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Induction of *bcl*-2 Expression by Phosphorylated CREB Proteins during B-Cell Activation and Rescue from Apoptosis

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Engagement of surface immunoglobulin on mature B cells leads to rescue from apoptosis and to proliferation. Levels of *bcl*-2 mRNA and protein increase with cross-linking of surface immunoglobulin. We have located the major positive regulatory region for control of *bcl*-2 expression in B cells in the 5′-flanking region. The positive region can be divided into an upstream and a downstream regulatory region. The downstream regulatory region contains a cyclic AMP-responsive element (CRE). We show by antibody supershift experiments and UV cross-linking followed by denaturing polyacrylamide gel electrophoresis that both CREB and ATF family members bind to this region in vitro. Mutations of the CRE site that result in loss of CREB binding also lead to loss of functional activity of the *bcl*-2 promoter in transient-transfection assays. The presence of an active CRE site in the *bcl*-2 promoter implies that the regulation of *bcl*-2 expression is linked to a signal transduction pathway in B cells. Treatment of the mature B-cell line BAL-17 with either anti-immunoglobulin M or phorbol 12-myristate 13-acetate leads to an increase in *bcl*-2 expression that is mediated by the CRE site. Treatment of the more immature B-cell line, Ramos, with phorbol esters rescues the cells from calcium-dependent apoptosis. *bcl*-2 expression is increased following phorbol ester treatment, and the increased expression is dependent on the CRE site. These stimuli result in phosphorylation of CREB at serine 133. The phosphorylation of CREB that results in activation is mediated by protein kinase C rather than by protein kinase A. Although the CRE site is necessary, optimal induction of *bcl*-2 expression requires participation of the upstream regulatory element, suggesting that phosphorylation of CREB alters its interaction with the upstream regulatory element. The CRE site in the *bcl*-2 promoter appears to play a major role in the induction of *bcl*-2 expression during the activation of mature B cells and during the rescue of immature B cells from apoptosis. It is possible that the CRE site is responsible for induction of *bcl*-2 expression in other cell types, particularly those in which protein kinase C is involved.

The induction of a humoral immune response is complex. It involves initial activation of resting B cells, subsequent proliferation of the activated precursors, and differentiation of the activated cells into mature immunoglobulin-secreting cells. The process of differentiation has been studied by using *Staphylococcus aureus* Cowan 1 to bind to the antigen receptor because it can induce B-cell DNA synthesis in a T-cell-independent manner (16, 48). One of the many changes in gene expression during B-cell activation is an increase in the expression of *bcl*-2 at the mRNA level (22, 45).

Engagement of the B-cell antigen receptor leads to differing outcomes depending on the stage of development of the B cell. Apoptosis plays a key role in this process. In the bone marrow, self-reactive immature surface IgM^+ IgD^- B cells are eliminated by apoptosis; this process leads to induction of tolerance. At the pro-B-cell stage, as immunoglobulin (Ig) gene rearrangement is occurring, the vast majority of cells die by apoptosis. Binding of antigen to the antigen receptor in immature B cells leads to death by apoptosis (13, 26). In cell line models, this is associated with a decrease in the level of *bcl*-2 mRNA (14). Anti-CD40 antibodies protect against apoptosis and result in an increase in the expression of *bcl*-2 (52).

Apoptosis plays a role in the development of mature lymphocytes. In the germinal centers, widespread apoptosis is observed. Engagement of surface Ig leads to rescue from apoptosis. Increased expression of *bcl*-2 is observed when germinal-center cells are prevented from undergoing apoptosis (33, 35). Binding of antigen to the B-cell receptor in resting B cells leads to proliferation. Binding of antigen to the B-cell receptor on either germinal-center cells or resting B cells leads to a similar pattern of second-messenger induction, including activation of Src and Src-related protein kinases (4, 60), phosphorylation on tyrosine residues of a number of substrates (3, 6, 20, 31), engagement of G proteins (19, 25, 39), increased inositol 1,4,5-triphosphate levels, increased intracellular calcium levels, and activation of protein kinase C (PKC) (5, 9). Activation of PKC leads to activation of NF-κB and AP-1, as well as other transcription factors (10, 34, 54).

Burkitt's lymphoma cells undergo apoptosis after ligation of surface IgM or treatment with a calcium ionophore (2, 7, 18, 23, 28, 53). Group I Burkitt's lymphoma cells display a surface phenotype characteristic of germinal-center cells, and they have been used as a model for germinal-center B cells (28). Activation of PKC by phorbol esters reduces apoptosis induced by calcium ionophores or surface IgM ligation (53). Apoptosis can also be triggered by inhibitors of PKC. High-level expression of Bcl-2 protects Burkitt's lymphoma cells from apoptosis induced by ionomycin or surface IgM cross-linking (28, 42). Treatment of Burkitt's lymphoma cells with phorbol esters causes an up-regulation of *bcl*-2 mRNA and protein (18). Tumor necrosis factor alpha protects Burkitt's lymphoma cell

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lines from apoptosis induced by ionomycin by up-regulation of *bcl*-2 by a mechanism that is dependent on PKC (18).

The major transcriptional promoter for *bcl*-2, P1, is located 1,386 to 1,423 bp upstream of the translation start site (50). This is a TATA-less, GC-rich promoter that displays multiple transcription state sites. A minor promoter, P2, which is used in some cell types, is located 1.3 kb downstream from the first one (50). Little information is available concerning the transcriptional control of the *bcl*-2 gene. A negative regulatory element upstream of the P2 promoter has been described previously (61). The proteins that bind to this element have not been identified, although p53 was shown to mediate downregulation of *bcl*-2 either directly or indirectly through a 195-bp fragment of this region (37). We have previously described three π1-binding sites which are negative regulators of *bcl*-2 expression in pre-B cells (8). Normal pre-B cells express very little *bcl*-2, and extensive cell death by apoptosis occurs at this developmental stage. Bcl-2 protein levels are increased in mature B cells. We have found that the three π1 sites are not functional in mature B cells (8). In the present study, we characterize the regulatory regions, including a cyclic AMP (cAMP)-responsive element (CRE), that are responsible for the positive regulation of *bcl*-2 expression in both pre-B cells and mature B cells.

Elevation of intracellular cAMP levels can result in either stimulation or repression of specific gene expression; most of these genes contain one or more CREs. The consensus CRE sequence is GTGACGTCA. cAMP binds to the regulatory subunit of PKA and releases the active catalytic subunit. This subunit phosphorylates the transactivation domain of CRE-binding protein (CREB), which then induces the expression of genes containing CREs. Mutation of the phosphoacceptor site (Ser-133) results in a dominant negative form of CREB (1, 51). A number of CREBs, including CREB, CRE modulator, and several activating transcription factors (ATFs), have been described. The CREBs are basic leucine zipper transcription factors and are active as either homo- or heterodimers. Some of the ATFs heterodimerize with members of the Jun/Fos family of proteins (for reviews, see references 30, 32, and 36).

We have elucidated the mechanism of increased *bcl*-2 expression during B-cell activation in mature B cells and during rescue from calcium-dependent apoptosis of immature B cells. We have found that a CRE mediates the increase in *bcl*-2 expression following surface Ig cross-linking in mature B cells or treatment with phorbol esters in both mature and immature B cells. Furthermore, we present evidence that PKC is responsible for the activation of CREB by phosphorylation. Optimal *bcl*-2 activation occurs when the phosphorylated CREB interacts with an upstream regulatory region, suggesting that phosphorylation changes the interaction of CREB with neighboring promoter elements.

MATERIALS AND METHODS

Plasmid constructs. A DNA fragment from *Bam*HI (−3934) to *Sac*I (−1287) of the human *bcl*-2 gene (a generous gift from Michael Cleary, Stanford University) was inserted into a luciferase reporter vector with *Pst*I linkers. Numbering of the *bcl*-2 sequence is relative to the translation start site. Progressive 5′ truncations of the *bcl*-2 sequence by restriction digestion gave rise to constructs spanning the sequence from *Sac*I (−1287) to *Xho*I (−2857), *Avr*II (−2337), *Not*I (−1794), *Sac*II (−1743), *Sac*II (−1693), *Sac*II (−1640), *Bsr*FI (−1526), and *Apa*I (−1337). Additional 5′ truncations were generated by PCR subfragment cloning to form constructs spanning the sequence from *Sac*I (−1287) to introduced *Bam*HI sites at −1473 and −1413 (see Fig. 1).

To study the activity observed between *Sac*II (−1640) and *Bsr*FI (−1526) in more detail, subfragments of this area generated by PCR cloning or oligonucleotide synthesis were inserted into a polylinker at the *Bsr*FI site in the −1526–*bcl*-2 plasmid constructed as described above. Progressive 5′-subfragment truncations spanning from −1526 to −1640, to −1611, to −1578, to −1564, and to −1552 were inserted in this polylinker. Interposition of the polylinker between contiguous *bcl*-2 sequences at site −1526 did not alter promoter activity when compared with analogous controls without polylinker (data not shown). A plasmid containing the wild-type upstream regulatory element (WtURE) was constructed in the same manner by inserting the PCR-cloned sequence −1640 to −1544 in the polylinker 5′ of the −1526–*bcl*-2 plasmid. A plasmid containing the wild-type downstream regulatory element (WtDRE), including the endogenous *bcl*-2 CRE site, was constructed by inserting the synthesized oligonucleotide sequence −1552 through −1535 in the polylinker 5′ of the −1526–*bcl*-2 plasmid. Plasmids with mutations in and near the CRE site were constructed similarly with the sequences shown in Table 1. The following base pairs were mutated (with plasmid name in parentheses): −1552 and −1548 (mC1,4); −1551 through −1549 (mC2–3); −1547 and −1545 (mC6,8); −1541 and −1540 (mC12,13); −1540 and −1539 (mC13,14); and −1540 through −1536 (mC13–17). A construct (MutCRE) with the CRE site changed from GTGACGTTA to GTGA<u>AT</u>TTA was prepared from the −1640 construct with the Quikchange site-directed mutagenesis kit (Stratagene). A construct containing two CRE sites was prepared by inserting CRE oligonucleotides with *Sal*I and *Xho*I ends into the *Sal*I site of the pGL2 promoter, a luciferase reporter gene linked to the simian virus 40 promoter (Promega). A similar construct containing two copies of the Ig heavy-chain enhancer π1 site has been described previously (8). All plasmid sequences were confirmed by the dideoxynucleotide method (Sequenase; U.S. Biochemical). The CRE-dependent plasmid (*Bgl*II CAT) and Rous sarcoma virus (RSV) CREB and RSV CREB M1 were obtained from Marc Montminy, Salk Institute. The *Bgl*II CAT construct contains the somatostatin gene promoter from −71 to +53 linked to the chloramphenicol acetyltransferase (CAT) reporter gene (40). RSV CREB expresses full-length CREB from the RSV promoter, and RSV CREB M1 expresses full-length CREB with serine 133 changed to alanine (21).

Cell lines and transient-transfection assays. Nall-1, a human pre-B-cell line, and DHL-9, a human mature B-cell line, were cultured in RPMI medium supplemented with 10% fetal calf serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 2 mM L-glutamine. Neither cell line has a translocation involving the *bcl*-2 locus. The Epstein-Barr virus-negative Burkitt's lymphoma cell line, Ramos, was grown in RPMI medium supplemented with 10% fetal calf serum. BAL-17 cells, a murine B-lymphoma cell line, were kindly provided by William Paul and Richard Asofsky, National Institutes of Health, Bethesda, Md. BAL-17 cells are a model for mature B cells and were cultured in RPMI medium supplemented with 5% fetal calf serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 2 mM L-glutamine, 10% NCTC 109, and 0.1% β-mercaptoethanol. Cell viability was determined by trypan blue exclusion.

DNA transfections were performed with cells in the log phase (5×10^5 to 6×10^5 cells per ml). The cells were washed with RPMI medium and resuspended at 3×10^7 cells per ml in RPMI medium containing 26 μg of DEAE-dextran per ml (17). A total of 10 to 20 μg of plasmid DNA was added, and electroporation was performed with a Bio-Rad gene pulser at 960 μF and 350 mV for DHL-9, 320 mV for Ramos, 300 mV for Nall-1, or 400 mV for BAL-17 cells. Transfected cells were cultured in 25 ml of supplemented RPMI medium for 48 h. Reporter gene activity was determined by the luciferase assay system (Promega), and luminescence was quantitated with an LKB 1251 luminometer. Variation in transfection efficiency was controlled for by cotransfection with 5 μg of an RSV long terminal repeat–β-galactosidase or a simian virus 40 promoter–β-galactosidase plasmid. Each assay was performed at least three times in duplicate with at least two different plasmid preparations. The normalized mean values with standard deviations were plotted or reported in tabular form. Phorbol 12-myristate 13-acetate (PMA), forskolin, ionomycin, or goat anti-mouse IgM was added at the concentrations indicated in Fig. 7C, 8A, and 9C. Cells were harvested after 18 h (Fig. 7C and 8A) or 6 h (Fig. 9C).

EMSA. The oligonucleotides used as probes for the electrophoretic mobility shift assay (EMSA) of the CRE region are shown in Table 1 (see below). The sequence of the CRE consensus oligonucleotide was GAACCGTGTGACGTC ACGCG. The oligonucleotides were synthesized with 5′ overhangs and end labeled with [α-^{32}P]dCTP and Klenow polymerase. Binding conditions were as follows: 12 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9), 4 mM Tris (pH 7.5), 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, 3 μg of poly(dI-dC), 1 ng (10^4 cpm) of end-labeled oligonucleotide probe, and 10 μg of protein from crude nuclear extract. Leupeptin (0.3 μg/ml), phenylmethylsulfonyl fluoride (5 mM), antipain (0.3 μg/ml), and aprotinin (2 μg/ml) were included in all nuclear extract buffers. Samples were incubated in the presence or absence of excess competitor oligonucleotides for 15 min at room temperature. Electrophoresis was performed at 30 mA at 4°C in a 0.5× Tris borate-EDTA–5% polyacrylamide gel. For the supershift EMSA, the binding-reaction mixture was incubated with antibody for 30 min at 4°C, labeled oligonucleotide was added, and the mixture was incubated for 15 min at room temperature. Antibodies specific to the following proteins were obtained from Santa Cruz Biotechnology for use in supershift EMSA: ATF-1/CREB (recognizes all CREB/ATF family proteins), ATF-1/ATF-2, ATF-2, ATF-3, CREB-1 (CREB), CREB-2, CREM-1, PEA3, c-jun, egr-1, ets1/ets2, and c-fos. An antibody specific for the phosphorylated serine 133 form of CREB was obtained from Upstate Biotechnology Inc. CREB interactions were studied by addition of purified truncated CREB (CREB bZIP 254–327 [Santa Cruz Biotechnology]) to the binding-reaction mixture prior to the addition of labeled oligonucleotide.

UV cross-linking and SDS-polyacrylamide gel electrophoresis. EMSA was performed as described above. UV cross-linking was performed as described previously (11) with a short-wavelength UV light box at 4°C for 60 min. An autoradiograph of the wet gel was used to locate the EMSA complexes. Regions of the gel containing the complexes were cut out, and the individual complexes were eluted at room temperature overnight in 50 mM Tris-HCl (pH 7.9)–0.1% sodium dodecyl sulfate (SDS)–0.1 mM EDTA–5 mM dithiothreitol–150 mM NaCl–0.1 mg of bovine serum albumin per ml. The eluted protein was precipitated with 4 volumes of acetone, washed with ethanol, and dried. It was then resuspended in Laemmli loading buffer, and SDS-polyacrylamide gel electrophoresis was performed. The ^{32}P-labeled proteins were visualized by autoradiography.

Methylation interference. The 5′-end-labeled oligonucleotide (either WtDRE or bCREO) was labeled with T4 kinase and methylated with 0.5% dimethyl sulfate for 2 min at room temperature. The sequence of WtDRE is shown in Table 1, and the sequence of bCREO was GAACCGTGTGACACGA. These oligonucleotides were used in EMSA as described above. The complexes were transferred to DEAE membrane, the membrane was exposed to film to locate the complexes, and regions containing the bound and free probes were excised. The DNA was eluted and cleaved with piperidine, and equal counts of bound and free samples were resolved in a 15% acrylamide sequencing gel.

S1 nuclease protection assay. BAL-17 cells were cultured in the presence of medium, 60 ng of PMA per ml, or 4 μg of anti-IgM per ml for 18 h at 37°C prior to isolation of RNA by the guanidine thiocyanate method (12). The murine *bcl*-2 probe (41) and a 1.8-kb *Bam*HI-*Hin*dIII genomic fragment of β_2-microglobulin end labeled at the *Eco*RI site of exon 2 (43) were hybridized with 10 μg of total RNA for 16 h at 55°C. Digestion with 200 U of S1 nuclease was performed for 1 h at 37°C. The protected fragments were separated on a 6% sequencing gel.

DNA fragmentation analysis. Ramos cells (5 × 10^6) were cultured for 18 h in medium with no addition or the addition of 1 μg of ionomycin per ml, 50 ng of PMA per ml, or both ionomycin and PMA. The cells were washed, centrifuged, and resuspended in 20 μl of 10 mM EDTA–50 mM Tris-HCl–0.5% sodium *N*-lauroyl sarcosinate–0.5 μg of protease K per ml and incubated for 1 h at 50°C. The samples were incubated for 1 h at 37°C after addition of 10 μl of RNase A (100 μg/ml) and then incubated for 5 min at 70°C. The samples were mixed with 10 μl of 10 mM EDTA plus 1% low-melting-point agarose and electrophoresed in 40 mM Tris-acetate–1 mM EDTA in a 1.2% agarose gel.

RESULTS

Identification of two positive regulatory regions in the *bcl*-2 promoter. The *bcl*-2 major transcriptional promoter, P1, is located between bp −1390 and −1440 upstream from the translation start site (50). *bcl*-2 sequence from −3934 to −1287 that contains the P1 promoter was inserted upstream of a promoterless luciferase reporter gene construct (Fig. 1A), and reporter gene activity in DHL-9 cells (a human mature B-cell line) was assessed. We were unable to demonstrate any transcript initiation at the P2 promoter (8). Constructs that contained both the P1 and P2 promoters yielded similar results, although the activity of the constructs with the P2 promoter was much lower because of the negative regulatory element just upstream of P2 (data not shown) (61). Progressive 5′ truncations of the *bcl*-2 sequence revealed a positive regulatory region between the *Sac*II site at −1640 and a *Bsr*FI site at −1526 (Fig. 1A). In DHL-9 cells, this area resulted in a 3.8-fold increase in reporter gene activity over the basal promoter (−1526) construct. This appeared to be the only positive regulatory region. Similar results were obtained with the pre-B-cell line, Nall-1 (data not shown). Comparison of the murine and human *bcl*-2 sequences revealed a high degree of homology in this area.

To study the activity observed between *Sac*II (−1640) and *Bsr*FI (−1526) in more detail, subfragments of this area were inserted upstream of the *Bsr*FI (−1526) site. Progressive 5′ truncations within this area revealed two positive regulatory regions in DHL-9 cells (Fig. 1B). The DRE, located between −1552 and −1526, was responsible for a 1.5-fold increase in basal promoter activity over that obtained with a P1 promoter control construct. This activity was preserved when a 3′ deletion narrowed this element to −1552 through −1534.

The remainder of the positive regulatory activity was located between −1611 and −1552; this area is the URE. Preliminary

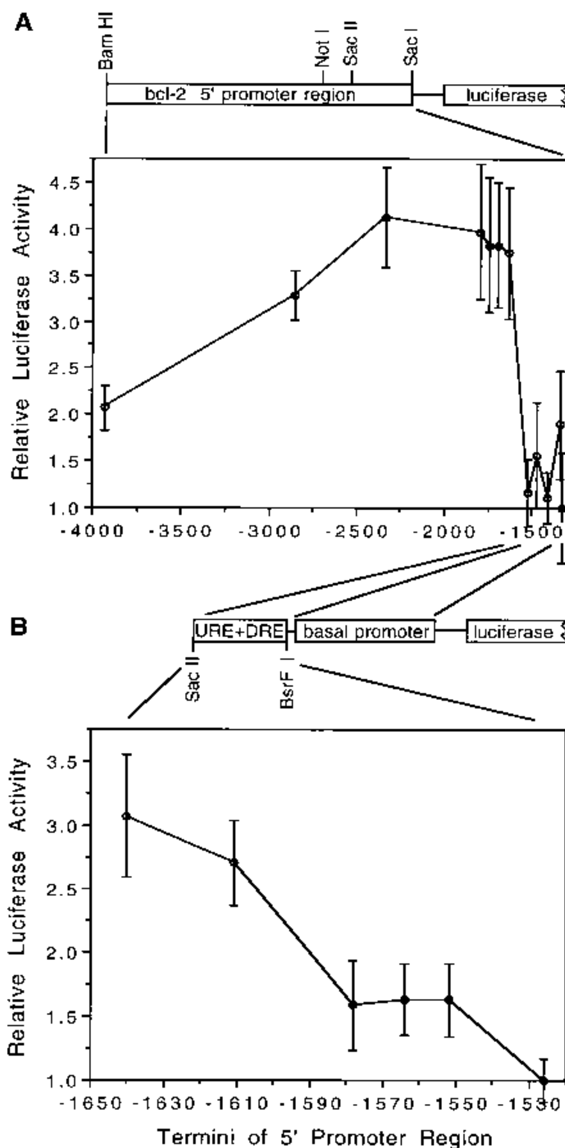

FIG. 1. Identification of a positive regulatory region in the *bcl*-2 promoter. (A) Diagram of the *bcl*-2 5′ sequences used in the constructs with relevant restriction enzyme sites indicated and luciferase activity of progressive 5′ deletions of the *bcl*-2 promoter region in DHL-9 cells. The results are shown relative to the luciferase activity of the basal promoter (−1526) construct, which was assigned a value of 1. (B) Diagram of the *bcl*-2 5′ sequences used in the constructs and results of transient-transfection analysis of the region between *Sac*II (−1640) and *Bsr*FI (−1526).

studies suggest that the 5′ end of the URE was located between −1611 and −1591. Constructs with deletions or additions of sequence between the two regions that disrupted the helical spacing resulted in promoter activities similar to that of the DRE alone (data not shown). These results suggest that disruption of the helical spacing between the URE and DRE leads to loss of the positive interaction between these elements. The URE has not been further characterized.

Sequence specificity of protein binding to the DRE. EMSA of nuclear extracts obtained from the B-cell lines DHL-9, Ramos, Nalm-6, and Reh with an oligonucleotide corresponding to *bcl*-2 sequence −1552 to −1525 or −1552 to −1535, both of which contain WtDRE, revealed four specific bands

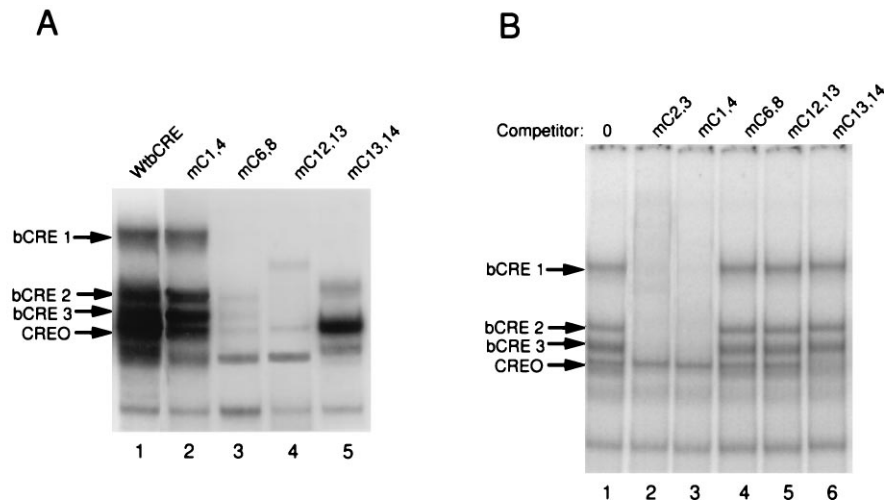

FIG. 2. EMSA with DRE oligonucleotides and DHL-9 nuclear extracts. (A) EMSA with the wild-type bCRE site and mutated sites. The sequences of the different DRE oligonucleotides are listed in Table 1. The arrows indicate the specific complexes; the two complexes that migrate faster than the CREO complex are nonspecific. (B) Competition with mutated CRE oligonucleotides. The indicated oligonucleotide is present at a 50-fold molar excess. The specific complexes are indicated by arrows.

(the results for DHL-9 are shown in Fig. 2). Sequence mutation analysis within the DRE identified two distinguishable protein-binding sites by EMSA (Fig. 2A) and by EMSA with competition with the mutated oligonucleotides (Fig. 2B). The results are summarized in Table 1. The 5′ site spanned the sequence from −1552 to −1541 and produced a single band on EMSA which corresponded to the fastest migrating of the four specific bands (Fig. 2, lane 5). The 3′ site spanned from −1547 to −1534 and produced one slowly migrating band and two more rapidly migrating bands (bands 1, 2, and 3 from most slowly to most rapidly migrating in Fig. 2). The 3′ site contains a complete CRE consensus sequence, while the 5′ site contains only part of the CRE. Consequently, they are referred to as the *bcl*-2 CRE (bCRE) site and the CRE-overlapping site (CREO site), respectively. EMSA with the CRE consensus oligonucleotide with *bcl*-2-flanking sequences yielded the same four complexes that were seen with the WtDRE oligonucleotide, and the two oligonucleotides competed against one another (data not shown). The CREO site does not appear to be homologous to any known transcription factor consensus-binding site.

Transient-transfection assays were performed with DHL-9 cells and reporter gene constructs that contained mutations in the CREO and bCRE sites in the absence of the URE site. As seen in Table 1, it appeared the bCRE site was responsible for most if not all of the positive regulatory activity in this area. The CREO site appeared to have only weak stimulatory activity.

The 5′- and 3′-binding sites were also distinguishable by in vitro methylation interference (footprinting) analysis, which confirmed the site lengths of −1552 to −1541 and −1547 to −1534, respectively (Fig. 3). Protein binding to the bCRE was strongly inhibited (>60% inhibition) by guanine methylation at sites −1545, −1543, −1541, and −1540. Weaker inhibition (35 to 60% inhibition) was seen at sites −1547 and −1535. Protein binding to the CREO site was strongly inhibited by methylation at sites −1549, −1548, −1547, and −1545. Weaker inhibition was seen at sites −1552 and −1543. This analysis confirmed an overlap between the bCRE and the CREO sites.

Characterization of the bCRE-binding proteins. To further study the proteins interacting with the bCRE site, UV cross-linking and SDS-polyacrylamide gel electrophoresis of the three upper bands obtained by EMSA with the mC1,4 oligo-

TABLE 1. Effect of mutations in the DRE on protein binding by EMSA and on transcriptional activity measured by transient transfections with promoter-luciferase constructs in DHL-9 cells

Construct[a]	Sequence of the DRE site[b]	EMSA binding		Luciferase activity
		CREO band	bCRE bands	
WtURE/DRE	GAACCGT**GTGACGTTA**CGCA	+++	+++	3.07 ± 0.48
MutCRE	GAACCGT**GTGAATTTA**CGCA	±	−	1.34 ± 0.26
WtURE	GAACCGT**GTACTAGTG**GACA	±	−	0.52 ± 0.14
WtDRE	GAACCGT**GTGACGTTA**CGCA	+++	+++	1.51 ± 0.24
mC2-3	GCCGCGT**GTGACGTTA**CGCA	+	+++	1.45 ± 0.18
mC1,4	TAACAGT**GTGACGTTA**CGCA	+	+++	1.58 ± 0.30
mC6,8	GAACCTT**TTGACGTTA**CGCA	±	±	1.14 ± 0.19
mC12,13	GAACCGT**GTGAATTTA**CGCA	±	−	0.98 ± 0.12
mC13,14	GAACCGT**GTGACTATA**CGCA	+++	−	1.17 ± 0.14
mC13-17	GAACCGT**GTGACTAGT**GGCA	+++	−	1.13 ± 0.18
Basal *bcl*-2 promoter control construct				1.00 ± 0.11

[a] A schematic representation of the expression vectors is shown in Fig. 7A.
[b] Boldface type highlights the CRE consensus sequence. Underlined nucleotides represent mutations from the wild-type sequence.

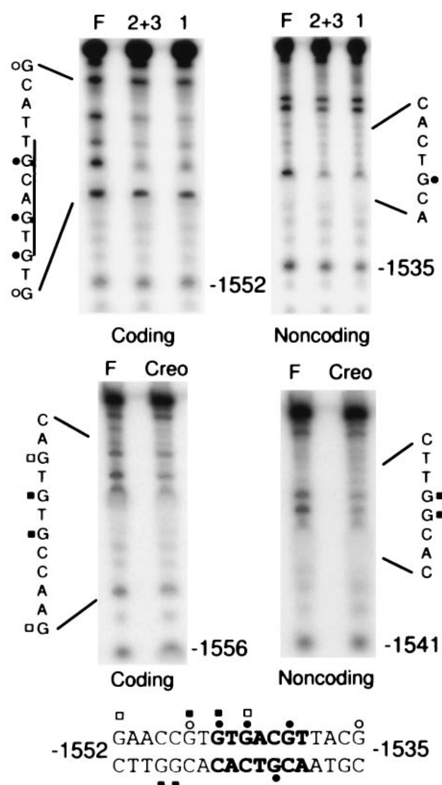

FIG. 3. Methylation interference analysis of protein-DNA complexes formed with DHL-9 nuclear extracts and the DRE oligonucleotides. The EMSA complexes analyzed are indicated above each lane. bCRE EMSA complexes 1 through 3, formed with the WtDRE oligonucleotide, are shown above the CREO EMSA complexes formed with the bCREO oligonucleotide. The guanine residues that showed strong protection are indicated by solid circles (CRE) or squares (CREO); those that showed weaker protection are indicated by open circles (CRE) or squares (CREO). The CRE site is underlined. The protected guanine residues are summarized in the sequence at the bottom of the figure, and the bCRE site is indicated in boldface letters.

nucleotide were performed (Fig. 4A). The fourth band obtained with this probe corresponded to the CREO site complex and was not studied.

UV cross-linking of the most slowly migrating bCRE band, band 1, obtained by EMSA with mC1,4 yielded two protein-DNA complexes migrating at 77 and 88 kDa (Fig. 4A, lane 3). Correcting for oligonucleotide size, proteins of approximately 63 and 67 kDa were predicted. In addition, this EMSA band yielded two faintly labeled protein-DNA complexes migrating at 130 and 170 kDa, which may represent dimeric forms of the faster-migrating proteins.

UV cross-linking of bCRE band 2 revealed a protein-DNA complex of 53 kDa (corrected protein size, 43 kDa) (Fig. 4A, lane 2). A faintly labeled protein-DNA complex of approximately 100 kDa, which may represent a homodimeric complex of the 43-kDa protein, was seen. The fastest migrating bCRE band, band 3, yielded two protein-DNA complexes of 45 and 53 kDa (corrected protein sizes, 34 and 43 kDa, respectively) (lane 1). Again, a faintly labeled protein-DNA complex of approximately 94 kDa was seen, which may represent a heterodimeric complex.

EMSA supershift analyses of the bCRE complexes were performed with antibodies directed against several known CREBs (ATF-1, ATF-2, ATF-3, CREB, CREB-2, and CREM-1) and several non-CREBs (PEA3, c-jun/AP-1, c-fos, egr-1, and ets1/ets2). Performing this analysis with the mC1,4 oligonucleotide probe demonstrated a supershift of bCRE band 1 with antibodies against ATF-2 and ATF-1/ATF-2. bCRE band 2 was supershifted with antibodies against CREB and ATF-1/CREB, and bCRE band 3 was supershifted only with antibody against ATF-1/CREB (Fig. 4B). None of the other antibodies tested shifted either the bCRE bands or the CREO band.

Western blot (immunoblot) analysis of DHL-9 nuclear extract revealed abundant expression of ATF-2 (reactive antigens found at 62, 67, and 170 kDa) and CREB (reactive antigen found at 43 kDa [data not shown]). By Western blot analysis, the ATF-1/ATF-2 antibody displayed weak cross-reactivity with the ATF-2 antigens, suggesting that the supershift

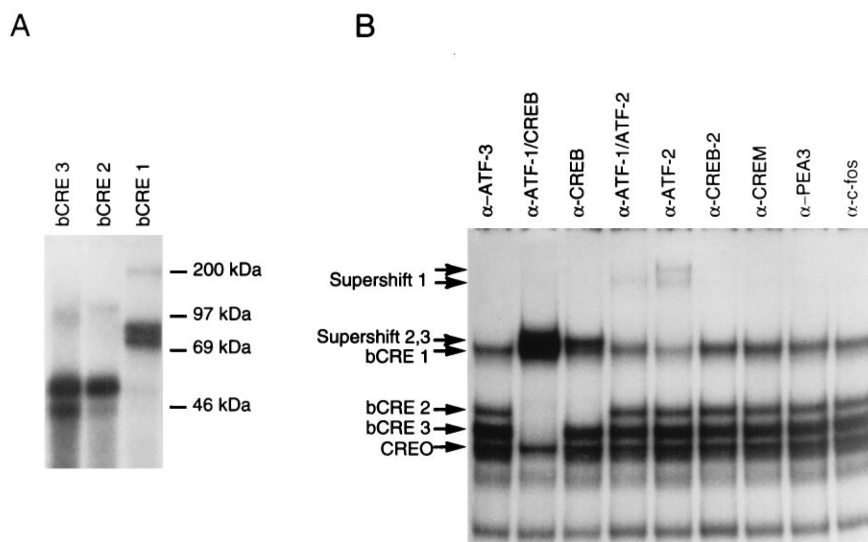

FIG. 4. Characterization of the proteins that bind to bCRE. (A) Denaturing SDS-polyacrylamide gel analysis of the UV cross-linked EMSA complexes formed with DHL-9 nuclear extract and the mC1,4 oligonucleotide. The EMSA complexes analyzed are indicated above each lane. The migration of the molecular mass markers is shown on the right. (B) EMSA supershift analysis of the complexes formed with the DRE oligonucleotide and DHL-9 nuclear extract. The arrows indicate the complexes and the corresponding supershifted complexes.

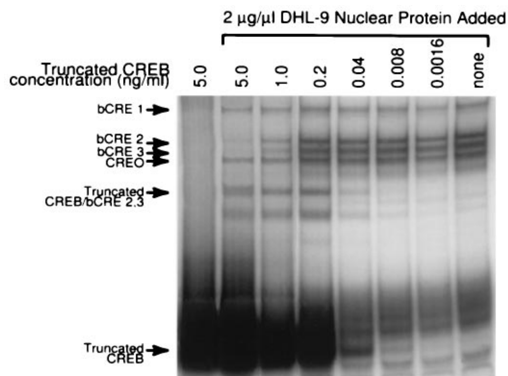

FIG. 5. Interaction of a purified truncated CREB with the CREBs in DHL-9 nuclear extract by EMSA with the mC1,4 oligonucleotide. Decreasing amounts of a purified truncated CREB were added to a constant protein concentration from DHL-9 nuclear extract (2 μg/μl). The arrows indicate the complexes formed with DHL-9 nuclear extract, the complex formed by the truncated CREB protein, and the complex of intermediate mobility formed with the truncated CREB and with CREB in DHL-9 nuclear extract. Quantitation of the bands was performed for the EMSA with 1.0 and 5.0 ng of truncated CREB per ml. There was no change in the relative intensity of the bCRE1 and CREO bands. With 1.0 and 5.0 ng of truncated CREB per μl, bCRE band 2 decreased by 18 and 70%, respectively, and bCRE band 3 decreased by 28 and 83%, respectively.

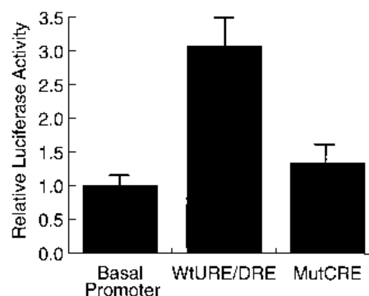

FIG. 6. Effect of mutation of the *bcl*-2 CRE site on promoter activity in DHL-9 cells. Luciferase activities of the WtURE/DRE and the construct with the mutated CRE site (MutCRE) are shown relative to the activity of the basal promoter.

activity seen with this antibody was a result of cross-reactivity with ATF-2 and not involvement of ATF-1 in the protein-DNA complex bCRE band 1. The ATF-1/CREB antibody is known to have polyspecific reactivity. By Western blot analysis, it was strongly reactive with CREB-1 and weakly reactive with several other antigens (of 35, 38, 49, 65, 74, and 138 kDa).

Members of the CREB/ATF family form homo- and heterodimers. To further confirm the involvement of CREB/ATF family members in the bCRE complexes, EMSA of the mC1,4 oligonucleotide was performed in the presence of decreasing amounts of a truncated CREB that contains the DNA-binding and dimerization domains (Fig. 5). High concentrations of this protein (>10 ng/μl) effectively blocked the binding of nuclear extract proteins to both the bCRE and CREO sites, confirming that these sites overlap and that the bCRE site can function as a CRE site (data not shown). Lower concentrations (0.008 to 5 ng/μl) of the truncated CREB produced one intermediate migrating band with concurrent relative diminution in the intensity of bCRE band 2 and 3 but not in the intensity of bCRE band 1 or of the CREO band (Fig. 5). These results confirmed that the proteins involved in bCRE bands 2 and 3 are capable of dimerization with CREB. As ATF-2 is a distant relative of CREB and has not been shown to dimerize with CREB, it is not surprising that no interaction between the truncated CREB and the bCRE band 1 was seen (36).

Thus, the *bcl*-2 CRE site appears to be able to form three complexes with B-cell nuclear extracts, each represented by a specific EMSA band. The most slowly migrating complex (bCRE band 1) appears to consist of dimerized 63- and 67-kDa forms of ATF-2. The middle complex (bCRE band 2) is apparently a homodimer of CREB, and the fastest-migrating complex (bCRE band 3) is a heterodimer of CREB and possibly ATF-1.

To confirm the importance of the CRE site in the full-length *bcl*-2 promoter, site-directed mutagenesis was used to create a 2-bp change in the CRE site in the WtURE/DRE construct (MutCRE). As shown in Fig. 6, the promoter activity decreased by 83% relative to the WtURE/DRE construct.

Induction of *bcl*-2 promoter activity during B-cell activation and rescue from apoptosis. Because the CRE is an inducible regulatory element, we wished to determine whether a CREB was involved in the increase in *bcl*-2 expression during B-cell activation. DHL-9 cells could not be used for these studies because they do not express surface IgM. The murine B-cell line, BAL-17, was chosen because it has been shown to recapitulate many of the characteristics of primary B cells after surface Ig receptor cross-linking. Although BAL-17 cells express relatively high levels of *bcl*-2, cross-linking of the surface Ig receptor or treatment with PMA leads to a further 2.5- to 3.5-fold increase in *bcl*-2 mRNA levels (Fig. 7B, lanes 2 and 3). To determine the effect on *bcl*-2 promoter activity, cross-linking of surface Ig receptor was performed with goat anti-IgM antibodies 24 h after transfection of the *bcl*-2-luciferase constructs. The *bcl*-2 promoter constructs used are shown in Fig. 7A. The activity of the *bcl*-2 promoter increased by threefold (Fig. 7C).

We used either dibutyryl-cAMP or forskolin to increase cAMP levels. This PKA pathway is involved in the phosphorylation of CREBs in many different cell types. As shown in Fig. 7C, forskolin did not produce an increase in *bcl*-2 promoter activity. Similar negative results were obtained with dibutyryl-cAMP (data not shown). Because CREB is phosphorylated by other kinases, including PKC, we treated the BAL-17 cells with PMA. PMA produced a threefold activation (Fig. 7C). The increase in activity due to PMA was very similar to that observed with surface Ig receptor cross-linking. Simultaneous stimulation with PMA and Ig cross-linking resulted in little additional stimulation of promoter activity, suggesting that the stimulation evoked by surface Ig cross-linking was almost entirely due to a PKC mechanism. Similar results were obtained with a control plasmid (*Bgl*II CAT) that contains the somatostatin gene promoter CRE site. Treatment of BAL-17 cells with PMA led to a threefold increase in CAT activity, while treatment with forskolin had no effect (data not shown). The reason for the failure of increased cAMP levels to activate a CRE site in these cells is unclear. It is possible that an inhibitor of PKA is present in the cell lines we used for these studies.

The increase in activity of the *bcl*-2 promoter with both anti-IgM and PMA was dependent on the presence of the CRE site. As shown in Fig. 7C, there was no change in the activity of either the WtURE or MutCRE constructs. A promoter construct with the URE deleted showed only a 60% increase in activity with PMA. These results suggest that the bCRE site is required for the increased promoter activity and that interaction of the URE and bCRE results in maximal stimulation of promoter activity. Thus, *bcl*-2 expression is activated by surface Ig receptor cross-linking or by PMA through the CRE site in the 5′-flanking sequence.

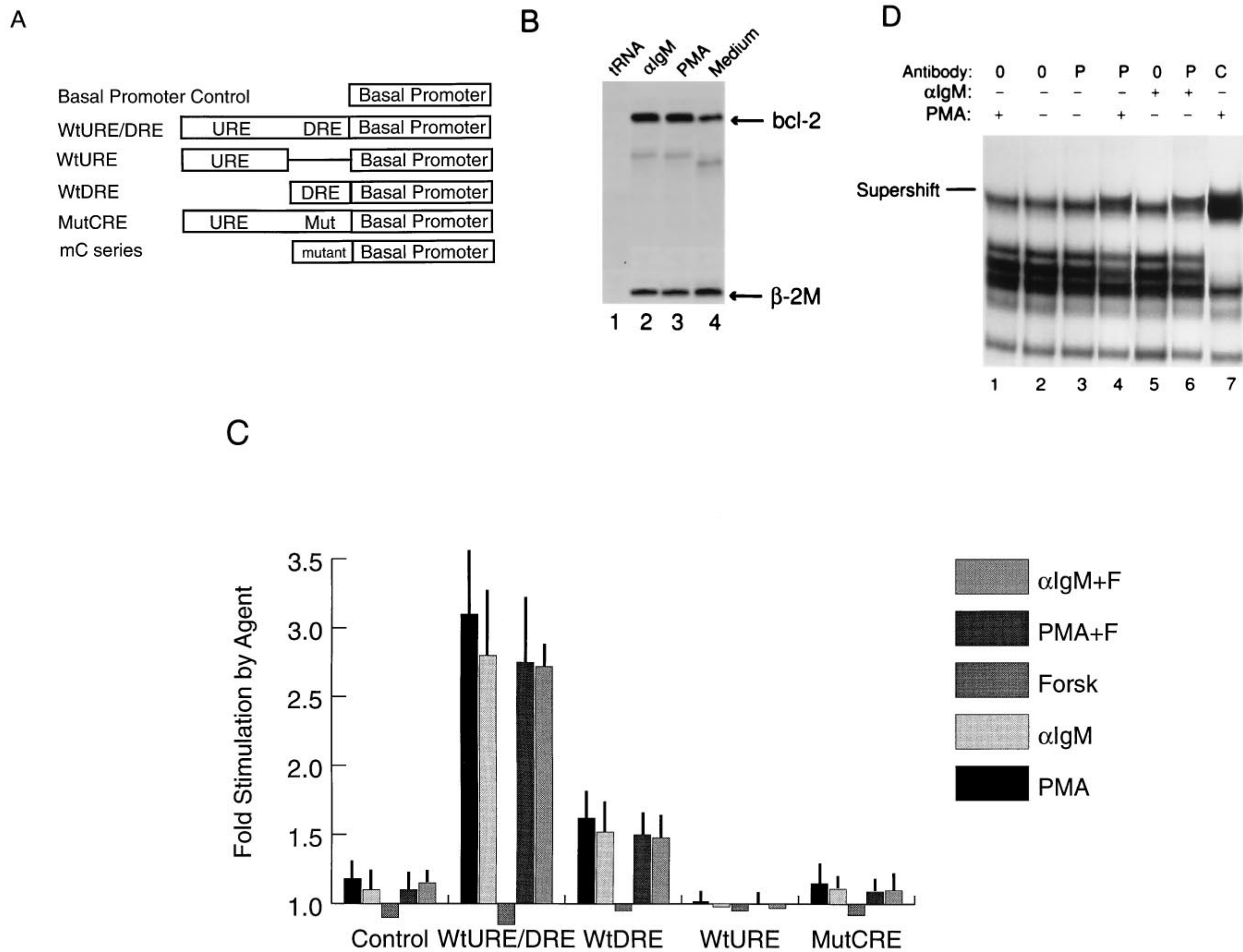

FIG. 7. Effect of B-cell stimuli on the activity of the CRE site in the *bcl*-2 promoter in BAL-17 cells. (A) Diagram of the *bcl*-2 promoter constructs used for transient-transfection analysis. (B) S1 analysis of *bcl*-2 mRNA levels in BAL-17 cells after treatment with anti-IgM (lane 2), PMA (lane 3), or no addition (lane 4). Lane 1 is tRNA only. Protected fragments of 600 (*bcl*-2) and 202 (β_2-microglobulin [β-2M]) nucleotides are shown. (C) Transient-transfection analysis of the *bcl*-2 basal promoter region (Control), the WtURE/DRE, WtDRE, WtURE, and MutCRE constructs after treatment of BAL-17 cells with 60 ng of PMA per ml, 4 μg of anti-IgM per ml, 10 μM forskolin, 60 ng of PMA per ml plus 10 μM forskolin, and 4 μg of anti-IgM per ml plus 10 μM forskolin for 18 h. (D) EMSA of the mC1,4 oligonucleotide with BAL-17 nuclear extract. Nuclear extracts were prepared after incubation with the indicated compound for 30 min. An antibody that recognizes all CREBs (lane C) or an antibody specific for the phosphorylated CREB (lanes P) was added prior to EMSA as indicated.

To confirm that CREB was phosphorylated by both surface Ig receptor cross-linking and PMA, we performed EMSA with an antibody specific for phosphorylated CREB. Figure 7D demonstrates that very little phosphorylated CREB is present in unstimulated BAL-17 cells. Treatment with either anti-IgM or PMA led to the appearance of a complex that was supershifted with the antibody specific for phosphorylated CREB (Fig. 7D, lanes 4 and 6). Thus, phosphorylation of CREB after surface Ig cross-linking is mediated by PKC and the phosphorylated CREBs activate the *bcl*-2 promoter.

BAL-17 cells express relatively high levels of *bcl*-2, so we wished to examine the up-regulation of *bcl*-2 in a mature B-cell line that expressed low levels of *bcl*-2. We treated the DHL-9 cell line with PMA for 18 h after transfection of the *bcl*-2–luciferase constructs. The activity of the *bcl*-2 promoter increased by ninefold, and the increase was dependent on the CRE site (Fig. 8A). As was the case for BAL-17 cells, maximal stimulation required the presence of the URE. Analysis of the CREBs in DHL-9 nuclear extracts revealed that phosphorylation on serine 133 occurred after treatment of the cells with PMA and to a lesser extent after treatment with forskolin (Fig. 8B, lanes 4 and 6). As was the case with BAL-17 cells, treatment of DHL-9 cells with forskolin did not result in an increase in *bcl*-2 promoter activity (data not shown).

To further demonstrate the importance of CREB in the regulation of the *bcl*-2 promoter, cotransfection experiments were performed with a wild-type CREB expression vector or a dominant negative CREB M1 expression vector. The dominant negative CREB contains an alanine at position 133 and cannot be phosphorylated (21, 51). As shown in Fig. 8C, cotransfection with either the wild-type CREB or CREB M1 resulted in a twofold increase in the constitutive activity of the *bcl*-2 promoter. With PMA stimulation, there was a slight increase in promoter activity with the wild-type CREB construct and a dramatic decrease with the CREB M1 construct. These changes were mediated through the *bcl*-2 CRE site, because there was no change in the activity of the promoter construct with mutated CRE site (Fig. 8C). Furthermore, a construct with two CRE sites linked to the simian virus 40 promoter showed changes similar to the *bcl*-2 promoter when

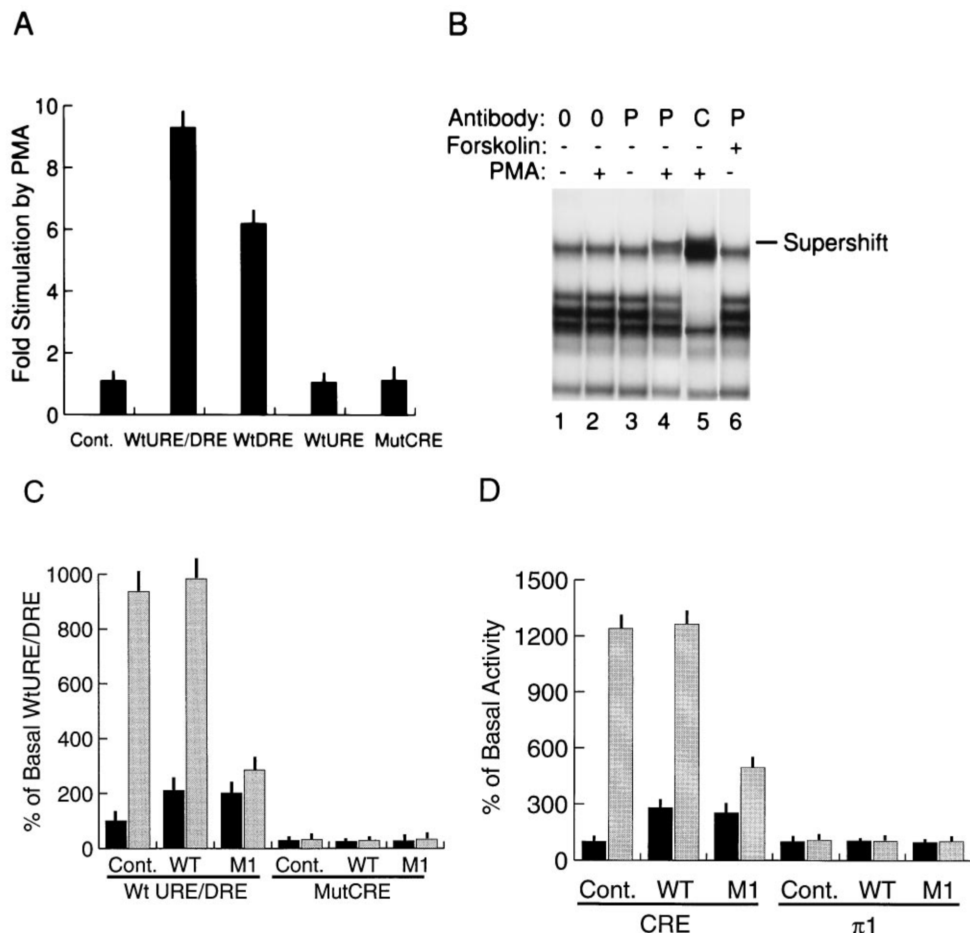

FIG. 8. Effect of PMA on the activity of the *bcl*-2 promoter in DHL-9 cells. (A) Transient-transfection analysis with the basal promoter (Cont.), WtURE/DRE, WtDRE, WtURE, and MutCRE constructs after treatment of DHL-9 cells with 50 ng of PMA per ml for 18 h. (B) EMSA of the mC1,4 oligonucleotide with DHL-9 nuclear extract. Nuclear extracts were prepared after incubation with the indicated compound for 30 min. An antibody that recognizes all CREBs (lane C) or an antibody specific for the phosphorylated CREB (lanes P) was added prior to EMSA as indicated. (C) Cotransfection experiments in DHL-9 cells with wild-type CREB and CREB M1 and the *bcl*-2 promoter constructs WtURE/DRE and MutCRE. The cells were untreated (■) or treated with 50 ng of PMA per ml for 18 h (▨) prior to harvesting. A 1-μg portion of the RSV CREB constructs (wild type [WT], dominant negative mutant (M1) or the empty expression vector (Cont.) and 10 μg of either WtURE/DRE or MutCRE were used in each transfection experiment. (D) Cotransfection experiments in DHL-9 cells with wild-type CREB and CREB M1 and a construct with two CRE sites linked to the simian virus 40 promoter (CRE) or two π1 sites linked to the simian virus 40 promoter (π1). The cells were untreated (■) or treated with 50 ng of PMA for 18 h (▨) prior to harvesting. A 1-μg portion of the RSV CREB constructs (wild type [WT], dominant negative mutant [M1]) or the empty expression vector (Cont.) and 10 μg of either the CRE or π1 construct were used in each transfection experiment. The basal activity of each promoter in the presence of the empty expression vector was assigned a value of 100.

wild-type and mutant CREB expression vectors were cotransfected (Fig. 8D). The CRE site was required since a construct with two π1-binding sites showed no change in activity with cotransfection of either wild-type or M1 CREB (Fig. 8D). These results provide additional support for the role of phosphorylated CREBs in the regulation of the *bcl*-2 promoter during B-cell activation.

We also wished to determine whether the CRE site in the *bcl*-2 promoter mediated the increase in *bcl*-2 mRNA that is seen in more immature B-cell lines that are rescued from apoptosis induced by anti-IgM or calcium ionophores. Treatment with phorbol esters prevents the apoptosis. We used the Epstein-Barr virus-negative Ramos cell line to avoid the confounding effects of Epstein-Barr virus gene products on *bcl*-2 expression. We confirmed by several different techniques including cell survival and DNA fragmentation analysis that ionomycin caused apoptosis in Ramos cells (Fig. 9A and B). Treatment with PMA protected the cells from apoptosis (Fig. 9A and B). Ramos cells were transfected with the *bcl*-2 promoter-luciferase constructs and treated with ionomycin, PMA, or ionomycin plus PMA for 6 h before being harvested. As shown in Fig. 9C, treatment with PMA or PMA plus ionomycin resulted in a 12-fold increase in *bcl*-2 promoter activity. There was no increase in promoter activity if the CRE site was mutated. Finally, we demonstrated that treatment of Ramos cells with PMA resulted in phosphorylation of CREBs at serine 133 and that treatment with forskolin had a smaller effect (Fig. 9D, lanes 4 and 5).

DISCUSSION

We have identified the major positive regulatory region of the *bcl*-2 promoter in B cells and demonstrated that a CRE site mediates both basal expression and induction of the *bcl*-2 promoter region in B cells following activation of PKC. CREBs have been shown to mediate both constitutive and kinase-inducible gene expression (29, 44, 58). Although negative regulatory elements have been described (8, 61), this is the first

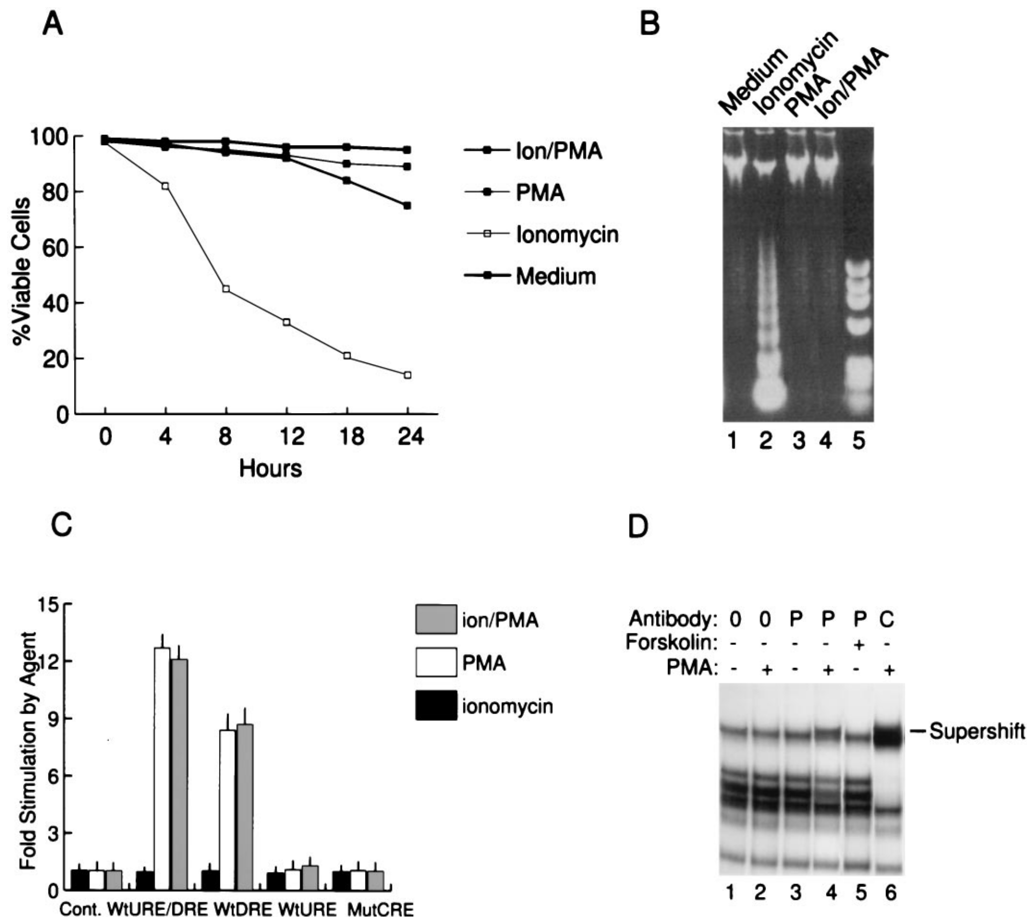

FIG. 9. PMA treatment of Ramos cells leads to rescue from ionomycin-induced apoptosis and up-regulation of the *bcl*-2 promoter. (A) Effect of 1 μg of ionomycin per ml, 25 ng of PMA per ml, or 1 μg of ionomycin per ml plus 25 ng of PMA per ml on the viability of Ramos cells. (B) Induction of apoptosis in Ramos cells at 12 h after the addition of medium, 1 μg of ionomycin per ml, 25 ng of PMA per ml, or 1 μg of ionomycin per ml plus 25 ng of PMA per ml. (C) Transient-transfection analysis with the basal promoter (Cont.), WtURE/DRE, WtURE, WtDRE, and MutCRE constructs after treatment of Ramos cells with 1 μg of ionomycin per ml, 25 ng of PMA per ml, or 1 μg of ionomycin per ml plus 25 ng of PMA per ml for 6 h. (D) EMSA of the mC1,4 oligonucleotide with Ramos nuclear extract. Nuclear extracts were prepared after incubation with the indicated compound for 30 min. An antibody that recognizes all CREBs (lane C) or an antibody specific for the phosphorylated CREB (lanes P) was added prior to EMSA as indicated. In the presence of 1 μg of ionomycin per ml, an identical EMSA pattern was obtained.

report of a positive regulatory element in the *bcl*-2 promoter. The constitutive positive region is active in both pre-B and mature B cells. We have previously demonstrated that the *bcl*-2 promoter is repressed in pre-B cells by π1-binding factors and that expression of *bcl*-2 is low in pre-B cells (8).

We describe two discrete positive regulatory regions, referred to as the URE and the DRE. Our preliminary results demonstrate that deletions or additions of sequence between the two sites that disrupt the helical spacing lead to loss of activity. Although we have not completed the characterization of the URE or identified the transcription factor that binds to the URE, it is possible that protein-protein interactions between CREB and the URE factor are important. There are many examples of interactions between two regulatory elements that can be destroyed by a change in helical spacing (27, 46, 49, 55). In addition, the CREB interacts with factors that bind to an adjacent AP-1 site in a manner dependent on helical spacing (24).

The results of in vitro methylation analysis revealed that the *bcl*-2 DRE consisted of two overlapping sites, CREO and bCRE. Transient-transfection analysis revealed that the bCRE site was associated with most of the activity in this region. EMSA with transcription factor antibodies, UV cross-linking followed by SDS polyacrylamide gel electrophoresis, and heterodimerization with a truncated CREB were performed to demonstrate that CREB family members bind to the bCRE site in vitro.

The presence of a CRE site in the *bcl*-2 promoter implies that the regulation of *bcl*-2 expression is linked to a signal transduction pathway in B cells. *bcl*-2 induction is seen in primary B cells with activation and presumably protects the cells from apoptosis. It has been demonstrated that CREB can be phosphorylated and transcriptionally activated by PKC (59). PKC phosphorylates CREB in B cells (57). This is of interest because it may represent another pathway involved in the activation of B cells. Cross-linking of surface Ig results in transactivation of a CRE-dependent promoter-CAT construct (56). Agents that increase cAMP levels did not activate the CRE-dependent promoter but could act synergistically with cross-linking of surface Ig.

We have shown that cross-linking of surface Ig receptor on mature B cells leads to increased *bcl*-2 promoter activity through a PKC-dependent pathway involving phosphorylation of CREB. This pathway may provide a mechanism for selection of long-lived antigen-reactive B cells by coupling antigen recognition to protection from apoptosis. Reagents that in-

crease cAMP levels have no effect on the activity of the DRE, either alone or in combination with PMA or anti-IgM. BAL-17 cells are derived from a malignant lymphoma and resemble partially activated mature B cells. They express relatively high levels of *bcl*-2 constitutively. It is possible that the magnitude of induction of the *bcl*-2 promoter would be greater in B cells that express lower levels of *bcl*-2 such as resting, mature B cells. In support of this, we find that the magnitude of induction of *bcl*-2 expression after phorbol ester treatment of another mature B-cell line, DHL-9, is approximately threefold greater than that seen in BAL-17 cells. A dominant negative CREB construct interferes with the PMA-induced activation of the *bcl*-2 promoter. Cotransfection of a wild-type CREB construct increased the constitutive activity by twofold. Stimulation with PMA resulted in a similar increase in activity of the *bcl*-2 promoter whether wild-type CREB was cotransfected or not. Since we find that 10 to 20% of the CREBs present in DHL-9 cells are phosphorylated in response to PMA (Fig. 8B and Western blotting data [not shown]), the increase in the level of unphosphorylated CREB may not result in more active phosphorylated CREB. Therefore, no increase in promoter activity with PMA would be observed. The dominant negative CREB appears to interfere with the function of the phosphorylated CREB, perhaps by forming inactive heterodimers.

We have demonstrated that a similar signal transduction pathway is involved in the up-regulation of *bcl*-2 expression in immature B cells given a survival signal. Treatment of Ramos cells with phorbol esters prevents apoptosis induced by ionomycin or by surface IgM cross-linking. *bcl*-2 mRNA is upregulated, and we show that expression of the *bcl*-2 promoter increases by 12-fold following phorbol ester treatment. This increase in expression is dependent on the CRE site in the *bcl*-2 promoter. CREBs in phorbol ester-treated Ramos cells are phosphorylated by a pathway involving PKC. These results are similar to those found in mature B cell lines and provide a demonstration of the physiological significance of signals through the *bcl*-2 promoter CRE site.

We have shown that the CRE site mediates the PKC-dependent increase in *bcl*-2 levels in B cells at two different stages of development. It will be interesting to determine whether the CRE site plays a role in *bcl*-2 induction in other cells in which Bcl-2 prevents apoptosis, including myeloid, breast, neural, and T cells. Recent data have demonstrated that phosphorylation of CREBs during T-cell activation occurs in a cAMP-independent manner and that the phosphorylation of CREB is inhibited by rapamycin in T cells (15). In addition, *bcl*-2 expression is inhibited by rapamycin (38). Our description of CRE site regulation of the *bcl*-2 promoter suggests that the inhibitory effect of rapamycin on *bcl*-2 expression may be due to its interference with CREB phosphorylation in T cells. Similarly, in a myeloid cell line that can be rescued from apoptosis by interleukin-3, the interleukin-3 receptor modulates *bcl*-2 mRNA through a PKC-dependent pathway (47). Our results raise the possibility that the PKC-CREB pathway we have identified is responsible for the induction of *bcl*-2 mRNA in these cells. It is intriguing to speculate that CREBs acting through the CRE site serve as a general mechanism for coupling surface receptor stimulation to protection from apoptosis by Bcl-2.

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