A-Myb Up-regulates Bcl-2 through a Cdx Binding Site in t(14;18) Lymphoma Cells*

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In follicular lymphoma, bcl-2 is translocated to the immunoglobulin heavy chain locus leading to deregulation of bcl-2 expression. We examined the role of Myb proteins in the regulation of bcl-2 expression in lymphoma cells. We showed that A-Myb up-regulates bcl-2 promoter activity. Northern and Western analyses demonstrated that A-Myb was expressed in the DHL-4 t(14;18) cell line. In t(14;18) cells and mature B cells, A-Myb up-regulated bcl-2 expression, whereas B- and c-Myb had little effect on bcl-2 gene expression. Deletion analysis of the bcl-2 5’-region identified a region responsive to A-Myb in t(14;18) cells. A potential binding site for the Cdx homeodomain proteins was located in this sequence. Analysis of the A-Myb-responsive region by UV cross-linking experiments revealed that a 32-kDa protein formed a complex with this region, but direct binding by Myb proteins could not be demonstrated. A-Myb could be recovered along with Cdx2 when nuclear extracts were passed over the Cdx site. Mutagenesis of the Cdx binding site abolished binding by the 32-kDa protein and significantly reduced the ability of A-Myb to induce bcl-2 expression. A strong induction of bcl-2 P2 promoter activity was observed in cotransfection studies of DHL-4 cells with the A-Myb and Cdx2 expression vectors, and increased endogenous Bcl-2 protein expression was observed in B cells transfected with A-Myb and/or Cdx2 expression constructs.

The bcl-2 gene was originally identified by its involvement in the t(14;18) translocation associated with human follicular lymphoma (1). The translocation of the bcl-2 gene from chromosome 18q to the immunoglobulin heavy chain locus at 14q results in the deregulated expression of bcl-2 (2). The overexpression of bcl-2 in t(14;18) lymphoma leads to high levels of bcl-2 mRNA and protein, which act to protect cells from apoptosis (3). During B cell development, bcl-2 is expressed at low levels in pre-B cells, in which extensive cell death occurs by apoptosis, whereas bcl-2 expression is higher in mature and activated B cells (3, 4). The mechanisms of normal and deregulated expression of bcl-2 remain unclear although recent studies have provided some insight. In t(14;18) cells, regulatory elements of both the bcl-2 promoter and the immunoglobulin heavy chain enhancers are believed to play a role in bcl-2 overexpression.

Two promoters mediate initiation of bcl-2 gene transcription. The 5’-promoter (P1) is located 1306–1423 base pairs upstream of the translational start site (5). This is a TATA-less, GC-rich promoter that displays multiple start sites. The 3’-promoter (P2) is located 1.3 kilobases downstream of the P1 promoter (5). The P2 promoter contains a TATA-box and CCAAT element. A number of negative regulatory sites have been described in the bcl-2 promoter region. We have previously demonstrated three \(1\) binding sites, which function as negative regulators of bcl-2 expression in pre-B cells (6). The WT1 protein has also been shown to repress bcl-2 activity in HeLa cells and B cells (7, 8).

In addition, a negative regulatory element upstream of the P2 promoter has been described, although the proteins that bind to this element have not been identified (9). The p53 tumor suppressor has been shown to mediate repression of bcl-2 directly or indirectly through a 195-base pair region (10). We have also characterized a cyclic AMP (cAMP)-responsive element that is responsible for the positive regulation of bcl-2 expression during the activation of mature B cells and during the rescue of immature B cells from calcium-dependent apoptosis (11). In chicken myeloid cells, v-Myb was reported to function as an anti-apoptotic factor by up-regulation of bcl-2 expression, whereas in murine T cells, both c-Myb and B-Myb were shown to induce bcl-2 promoter activity (12–14). It is not clear if the Myb transcription factors have a similar function in human B cells.

From our studies of the deregulation of bcl-2 in t(14;18) lymphoma cells, we have shown that the cAMP-response element site in the 5’-flanking sequence of the translocated bcl-2 gene is occupied (15). The cAMP-response element-binding protein family of proteins was shown to bind to this site in vitro, and the maximal increase in bcl-2 activity mediated by the immunoglobulin heavy chain enhancers in transient transfection assays was dependent on an intact cAMP-response element site. We have also described an in vivo footprint over a WT1 site on the normal silent bcl-2 allele; this site displayed a negative regulatory activity (8). The site was not occupied on the translocated allele, and the presence of the immunoglobulin enhancers prevented the repression by WT1.

We now describe the positive regulation of bcl-2 promoter activity by the A-Myb transcription factor. The Myb family of transcription factors, which includes the structurally related A-, B-, and c-Myb genes, is thought to play a pivotal role in differentiation and proliferation of various cells. Each member is able to transactivate promoters with the consensus sequence PyAAC(G/T)G (16–19). c-Myb is expressed predominantly in immature and rapidly dividing hematopoietic cells. B-Myb is...
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expressed in many cell types (20, 21), whereas A-Myb expression is limited to certain stages of reproductive tissues, some neural cells, and a subset of normal and neoplastic B lymphocytes (22–26). In this study, we show that A-Myb and B-Myb, but not c-Myb, are expressed in t(14;18) lymphoma cells. We also demonstrate that A-Myb is an effective activator of the bcl-2 promoter in t(14;18) and mature B cells, but it does not bind to a consensus sequence within the bcl-2 promoter. We show that A-Myb mediates positive regulatory activity through a Cdx homeodomain protein binding site and that A-Myb and a Cdx family member, Cdx2, are components of the complex formed at this sequence. By cotransfection studies, we demonstrate that the expression of A-Myb and Cdx2 results in a marked increase in bcl-2 promoter activity. The endogenous Bcl-2 protein is also increased when A-Myb and/or Cdx2 are transfected into B cells. Because the role of Bcl-2 in protection from apoptosis is well established in B cells, A-Myb functions as an anti-apoptotic factor through its ability to up-regulate Bcl-2 expression.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—The bcl-2-promoter-luciferase constructs have been described previously (11). Deletions of the bcl-2 5′-flanking sequence were made by polymerase chain reaction subfragment cloning or by insertion of restriction enzyme sites using site-directed mutagenesis methods (CLONTECH and Stratagene) followed by subfragment cloning. Constructs with mutations in the Cdx or Myb binding sites were generated from the −748 construct using the Chameleon double-stranded site-directed mutagenesis kit (Stratagene). The oligonucleotide sequences used as the mutagenic primers are as follows with the transcription factor binding site underlined and the mutated bases in boldface: Cdx Mut: GGGGACCGACCTTTTATCTTTCACTTTCCGGTTGTGTTTTTACAACGTACG; 5′-Myb Mut: GGGGGAGATTCTCGAGGAGGTTCAGGAGCCCCCTGAGCACG; 3′-Myb Mut: GGGGGTGGCTTCTCGAACCTTCTCTGGG. The construct with mutations in the Cdx or Myb binding sites was generated from the 5′-Myb Mut construct. The oligonucleotide sequence used as the mutagenic primer was: GGGGGAGATTCTCGAGGAGGTTCAGGAGCCCCCTGAGCACG. All plasmid sequences were confirmed by sequencing.

The rat Cdx2 expression vector was a kind gift from Dr. Eric Sibley (Stanford University). The human A-, B-, and c-Myb and the rat Cdx2 expression vectors consisted of the full-length coding region under the control of the SV40 promoter or the cytomegalovirus immediate early promoter. The Cdx2-myc tag construct (Cdx2-M) was made by polymerase chain reaction subfragment cloning or by insertion of restriction enzyme sites using site-directed mutagenesis methods (CLONTECH and Stratagene). The oligonucleotide sequence used as the mutagenic primer was: GGCGGAGATTCTCGAGGAGGTTCAGGAGCCCCCTGAGCACG. All plasmid sequences were confirmed by sequencing.

The double-stranded site-directed oligonucleotides used for EMSA of the A-Myb-responsive region in the bcl-2 promoter are shown below with the Cdx binding site underlined and the mutated bases in boldface.

**Plasmid Constructs**

| SEQUENCE 1 | MUTATED CDNAX |
|------------|--------------|
| GGGATTCTCGAGGAGGTTCAGGAGCCCCCTGAGCACG | GGGATTCTCGAGGAGGTTCAGGAGCCCCCTGAGCACG |

**Isolation of Nuclear Proteins Binding to Biotinylated Probes**—Oligonucleotides representing the rat Cdx 2 Cdx and mutated Cdx (Mut Cdx) sequences shown above were synthesized, and 5 μg of the sense strand of each sequence was labeled with [α-32P]dCTP and Klenow polymerase. The binding reaction mixture contained 10μM Tris-HCl (pH 7.5), 30 mM NaCl, 0.5 mM EDTA, 1 mM diethiothreitol, 5% glycerol, 1 μg of poly(dI-dC), 6 μg of bovine serum albumin, 0.5 ng (10 4cpm) of end-labeled DNA oligonucleotide probe, and 5–10 μg of protein from crude nuclear extract. Electrophoresis was performed as described previously (30).

**UV Cross-linking and SDS-Polyacrylamide Gel Electrophoresis**—EMSAs was performed as above with the binding reaction scaled up 5-fold. UV cross-linking and SDS-polyacrylamide gel electrophoresis were performed as described previously (30).

**Selection of Transfected Cells**—DHL-9 or DH1-4 cells were transfected using the previously described procedure with 10 μg of the pHook2 vector (Invitrogen) and 5 μg of A-Myb and/or 5 μg of Cdx2-M vectors or an equivalent amount of empty expression vector. After incubation for 24 h at 37°C, the transfected cells were selected for sFV expression using hapten-coated magnetic beads (Invitrogen). Equal numbers of transfected or nontransfected control cells were lysed in Laemmli buffer and heated at 95°C for 5 min. The lysates were separated by SDS-PAGE and transferred onto nitrocellulose. Western de-
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**RESULTS**

Expression of A-, B-, and c-Myb in Different B Cell Lines—To elucidate the role of the Myb family of transcription factors in the up-regulation of bcl-2 gene expression, we determined the pattern of Myb expression in malignant B cells. We tested for A-, B-, and c-myb RNA expression in the four B cell lines Nalm-6, DHL-9, DHL-4, and Raji by Northern blot analysis. Using radiolabeled probes specific for either A-, B-, or c-Myb, we found that A-Myb was expressed in the two cell lines containing translocations, DHL-4 and Raji, but A-myb was not expressed in Nalm-6 or DHL-9 cells (Fig. 1A). In contrast to A-Myb, c-myb was not expressed in DHL-4 or Raji cells but was expressed in Nalm-6 and DHL-9 cells (Fig. 1A). B-Myb was expressed in all four cell lines (Fig. 1A).

To determine if myb RNA levels correlated with Myb protein levels, we examined a number of B cell lines for A-, B-, and c-Myb protein expression by Western blot analysis. In testing for A-Myb expression, the BJAB Burkitt’s cell line and the IM-9 myeloid cell line were used as positive and negative controls, respectively. Similar to the Northern data, A-Myb was expressed in DHL-4 cells and Raji but not in DHL-9 or Nalm-6 cells (Fig. 1B). The Nalm-6, DHL-9, DHL-4, and Raji cells were examined for B- and c-Myb protein expression. As shown in Fig. 1B, the pattern of B- and c-Myb protein expression exactly paralleled the pattern of RNA expression.

A-Myb Is a Positive Regulator of the bcl-2 Promoter in B Cells—A schematic representation of the human bcl-2 5’-flanking and 5’-untranslated regions is shown in Fig. 2A. Sequence analysis revealed two potential binding sites for Myb proteins in the bcl-2 P2 promoter. To determine if the Myb transcription factors were capable of regulating the bcl-2 5’-(P1) and 3’-(P2) promoters, cotransfection experiments were performed.

Transfection of DHL-9 cells with the construct containing both the bcl-2 P1 and P2 promoters and an A-Myb expression vector resulted in a 7-fold increase in luciferase activity over the activity of the promoter construct when cotransfected with an empty expression vector (Fig. 2B). When the same construct was transfected with a B- or c-Myb expression vector, there was little change in the promoter activity (Fig. 2B). When a construct containing the bcl-2 P2 promoter was transfected with the A-Myb expression vector into DHL-9 cells, the result was a 6- to 7-fold increase in luciferase activity, which was similar to that of the construct containing both the P1 and P2 promoters (Fig. 2B). Transfection of the same construct with the B-Myb expression vector showed less than a 3-fold induction of luciferase activity, whereas transfection with the c-Myb expression vector resulted in less than a 2-fold increase (Fig. 2B). These data suggested that B- and c-Myb, both of which are expressed in DHL-9 cells, have very little effect on bcl-2 promoter activity. Although A-Myb is not expressed in DHL-9 cells, it was a strong inducer of the bcl-2 promoter, primarily acting through the P2 promoter. Because A-Myb is expressed in DHL-4 cells but not in DHL-9 cells, our studies focused on DHL-4 cells.

To assess the activity of the Myb proteins on the bcl-2 promoter in t(14;18) cells, the same constructs were transfected into the DHL-4 cell line. In these cells, A-Myb acted as a powerful inducer of the bcl-2 promoter. When the construct containing the P1 and P2 promoters was transfected with the A-Myb expression vector into DHL-4 cells, the result was a 30-fold increase in luciferase activity (Fig. 2C). A-Myb also increased the activity of the bcl-2 P2 promoter by more than 30-fold (Fig. 2C). Unlike A-Myb, B- and c-Myb had no significant effect on the bcl-2 P2 promoter activity in DHL-4 cells (Fig. 2C). These results suggested that A-Myb, rather than B- or c-Myb, was the functional Myb family member with respect to bcl-2 promoter activity in DHL-4 cells. Both the P1 and P2 promoters are utilized in DHL-4 cells, and A-Myb acts primarily through the P2 promoter.

Identification of the Regions in the bcl-2 Promoter Responsive to A-Myb in t(14;18) Cells—To identify the region of the bcl-2 P2 promoter responsive to A-Myb, deletion constructs of this promoter were transfected with an A-Myb expression vector into DHL-4 cells. Sequence analysis of the P2 promoter revealed a TATA-box, a CCAAT element, an octamer site, and two potential Myb binding sites (Fig. 3A). Surprisingly, results from the transfection studies showed that the region mediating induction by A-Myb was between −297 and −324, which is upstream of the Myb consensus sequences (Fig. 3B). When this region was deleted, induction of the bcl-2 P2 promoter by A-Myb was dramatically reduced (Fig. 3B). Comparison of this region with a transcription factor data base revealed a potential binding site for the Cdx homeodomain protein family members flanked by stretches of AT-rich sequences. These results suggested that induction of the bcl-2 P2 promoter by A-Myb was not mediated by the Myb binding sites but possibly through a homeodomain protein binding site.

Characterization of the Protein That Interacts with the A-Myb-responsive Region in the bcl-2 P2 Promoter—To determine if a protein interacted specifically with the A-Myb-responsive region of the bcl-2 P2 promoter, EMSA was performed using an oligonucleotide encompassing the sequence. Two specific complexes and a few nonspecific complexes formed when the labeled oligonucleotide was incubated with DHL-4 nuclear extract (Fig. 4A, lane 1). Formation of the specific complexes could be prevented by competition with a 100-fold or greater
molar excess of unlabeled cold oligonucleotide (Fig. 4A, lanes 2–4), but not by a 500-fold molar excess of a nonspecific oligonucleotide (Fig. 4A, lane 8). To assess the involvement of the Cdx sequence in the specific complex formation, an oligonucleotide containing a change of the core Cdx binding site from ATTA to GCGG was used as a competitor. As shown in Fig. 4A, lanes 5–7, this oligonucleotide was not able to compete for the two specific complexes formed by the wild-type sequence, even at a 500-fold molar excess. When the mutant oligonucleotide was labeled and incubated with DHL-4 nuclear extract, no specific complexes were formed (Fig. 4A, lanes 9–12).

EMSA followed by UV cross-linking and SDS denaturing gel electrophoresis was performed to determine the composition of the two specific complexes. In each case, a single protein was observed (Fig. 4B, lanes 1 and 2). After correction for the bound oligonucleotide, the molecular mass was 32 kDa (Fig. 4B, lanes 1 and 2). This is approximately the size of the Cdx homeodomain proteins. There was no antibody available against Cdx so we were not able to confirm by Western blot analysis that this protein was a Cdx family member. We also do not have an antibody to supershift A-Myb so we could not confirm by EMSA that A-Myb was in the complex binding to the Cdx site.

We also attempted to characterize the faster migrating EMSA complexes because these displayed such strong binding. Loss of these complexes in the EMSA studies could only be achieved by mutation of the Cdx binding site as well as the
AT-rich sequences flanking the Cdx site. EMSA followed by UV cross-linking and SDS denaturing gel electrophoresis revealed that the complexes involved a protein of 29 kDa. We suspect that this protein may be one of the high mobility group family members, because these proteins bind to AT-rich regions, and they display relatively nonspecific binding. High mobility group 1 has a molecular mass of 29 kDa.

Studies with in vitro translated A-Myb and Cdx2-M did not reveal any interaction between these two proteins. To determine whether an interaction required DNA or other nuclear proteins, we prepared nuclear extracts from DHL-4 cells transfected with Cdx2-M. The nuclear extract was incubated with the biotinylated Cdx or Mut Cdx oligonucleotide immobilized on strepavidin-coated magnetic beads. After washing the beads, the bound nuclear proteins were separated by SDS-PAGE and analyzed by Western blot. As shown in Fig. 4C, Cdx2-M bound to the Cdx oligonucleotide but not to the oligonucleotide with the mutated Cdx2 site. Endogenous A-Myb but not B-Myb was present in the protein complex that bound to the wild-type Cdx site. These results strengthen our conclusion that the UV cross-linked protein of 32 kDa is Cdx2 and demonstrate that A-Myb is a component of the protein complex formed at the Cdx binding site.

A Cdx Binding Site Mediates the Positive Regulatory Activity of A-Myb on the bcl-2 P2 Promoter in t(14;18) Cells—To determine if the Cdx binding sequence was responsible for the induction of the bcl-2 P2 promoter activity by A-Myb, a construct containing a mutant Cdx site was generated (Fig. 5A). Constructs containing mutations in either of the two Myb consensus sequences, or both, were also generated to eliminate the possibility that these sites were necessary for induction of the bcl-2 P2 promoter by A-Myb (Fig. 5A). Although some loss of basal transcriptional activity was observed with the construct containing the mutated Cdx binding site, no change was observed in basal activity when the Myb binding sites were mutated. As shown in Fig. 5B, disruption of the Cdx binding site resulted in a drastic loss in the ability of A-Myb to induce the P2 promoter in DHL-4 cells (Fig. 5B). In contrast, the constructs containing mutations in the Myb sequences displayed no loss in their induction by A-Myb (Fig. 5B). Constructs containing mutations in the AT-rich sequences flanking the Cdx binding site were also tested by transient transfection assay, but these mutations showed no loss in bcl-2 promoter activation by A-Myb.

Expression of Cdx2 in Different B Cell Lineages—The expression of the two cloned human Cdx family members, Cdx1 and Cdx2, in normal tissues has been limited to the intestinal epithelium (31). In consideration of the results showing the importance of a Cdx binding site in the induction of the P2 promoter by A-Myb, we wanted to determine if a Cdx family member was expressed in our malignant B cell lines. Using a radiolabeled probe generated from the rat cdx2 sequence, we tested for cdx expression in the Nalm-6, DHL-9, DHL-4, and Raji B cell lines by Northern blot analysis. The colon carcinoma cell lines SW480 and HT-29 were used as positive and negative controls, respectively. As shown in Fig. 6, the rat cdx2 probe

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2 C. A. Heckman, J. W. Mehew, and L. M. Boxer, unpublished data.
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The specific complexes formed with the wild-type probe are indicated by arrows, C1 and C2. Lanes 9–12 represent a labeled probe with a mutation (M) in the Cdx binding site incubated with DSL-4 nuclear extract and the indicated 100-fold molar excess of competitor (50 ng). B, denaturing SDS-polyacrylamide gel electrophoresis of the UV cross-linked complexes formed with the bcl-2 P2 promoter A-Myb-responsive region and DSL-4 nuclear extract. Protein from the faster migrating complex, C1, is shown in the first lane, and protein from the slower migrating complex, C2, is shown in the second lane. The positions of the molecular mass markers are shown. After correction for bound oligonucleotide, the molecular mass of the protein for each complex was 32 kDa. C, recovery of nuclear proteins that interact with the Cdx binding site. Nuclear extracts from DSL-4 cells transfected with Cdx2-M were incubated with Cdx (lane 2) or Mut Cdx (lane 3) oligonucleotides conjugated to magnetic beads. The proteins associated with these sequences were separated by SDS-PAGE. The recruitment of A-Myb, B-Myb, and Cdx2-M to the Cdx binding site was assessed by immunoblotting with anti-A-Myb, anti-B-Myb, or anti-c-Myc antibodies, respectively. The first lane represents 100 μg of nuclear extract and shows that A-Myb, B-Myb, and Cdx2-M are expressed in the transfected cells.
These results demonstrate, however, that the endogenous Bcl-2 protein in human B cells can be up-regulated by A-Myb and/or Cdx2 transfection.

The DNA Binding and Transactivation Domains of A-Myb Are Required for Induction of the bcl-2 P2 Promoter—The A-Myb transcription factor is comprised of a DNA binding domain, a transactivation domain, and a negative regulatory region at the C terminus (27). To assess the importance of each of these regions in the induction of the bcl-2 P2 promoter, we transfected DHL-4 cells with a wild-type A-Myb gene, a deletion of the C terminus, a deletion of the transactivation domain, a deletion of the DNA binding domain, the DNA binding domain alone, and the DNA binding domain attached to the VP16 transactivation domain (Fig. 9A). Expression of all the A-Myb mutants was verified, and these constructs have also been tested for expression in previous studies (19, 27, 28). As shown in Fig. 9B, transfection of DHL-4 cells with the bcl-2 P2 promoter construct and the A-Myb C terminus truncation resulted in a 3.1-fold increase in relative luciferase activity as compared with the wild-type A-Myb vector (Fig. 9B). The A-Myb transactivation and DNA binding domains were both necessary for induction of the P2 promoter. Transfection results showed a 3-fold loss in A-Myb induction with the transactivation domain deletion construct and a 13-fold loss with the DNA binding domain deletion construct (Fig. 9B). The DNA binding domain alone had little activity, but the construct with the VP16 transactivation domain showed increased activity (Fig. 9B).

FIG. 5. Effect of mutations in the bcl-2 P2 promoter on induction by A-Myb. A, diagram of the bcl-2 P2 promoter and the constructs used in the transient transfection assays. All mutant constructs were derived from the −748 construct. B, results of transient cotransfection experiments with wild-type or mutant bcl-2 P2 promoter constructs and the A-Myb expression vector. The data were plotted as fold activation of the bcl-2 promoter construct by A-Myb over the activity of the bcl-2 promoter when transfected with an empty expression vector. WT, wild type.

FIG. 6. Cdx2 RNA expression in human B cell and colon carcinoma cell lines. RNA from the Nalm-6, DHL-9, DHL-4, and Raji B-cell lines and the HT-29 and SW480 colon carcinoma cell lines was isolated and separated on a 1% agarose-formaldehyde gel. The RNA was blotted onto a nylon membrane, which was hybridized with a 32P-labeled fragment of the rat cdx2 cDNA. The β-actin cDNA probe was used to ensure equivalent loading of RNA.

These results demonstrate, however, that the endogenous Bcl-2 protein in human B cells can be up-regulated by A-Myb and/or Cdx2 transfection.

In these studies, we demonstrated by Northern and Western blot analyses that the Myb family of transcription factors has a defined pattern of expression in a number of malignant B cell lines. We showed that A-Myb is expressed in the DHL-4 and Raji cell lines, which have the t(14;18) and t(8;14) translocations, respectively, but not in the pre-B cell line, Nalm-6, or the mature B cell line, DHL-9. In contrast, c-Myb is expressed in Nalm-6 and DHL-9 cells but not in DHL-4 or Raji cells. B-Myb is expressed in all four cell lines tested. These results are consistent with previous studies that showed A-Myb expression...
A-Myb expression vector (hatched bars), 6506
nous Bcl-2 protein in DHL-9 cells. Protein extracts from 5 expressing cells were selected using hapten-coated magnetic beads. lanes 3–6 transfected with the pHook2 vector and A-Myb and/or Cdx2-M or an empty expression vector. Further, A-Myb expression vectors over the activity of the promoter when transfected with the different expression vectors over the activity of the promoter construct when transfected with the empty expression vector.

Results of transient transfection of DHL-4 cells with the −748 wild-type bcl-2 P2 promoter construct (WT), the −748 construct with a mutation in the Cdx binding site (Cdx Mut), the −297 construct, which is missing the Cdx binding site, or the minimal −92 bcl-2 P2 promoter construct, with the A-Myb expression vector (hatched bars), the Cdx2 expression vector (dotted bars), or both A-Myb and Cdx2 expression vectors (black bars). The data were plotted as fold activation of the bcl-2 promoter construct when transfected with the different expression vectors over the activity of the promoter construct when transfected with the empty expression vector.

Effect of transfection of A-Myb and Cdx2 on endogenous Bcl-2 protein in DHL-9 cells. DHL-9 cells were transiently transfected with the pHook2 vector and A-Myb and/or Cdx2-M or an empty expression vector (lanes 3–6). After incubation for 24 h, cells expressing protein were selected using hapten-coated magnetic beads. Protein extracts from 5 × 10⁶ cells were separated by SDS-PAGE and transferred to nitrocellulose. Extracts from nonelectroporated and electroporated DHL-9 cells were used as controls (lanes 1 and 2). Bcl-2 expression was assessed by an anti-Bcl-2 antibody. An anti-β-actin antibody was used as a protein-loading control. Although not shown, the blot was also probed with antibodies against A-Myb and Myc to confirm that A-Myb and Cdx2-M were expressed in the transfected cells.

Bcl-2 expression is higher in normal mature B cells than in pre-B cells, and bcl-2 is overexpressed in t(14;18) lymphomas, and these cells are resistant to the induction of apoptosis (2–4). We demonstrated that A-Myb acts as a powerful inducer of bcl-2 activity in mature B cells and t(14;18) cells. In light of the well-established role of Bcl-2 in protection of B cells from apoptosis, A-Myb can be considered to function as an antiapoptotic factor.

The region within the human bcl-2 P2 promoter that was responsive to A-Myb was found to be −324 to −297 upstream of the bcl-2 translational start site. This region consists of a Cdx binding site flanked by AT-rich sequences. In the absence of this sequence, A-Myb showed only minimal induction of the bcl-2 P2 promoter. EMSA with an oligonucleotide of the Cdx binding site and DHL-4 nuclear extract revealed two specific complexes. Mutation of the Cdx sequence abolished formation of both complexes. In addition, mutation of the Cdx binding site in the bcl-2 promoter construct resulted in the loss of the ability of A-Myb to induce bcl-2 activity. In contrast, mutation of the Myb consensus sequences had no effect on A-Myb induction of the P2 promoter. These data demonstrate that a Cdx binding site rather than a Myb binding site is necessary for induction of the bcl-2 P2 promoter by A-Myb.

UV cross-linking followed by SDS-polyacrylamide gel electrophoresis of both specific EMSA complexes revealed a single protein with a molecular mass of 32 kDa. This is within the size range of the two characterized human Cdx family members, Cdx1 and Cdx2. If A-Myb directly interacted with this sequence, we would have expected a protein of 91 kDa. It is possible that the more slowly migrating EMSA complex is formed by Cdx and A-Myb with only Cdx contacting the DNA. Whereas we have an A-Myb antibody for Western blot analysis, we do not have an A-Myb antibody that produces a supershift in EMSA, so we cannot confirm this hypothesis.

We were able to demonstrate that A-Myb and Cdx2-M were present in the nuclear protein complex that bound to the Cdx site. This supports our transfection results that showed that A-Myb acts through the Cdx binding site to induce bcl-2 promoter activity. However, we could not demonstrate a direct interaction between these two proteins when they were translated in vitro, even in the presence of the Cdx oligonucleotide. These results suggest that there may be one or more other nuclear proteins involved in this complex and that the interaction of A-Myb with Cdx2 may not be direct. Because we were not able to demonstrate that B-Myb bound to the Cdx site, it appears that only A-Myb is recruited to the Cdx-2 complex on the bcl-2 promoter. This finding would explain why A-Myb is the only member of the Myb family that induces expression of the bcl-2 promoter in DHL-4 cells.

By Northern blot analysis, we demonstrated that Cdx2 or a Cdx family member is expressed in the Nalm-6, DHL-9, DHL-4, and Raji B-cell lines. This is surprising, because other studies have shown Cdx1 and Cdx2 expression to be confined to the intestinal epithelium (31). Only one study has demonstrated expression of Cdx2 in non-intestinal tissue. In that case, a patient with acute myelogenous leukemia with a t(12;13) translocation displayed a fusion between the ETV6 and Cdx2 genes with expression of a chimeric ETV6-Cdx2 fusion protein and normal Cdx2 transcripts (34). Cdx2 expression was also found in the leukemic cells from a patient with chronic myeloid leukemia in transformation (34). From our results, we can state that a Cdx family member is expressed in our malignant B cell lines, and a protein that is the same size as the Cdx family members interacts with the region of the bcl-2 P2 promoter that is responsive to A-Myb. In addition, transfected Cdx2-M interacts with the bcl-2 Cdx site.

In cotransfection studies with Cdx2 and A-Myb expression vectors, we were able to demonstrate a significant induction of the wild-type bcl-2 P2 promoter, but much less activity was
seen with a promoter construct containing a mutation or deletion of the Cdx binding site or with a minimal P2 promoter construct. In addition, we observed that the expression of both Cdx2 and A-Myb resulted in much higher \( \textit{bcl-2} \) promoter activity compared with the activity seen when either expression vector was used alone. We believe it is likely that a Cdx protein and A-Myb are included in a complex that binds to the Cdx site in the \( \textit{bcl-2} \) P2 promoter. Our in vitro binding studies demonstrate that both A-Myb and Cdx2-M interact with the Cdx site. Although it is possible that A-Myb acts on the promoter of a Cdx gene and this leads to increased Cdx expression, we think this is unlikely to account for the effect of A-Myb on the \( \textit{bcl-2} \) promoter. We have seen activation of the \( \textit{bcl-2} \) promoter by transfection of an A-Myb expression vector at short time intervals. In addition, Cdx2 is already expressed in DHL-4 cells, and by itself, it is a weak transactivator of the \( \textit{bcl-2} \) P2 promoter even when the level of Cdx2 protein is increased by transfection of the Cdx2 expression vector. Our results with the \( \textit{bcl-2} \) promoter reporter construct are supported by the demonstration that transfection of A-Myb and/or Cdx2 expression vectors increased expression of endogenous Bcl-2, although we were not able to demonstrate cooperativity. Transfection of the A-Myb expression vector resulted in a greater induction of Bcl-2 protein than did transfection of the Cdx2 expression vector. Because DHL-9 cells express no A-Myb, this result demonstrates the important role of the A-Myb protein in induction of endogenous Bcl-2 protein levels. The rather high level of Cdx2 in DHL-9 cells may account for our inability to demonstrate cooperativity with A-Myb in the induction of the endogenous Bcl-2 protein.

There is precedent for the formation of a complex between a Myb protein and a homeodomain protein. Bas1p is a Myb-related transcription factor and Bas2p is a homeodomain protein. They form a complex and regulate adenine biosynthetic genes in \textit{Saccharomyces cerevisiae} (35). c-Myb is known to interact with several different transcription factors, although usually this occurs with both factors binding to their DNA consensus sites. It has been reported that c-Myb can interact synergistically with the Epstein-Barr virus BZLF1 leucine zipper transactivator in lymphoid cells. This interaction occurs on the BZLF1 DNA binding site without DNA binding by c-Myb, although the DNA binding domain of c-Myb is required for the synergistic effect (36). It is also clear that c-Myb can transactivate promoters that lack Myb binding sites probably through interactions with particular types of TATA boxes (37). The DNA binding domain of c-Myb is not required for this activity.

The structure of the A-Myb transcription factor can be separated into three different domains: the N-terminal region, which consists of the DNA binding domain, a central transactivation domain, and a negative regulatory region within the C-terminal truncation. The A-Myb protein and deletion constructs of A-Myb used in the transient transfection experiments. B, results of transient transfection assays with the \(-748\) \( \textit{bcl-2} \) P2 promoter construct and the indicated A-Myb wild-type or deletion expression vector. The data were plotted as fold activation of the \( \textit{bcl-2} \) promoter by the different A-Myb expression vectors over the activity of the \( \textit{bcl-2} \) promoter construct when transfected with the empty expression vector.

![Regions of A-Myb required for the induction of the \( \textit{bcl-2} \) P2 promoter in DHL-4 cells. A, diagram of the wild-type (WT) A-Myb protein and deletion constructs of A-Myb used in the transient transfection experiments. B, results of transient transfection assays with the \(-748\) \( \textit{bcl-2} \) P2 promoter construct and the indicated A-Myb wild-type or deletion expression vector. The data were plotted as fold activation of the \( \textit{bcl-2} \) promoter by the different A-Myb expression vectors over the activity of the \( \textit{bcl-2} \) promoter construct when transfected with the empty expression vector.](http://www.jbc.org/)

**FIG. 9**
terminus (27). We have demonstrated that the DNA binding and transactivation domains are both required for induction of the bcl-2 P2 promoter by A-Myb, whereas the C terminus has a negative effect on induction. We have not been able to demonstrate a direct interaction of the Myb family members with any Myb binding sites in the bcl-2 promoter. Although the DNA binding domain of A-Myb is required, it is possible that this region also mediates protein-protein interactions (28) or serves as a transactivator. In this case, A-Myb could interact with another protein that binds to Cdx, which then directly contacts the DNA. Because B-Myb was not present in the complex with Cdx2, it is likely that sequences that are not homologous between the two Myb family members are involved in the interaction with the Cdx complex.

It has been suggested that v-Myb and c-Myb can regulate bcl-2 expression in chicken myeloid cells and murine thymoma cells, respectively, by direct interaction of the Myb protein with its consensus sequence in the promoter (12, 13). Increased expression of bcl-2 correlated with resistance to apoptosis in these cells, so the Myb proteins were shown to function as antiapoptotic factors. Although v-Myb was a relatively weak activator of the chicken bcl-2 promoter in myeloid cells, the E26 Myb-Ets fusion protein was a much stronger transactivator (12). Some, but not all, of this activity was mediated by four Myb binding sites in the bcl-2 promoter (12, 13). It was not clear if this binding site was essential for the action with the Cdx complex.

In this study we demonstrated that A-Myb, rather than B- or c-Myb, is the active positive regulator of bcl-2 activity in human malignant B cells. We have shown that the mechanism A-Myb utilizes to induce bcl-2 P2 promoter activity is indirect and involves the binding site of the Cdx homeodomain proteins. Further studies are being done to determine whether other proteins are involved in this complex with Cdx and A-Myb and to discern their role in the transformed phenotype of t(14;18) lymphoma cells.

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A-Myb Up-regulates Bcl-2 through a Cdx Binding Site in t(14;18) Lymphoma Cells
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