3C). The overlapping GC-rich sites alone were E2-responsive (pCD7), and mutation and deletion analyses were used to determine contributions of the upstream GC-rich and E2-binding sites. E2 did not significantly induce luciferase activity in cells transfected with pCD5m1 (mutation

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**FIG. 1.** Induction of cyclin D1 mRNA and protein levels by E2. A, cyclin D1 mRNA. ZR-75 cells were treated with 10 nM E2 for different times (0.5–24 h), and cyclin D1 mRNA levels were determined by Northern blot analysis as described under “Materials and Methods.” Induction was observed 30 min after treatment and remained elevated for the treatment period. B, cyclin D1 protein. Cyclin D1 protein levels induced by E2 were determined by Western blot analysis as described under “Materials and Methods,” and increased (maximum 3.3-fold) levels were observed 2 h after treatment with E2, and these were decreased to near background levels after 24 h.
of upstream and downstream overlapping GC-rich sites), whereas small but significant induction was observed in cells transfected with pCD5m2 (mutation only of the overlapping GC-rich site). These data suggest that the upstream GC-rich site is also E2-responsive, whereas the E2F-binding sequence is not required, and this was confirmed by comparing E2-induced transactivation in cells transfected with pCD6 or pCD6m in which significant induction was observed only with pCD6. Results obtained for these deletion/mutant constructs (Fig. 3) indicate that the GC-rich motifs in the H11002172 to H11002100 region of the cyclin D1 gene are important for hormonal activation by ERα/Sp1. Transfection of pCD5 into insect SL-2 cells followed by cotransfection with expression plasmids for Sp1 and hERα showed that only Sp1 enhanced reporter gene activity, and this was consistent with interaction of Sp1 protein with the GC-rich element in cyclin D1 promoter. In SL-2 cells cotransfected with both hERα and Sp1 expression plasmids, there was a slight enhancement of activity using 100 ng of both expression plasmids. Similar interactions between hERα and Sp1 have been observed previously in SL-2 cells transfected with constructs containing GC-rich promoter inserts from the bcl-2 and vascular endothelial growth factor genes (35, 61).

Hormonal Activation of the CRE in the Cyclin D1 Gene Promoter—pCD8 contains the −107 to +100 region of the cyclin D1 promoter, and E2 induces reporter gene activity in ZR-75 cells transfected with pCD8 and ERe expression plasmid (Fig. 4A). E2 also induces activity in the absence of cotransfected ERα (<2-fold); however, the induction response is markedly enhanced by cotransfection with ERα, and this has been observed previously (35) for regulation of an E2-responsive CRE in the bcl-2 gene promoter. The induction response in ZR-75 cells transfected with pCD8 was inhibited after cotreatment with the PKA inhibitor SQ22536 (200 μM), whereas the inhibitor alone had no effect on reporter gene activity compared with solvent control. This contrasts with previous studies on the E2 responsiveness of this region of the cyclin D1 promoter where inhibition of PKA did not block E2 activation, and induction of cAMP by forskolin did not increase reporter gene activity (34). Thus, hormonal activation of the CRE in the cyclin D1 gene promoter was not cAMP-dependent in HeLa cells, and transactivation through this element correlated with hormonal induction of c-Jun and dimerization with ATF-2 (34). Activation of CREB by E2 in ZR-75 cells was further investigated in cells transfected with a pGal45 promoter construct and a CREB-Gal4 chimeric protein containing the yeast Gal4 DNA-binding domain (amino acids 1–147) fused to CREB (amino acids 4–285) (Fig. 4B). Both E2 (50 nM) and CT induced a >5–6-fold increase in luciferase activity, whereas induction was not observed in cells transfected with the empty vector pGal45 alone or in combination with cotransfected ERe. The role of cAMP-PKA activation of cyclin D1 promoter in ZR-75 cells was confirmed by showing that CT also induced luciferase activity in cells transfected with pCD8 (Fig. 4C). In contrast, pCD5 which contains GC-rich sites that are 5′ to the CRE site was not affected by CT. Thus, both E2 and CT induced luciferase activity in cells transfected with pCD8 or CREB-Gal4. Results in Fig. 4D show that
in ZR-75 cells transfected with pCD8, the hormone-induced response is inhibited by cotransfection with dominant-negative KCREB expression plasmid, whereas KCREB did not affect hormone-induced transactivation of a construct (pCD6) that did not contain the CRE (data not shown).

Protein interactions with the CRE in the cyclin D1 gene promoter were investigated in EMSAs using nuclear extracts from estrogen-treated ZR-75 cells and 32P-labeled oligonucleotide (Fig. 4E). Incubation of 32P-labeled cyclin D1 promoter with nuclear extracts gave two major bands (B1 and B2) (lane 1), and the more mobile band B2 may represent a complex of unresolved bands. Incubation with a 200-fold excess of unlabeled consensus CRE decreased band intensities of the 32P-labeled cyclin D1 promoter complexes (lane 2); however, unlabeled mutant CRE also decreased intensity of band 2 (lane 3). Decreased intensity of band 2 was also observed in competition studies using consensus AP1, ERE, and GC-rich oligonucleotides (data not shown) suggesting that this complex may be due, in part, to nonspecific binding. Supershift experiments with antibodies to ATF1, CREB1, and CREB2 indicated that ATF-1 formed a weak supershifted band with the 32P-labeled cyclin D1 promoter complex (lane 4), and CREB1 antibody slightly decreased intensity of this band (lane 5). Interactions of CREB1, CREB2, and CREM1 antibodies with the 32P-labeled cyclin D1 promoter complex were investigated in several experiments, and a weak supershifted band could only be detected with CREB1 antibody (data not shown).

Protein/DNA Interactions with GC-rich Regions of the Cyclin D1 Promoter—The comparative binding of ZR-75 nuclear extracts with 32P-labeled cyclin D1 promoter and 32P-labeled Sp1 oligonucleotide (Fig. 5A) was investigated in gel mobility shift assays. Incubation of 32P-labeled cyclin D1 promoter with nuclear extracts gave a retarded band (Fig. 5A, lane 1 (arrow)), and competition with 100-fold excess unlabeled Sp1 oligonucleotide (lane 2) decreased intensity of the band, whereas mutant Sp1 oligonucleotide did not affect retarded band intensity (lane 3). Incubation of 32P-labeled cyclin D1 promoter with nuclear extracts from ZR-75 cells gave a retarded band (lane 4) with a mobility similar to that observed using 32P-labeled Sp1. Intensity of the 32P-labeled cyclin D1 promoter band was decreased after competition with 100-fold excess unlabeled Sp1 oligonucleotide (lane 5), whereas mutant Sp1 oligonucleotide did not affect retarded band intensity (lane 6). In a separate experiment, coinubcation with 100-fold excess unlabeled CD01

FIG. 3. Role of GC-rich Sp1-binding sites. A, wild-type and deletion mutants of ERα. ZR-75 cells were transfected with pCD1 and ERα and HE11, HE19, or HE15, and hormonal activation was determined as described under “Materials and Methods.” Significant (*p < 0.05) induction by E2 is indicated by an asterisk. B, deletion analysis of the −163 to +130 region. ZR-75 cells were transfected with pCD4, pCD5, pCD6, pCD7, or pCD8. Hormone-induced activity was determined as described under “Materials and Methods,” and significant (p < 0.05) induction by E2 is indicated by an asterisk. C, mutation/deletion analysis. ZR-75 cells were transfected with pCD4, pCD5, pCD5m1, pCD5m2, pCD5m3, pCD6, and pCD6m1. Hormone-induced activity was determined as described under “Materials and Methods,” and significant (p < 0.05) induction is indicated by an asterisk. D, transfection of pCD5 in SL-2 cells. pCD5 was transiently transfected in SL-2 cells and cotransfected with expression plasmids for Sp1 or ERα (+10 nM E2) or their combination, and luciferase activity was determined as described under “Materials and Methods.” Significant (p < 0.05) induction is indicated by an asterisk.
Estrogen Regulation of Cyclin D1

**Fig. 4.** Role of the CRE in estrogen activation. A, PKA inhibitor. ZR-75 cells were transfected with pCD8 treated with E2, SQ22538, and their combination, and luciferase activity determined as described under “Materials and Methods.” Activity was significantly (*p < 0.05) increased by E2, and this response was significantly (**p < 0.05) decreased by SQ22538. B, CREB-Gal4 activation. ZR-75 cells were treated with MeSO (control), 50 nM E2, or CT, transfected with pGal4, alone or in combination with CREB-Gal4 or ERα expression plasmid, and luciferase activity determined as described under “Materials and Methods.” Significant (p < 0.05) induction is indicated by an asterisk. C, activation of pCD8 by CT. ZR-75 cells were transfected with pCD8 or pCD5, treated with 10 or 100 nM CT, and luciferase activity was determined as described under “Materials and Methods.” Significant (*p < 0.05) induction is indicated by an asterisk. D, dominant-negative CREB (KCREB). Cells were transfected with pCD8 treated with E2 and cotransfected with different amounts of KCREB which significantly (**p < 0.05) decreased E2-induced luciferase activity. E, nuclear extract binding to 32P-CD02 (–86 to –51). Nuclear extracts from E2-treated ZR-75 cells were incubated with 32P-CD02, and the effects of wild-type and mutant CRE oligonucleotides and antibodies to ATF1, CREB1, and CREB2 on retarded band formation were determined as described under “Materials and Methods.” Two major bands (B1 and B2) were detected, and ATP1 antibodies gave a shifted band (SS) using 32P-CD02; a weak shifted band could also be detected using CREB1 antibodies when the gel was overexposed (data not shown). c-Jun antibodies did not give a shifted band with nuclear extracts and 32P-CD02, whereas recombinant c-Jun bound to a consensus 32P-AP1 gave a shifted band (data not shown). Nonspecific IgG did not affect retarded band intensities (data not shown).
and Sp1 antibodies did not immunoprecipitate a region of exon 2 of the cathepsin D gene promoter (negative control) (Fig. 6D).

**DISCUSSION**

Cyclin D1 is overexpressed in ~50% of primary mammary tumors, and some studies (21–23) report that overexpression in advanced malignant tumors can be greater than 80%. Transgenic mouse studies indicate that cyclin D1 plays an important role in normal mammary development, and in mice that overexpress cyclin D1 alone or in combination with overexpression of other oncogenes, there is a more rapid onset of mammary tumor formation (62, 63). Cyclin D1 expression and function in breast cancer cells is complex. For example, cyclin D1 protein physically interacts with ERα, and ectopic expression of cyclin D1 enhances expression of reporter gene activity in cells transfected with a construct (pERE) containing a consensus estrogen-responsive element (ERE) promoter insert (30, 31). Cyclin D1/ERα interactions are also enhanced by nuclear coactivators and P/CAF, and this latter response is related, in part, to the histone acetylase activity of P/CAF (32, 33). The importance of cyclin D1/ERα interactions on the regulation of hormone-induced endogenous genes is unknown, and cyclin D1 is not induced by E2 in ER-negative breast cancer cells stably transfected with ERα (64). This latter response is consistent with the unusual growth inhibitory activity of E2 in most ER-negative cell lines stably transfected with ERα (65).

E2 induces cyclin D1 gene expression and protein in ER-positive breast cancer cells (e.g. Fig. 1), and this is paralleled by activation of other genes and gene complexes required for cell cycle progression. A recent study also demonstrated that E2 induced reporter gene activity in MCF-7 cells transfected with a construct containing the −944 to +139 region of the cyclin D1 gene promoter, and deletion analysis studies in HeLa cells identified an E2-responsive CRE between −96 and −29 region of the cyclin D1 promoter (34). Our studies show that E2 also induces cyclin D1 gene expression in ER-negative ZR-75 breast cancer cells, and analysis of the cyclin D1 gene promoter (Figs. 1–3) has identified two major regions that are required for E2 responsiveness. The downstream −100 to +30 sequence contains a CRE, and it was reported that activation through the CRE was not due to cAMP-dependent pathways but to induction of c-jun and subsequent interaction of c-Jun/ATF-2 heterodimers with the CRE (34). In contrast, activation of the CRE in ZR-75 cells was blocked by the PKA inhibitor SQ22536 and inhibited by ectopic expression of a dominant-negative form of CREB (Fig. 4). These results demonstrate that activation of the cAMP-PKA pathway contributes to induction of cyclin D1, and this is consistent with previous studies showing that E2 induces activity from CRE constructs in breast cancer and other cancer cell lines (35, 66–69). Differences observed in this study with a previous report (34) on hormonal activation of the downstream −107 to +100 region of the cyclin D1 promoter are probably due to cell context (ZR-75 versus HeLa/MCF-7 cells).

Hormonal activation of pCD1 was observed in cells transfected with wild-type hERα and HE11 (DBD-deletion mutant) suggesting that cyclin D1 induction may include DNA-independent actions of ER that could include ERα/Sp1 or ERα/AP1 interactions with GC-rich or AP1 sites, respectively (35, 49–60). Deletion and mutation analysis of the cyclin D1 gene promoter showed that a single and two overlapping GC-rich sites at −143 and −123/−123 were E2-responsive, and results of EMSAs and studies in SL2 cells suggest that hERα/Sp1 also plays a role in activation of cyclin D1 by Sp1 (Figs. 2 and 5). We have also used the ChIP assay (Fig. 6) to show that both ERα and Sp1 antibodies immunoprecipitate this region (−204 to +32) of the cyclin D1 promoter after treatment with E2 for 30 min (Fig. 6C). Ongoing studies with several GC-rich gene pro-
motors gave similar results, and maximal band intensities were observed 15–45 min after treatment (data not shown). In longer term studies (3.5 h), we observed ERα/Sp1 clearance from the promoter and the temporal variability in ERα interactions with the GC-rich region of the cyclin D1 promoter differed from results of a recent report on occupancy of the cathepsin D gene promoter by ERα and other nuclear factors (70). The reasons for these differences are unknown since both gene promoters can be activated by ERα/Sp1 (this study and Refs. 46 and 71), and we are currently investigating the dynamics of gene promoter-specific assembly of ERα and Sp1 transcription factors.

Our results demonstrate that hormonal regulation of cyclin D1 is due to both ERα/Sp1 and activation of cAMP-PKA through non-genomic ER pathways. Induction of bcl-2 gene expression by E2 is also dependent on GC-rich motifs and a CRE that is also proximal to one another (32). In contrast, the GC-rich/CRE motifs in the bcl-2 gene promoter are distal (−1578 to −1534) from the transcription start site, and this may be important for temporal and cell context-dependent differences in hormone responsiveness of bcl-2 and cyclin D1. Previous studies have shown that the GC-rich sites play an integral role in cyclin D1 expression (8, 11, 14, 18). For example, in vascular endothelial cells, ras-dependent activation of Sp1 is important for induction of cyclin D1 (8), whereas in colon cancer cells, cyclin D1 expression is repressed by gut-enriched Krüppel-like factor interaction with the proximal GC-rich site (11). Cyclin D1 protein also binds Sp1 and represses Sp1-dependent promoter activity (72), and it is possible that cellular levels of this protein could be regulated, in part, by a feedback mechanism involving cyclin D1/Sp1 interactions at the GC-rich promoter sites. These data confirm the importance of GC-rich motifs in modulating differences in cyclin D1 activation in various cell lines.

There are an increasing number of E2-responsive genes regulated by ERα/Sp1, and these include E2F1, bcl-2, progesterone receptor, retinoic acid receptor α, cathepsin D, c-fos, insulin-like growth factor-binding protein 4, adenosine deaminase, thymidylate synthase, DNA polymerase α, telomerase, progesterone receptor, epidermal growth factor receptor, and the receptor for advanced glycation end products (35, 51–61, 73–76). Other members of the nuclear receptor superfamily including retinoic acid X receptors, progesterone receptor, chick ovalbumin upstream promoter transcription factor, and the androgen receptor also bind Sp1 and transactivate through GC-rich promoter elements (77–81). This study demonstrates that ERα/Sp1 plays a role in hormonal activation of cyclin D1 gene expression in ZR-75 cells, and our results show differences in promoter activation even between ER-positive ZR-75 (this study) and MCF-7 (34) breast cancer cell lines. Current studies in this laboratory are focused on the role of Sp1 and other Sp-like proteins in regulation of hormone-induced gene expression and the importance of cell context and other proteins such as coactivators in mediating these responses.

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Estrogen Regulation of Cyclin D1 Gene Expression in ZR-75 Breast Cancer Cells Involves Multiple Enhancer Elements
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