Mechanisms of Transcriptional Activation of bcl-2 Gene Expression by 17β-Estradiol in Breast Cancer Cells

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Lian Dong†, Weili Wang†, Fan Wang†, Matthew Stoner‡, John C. Reed§, Masayoshi Harigai§*, Ismael Samudio, Michael P. Kladde, Cary Vyhlidal, and Stephen Safe‡**

From the †Department of Veterinary Physiology and Pharmacology and ‡Department of Biochemistry and Biophysics, Texas A&M University, College Station, Texas 77843 and the §Burnham Institute, La Jolla, California 92037

Programmed cell death or apoptosis plays an important role in maintaining cellular homeostasis to ensure the balance between the rates of new cell formation and cell loss (1–6). The protooncogene bcl-2 was first discovered in follicular lymphoma, where its translocation into the immunoglobulin locus resulted in overexpression of the bcl-2 gene product (7–11). bcl-2 has been extensively characterized as an inhibitor of apoptosis, and members of the bcl-2 gene family both inhibit and promote cell death (2–6). The bcl-2 gene is overexpressed in many tumors including breast cancer; however, the precise role of bcl-2 in tumor development is not well understood (12–22).

Human breast cancer cell lines have been extensively used as models for understanding the role of bcl-2 in development and growth of breast tumors and their response to chemotherapeutic drugs. Several lines of evidence suggest that resistance of breast cancer cells to treatment with chemotherapeutic drugs is linked to bcl-2 expression in these cells (23–28). For example, intracellular expression of single chain antibodies to bcl-2 in MCF-7 human breast cancer cells decreased bcl-2 levels and increased the sensitivity of these cells to drug-induced cytotoxicity (24). It was also shown that stable overexpression of bcl-x, a dominant negative inhibitor of bcl-2, also increased the sensitivity of MCF-7 cells to growth inhibitory effects of the chemotherapeutic agents VP-16 and taxol (7). Expression of bcl-2 and drug resistance of breast cancer cells is also hormone-dependent (23, 29–31). Estrogens induce bcl-2 gene and/or protein expression in T47D, ZR-75, and MCF-7 cells, and these responses are inhibited by androgens (31) and progesterins (30). Both androgens and progestins alone decrease bcl-2.

This study probes the molecular mechanism of E2-induced bcl-2 gene expression in T47D and MCF-7 breast cancer cell lines by analysis of constructs containing bcl-2 gene promoter inserts. A 70-bp distal promoter fragment (−1603 to −1534) was identified as estrogen-responsive, and this region of the promoter did not contain perfect or imperfect estrogen-responsive elements (EREs). Further analysis identified fragments of 25 (−1603 to −1579) and 21 (−1554 to −1534) bp that were estrogen-responsive. The more distal G-rich sequence bound Sp1 protein at two sites, and transcriptional activation by E2 was associated with ER/Sp1 interactions with cis-genomic Sp1 binding sites. In contrast, the E2-responsive 21-bp sequence contained a cAMP response element (CRE) that bound ATF-1 and CREB-1, and transactivation was associated with induction of cAMP by E2. Thus, transcriptional activation of bcl-2 by E2 in MCF-7 and T47D cells involves at least three different distal cis-genomic elements and does not require direct binding of ER to the bcl-2 gene promoter.

MATERIALS AND METHODS

Chemicals, Cells, and Antibodies—MCF-7 and T47D cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained in minimum essential medium (MEM) with 1 mM sodium pyruvate, 1 g of glucose, and 6 μg of insulin per liter (for MCF-7 cells) or α-MEM for T47D cells. Media for these cells were supplemented with 5% fetal bovine serum plus 10 ml/liter antibiotic-antimycotic solution. Cells were grown in 150-cm² culture flasks in an

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‡ Present address: Dept. of Rheumatology, Tokyo Women’s Hospital, Tokyo 162, Japan.

§ Sid Kyle Professor of Toxicology, To whom correspondence should be addressed: Dept. of Veterinary Physiology and Pharmacology, Texas A&M University, College Station, TX 77843-4466. Tel.: 409-845-5988; Fax: 409-862-4929; E-mail: s-safe@cvm.tamu.edu.

* The abbreviations used are: E2, 17β-estradiol; bp, base pair(s); kb, kilobase pair(s); CAT, chloramphenicol acetyltransferase; MEM, minimal essential medium; PCR, polymerase chain reaction; ER, estrogen receptor; CRE, cAMP-responsive element; ERE, estrogen-responsive element.
air: carbon dioxide (95:5) atmosphere at 37 °C. After reaching confluence, cultures were trypsinized and washed once with culture medium. Cells were passed into fresh culture flasks at the ratio of 1:2. Sp1, Sp3, ATP-1, CREB-1, CREB-2, CREM, and ER, antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The cAMP inhibitor H8 (N-(2-methylamyl)-ethyl-5-isouquinoline sulfonamide) was purchased from ICN Biomedicals, Inc. (Aurora, OH). Dulbecco’s modified Eagle’s medium’s F-12 medium without phenol red, α-MEM, phosphate-buffered saline, acetyl-coenzyme A, E2, and 100 μM antioxidant/antiinflammatory solution were purchased from Sigma. Fetal calf serum was obtained from Gibco (Paisley, UK). MEM was purchased from Life Technologies, Inc. [32P]ATP (3000 Ci/mmol) and [3H]Chloramphenicol (53 μCi/mmol) were purchased from NEN Life Science Products. Poly(dI·dC), restriction enzymes (HindIII, KpnI, and BamHI), and T4-polynucleotide kinase were purchased from Boehringer-Mannheim Biochemicals. Recombinant Sp1 and ER, proteins and the β-galactosidase enzyme assay system were purchased from Promega (Madison, WI). The human estrogen receptor (hER) expression plasmid was kindly provided by Dr. Ming-ker Tsai (Baylor College of Medicine, Houston, TX). All other chemicals and biochemicals were the highest quality available from commercial sources. Oligonucleotides were synthesized by the Gene Technology Laboratory, Texas A&M University (College Station, TX) (Table I).

**Table I**

| Synthetic oligonucleotides (location in bcl-2 gene promoter) | Sequences (sense strand)* |
|---------------------------------------------------------------|----------------------------|
| bcl-2 (1603 to 1579)                                          | 5'-CTG TGG +CC +GC +GG-3'  |
| bcl-2 (1554 to 1534)                                          | 5'-CTG TGG +CC +GC +GG-3'  |
| bcl-2 (1578 to 1534)                                          | 5'-CTG TGG +CTG TGG TGG-3' |
| bcl-2 (1603 to 1579)                                          | 5'-CTG TGG +CC +GC +GG-3'  |
| mt4-bcl-2 (1554 to 1534)                                      | 5'-CTG TGG +CTG TGG TGG-3' |

* The GC-rich sites and CRE sites are shown in bold and base mutations are underlined.

**Plasmids and Cloning**—The mutant CREB inhibitory expression plasmid (KCREB) and protein kinase A expression plasmid (pPKA) were kindly provided by Drs. Elaine Lewis and Richard Maurer, Oregon Health Science Center (Portland, OR). The plasmid bcl-2 promoter-CAT contains pbcl-2a, pbcl-2b, and pbcl-2c have been described previously (32). pbcl-2d was constructed in this laboratory by deletion of 1.2 kb (XhoI/SstI) (see Fig. 2) from pbcl-2b. Plasmids pbcl-2e, pbcl-2f, pbcl-2g, pbcl-2h, and pbcl-2i were created by PCR extension technique. All the forward primers contained a HindIII site and the reverse primers contained a KpnI site. R120 and F120 primers were used to obtain the pbcl-2h insert; R and F1, R and F2, and R and F3 primers were used to obtain inserts for pbcl-2g, pbcl-2f, and pbcl-2e, respectively. The insert sequences are given below.

R120: 5'-GGG AAG CCT GAG CTC CCG CCG CGG-3'
F120: 5'-GGG GGT ACC CTC TCC GGG GCC CCG-3'
R: 5'-GGG AAG CCT GAG CCG CCG CCG CAG-3'
F1: 5'-GGG GGT ACC GAC AGC GCC GCC CCC CAG-3'
F2: 5'-GGG GGT ACC AAA CCG GTC GGC GGG GAG-3'
F3: 5'-GGG GGT ACC TCG CGC TGG AGA GAG-3'

**Sequences 1–6**

Fragments were amplified with Vent DNA polymerase and the plasmid p18–21 as a template. After gel purification, the PCR products were digested with HindIII and KpnI and then subcloned into pUC8V5CAT. The oligonucleotides from the human bcl-2 promoter listed above were cloned into the pBLCAT2 at the HindIII and BamHI sites to give pbcl-2j, bcl-2k, bcl-2m1, bcl-2m2, bcl-2m3, bcl-2l, bcl-2m, mt1-pbcl-2m, mt2-pbcl-2m, mt3-pbcl-2m, and mt4-pbcl-2m plasmids, respectively. pPacSp1 (provided by Dr. R. Tjian, University of California, Berkeley) was digested with XhoI, and the phosphate group was removed by treatment with calf intestinal alkaline phosphatase. After being treated with 10 μg EDTA and heated at 80 °C for 15 min, the reaction was extracted with phenol/chloroform (two times) and the vector was separated from the Sp1 insert by electrophoresis and gel extraction. hER was released from the hER expression plasmid by digestion with EcoRI. The hER fragment was filled by dATP and dTTP, and an XhoI linker was ligated to the blunted hER fragment. After digestion with XhoI, the hER fragment was ligated to the pPac vector, which was treated with XhoI and calf intestinal alkaline phosphatase as described above. All ligation products were transformed into DH5α competent Escherichia coli cells, plasmids were isolated, and correct clonings and orientation were confirmed by restriction enzyme mapping and DNA sequencing using Sequitherm cycle sequencing kit from Epicentre Technologies (Madison, WI). Plasmid preparations for transfections were carried out using a Qiagen Plasmid Midi Kit.

**Transient Transfection Assays**—Cells were plated in 100-mm culture dishes, grown to 60% confluence, and transfected by the calcium phosphate method (33) with various amounts of the appropriate plasmids. For each 100-mm dish, 5–10 μg of plasmid was mixed with 500 μl of 2× HBC (NaCl 1.635% , Hepes 1.188% , NaHPO4 0.02%, pH 7.05–7.12 and 62 μl of CaCl2 (2 μ) to give a final volume of 1 ml with distilled water. The mixture of DNA and CaCl2 was added to 2× HBS dropwise with gentle vortexing and allowed to settle at 20 °C for 30 min. DNA was added to the medium dropwise, and formation of fine particles was avoided. Cells were microscopically counted during DNA-cellula interaction at 6 h and then shocked with 20% glycerol for 90 s. Medium was then changed, and cells were treated with appropriate chemicals for 24–48 h. Cells were then washed with phosphate-buffered saline and scraped from the plates. Cell lysates were prepared in 0.3 ml of 0.25 M Tris-HCl (pH 7.5) with three freeze-thaw-sonication cycles (3 min/each). After centrifugation at 4 °C for 15 min, the supernatant was incubated at 36 °C for 7 min to remove endogenous deacetylase activity and protein (100–150 μg) was incubated in a reaction mixture containing 0.25 M Tris, pH 7.5, 1 μM acetyl-CoA, 0.2 mM of [14C]Chloramphenicol at 37 °C for various times. After incubation, acetylated metabolites were isolated by extraction with ethyl acetate and separated by thin layer chromatography in chloroform: methanol (95:5). The plate was air-dried, and radioactive metabolites were quantified using a Betascope 603 Bioluminescence (In-telligen, Mountain View, CA). Cells were cotransfected with 2.0 μg of a β-galactosidase-LacZ plasmid (InVitrogen, Carlsbad, CA), and the results were used to normalize CAT activities in various treatment groups.

**Schneider Cell Maintenance and Transfection**—Cells were grown at room temperature in T-150 flasks in Schneider’s medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum (heat-inactivated at 56 °C for 30 min) and 10% antibiotic/antimycotic solution. Two ml of cells/well were pipetted to six-well plates, and after incubation for 24 h at room temperature, cells were transfected with 0.5 ml of transfection mixture containing 1 μg of pbcl-2k reporter plasmid, 1 μg of β-galactosidase, 250 μl of 2× HBS, 25 μl of 2.5 μM CaCl2, with different amounts of pPacSp1 or pPacER plasmids. The empty vector, pPac, was used to make the total amount of plasmid 4.1 μg/Incubation. After incubation for 20 h at room temperature, cells were treated with 10 μM trastuzumab (Herceptin, Genentech, South San Francisco, CA).
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FIG. 1. Transcriptional activation of bcl-2 by E2. A, mRNA levels. MCF-7 or T47D cells were treated with Me2SO or 10 nM E2 for 12 h; mRNA was isolated and quantitated as described under "Materials and Methods." E2 significantly induced bcl-2 mRNA levels in both cell lines (p < 0.05), and results are expressed as means ± S.E. for at least three separate determinations. B, CAT activity in T47D cells. The constructs were transiently transfected into T47D cells, treated with Me2SO or E2, and CAT activity was determined as described under "Materials and Methods." Significant induction (p < 0.05) by E2 was observed for all constructs, and results are expressed as means ± S.E. for at least three separate determinations for each treatment group. C, CAT activity in T47D cells. The constructs were transiently transfected into T47D cells, treated with Me2SO or E2, and CAT activity was determined as described under "Materials and Methods." Significant induction (p < 0.05) by E2 was observed only for pbl-2d and pbl-2e, and results are expressed as means ± S.D. for at least three separate determinations for each treatment group.

E2 or solvent carrier (ethanol) for about 48 h and harvested by scraping. Data are combined from two separate experiments using the same experimental protocols, and transfection efficiency was normalized with β-galactosidase activity as described above.

Reverse Transcription-PCR for Determination of mRNA Level—Total RNA was isolated from the guanidinium thiocyanate/acid phenol extraction method (34); 200 ng of RNA was reverse transcribed using murine leukemia virus reverse transcriptase obtained from Perkin Elmer. The primer size of bcl-2 mRNA was 385 bp; sequence was as follows.

Forward: 5′-ACT TGT GGC CCA GAT AGC CAC GCA G-3′
Reverse: 5′-CGA CTT CGG CAT GTC CAG CCA G-3′

SEQUENCE 7

Nuclear Extract Preparation and Gel Mobility Shift Assay—Nuclear extracts were prepared from MCF-7 cells treated with Me2SO (0.1% v/v) or 10 nM E2 for 4 h utilizing cells maintained in serum-free medium for 3 days. Oligonucleotides were annealed and labeled at the 5′ end using T4-polynucleotide kinase and [γ-32P]ATP. Gel electrophoretic mobility shift assays were performed by incubating 5–40 ng of pure Sp1 protein in 25 μl of 1× binding buffer (6% glycerol, 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 10 mM Tris-HCl, pH 8.0), 0.1 mg/ml BSA. After incubation for 10 min at 4 °C, [32P]-labeled oligonucleotide (50,000 cpm) was added to the reaction mixture in the presence of 1 μg of poly(dI-dC) and incubated for an additional 15 min at 25 °C. Excess unlabeled DNA was added 5 min before adding [32P]-labeled oligonucleotides. The following procedure was used for ERα enhanced Sp1 binding studies. (a) 200–800 fmol of pure hER protein in 1× binding buffer containing 40 ng E2 and BSA were added and incubated for 15 min at 4 °C; (b) 5–20 ng of Sp1 protein was added to the mixture and incubated on ice for 5 min; (c) [32P]-labeled oligonucleotides (50,000 cpm) were added to the reaction mixture in the presence of 1 μg of poly(dI-dC), and the mixture was incubated for an additional 15 min at 20 °C. Nuclear extracts from control (Me2SO) or E2-treated cells were incubated for 15 min at 0 °C in HEGD (2 mM Hepes, 1.5 mM EDTA, 1.0 mM dithiothreitol, 10% glycerol (ν/ν), pH 7.6) buffer with 1 mg of poly(dI-dC) to bind nonspecific DNA-binding proteins, and 200–500-fold excess of unlabeled wild-type or mutant oligonucleotide competitors for the competition experiments. Following addition of [32P]-labeled DNA, the mixture (final volume: 20 μl) was incubated for an additional 20 min at 20 °C. For experiments using saturation concentrations of Sp1 protein (10–30 ng) alone or in combination with 200 fmol of ERα protein, the ERα and Sp1 proteins were added simultaneously (early addition) or ERα was added 5 min after the radiolabeled probe (late addition). For gel supershift experiments, antibodies were added after standard gel mobility shift assay procedure and reactions were further incubated for 20–30 min at 20 °C. Samples were loaded onto a 5% polyacrylamide gel (acylamide-bisacrylamide ratio, 30:0.8) and run in 1× TBE buffer (0.09 M Tris, 0.09 boric acid, and 2 mM EDTA, pH 8.3) at 200 V at 4 °C. Protein-DNA binding was visualized by autoradiography and quantitated by densitometry using the Molecular Dynamics Zero-D software package (Molecular Dynamics, Sunnyvale, CA) and a Sharp JX-530 scanner (Mahwah, NJ) and subjected to autoradiography using

![Fig. 1](image-url)
Recombinant Sp1 protein was incubated with 32P-labeled oligonucleotide eliminated retarded band formation (data not shown).

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\text{bcl-2}; \text{competition with 100-fold excess of a consensus Sp1 oligonucleotide as indicated under "Materials and Methods." A retarded band was observed using only radiolabeled bcl-2 and bcl-2k but not Sp1 protein plus increasing concentrations of ER protein. The protein-DNA binding reactions were incubated on ice for 5 min, and then equilibrated to room temperature for 10 min; 2 μl of 1:2 dilution of purified SsI (New England Biolabs) was then added to the equilibrated reactions, which were then incubated at 30 °C for 5 min. After 15 min at 75 °C, 10 μl of freshly made deamination denaturation buffer (0.9 N NaOH, 25 mM EDTA, 0.2 mg/ml sheared salmon sperm DNA) was added. Following 5 min at 95 °C, 200 μl of a saturated solution of sodium metabisulfite (2 M solution) was added and the samples were processed as described (35, 36). The primers used to amplify from the purified deaminated plasmid DNA were bcl-2 B1 (5'-TCCACAAACCTAAACAAAAAACC-3') and bcl-2 B2 (5'-GTTTTTTTTATTTTATTTTTTTG-3'). PCR products were purified and cleaned up using the Wizard PCR prep kit from Promega Corp. Purified PCR products were sequenced with radiolabeled bcl-2 B1 primer in the presence of a 5 μM solution of dATP, dCTP, and dGTP using 50 μM ddGTP as the stop nucleotide. Sequitherm 10× buffer and Sequitherm thermostable DNA polymerase (Epicentre Technologies, Madison, WI) were used for the sequencing reactions. Sequencing reactions were run on 6% polyacrylamide-urea sequencing gels. The dried gels were exposed to a phosphor screen for 12 h and analyzed on a Molecular Dynamics Storm instrument.

**RESULTS**

**Transcriptional Activation of bcl-2 mRNA and Promoter-Reporter Constructs by E2**—Fig. 1A summarizes the effects of E2 on bcl-2 mRNA levels in MCF-7 and T47D cells, and these data are comparable to those previously reported (23, 29–31). Deletion analysis of the 7.0-kb fragment from the bcl-2 gene promoter (Fig. 1B) focused on the −3.0 kb region utilizing transient transfection studies in T47D cells. These plasmids contained bcl-2 gene promoter inserts in pUCSVO-CAT containing an SV40 early-region promoter 5’ of the HindIII site (32), and the construct was readily transfected into T47D but not MCF-7 cells due to poor conversion in these cells. Results obtained for bcl-2a–bcl-2d showed that treatment with E2 resulted in a 3.4–6.9-fold increase in CAT activity and E2 responsiveness was retained in a promoter region from −1647 to −1293. Further analysis of a series of constructs containing bcl-2 gene promoter inserts from within the −1647/+1289 region (bcl-2d–bcl2i; Fig. 1C) indicated that hormone responsiveness was localized within a minimal 70-bp sequence from −1603 to −1534.

Synthetic oligonucleotides within −1603/−1534 region of the bcl-2 gene promoter were cloned into pBLCAT3, transiently transfected into MCF-7 cells, and treated with 10 nM E2 or Me2SO. The results (Fig. 2A) indicate that E2 responsiveness of the 71-bp construct (bcl-2j) is associated with a 25-bp up-protein-DNA binding as indicated under “Materials and Methods.” Sp1 antibody supershifted the major Sp1-DNA complex (lanes 3 and 4), and a weak supershifted Sp3-DNA complex could also be detected.
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Interactions of Sp1 and ERα Proteins with Oligonucleotides Derived from the bcl-2 Gene Promoter (−1603 to −1534) and Transactivation in Schneider SL-2 Cells—The E2-responsive 70-bp sequence (−1603 to −1534) contains at least two G-rich regions; therefore, binding of Sp1 protein was investigated in gel mobility shift assays. The results illustrated in Fig. 3A show that purified recombinant Sp1 protein binds to a consensus [32P]Sp1 oligonucleotide to form a retarded band (lane 1). Similar results were obtained for the 70-bp bcl-2j (−1603 to −1534) oligonucleotide (lane 3) and G-rich 25-bp bcl-2k (−1603 to −1579) sequence (lane 5) but not for the downstream 45-bp bcl-2l (−1578 to −1534) oligonucleotide (lane 4). These results confirm that human Sp1 protein is capable of binding the G-rich 25-bp bcl-2k oligonucleotide; moreover, incubation of MCF-7 cell nuclear extracts with [32P]bcl-2l gave a series of retarded bands (Fig. 3B, lanes 1–4) typical of Sp1/Sp3 protein complexes when tested for binding to other GC-rich sequences (37–40). Coincubation with Sp1 (lanes 3 and 4) or Sp3 (lanes 5 and 6) antibodies gave supershifted bands confirming Sp1/Sp3 interactions with [32P]bcl-2l. The Sp1-DNA complex was the major retarded band (lanes 2 and 3) as determined by Sp1 binding and Sp1 antibody supershift experiments (lanes 3 and 4), whereas weaker Sp3-DNA supershifted complexes were observed, producing only diffuse bands (lanes 5 and 6).

Binding of human recombinant Sp1 protein to [32P]bcl-2k oligonucleotide was concentration-dependent over a range of Sp1 protein concentrations from 5–20 ng incubation (lanes 2–4) and intensity of the retarded band complex was decreased after coincubation with unlabeled wild-type (lane 5) but not mutant (lane 6) Sp1 oligonucleotides. Binding of [32P]bcl-2k1 (lanes 7–9) and [32P]bcl-2km2 (lanes 10–12) to recombinant Sp1 protein was also observed; it was apparent that the former oligonucleotide exhibited lower binding affinity for comparable amounts of Sp1 protein as higher amounts (20–40 ng) were required to observe retarded complexes (bound DNA). The effects of ERα on Sp1-DNA complex formation were also investigated (Fig. 4B) using [32P]-labeled wild-type (lanes 1–4) and mutant bcl-2k oligonucleotides (lanes 5–10). Although absolute band intensities were variable, the intensity of the Sp1-DNA complex (bound DNA) after incubation of [32P]bcl-2k, [32P]bcl-2km1, and [32P]bcl-2km2 with Sp1 protein and increasing amounts of ERα protein (0.2–0.8 pmol) resulted in a relative 2–4-fold increase in the intensity of the Sp1-DNA retarded band. All of these enhanced binding studies utilized subsaturating amounts of Sp1 protein. Therefore, the gel mobility shift

Fig. 4. Sp1 binding to G-rich sites and enhancement by ERα protein. A, binding of Sp1 protein to wild-type and mutant bcl-2k oligonucleotides. Recombinant Sp1 protein was incubated with [32P]-labeled oligonucleotide as indicated, and protein-DNA binding was analyzed by gel mobility shift assays as indicated under "Materials and Methods." A retarded band was observed using wild-type and mutant [32P]bcl-2k oligonucleotides; however, higher amounts of Sp1 protein were required using [32P]bcl-2km1 due to weaker binding. B, enhanced Sp1-DNA binding by ERα protein. Recombinant Sp1 protein was incubated with [32P]-labeled oligonucleotides and different amounts of ERα, as indicated, and protein-DNA binding was analyzed by gel mobility shift assays as indicated under "Materials and Methods." A retarded band was observed using wild-type and mutant bcl-2k oligonucleotides and 0.2–0.8 pmol of ERα caused a dose-dependent increase (2–6-fold) in Sp1-DNA complex formation. Competition with 100-fold excess of a consensus Sp1 oligonucleotide eliminated retarded band formation (data not shown).
assays were then repeated with a consensus Sp1 oligonucleotide and saturating concentrations of Sp1 protein followed by early or late addition of ERα protein (Fig. 5A). Supershifted bands were not observed using these conditions; moreover, similar results were obtained using nuclear extracts from Schneider Drosophila SL2 cells transfected with ERα or Sp1 expression plasmid (Fig. 5B). The failure to observe supershifted ERα/Sp1-DNA ternary complexes in these studies was similar to previous reports using GC-rich oligonucleotides from other E2-responsive gene promoters (41–45).

SssI is a Cpg viral methylase that we have utilized to further probe ERα/Sp1 interactions. The bcl-2k region of the bcl-2 gene promoter contains a CpG site and in SssI in vitro footprinting studies (Fig. 5C), pure Sp1 protein alone (lanes 2–4) only slightly protected the CpG site (○) in bcl-2k. Increasing concentrations of pure human recombinant ERα only slightly increased protection at the CpG site (lanes 5 and 6); however, incubation with ERα (100 and 200 fmol) plus the lowest amount of Sp1 protein (20 ng) completely protected this site and confirmed interactions between ERα and Sp1 proteins in this region of the bcl-2 gene promoter. However, the extent of protection is likely to span all of this region, and sites proximal to it since the Sp1 enhancement is also seen 36 bases downstream of bcl-2k (○). A ghost CpG band, which does not correspond to any known CpG in this sequence and is a likely result of a PCR artifact, appears at random in lanes 1, 5, and 7 (∗).

Interactions of ERα and Sp1 protein in the bcl-2k region of the promoter were further investigated in Schneider SL2 cells that do not express these transcription factors. In cells transfected with pCbl2k-2 and different amount of Sp1 expression plasmid alone (10–2000 ng), there was a 30-fold increase in CAT activity. In cells cotransfected with Sp1 (100 ng) and ERα (2–50 ng) expression plasmids, there was a maximal 3.6-fold increase in CAT activity (ERα = 40 ng) compared with cells transfected with Sp1 plasmid alone. ERα expression plasmid alone (50 ng) did not affect basal CAT activity (data not shown). Thus, the functional interactions of ERα and Sp1 observed at G-rich sites in the bcl-2 gene promoter in human breast cancer cells (Fig. 2) are also observed in Schneider SL2 cells.

**Fig. 5. Further analysis of ERα/Sp1 interactions.** Saturating amounts of recombinant Sp1 protein (10–30 ng) (A) alone or in combination with ERα protein (200 fmol) and nuclear extracts from Schneider SL2 cells transiently transfected with Sp1 and ERα expression plasmids (B) were incubated with consensus [32P]-labeled Sp1 oligonucleotide and analyzed by gel mobility shift assays as described under “Materials and Methods.” Supershifted bands were not detected in any of the coincubation studies or at higher doses of ERα protein (data not shown). C, in vitro footprinting experiments. In vitro footprinting of the G-rich bcl-2k region of the promoter was determined by SssI-dependent methylation of CpG sites after coincubation with recombinant Sp1 and ERα proteins and their combination as described under “Materials and Methods.” Incubation with Sp1 (lanes 2–4) or ERα (lanes 5 and 6) proteins alone caused minimal effects on methylation of the CpG site (○), whereas coincubation of Sp1 plus ERα (lanes 7 and 8) protected this site from methylation. A ghost CpG band that does not correspond to any known CpG in this sequence and is a likely result of a PCR artifact appears at random in lanes 1, 5, and 7 (∗). D, transactivation in Schneider SL2 cells. SL2 cells were transfected with pCbl2k and expression plasmids for Sp1 and ERα (or their combination), and CAT activity was determined as described under “Materials and Methods.” There was a 1.7-, 2.7-, 30-, and 30-fold increase in CAT activity in cells transfected with 10, 100, 1000, and 2000 ng of Sp1 expression plasmid alone compared with untreated (control) cells (mean of two separate experiments). In cells transfected with Sp1 expression plasmid alone (arbitrarily set at 100%), cotreatment with 10–8 M E2 and 2, 10, or 50 ng of ERα expression plasmid resulted in a 2.8 ± 0.13-, 3.6 ± 0.15-, and 3.4 ± 0.30-fold, respectively, increase in CAT activity. Results are expressed as means ± S.E. for three replicates for each treatment group.
Fig. 6. Binding of nuclear extracts to \[^{32}P\]bcl-2m. \(A\), oligonucleotide competition and direct binding studies. \[^{32}P\]bcl-2m was incubated with nuclear extracts from E2-treated MCF-7 cells (lane 1) and a series of unlabeled oligonucleotides (100-fold excess) (lanes 2–10) and gel mobility shift assays were determined as described under “Materials and Methods.” Five major bands were detected (B1–B5), and excess wild-type bcl-2m competitively decreased their formation. Unlabeled mutant bcl-2m oligonucleotides competitively decreased some of these bands (lanes 3, 4, and 6); however, mt3-bcl-2m exhibited activity similar to wild-type oligonucleotide. Wild-type consensus CRE decreased intensities of bands 1 and 3–5 (lane 7), whereas mutant CRE (lane 8), Sp1 (lane 9), and ERE (lane 10) oligonucleotides exhibit minimal effects. \(B\), antibody supershift studies. \[^{32}P\]bcl-2m was incubated with nuclear extracts from E2-treated MCF-7 cells (lane 1) and a series of protein antibodies (lanes 2–8) and non-specific IgG (lane 9). Gel mobility shift assays were determined as described under “Materials and Methods.” CREB-1 antibody supershifted bands 3 and 4 and decreased intensity of band 5 (lane 2) and one of the ATF-1 antibodies (lane 4) also supershifted in bands 3 and 4. Supershifts were not observed for the CREB-2, CREM-1, Sp-1, or ER\(_a\) antibodies (lanes 5–8).

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-1534): Interactions with ATF-1 and CREB-1—In addition to binding of transcription factors Sp1 and ER\(_a\) to upstream G-rich sequences, interactions of \[^{32}P\]bcl-2m (−1554 to −1534) with other nuclear proteins was also investigated. Preliminary studies showed that ER\(_a\), Sp1 or their combination did not form a complex with this oligonucleotide (data not shown). The results in Fig. 6A summarize binding of nuclear extracts from E2-treated MCF-7 cells with \[^{32}P\]bcl-2m (the 21-bp downstream sequence). At least five retarded bands were detected (B1–B5) (lane 1). Competition with 100-fold excess unlabeled bcl-2m resulted in decreased intensities of all retarded bands (lane 2). Competition with unlabeled oligonucleotides mutated within the bcl-2m (−1550/−1545) sequence only slightly reduced the intensity of retarded band complexes (lanes 3 and 4).

In contrast, mt3-bcl-2m, containing a mutation of the core DRE sequence, competitively decreased all bands (B1–B5; similar to wild-type bcl-2m) (lane 5). Unlabeled CRE oligonucleotide decreased intensity or eliminated B1, B3, B4, and B5 (but not B2) (lane 7), whereas mutant CRE, Sp1, and ERE oligonucleotides were relatively inactive as competitors (lanes 8–10, respectively).

Direct binding studies using \[^{32}P\]-labeled bcl-2m, mt1-bcl-2m, mt2–2bcl-2m, and mt3-bcl-2m and MCF-7 nuclear extracts (lanes 11–14) corroborated the results obtained by competitive binding studies. The five bands formed with wild-type bcl-2m were decreased in intensity after incubation with mt1-bcl-2m and mt2-bcl-2m, whereas all five bands were observed with \[^{32}P\]mt3-bcl-2m (lane 14). In addition, a new band (B1’) was
observed with $^{32}$Pmt2-bcl-2m (lane 3). Antibody supershift experiments (Fig. 6B) were determined with nuclear extracts incubated with $^{32}$Pbcl-2m alone (lane 1) or in combination with antibodies to ATF-1 (a and b), CREB-1, CREB-2, CREM-1, Sp1, Er$\alpha$ (lanes 2-8), or nonspecific IgG (lane 9). The results show that only ATF-1 and CREB-1 formed supershifted complexes (Supershift →).

Cyclic AMP Responsiveness of pbcl-21 (−1578 to −1534)—The finding that CREB-1 and ATF-1 bound to $^{32}$Pbcl-2m oligonucleotide prompted us to investigate the cAMP responsiveness of the 21-bp downstream sequence using transient transfection experiments and the pbcl-21 reporter plasmid that contains the −1578 to −1534 region of the promoter (Fig. 7). The results showed that 8-bromo cAMP significantly induced CAT activity, and similar results were observed after cotransfection with an expression plasmid for protein kinase A (pPKA). Thus, both cAMP and E2 (Fig. 2) induced pbcl-21 in MCF-7 cells. In contrast, cotransfection with a dominant negative form of CREB (KCReB expression plasmid) significantly decreased CAT activity. In addition, the cAMP inhibitor, H8, blocked transcriptional activation by E2 in cells transfected with pbcl-21. These results demonstrate that E2 responsiveness of the −1578 to −1534 region of the bcl-2 gene promoter is due to up-regulation of the cAMP/protein kinase A pathway.

DISCUSSION

bcl-2 gene or protein expression in breast cancer patients correlated positively with ER$\alpha$-positive tumors and is a prognostic factor for increased disease-free survival for breast cancer patients (17–21). In contrast, bcl-2 plays a role in drug resistance of ER$\alpha$-positive breast cancer cells due, in part, to inhibition of drug-induced apoptosis (23–28). Other studies have shown that the tumor suppressor gene p53 can inhibit expression of bcl-2 (32, 46), and this also correlates with an inverse relationship observed between bcl-2 and p53 proteins in breast tumors determined by immunohistochemical analysis (19). Thus, the role of bcl-2 in breast cancer may be complex and dependent on tumor stage, chemotherapeutic regimens and coexpression of other factors critical for cancer cell growth, differentiation and apoptosis.

E2 induces bcl-2 gene expression in ER$\alpha$-positive breast cancer cells (23, 29–31) (Fig. 1). In this study, we have investigated the molecular determinants associated with this hormone-induced response of the bcl-2 gene. Analysis of the bcl-2 gene promoter in MCF-7 and T47D breast cancer cells (Fig. 1) resulted in identification of an E2-responsive distal region at −1647 to −1289 and results of further 5′ and 3′ deletions identified a 70-bp sequence (−1603/−1532) that retained hormone-inducibility in transient transfection assays in both T47D and MCF-7 cells (Figs. 1 and 2). The 70-bp oligonucleotide (bcl-2j) did not contain a perfect (or imperfect) palindromic ERE and gel mobility shift assays showed that $^{32}$Pbcl-2j did not bind ER$\alpha$ to form a retarded band (data not shown). Subsequent analysis of the 70-bp region indicates that E2 responsiveness is complex and requires both upstream (−1603 to −1579) and downstream (−1554 to −1534) sequences (Fig. 2A), suggesting possible regulation by multiple transcription factors and cis-elements.

The upstream −1603 to −1579 region of the bcl-2 gene contains two G-rich sequences and mutations of both elements are required for loss of E2-induced transactivation. In gel mobility shift assays, both $^{32}$Pbcl-2j (−1603 to −1534) and $^{32}$Pbcl-2k (−1603 to −1579) form a retarded band with Sp1 protein using either recombinant Sp1 protein or nuclear extracts from MCF-7 cells (Fig. 3). The two Sp1 binding sites at −1601 (5′-GGGCGTGG-3′) and −1588 (3′-GGAGGGG-5′) differ from the consensus GC-rich motif; however, both G-rich motifs have previously been identified as Sp1 binding sites in human CD14, rat luteinizing hormone, rabbit lung surfactant protein B, and rhesus growth hormone-variant gene promoters (37–40). Functional ER$\alpha$-Sp1 interactions with the two G-rich sequences in the bcl-2 gene promoter were also confirmed in transient transfection assays in Schneider SL-2 cells in which ER$\alpha$ enhanced Sp1 action (3.6-fold) in cells transfected with pbcl-2k (Fig. 5). ER$\alpha$/Sp1 action at GC-rich sites have been identified in the heat shock protein 27, cathepsin D, retinoic acid receptor a1, c-Fos, and adenosine deaminase gene promoters (41–45), and PR/Sp1 plays a role in hormone-induced p21 gene expression (47).

Typically, ER$\alpha$ enhances Sp1-DNA binding in gel mobility shift assays but does not form a supershifted ternary complex (41–45), and the results in Figs. 3 and 4 show enhanced Sp1 binding to $^{32}$Pbcl-2k after coincubation with ER$\alpha$ as previously reported for other GC-rich sequences. In previous studies, enhanced Sp1-DNA binding by ER$\alpha$ was observed using nonsaturating concentrations of Sp1 protein, and we hypothesized that failure to observe a supershifted complex may be due to low (nonsaturating) Sp1 protein concentrations. However, coincubation of ER$\alpha$ with saturating amounts of Sp1 protein or coincubation of Sp1 and ER$\alpha$ expressed in Schneider SL-2 did not result in formation of a supershifted complex (Fig. 5). Protein-enhanced binding of other transcription factors to their cognate cis-genomic elements has been reported for several nuclear proteins and usually involves enhanced rate of retarded band formation or a decreased rate of protein-DNA dissociation (43, 48–54). Methylation of CpG sites by the viral methylase has been utilized as a highly sensitive technique for footprinting weak protein-DNA interactions and, therefore, we used this method to examine ER$\alpha$/Sp1 interactions in the E2-responsive G-rich sequence of the bcl-2 gene promoter (Fig.
The results show that ERα or Sp1 protein alone exhibit minimal binding at the CpG site (●); however, after coimmunoprecipitation with both proteins, this site was completely protected and methylation was not observed at this site. These results provide important new confirmation of ERα-Sp1 interactions at G/C-rich sites and complement results of previous studies on this transcription factor complex (41–45).

The downstream E2-responsive region at −1578 to −1534 was further localized to a 21-bp sequence at −1554 to −1534 and results of gel mobility shift assays indicated that neither Sp1 (Fig. 3) or ERα (data not shown) proteins bound to this region of the bcl-2 gene promoter. The 21-bp sequence contains this transcription factor complex (41–45).

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