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π1 Binding Sites Are Negative Regulators of *bcl*-2 Expression in Pre-B Cells

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The *bcl*-2 gene is differentially regulated during B-cell development, with low-level expression in pre-B cells and higher-level expression in mature B cells. These changes correlate with susceptibility to cell death by apoptosis and suggest that the Bcl-2 protein may play a role in the control of cell death during B-cell development. We have identified two negative regulatory regions in the human *bcl*-2 5′ flanking and 5′ untranslated regions in pre-B cells; these regions have no significant function in mature B cells. Further investigation of these regions revealed two pre-B-cell-specific enhancer elements (π1 sites) in the 5′ negative regulatory region and one in the 3′ negative regulatory region. Mutational analysis confirmed that these three sites functioned as negative regulators of the *bcl*-2 promoter in the pre-B-cell line Nalm-6. Electrophoretic mobility shift assays with each of the three sites demonstrated a complex of identical mobility to that formed with the immunoglobulin heavy-chain enhancer π1 site. UV cross-linking experiments revealed that a protein with a molecular mass of 58 kDa bound to the three *bcl*-2 sites and to the immunoglobulin enhancer site. This protein reacted with an antibody against Ets family proteins. Constructs with the isolated π1 sites linked to the simian virus 40 promoter were used in transient transfection experiments in the pre-B-cell line. The *bcl*-2 sites decreased expression of the simian virus 40 promoter, while the immunoglobulin enhancer site increased its expression. The π1 sites in the *bcl*-2 gene may play a role in the developmental regulation of *bcl*-2 expression during B-cell differentiation.

The *bcl*-2 gene product is a 25-kDa membrane protein that functions to prevent apoptosis (11, 35). The *bcl*-2 gene was originally identified by its involvement in the t(14;18) translocation which is associated with human follicular lymphoma (38). The translocation of *bcl*-2 to the immunoglobulin heavy-chain (IgH) locus leads to deregulated expression of *bcl*-2, and high levels of *bcl*-2 mRNA are detected in cells with the t(14;18) translocation (2, 6). Transgenic mice containing a *bcl*-2–immunoglobulin minigene show a polyclonal expansion of B cells which displays prolonged cell survival but no increase in cell cycling; progression to high-grade lymphomas is seen in these mice (23). Increased *bcl*-2 expression also prolongs the survival of several hematopoietic cell lines following growth factor deprivation (28, 35).

The normal *bcl*-2 gene has three exons with an untranslated first exon. The second intron is extremely large (225 kb) (34). The major transcriptional promoter, P1, is located 1,386 to 1,423 bp upstream of the translation start site (32). This is a TATA-less, GC-rich promoter that displays multiple start sites. Transcripts initiating at a minor promoter, P2, located 1.3 kb downstream from the first one have been identified in some tissues (32). The transcriptional control of the *bcl*-2 gene is not well understood. A negative regulatory element (NRE) upstream of the P2 promoter has been described previously (37). 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 segment of this region (25).

Human and murine malignant cell lines have been reported to express higher levels of Bcl-2 at the pre-B-cell stage compared with those at the mature B-cell stage (6, 7). In normal B cells, however, Bcl-2 is expressed at higher levels in mature B cells. Levels of Bcl-2 protein have been shown to be important during normal B-cell maturation. A large fraction of B cells dies at the pre-B-cell stage, presumably because of the failure to express a functional immunoglobulin receptor (3, 29). It has been shown recently that Bcl-2 is not expressed at the pre-B-cell stage when extensive cell death occurs (24); the cell death at this stage most likely reflects the elimination of cells that fail to express a pre-B-cell receptor complex. A second selection step occurs at the IgM^+ IgD^- B-cell stage. Immature B cells expressing IgM receptors for self undergo clonal deletion or clonal anergy (5, 8, 27). Immature B cells also express low levels of Bcl-2 (24). Mature B cells have been shown to express much higher levels of Bcl-2 (24). Bcl-2 expression is diminished in germinal centers where apoptotic B-cell death is occurring (12, 30). These findings suggest that the level of Bcl-2 protein correlates inversely with the susceptibility to cell death by apoptosis.

The similar expression patterns of *bcl*-2 at the mRNA and protein levels in bone marrow B-cell preparations are consistent with regulation at the transcriptional level (9, 19, 24). Thus, it is likely that *bcl*-2 expression is regulated at the transcriptional level during B-cell differentiation and that the Bcl-2 protein is a signal controlling cell death during B-cell development. The present study describes a pre-B-cell-specific transcription factor that negatively regulates *bcl*-2 expression.

The π site was originally defined in the IgH enhancer (20, 21). This element was further dissected into two regions, π1 and π3, with the π1 element demonstrating essentially all of the enhancer activity (20). The π1 binding factor was shown to be present in murine pre-B cells but not in mature B cells, and this region of the enhancer was active only at the pre-B-cell stage (20). The sequence of the immunoglobulin π1 site (GCAGGAAGC) was identified in the promoters of several

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TABLE 1. Double-stranded oligonucleotides of the *bcl*-2 and IgH π1 sites

Oligonucleotide	Sequence[a]	Location[b] (bp)
b1	GGCCGCGCGGGAGGCCTGTGC CCGGCGCGCCCTCCGGACACG	−1795 to −1775
b2	GCCCCCGGCGGGACGCGCCAC CGGGGGCCGCCCTGCGCGGTG	−1675 to −1655
b3	GCCAGGCGCGTCCTGCCTTCA CGGTCCGCGCAGGACGGAAGT	−1047 to −1027
Ig	AGCTGGCAGGAAGCAGGTCAT TCGACCGTCCTTCGTCCAGTA	
mb1	GGCCGCGCcttgGGCCTGTGC CCGGCGCGgaacCCGGACACG	
mb2	GCCCCCGGCcttgCGCGCCAC CGGGGGCCGgaacGCGCGGTG	
mb3	GCCAGGCGCGcaagGCCTTCA CGGTCCGCGCgttcCGGAAGT	
NS	AGCAGCTTTTCGGAAAATGC TCGTCGAAAAGCCTTTTACG	−1019 to −1000

[a] The π1 sites are underlined; mutated bases are in lowercase letters. The π1 site in b3 is on the antisense strand.
[b] Relative to the translation start site.

pre-B-cell-specific genes. Because of the core GGA sequence, it was suggested that an Ets family protein bound to this site (20). Several Ets proteins, including ERP, ERG-3, Ets-1, and Fli-1, have been shown to bind to this site in vitro (22, 26, 31). Which Ets protein is responsible for the functional activity of the π site in pre-B cells remains unclear, but both ERP and ERG-3 are expressed in pre-B cells, and they are downregulated as the cells differentiate into mature B cells.

Our results identify three π1 sites that negatively regulate *bcl*-2 transcription in the pre-B-cell line Nalm-6 but not in the mature B-cell line DHL-9. In addition, we demonstrate that an Ets family protein binds to these sites.

MATERIALS AND METHODS

Oligonucleotides. The double-stranded oligonucleotides of the *bcl*-2 π1 sites (b1, b2, and b3) and the IgH enhancer π1 site (Ig), the mutant *bcl*-2 π1 sites (mb1, mb2, and mb3), and an irrelevant sequence (NS) are shown in Table 1. For electrophoretic mobility shift assay (EMSA) analysis, the oligonucleotides were synthesized with 5′ overhangs. For cloning of b2, b3, and Ig, the oligonucleotides were synthesized with *Xho*I and *Sal*I ends.

Plasmid constructs. DNA fragments from *Sac*II (−1640) to *Eco*47III (−1063), *Not*I (−1796) to *Eco*47III, *Sac*II to *Nsi*I (−999), and *Not*I to *Nsi*I of the human *bcl*-2 sequence (a generous gift from M. Cleary, Stanford University) were inserted into a luciferase reporter vector to create p[S-E], p[N-E], p[S-N], and p[N-N] constructs, respectively (Fig. 1). Numbering of the *bcl*-2 sequence is relative to the translation start site. The p[S-D] construct was a ligation product of p[S-E] and a 45-bp fragment (*Eco*47III to −1018) synthesized by PCR. An oligonucleotide, GGCCGCGCcttgGG (the mutated bases are in lowercase), was used to replace the *Not*I-to-*Stu*I (−1782) sequence of p[N-E] to generate p[N-E(mb1)]. The other two mutant constructs, p[N-E(mb2)] and p[S-N(mb3)], were made by the recombinant PCR approach described by Higuchi (10). The primers used were TGTGCCCCCGGCcttgCGCGCCACTCCC (mb2) and GGGCCAG GCGCGcaagGCCTTCATTTAT (mb3). The π1 sites are underlined, and the mutated bases are in lowercase. The p[N-E(mb1b2)] double mutant was made by the same procedure as p[N-E(mb2)], except that p[N-E(mb1)] was used as the template. Three π1 site-containing oligonucleotides (b2 and b3 of *bcl*-2 and Ig of IgH) with *Sal*I and *Xho*I ends were inserted as monomers or dimers into the *Xho*I site of the pGL2-promoter, a luciferase reporter gene linked to the simian virus 40 (SV40) promoter (Promega).

Cell lines and transient transfection assays. Nalm-6, a human pre-B-cell line, and DHL-9, a human mature B-cell line, were cultured in RPMI medium with 10% fetal calf serum. DNA transfections were performed as previously described (14) with cells in log phase (5×10^5 cells per ml). The Rous sarcoma virus–β-galactosidase plasmid was used to control for variations in transfection efficiency. Cells were washed and resuspended in unsupplemented RPMI medium to a final concentration of 2×10^7 cells per ml and incubated for 10 min at room temperature after the addition of 20 μg of DNA plus 10 μg of DEAE-dextran (4). Electroporations were carried out with a Bio-Rad gene pulser at 370 mV and 960 μF. 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 the luminescence was quantitated with an LKB 1251 luminometer. Each assay was performed at least five times in duplicate with at least three different plasmid preparations. The average values with the standard deviations are plotted.

EMSA. The oligonucleotides used as probes for the EMSA are shown above. The oligonucleotides were synthesized with 5′ overhangs and end labeled with [α-^{32}P]dCTP and Klenow polymerase. The binding solution was as follows: 10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 1.75% polyvinyl alcohol, 5% glycerol, 2 μg of bovine serum albumin (BSA), 2 μg of poly(dI-dC), 0.5 ng (10^4 cpm) of end-labeled DNA oligonucleotide probe, and 2 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 competitor oligonucleotides for 15 min at room temperature and run on a 4% polyacrylamide gel in 25 mM Tris (pH 8.5)–190 mM glycine–1 mM EDTA. Electrophoresis was performed at 220 V at 4°C for 2 h and 40 min. For the competition studies, various molar excesses of an unlabeled competitor oligonucleotide were added to the binding reaction mixture. As a nonspecific competitor, an oligonucleotide (NS) containing the sequence downstream of the *bcl*-2 b3 site from −1019 to −1000 was used.

UV cross-linking and SDS-polyacrylamide gel electrophoresis. EMSA was performed as described above. The autoradiograph of the wet gel was used to locate the EMSA complexes. UV cross-linking was performed essentially as described elsewhere (1) with a short-wavelength UV light box at 4°C for 90 min. Regions of the gel containing the complexes were cut out, and the individual complexes were eluted at room temperature overnight in a mixture of 50 mM Tris-HCl (pH 7.9), 0.1% sodium dodecyl sulfate (SDS), 0.1 mM EDTA, 5 mM dithiothreitol, 150 mM NaCl, and 0.1 mg of BSA per ml. The eluted protein was precipitated with 4 volumes of acetone, washed with ethanol, and air dried. After resuspension in Laemmli loading buffer, SDS-polyacrylamide gel electrophoresis was performed. The Amersham enhanced chemiluminescence kit was used for Western blot (immunoblot) analysis. The polyclonal antibody against Ets-1/Ets-2 which recognizes all Ets family proteins was obtained from Santa Cruz Biotechnology.

RESULTS

Identification of two negative regulatory regions in the *bcl*-2 promoter in pre-B cells. The *bcl*-2 5′ flanking region and 5′ untranslated region are shown schematically in Fig. 1A. Several deletions were made through the 5′ flanking and untranslated sequence to localize regions of functional importance in pre-B cells (Fig. 1B). We examined the effects of these deletions in constructs that contained both the 5′ (P1) and 3′ (P2) *bcl*-2 promoters and in constructs which contained only the P1 promoter. We were unable to demonstrate any transcript initiation at the P2 promoter, and we have confirmed the finding of an NRE just upstream of the P2 promoter (37). Although the overall results are similar, we have investigated in detail the constructs which contain only the P1 promoter because the activity is higher in the absence of the NRE and because we are unable to demonstrate transcript initiation at the P2 promoter.

A deletion of 156 bp from the 5′ end (−1796 to −1641) resulted in a six- to sevenfold increase in luciferase activity in the pre-B-cell line Nalm-6 (Fig. 2A, compare p[N-E] with p[S-E]). In the presence of a 64-bp 3′ region (−1062 to −999), the change in activity with deletion of the 156-bp 5′ region was only 2.5-fold (Fig. 2A, compare p[N-N] with p[S-N]). These results suggested that the presence of the 3′ 64-bp segment influenced the activity of the 5′ region. Deletion of the 3′ 64-bp region in the presence of the 5′ 156-bp region resulted in a twofold increase in luciferase activity (Fig. 2A, compare p[N-N] with p[N-E]). Deletion of the 64-bp region at the 3′ end in

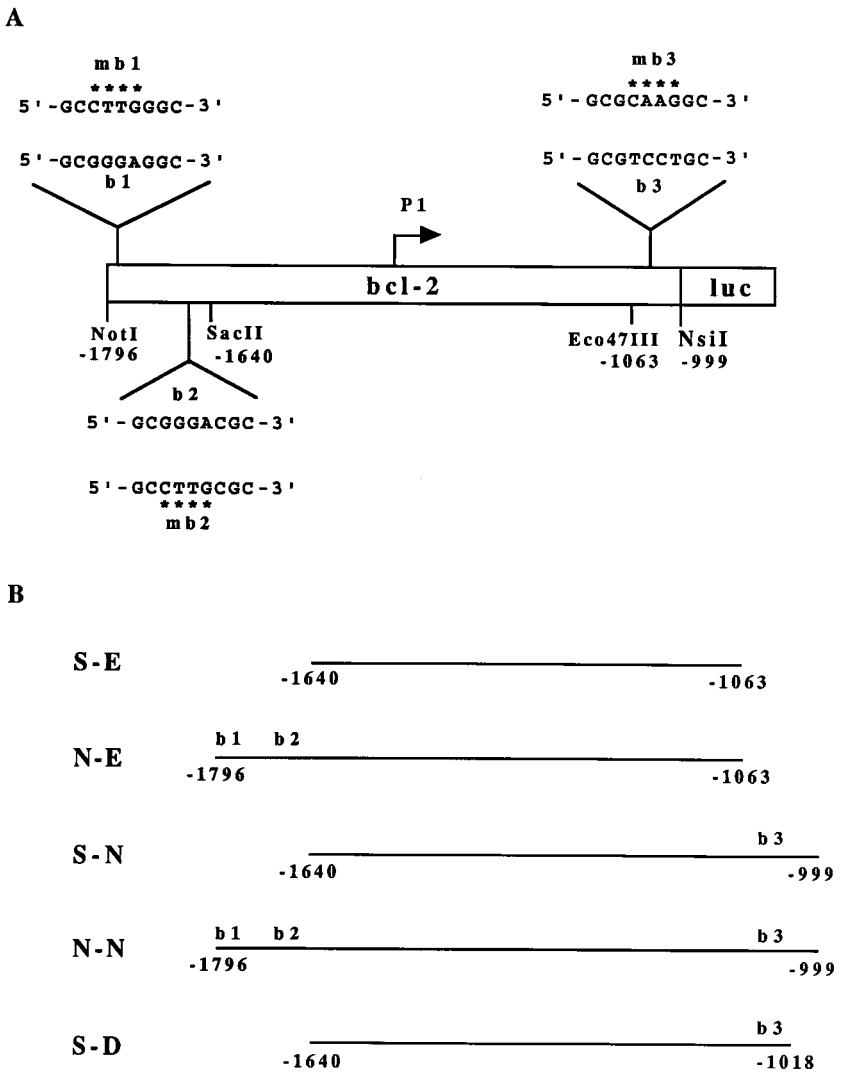

FIG. 1. Map of the *bcl*-2 promoter region and schematic diagram of the *bcl*-2–luciferase constructs. (A) Restriction map of *bcl*-2 promoter showing the locations of the restriction sites used for generating the *bcl*-2–luciferase constructs. The positions of the three π1 sites, b1, b2, and b3, are indicated. The mutated nucleotides in mb1, mb2, and mb3 are marked by asterisks. The arrow denotes the position of the 5′ transcription start site (P1). Numbering of the *bcl*-2 sequence is relative to the translation start site. (B) The regions of the *bcl*-2 sequence used in each of the luciferase constructs are shown schematically.

the absence of the 5′ 156-bp region resulted in a fivefold increase in luciferase activity (Fig. 2A, compare p[S-N] with p[S-E]). Again, the presence of the 5′ region appeared to influence the activity of the 3′ region. Deletion of either the 5′ region or the 3′ region resulted in approximately equal luciferase activity (Fig. 2A, compare p[S-N] with p[N-E]). Removal of both 5′ and 3′ regions simultaneously resulted in an 11-fold increase in luciferase activity (Fig. 2A, compare p[N-N] with p[S-E]). These results suggested that two discrete regions of the *bcl*-2 promoter were involved in negative regulation of *bcl*-2 expression in pre-B cells.

The same constructs were transfected into the mature B-cell line, DHL-9, to examine their function. Deletion of either the 5′ or 3′ region or both resulted in an increase in activity of less than 25% (Fig. 2B, compare p[N-N] with p[S-E]). The function of these two regions of the *bcl*-2 promoter appeared to be of greater consequence in pre-B cells than in mature B cells.

The π1 sequence is an NRE in the *bcl*-2 gene promoter. Three potential π1 sites, designated b1, b2, and b3 in Fig. 1A, were identified in the *bcl*-2 promoter. To determine whether the π1 sequence was responsible for the negative regulatory activity of the 156-bp 5′ and 64-bp 3′ regions, mutant constructs, p[N-E(mb1)], p[N-E(mb2)], and p[S-N(mb3)], with mutated sequences for the b1, b2, and b3 sites, respectively, were generated (Fig. 1A). Disruption of either the b1 or b2 site caused a drastic increase in the luciferase activity (Fig. 3A). Mutation of both the b1 and b2 sites resulted in an increased activity that was approximately 85% of the activity shown by the deletion construct p[S-E]. These mutant constructs demonstrated that the π1 sequences (b1 and b2) accounted for the negative regulatory activity of the 156-bp 5′ region, with b2 playing the major role. Mutation of the b3 site in the 3′ region also resulted in an increase in the transcriptional activity. However, the activity of the mb3 mutant was only half of the activity of the deletion construct p[S-E] (Fig. 3B). Another deletion construct, p[S-D], was made to examine regions other than the π1 site which could play a role in the negative regulatory activity associated with the 3′ region. The construct p[S-D] was

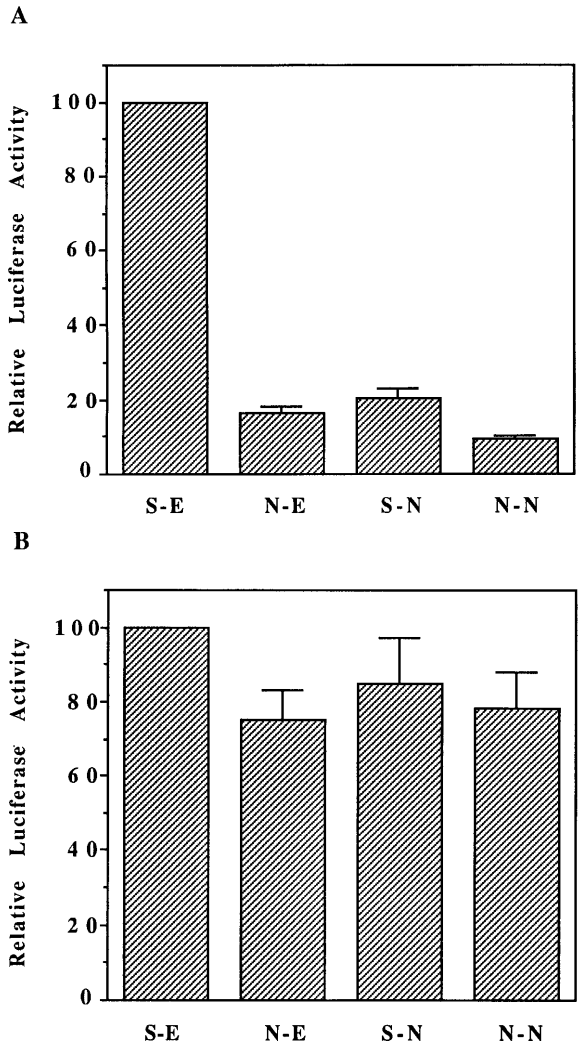

FIG. 2. Localization of two negative regulatory regions in the *bcl*-2 promoter in pre-B cells. Transient transfection analyses of the *bcl*-2 promoter constructs in Nalm-6 pre-B cells (A) and DHL-9 mature B cells (B). For both panels, cells were transfected with 20 μg of the p[S-E], p[N-E], p[S-N], or p[N-N] construct. The results are shown relative to the luciferase activity of construct p[S-E], which was assigned a value of 100.

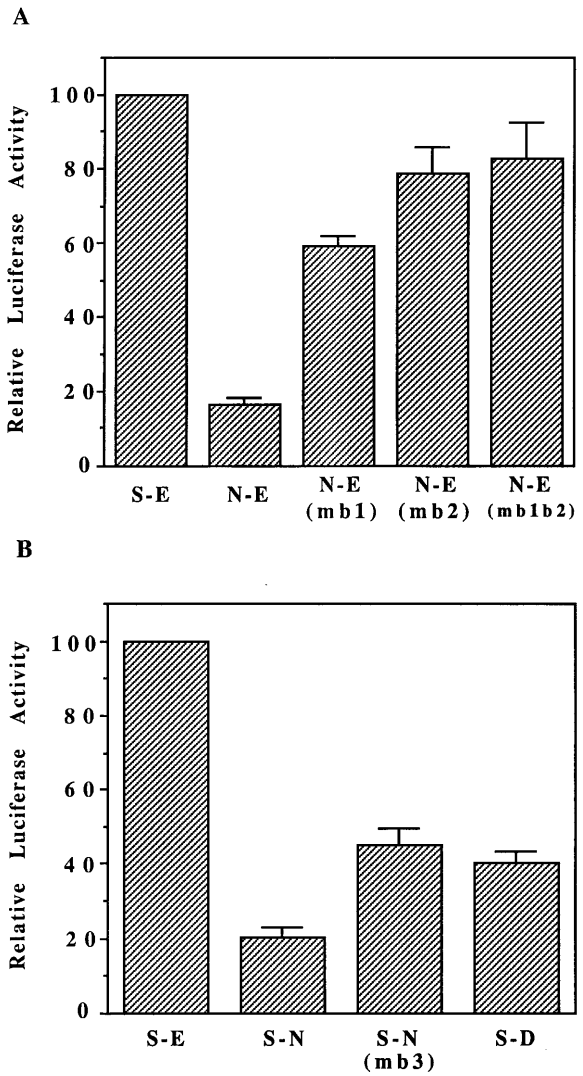

FIG. 3. Functional analyses of the π1 sequences in the two negative regulatory regions of the *bcl*-2 promoter in Nalm-6 cells. (A) Transient transfection results with constructs with mutated π1 sites (mb1 and mb2) in the 5′ negative regulatory region. The mutated bases are indicated in Fig. 1A. The activity of p[S-E] is set at 100. The activity of p[N-E] is shown for comparison with the mutated π1 sites in the construct p[N-E]. p[N-E(mb1)] and p[N-E(mb2)] are constructs with mutated b1 and b2 sites, respectively; p[N-E(mb1b2)] is a construct with both b1 and b2 sites mutated. (B) Transient transfection analysis with a construct with a mutated π1 site, mb3, in the 3′ negative regulatory region. The b3 site was mutated in the construct p[S-N] to yield p[S-N(mb3)]. The construct p[S-D] was derived from p[S-N] by deletion of a 19-bp fragment downstream of the b3 site. The activity of p[S-E] is set at 100, and the results are shown relative to it.

derived from p[S-N] by deletion of a 19-bp segment (−1017 to −999) downstream of the b3 site. This construct yielded a 40% increase in transcriptional activity (Fig. 3B). These results indicate that there is another negative regulatory region downstream of the b3 site; this site has not been further characterized.

An Ets family protein interacts with the *bcl*-2 π1 sequences. To characterize nuclear factors that interact with the *bcl*-2 π1 sequences, three oligonucleotides containing the b1, b2, and b3 sites were radioactively labeled and used as probes in EMSAs. When incubated with nuclear extracts from Nalm-6 cells, five major complexes of retarded mobility were formed with each probe (Fig. 4). A probe with a mutated π1 site (mb3) was also used in the EMSA. As shown in Fig. 4, lane 2, the complex marked with an arrow disappeared while the other four complexes were unchanged (Fig. 4, compare lanes 2 and 1). The IgH π1 site was also used as a probe in an EMSA, and a complex of similar mobility was observed (marked with the arrow in Fig. 4). The EMSA patterns with the b2 and Ig oligonucleotides differed somewhat from the pattern seen with the b1 and b3 oligonucleotides, but the complex marked with an arrow in Fig. 4 had an identical mobility with all four oligonucleotides, b1 to b3 and Ig. We have not characterized further the proteins responsible for the formation of the other EMSA complexes.

To further demonstrate the specificity of this EMSA complex, competition experiments were performed with oligonucleotides containing the *bcl*-2 and IgH π1 sequences. Formation of this complex with the b1 probe could be eliminated by a 50-fold molar excess of b1, b3, and Ig but not by 200-fold molar excess of nonspecific oligonucleotide (Fig. 5A). Like-

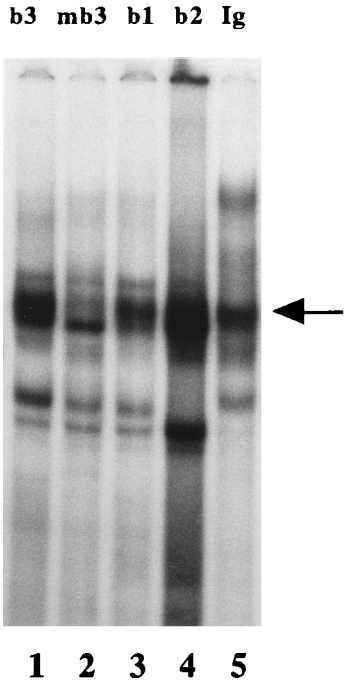

FIG. 4. EMSA of the π1 site oligonucleotides with pre-B-cell nuclear extracts. End-labeled wild-type and mutated π1 site oligonucleotides b1, b2, b3, mb3, and Ig (0.5 ng, 10^4 cpm) were incubated with 2 μg of nuclear extract from Nalm-6 cells. The position of the complex formed with the π1 site is indicated by an arrow.

wise, the b1, b2, and b3 sites could be cross-eliminated by each other or by the IgH π1 site (Fig. 5B and C). Under identical conditions, the oligonucleotide containing the mutated b3 site was shown to be an inefficient competitor (Fig. 5B, lanes 6 and 7; Fig. 5C, lanes 4 and 5), and the same was true for both mb1 and mb2. All of the *bcl*-2 π1 sites, b1, b2, and b3, were able to compete with the labeled IgH π1 probe in the EMSA (data not shown).

The π1-binding protein was found in murine pre-B cells but not in mature B cells (20). We did not observe the π1 EMSA complex with the *bcl*-2 π1 binding sites when nuclear extract from a human mature B-cell line, DHL-9, was used (data not shown).

On the basis of the similarity of the IgH π1 site to recognition sequences for Ets family proteins, it was suggested that the protein interacting with the π1 site was an Ets-related transcription factor. To characterize the protein that interacted with the *bcl*-2 π1 sites, UV cross-linking of the EMSA complexes followed by denaturing polyacrylamide gel electrophoresis was performed. A single protein with a molecular mass of 76 kDa was identified (Fig. 6A, lanes 1 to 3). The migration of this protein was identical to that of the protein that bound to the IgH π1 site (Fig. 6A, lane 4). Western analysis of the same gel with an antibody reactive against all Ets family proteins revealed a 76-kDa protein (Fig. 6B). This protein comigrated with the ^{32}P-labeled protein in Fig. 6A. These results suggested that an identical or at least immunologically indistinguishable Ets protein of similar size interacted with the *bcl*-2 and IgH π1 sequences. To determine the size of the protein without the covalently linked oligonucleotide, Western analysis was performed on the EMSA complexes in the absence of UV cross-linking. As shown in Fig. 6C, the π1-binding protein had a molecular mass of approximately 58 kDa. Although no protein-DNA complex was visible on EMSA with the π1 probes and DHL-9 nuclear extract, we analyzed the region of the EMSA gel corresponding to the location of the complex formed with pre-B-cell nuclear extracts. As shown in Fig. 6D, no protein of 76 kDa was present in the denaturing polyacrylamide gel analysis of the region of the EMSA gel with the b3 oligonucleotide and DHL-9 nuclear extract (lane D). In addition, no reactivity with the Ets antibody was observed when Western analysis was performed on this sample (data not shown).

***bcl*-2 π1 sequences repress the transcriptional activity of a heterologous promoter.** To determine whether the negative regulatory activity of the *bcl*-2 π1 sites was due to their minor sequence deviations from the IgH π1 site or whether the activity was promoter specific, we synthesized π1 oligonucleotides linked to the SV40 promoter. We confirmed that the *bcl*-2 π1 sites function in an orientation-independent manner (Fig. 7, b2[1f] and b2[1r]). A single copy of the b2 π1 site decreased the activity of the SV40 promoter by 25 to 30% in Nalm-6 cells. Two copies of the b2 or b3 site decreased the luciferase activity of the parental construct by 50% (Fig. 7, b2[2] and b3[2]). In contrast to these results, two copies of the IgH π1 sequence enhanced the transcriptional activity of the same promoter more than twofold (Fig. 7, Ig[2]). Thus, at least some of the opposing transcriptional activity of the *bcl*-2 and IgH π1 sequences appears to be due to sequence differences and is independent of the promoter context.

DISCUSSION

We have identified two regions of the *bcl*-2 gene that function as negative regulatory elements. The negative regulatory activity has been localized to three π1 sites. The two π1 sites, b1 and b2, in the 5′ negative regulatory region appeared to account for all of the activity of this region. The b3 site in the 3′ negative regulatory region contributed approximately 50% of the activity of this region. These sites were active in the pre-B-cell line, Nalm-6, but not in the mature B-cell line, DHL-9. EMSA with each of the π1 oligonucleotides and Nalm-6 nuclear extract revealed several complexes. Mutation of the π1 site led to the disappearance of one complex. In transient transfection studies, the same mutation resulted in an increase in activity. These data demonstrate that mutations of the π1 site that abrogate its negative regulatory activity also eliminate protein binding.

The *bcl*-2 π1 sites cross-competed with each other and with the IgH enhancer π1 site on EMSA. UV cross-linking of this complex followed by SDS-polyacrylamide electrophoresis revealed a protein with a molecular mass of 58 kDa after correction for the bound oligonucleotide. Western analysis with an Ets antibody confirmed that this protein was a member of the Ets family. As was the case for the IgH enhancer with murine B cells (20), this EMSA complex was not formed with nuclear extract from the mature B-cell line, DHL-9. Although it is not clear which Ets protein binds to the IgH enhancer π1 site in vivo, several Ets proteins, including Ets-1, Fli.1, ERG-3, and ERP, have been shown to bind in vitro (22, 26, 31). Both ERG-3 and ERP are expressed at higher levels in pre-B cells than in mature B cells. It is possible that one of these two Ets proteins binds to the *bcl*-2 π1 sites in vivo, although the protein that we have identified from EMSA analysis has a molecular mass somewhat higher than that predicted from the sequence of either ERG-3 or ERP.

In contrast to the positive regulation displayed by the IgH enhancer π site, the *bcl*-2 π1 sites were negative regulatory

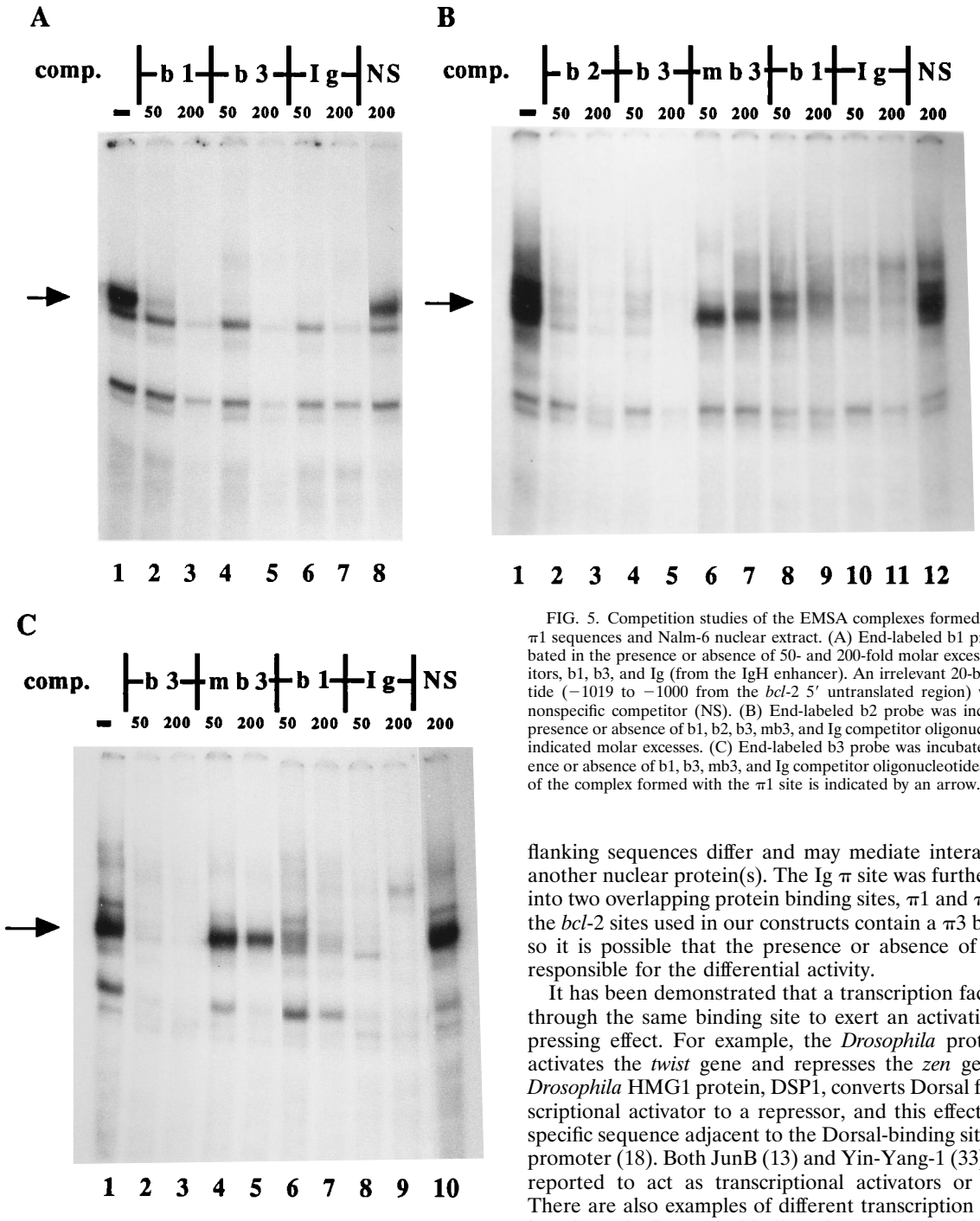

FIG. 5. Competition studies of the EMSA complexes formed with the *bcl*-2 π1 sequences and Nalm-6 nuclear extract. (A) End-labeled b1 probe was incubated in the presence or absence of 50- and 200-fold molar excesses of competitors, b1, b3, and Ig (from the IgH enhancer). An irrelevant 20-bp oligonucleotide (−1019 to −1000 from the *bcl*-2 5′ untranslated region) was used as a nonspecific competitor (NS). (B) End-labeled b2 probe was incubated in the presence or absence of b1, b2, b3, mb3, and Ig competitor oligonucleotides at the indicated molar excesses. (C) End-labeled b3 probe was incubated in the presence or absence of b1, b3, mb3, and Ig competitor oligonucleotides. The position of the complex formed with the π1 site is indicated by an arrow.

elements when linked to a heterologous promoter. It should be noted, however, that the magnitude of the change was less with a heterologous promoter than with the endogenous *bcl*-2 promoter. Sequence differences between the π1 sites may play an important role in the differential transcriptional activity observed between the *bcl*-2 and IgH enhancer π1 sites, but it is likely that the promoter context is also important for determining the transcriptional activity of the π1 sites. The b3 π1 sequence differs from the Ig enhancer π1 site by only a single nucleotide, and the b1 and b2 sites differ from the Ig π1 site by two nucleotides. It is possible that these minor changes are responsible for the difference in activity. Alternatively, the flanking sequences differ and may mediate interactions with another nuclear protein(s). The Ig π site was further dissected into two overlapping protein binding sites, π1 and π3. None of the *bcl*-2 sites used in our constructs contain a π3 binding site, so it is possible that the presence or absence of this site is responsible for the differential activity.

It has been demonstrated that a transcription factor can act through the same binding site to exert an activating or a repressing effect. For example, the *Drosophila* protein Dorsal activates the *twist* gene and represses the *zen* gene (18). A *Drosophila* HMG1 protein, DSP1, converts Dorsal from a transcriptional activator to a repressor, and this effect requires a specific sequence adjacent to the Dorsal-binding site in the *zen* promoter (18). Both JunB (13) and Yin-Yang-1 (33) have been reported to act as transcriptional activators or repressors. There are also examples of different transcription factors acting through the same binding site to differentially regulate target gene expression (15, 36). In addition, a number of overlapping binding sites for positive and negative regulatory factors have been described (16, 17). Further studies will be required to determine the mechanisms mediating the activator or repressor functions of the π1 binding sites.

An NRE has been identified in the 5′ untranslated region of *bcl*-2 between bp −279 and −85 (37). The NRE was able to inhibit expression from the *bcl*-2 P1 promoter or from a heterologous promoter in a position-dependent manner (37). More recently, the NRE was shown to contain a p53-dependent element that functioned in an orientation- and position-independent fashion (25). Whether p53 acted directly or indi-

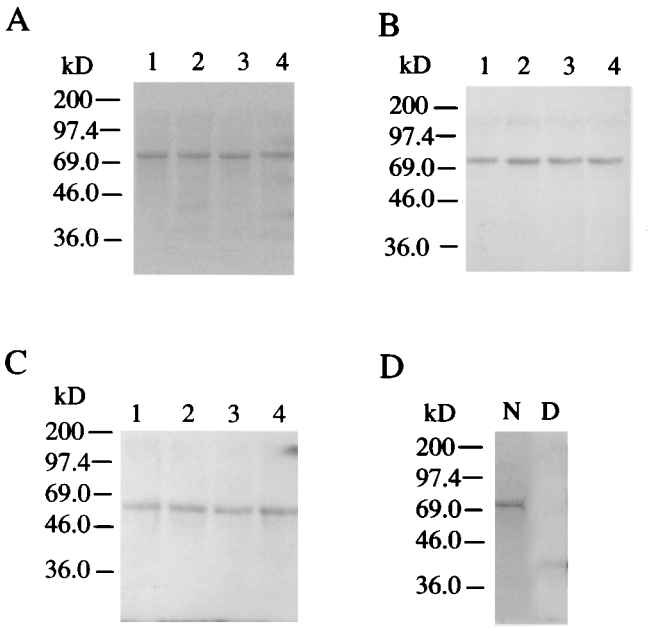

FIG. 6. An Ets family protein binds to the *bcl*-2 π1 sequences. (A) Denaturing SDS-polyacrylamide gel analysis of the UV cross-linked EMSA complexes formed with the π1 sites b1, b2, b3, and Ig in lanes 1, 2, 3, and 4, respectively. A single protein with a molecular mass of 76 kDa is seen. The migration of the molecular mass markers is shown on the left. (B) Western analysis with the Ets-1/Ets-2 antibody of the cross-linked EMSA complexes shown in panel A. The bands on the Western blot comigrate with the ^{32}P-labeled bands shown in panel A. (C) Western analysis with the Ets-1/Ets-2 antibody of the non-cross-linked EMSA complexes formed with the π1 sites b1, b2, b3, and Ig (lanes 1 to 4, respectively). In the absence of the bound oligonucleotide sequences, the molecular mass of the π1 site-binding protein is 58 kDa. (D) Denaturing SDS-polyacrylamide gel analysis of the UV cross-linked EMSA complex formed with the π1 site oligonucleotide b3 and Nalm-6 nuclear extract (lane N) and the corresponding region of the gel (no protein-DNA complex was present) from EMSA with the b3 site and DHL-9 nuclear extract (lane D).

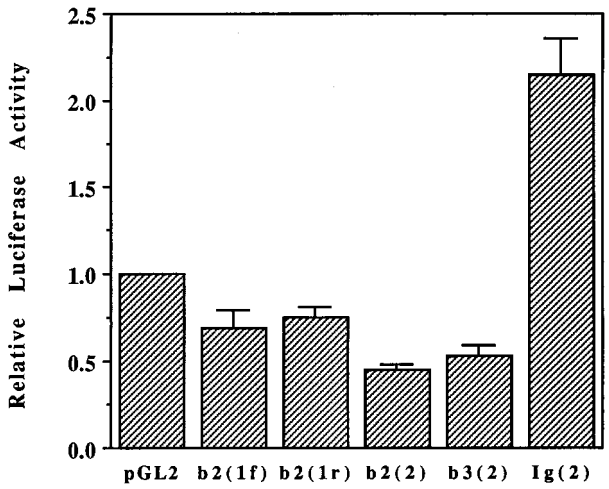

FIG. 7. Effect of the *bcl*-2 and IgH π1 sequences on the SV40 promoter activity. Transient transfection analyses of the following constructs: b2(1f), one copy of the b2 oligonucleotide in the forward orientation; b2(1r), one copy of the b2 oligonucleotide in the reverse orientation; b2(2), b3(2), and Ig(2), two copies of the b2, b3, and Ig oligonucleotides, respectively. All of the oligonucleotides were linked to the SV40 promoter in the pGL2 construct. Transfections were done in Nalm-6 cells, and the results were calculated relative to the luciferase activity of the pGL2 construct, which was assigned a value of 1.

rectly to downregulate *bcl*-2 expression through this element was not determined. The three π1 sites that we have identified are located from 743 to 1,510 bp upstream of the NRE and are active in either the presence or the absence of the NRE sequence in Nalm-6 cells.

The *bcl*-2 π1 sites may play a role in the developmental regulation of the *bcl*-2 gene. In normal B cells, Bcl-2 levels are low in pre-B cells and increase as the cells differentiate. Our preliminary studies suggest that the same region of the *bcl*-2 promoter is responsible for positive regulation in both pre-B and mature B cells. It is possible that the π1 sites override the positive regulatory region in pre-B cells. It should be noted that, as is usually the case, our transfection analyses were done in malignant cell lines. It is likely that these malignant cells display abnormal regulation of the endogenous *bcl*-2 gene because it is expressed in malignant pre-B cells. We have found that a transfected *bcl*-2 promoter displays much higher activity in the mature B cell, DHL-9, compared with that in the pre-B cells, Nalm-6, after controlling for differences in transfection efficiency. These results are analogous to the situation in normal B cells and suggest that malignant lines represent an adequate model system to study *bcl*-2 transcriptional regulation.

In summary, we have identified three π1 sites that negatively regulate *bcl*-2 expression in pre-B cells but not in mature B cells. An Ets family protein binds to these sites and to the IgH enhancer π1 site. The *bcl*-2 π1 sites may play a significant role in the regulation of expression of the *bcl*-2 gene during B-cell differentiation.

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