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The *bcl*-3 Proto-Oncogene Encodes a Nuclear IκB-Like Molecule That Preferentially Interacts with NF-κB p50 and p52 in a Phosphorylation-Dependent Manner

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The product of the putative proto-oncogene *bcl*-3 is an IκB-like molecule with novel binding properties specific for a subset of the *rel* family of transcriptional regulators. In vitro, Bcl-3 protein specifically inhibited the DNA binding of both the homodimeric NF-κB p50 subunit and a closely related homolog, p52 (previously p49), to immunoglobulin κ NF-κB DNA motifs. Bcl-3 could catalyze the removal of these proteins from DNA. At concentrations that significantly inhibited DNA binding by homodimeric p50, Bcl-3 did not inhibit binding of reconstituted heterodimeric NF-κB (p50:p65), a DNA-binding homodimeric form of p65, or homodimers of c-Rel. Phosphatase treatment of Bcl-3 partially inactivated its inhibitory properties, implicating a role for phosphorylation in the regulation of Bcl-3 activity. Bcl-3, like p50, localizes to the cell nucleus. In cells cotransduced with Bcl-3 and p50, both molecules could be found in the nucleus of the same cells. Interestingly, coexpression of Bcl-3 with a p50 mutant deleted for its nuclear-localizing signal resulted in the relocalization of Bcl-3 to the cytoplasm, showing that the proteins interact in the cell. These properties contrast Bcl-3 to classically defined IκB, which maintains heterodimeric NF-κB p50:p65 in the cytoplasm through specific interactions with the p65 subunit. Bcl-3 appears to be a nuclear, IκB-related molecule that regulates the activity of homodimeric nuclear p50 and its homolog p52.

IκB is a physiologic controller of the activity of the NF-κB transcription factor (2, 3, 13). It is found in the cytoplasm of cells complexed with NF-κB, a heterodimer of the Rel-related proteins p50 and p65. IκB interacts with NF-κB through residues in the Rel homology domain of the p65 subunit of NF-κB (31). Upon stimulation of cells with a variety of agents, IκB dissociates from the complex and NF-κB translocates to the nucleus, where it directs the expression of a number of genes, including immunoglobulin κ (Igκ) (34), interleukin-6 (25), tumor necrosis factor alpha, beta interferon (24), and human immunodeficiency virus (28) genes. Two species of IκB have been described: IκB-α (36 kDa) and IκB-β (45 kDa) (45). Cloned mammalian IκB-α, a product of the *Mad*-3 gene (17), and pp40, the avian IκB-like inhibitor of c-Rel activity (15), contain a repeated 33- to 35-amino-acid residue motif termed an ankyrin domain. Ankyrin domains are also found in NF-κB p50 precursor p105 (14, 23) as well as in other proteins such as Bcl-3 (32), CDC10 (1), SWI4/SWI6 (6), the *Drosophila* Notch-related proteins (Xotch, Rotch, Tan-1, and Int-3 [6, 8, 10, 41, 42]), Lin-12, Glp-1 (44), and erythrocyte ankyrin (27).

Two different classes of ankyrin-containing *rel*-binding proteins have been characterized previously. The first, a product of the cDNA clone *MAD*-3, encodes the α isoform of IκB (45), as defined by amino acid analysis (9). IκB-α binds specifically to the p65 subunit of NF-κB (17, 31, 39) and is the apparent murine homolog of pp40, the associated phosphoprotein of c-Rel (15). The second ankyrin-containing protein, IκB-γ, preferably inhibits the DNA binding of p50 homodimers (19, 26). p50 derives from the p105 precursor by cleavage, and IκB-γ is the protein product of an alternatively spliced mRNA arising from the p105 locus, corresponding to the carboxy-terminal half of p105. As part of p105, IκB-γ appears to participate in internal inhibition of the *rel* domain in p105 as well as in masking of the nuclear translocation signal of p105 (5, 18).

We noted that the predicted ankyrin domains of the previously cloned proto-oncogene *bcl*-3 can be aligned with greater significance to IκB-like molecules than with ankyrin-containing molecules having no known IκB function (30). *bcl*-3 has been described as a putative proto-oncogene found at a 14:19 translocation breakpoint in the IgH-α region (32) in a small number of adult chronic lymphocytic leukemias, neoplasms of the $CD5^+$ B-cell subset (36). An interesting feature of Bcl-3 is the high density of serine and proline residues present at the carboxyl terminus of the molecule. The normal cellular functions of *bcl*-3 and its role in leukemogenesis remain to be determined.

We find that baculovirus-produced Bcl-3 specifically inhibits the DNA binding of homodimeric p50 and its close homolog p52. Surprisingly, Bcl-3, at concentrations that inhibited >90% of p50 homodimeric complexes, did not efficiently inhibit the DNA binding of heterodimeric Rel complexes that contain p50, such as reconstituted p50:p65. This is in contrast to the activity of IκB-α, which is capable of inhibiting both homodimers and heterodimers of complexes containing c-Rel and p65. In contrast to the proposed role of IκB as a regulator of cytoplasmic-nuclear partitioning for NF-κB, we found that Bcl-3 is an intrinsically nuclear protein that associates with p50 in vivo. Thus, it is possible that Bcl-3 plays a broader role as a regulator of p50 transcriptional activity. While this report was in preparation, Wulczyn et al. (43) reported evidence on the properties of

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Bcl-3, some of which are consistent and others of which are in contrast to those presented here (see Discussion).

MATERIALS AND METHODS

Proteins. SF9 cells were grown in Grace's medium supplemented with 10% fetal calf serum. p50, p65, and p65-Δcarboxyl were made in a baculovirus expression system and purified according to Fujita et al. (12). NF-κB p50:p65 was prepared similarly. p52 was a kind gift of Colin Duckett and Gary Nabel (33). c-Rel was prepared in a baculovirus expression system and purified similarly to the Rel-related proteins (12). Bcl-3 was made by a baculovirus expression system and was purified by standard column chromatography (T. Fujita) to near homogeneity. IκB-α was fused after the initiating ATG to glutathione synthetase (GST) and purified by glutathione agarose affinity chromatography (26). The *Drosophila* proteins cactus and notch, also glutathione *S*-transferase (GST) fusions, were a kind gift of Simon Kidd; Cactus protein was previously shown to be functional in the inhibition of Dorsal protein binding to *dorsal* DNA-binding sites (22b). Rabbit polyclonal antibody to the baculovirus-derived p50 and *Bcl*-3 purified in this study was prepared and used as described in the text.

Immunofluorescence. NIH 3T3 cells seeded onto glass coverslips were infected with retroviruses produced by a transient-transfection protocol (32a). A *bcl3* cDNA was inserted into the *Nco*I site of the defective retrovirus construct MFG (a kind gift from P. Robbins, University of Pittsburgh, and R. Mulligan, Whitehead Institute) by using synthesized linkers corresponding to the DNA sequence from the second residue to the unique *Xma*I site in *bcl*-3. At the 3′ end, *bcl*-3 was fused to the *Bam*HI site of MFG, using the first *Bst*YI site 3′ of the *bcl*-3 terminator codon (in the case of native *bcl*-3); the resulting construct was designated MFG-*bcl*-3. An influenza virus hemagglutinin (HA) epitope tag was added to Bcl-3 by using a synthesized linker corresponding to sequence from the unique *Rsa*I site of *bcl*-3 to the last codon that included a fused HA epitope, a new terminator, and a *Bam*HI site; the resulting construct was designated MFG-*bcl*-3-HA. Two days postinfection cells, were stained according to Van Etten et al. (40) in a two-step procedure; the primary antibody was monoclonal anti-HA epitope antibody 12CA5 (Berkeley Antibody Co., Berkeley, Calif.), and the secondary antibody was a rhodamine-conjugated donkey Fab_2 anti-mouse IgG. p50 tagged at the amino terminus with HA epitope was inserted in the pZip-NeoSV(X)-1 retrovirus (7), using the *Xho*I and blunted *Cla*I sites (thus removing the *neo*, pBR origin of replication, and simian virus 40 origin of replication sequences); the resulting construct was designated pZip-HA-p50. A retrovirus directing the expression of wild-type p50 was derived from pZip-HA-p50 by replacing the Rel region with the wild-type *Nco*I fragment containing all of the Rel homology region and some regions carboxy terminal to it; the resulting construct was designated pZip-p50. A retrovirus directing the expression of a nuclear-localizing mutant of p50, designated pZip-p50-ΔNLS, was constructed similarly from a p50 construct deleted of residues 351 to 367. A polyclonal antibody to Bcl-3 from immunized rabbits was used at a 1:300 dilution in immunofluorescence staining of cells infected with MFG-*bcl*-3. Polyclonal fluorescein isothiocyanate-labeled donkey anti-rabbit IgG was used at 1:250 to reveal Bcl-3 overexpression in NIH 3T3 cells.

EMSA and phosphatase treatment. Approximately 1 ng of each Rel protein was mixed with the indicated ankyrin motif-containing protein and incubated for 5 min at room temperature prior to the addition of radiolabeled probe. All buffers were as described previously (2). After incubation for an additional 5 min, the samples were analyzed by EMSA (2). For phosphatase treatment experiments, Bcl-3 was incubated with an equivalent amount of calf intestine phosphatase (CIP). A portion was subjected to denaturing sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The rest was diluted for use in assay of inhibitory effect on p50 DNA binding. Destruction of Bcl-3 protein by proteolytic contaminants of the CIP was not observed.

Western immunoblotting and immunoprecipitation. Cells infected with retroviruses (32a) directing the expression of HA-tagged Bcl-3 were homogenized in sample buffer (1% SDS, 50 μM β-mercaptoethanol, 0.2% bromophenol blue), and insoluble material was removed by centrifugation. Proteins were electrophoresed through SDS–12.5% polyacrylamide gels, and proteins were electrotransferred to a nitrocellulose support. Immunoblots were blocked with 5% nonfat milk antibody proteins. Primary antibody was protein A-purified monoclonal 12CA5 (anti-HA; Berkeley Antibody Co.) at a 1:1,000 dilution. Bands were revealed with protein A-alkaline phosphatase conjugate at 1:5,000. For immunoprecipitations, 293 cells that were transfected by calcium phosphate precipitation of DNA were labeled for 6 h with 500 μCi of [^{35}S]methionine. Cells were lysed in immunoprecipitation buffer (20 mM Tris [pH 8.0], 137 mM NaCl, 1% Nonidet P-40, 10% glycerol, and 2 mM EDTA, with the protease inhibitors aprotinin, benzamidine, phenylmethylsulfonyl fluoride, antipain, and tosylsulfonyl phenylalanyl chloromethyl ketone). Cells were vigorously vortexed and then briefly sonicated; insoluble material was removed by centrifugation. Then 2×10^6 incorporated counts were diluted into 1 ml of immunoprecipitation buffer and precleared with Sepharose-protein A beads for 1 h. Primary antibodies (both polyclonal anti-p50 and polyclonal anti-Bcl-3) at 1:500 were added and allowed to incubate overnight at 4°C with rocking. Protein A beads (precoated with cold 293 cell lysate) were added for 1 h. Beads were washed five times in immunoprecipitation buffer. Sample buffer was added to washed beads and heated to 100°C briefly, and proteins were visualized by autoradiography after SDS-PAGE separation.

RESULTS

Bcl-3 is an IκB-like inhibitor of $(p50)_2$ binding to Igκ NF-κB DNA motifs. To examine the ability of Bcl-3 to inhibit the DNA-binding activity of Rel-related proteins, we produced recombinant full-length murine Bcl-3 protein by using a baculovirus expression system, purified it to near homogeneity and tested its activity in an EMSA. We first tested the inhibitory properties of Bcl-3 on the DNA-binding properties of homodimers of p50 and heterodimeric p50:p65 NF-κB (Fig. 1A). Titration of increasing amounts of baculovirus Bcl-3 resulted in the inhibition of $(p50)_2$ binding to the Igκ κB DNA motif and had no apparent inhibitory effect upon reconstituted NF-κB binding. The molar ratio that gave 90% inhibition of $(p50)_2$ binding to Igκ motifs was approximately 5 Bcl-3:1 $(p50)_2$.

We next tested the specificity of the Bcl-3 inhibitory effect upon other homo- and heterodimeric cloned Rel homologs: murine p50 (12, 14), a DNA-binding truncated form of murine p65 (12, 31), c-Rel (15), and the 52-kDa derivative of human lyt-10/p100 (p52; previously designated p49) (29, 33). Five nanograms of Bcl-3 inhibited the binding of 1 ng of p50

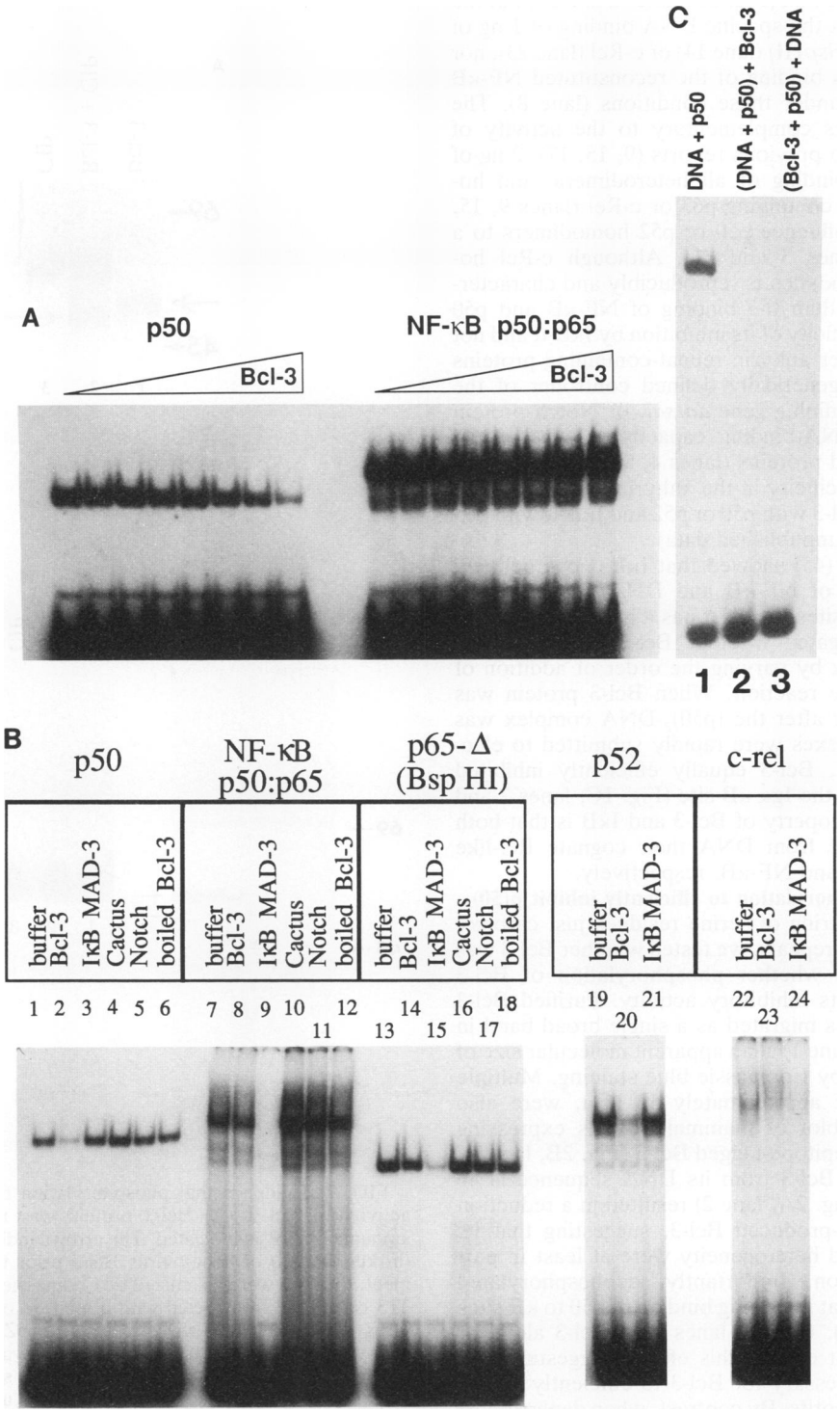


FIG. 1. Evidence that Bcl-3 preferentially inhibits the DNA binding of NF-κB p50 and NF-κB p52. A radiolabeled oligonucleotide probe containing the NF-κB motif from the Igκ intronic enhancer was used in EMSA with the indicated amounts of Rel-related DNA-binding proteins and the indicated amounts of ankyrin motif-containing proteins. Ankyrin proteins were premixed, except where indicated, with Rel proteins, allowed to incubate, and then added to radiolabeled probe. (A) Twofold serial dilutions from 0.2 to 25 ng to determine the amount (molar ratio) of Bcl-3 protein that inhibits 5 ng of $(p50)_2$ versus the amount of Bcl-3 that inhibits NF-κB p50:p65. (B) Test of ankyrin protein inhibitory activities upon other Rel complexes. Inhibition trials were carried out with either approximately 5 ng of Bcl-3, 5 ng of GST-IκB-α, 100 ng of GST-cactus, 100 ng of GST-notch, or 5 ng of Bcl-3 boiled for 10 min. Proteins added are as indicated above the lanes; all Rel-related proteins were added to approximately 1 ng. (C) Test for active inhibition by Bcl-3 of p50 DNA binding. Lanes: 1, p50 plus Igκ site; 2, p50 plus Igκ κB site premixed for 20 min, with subsequent addition of Bcl-3; 3, p50 and Bcl-3 premixed for 20 min, with subsequent addition of the Igκ κB DNA-binding motif. Reactions were then immediately electrophoresed in EMSA.

or p52 homodimers to DNA (Fig. 1B, lanes 2 and 20) but did not significantly inhibit the specific DNA binding of 1 ng of homodimers of p65-Δ(*Bsp*HI) (lane 14) or c-Rel (lane 23), nor did it inhibit the DNA binding of the reconstituted NF-κB heterodimer p50:p65 under these conditions (lane 8). The response to Bcl-3 was complementary to the activity of IκB-α. Consistent with previous reports (9, 15, 17), 2 ng of IκB-α inhibited the binding of all heterodimeric and homodimeric complexes containing p65 or c-Rel (lanes 9, 15, and 24) but did not influence p50 or p52 homodimers to a significant degree (lanes 3 and 21). Although c-Rel homodimer binding to Igκ sites is reproducibly and characteristically less efficient than the binding of NF-κB and p50 homodimers, the specificity of its inhibition by IκB-α and not by Bcl-3 is clear. Other ankyrin repeat-containing proteins such as Cactus, the genetically defined controller of the *Drosophila* axis-determining gene *dorsal*, or Notch protein did not affect the DNA-binding capacity of any of the mammalian Rel-related proteins (lanes 4, 5, 10, 11, 16, and 17). Thus, there is specificity in the ankyrin motif-mediated interactions of both Bcl-3 with p50 or p52 and IκB-α with p65 or c-Rel (Fig. 1B and unpublished data).

Zabel and Baeuerle (45) showed that IκB-α can actively dissociate a complex of NF-κB and DNA. Because p50 binding to various κB sites on DNA has a half-life of greater than 1 h, we investigated whether Bcl-3 could actively dissociate the complex by varying the order of addition of the components in the reaction. When Bcl-3 protein was added either before or after the $(p50)_2$-DNA complex was formed and the complexes were rapidly submitted to electrophoretic separation, Bcl-3 equally efficiently inhibited $(p50)_2$ interaction with the Igκ κB site (Fig. 1C, lanes 2 and 3). Thus, a common property of Bcl-3 and IκB is that both can actively dissociate from DNA their cognate Rel-like protein targets, $(p50)_2$ and NF-κB, respectively.

Bcl-3 requires phosphorylation to efficiently inhibit $p(50)_2$. Because Bcl-3 has a string of serine residues just carboxy terminal to the ankyrin repeats, we tested whether Bcl-3 was a phosphoprotein and whether phosphorylation of Bcl-3 might play a role in its inhibitory activity. Purified Bcl-3 produced in insect cells migrated as a single broad band in SDS-PAGE (Fig. 2A, lane 1) at an apparent molecular size of 52 kDa, as visualized by Coomassie blue staining. Multiple isoforms, migrating at approximately 52 kDa, were also visible in a Western blot of mammalian cells expressing retrovirus transduced epitope-tagged Bcl-3 (Fig. 2B, lane 2). The predicted size of Bcl-3 from its DNA sequence is 46 kDa. CIP treatment (Fig. 2A, lane 2) resulted in a reduction in size of baculovirus-produced Bcl-3, suggesting that its large apparent size and heterogeneity were at least in part due to phosphorylation. Importantly, dephosphorylated Bcl-3 was less efficient at inhibiting binding of p50 to κB sites (Fig. 2C, lanes 5 to 7). Control lanes with Bcl-3 alone or CIP-treated p50 do not exhibit this effect, suggesting that phosphorylation is necessary for Bcl-3 to efficiently inhibit p50 binding to Igκ κB motifs. By contrast, when dephosphorylated, IκB-α inhibits the DNA-binding activity of NF-κB (22, 45) through specific interaction with the p65 subunit (31). Only IκB-β has been previously shown to require phosphorylation (22). Thus, Bcl-3, which has several potential protein kinase C and creatine kinase II phosphorylation sites predicted from its sequence (32), might also require phosphorylation to be active. Phosphorylation could account for the aberrant migration and multiple isoforms observed.

Bcl-3 associates with p50 and localizes to the nucleus. To

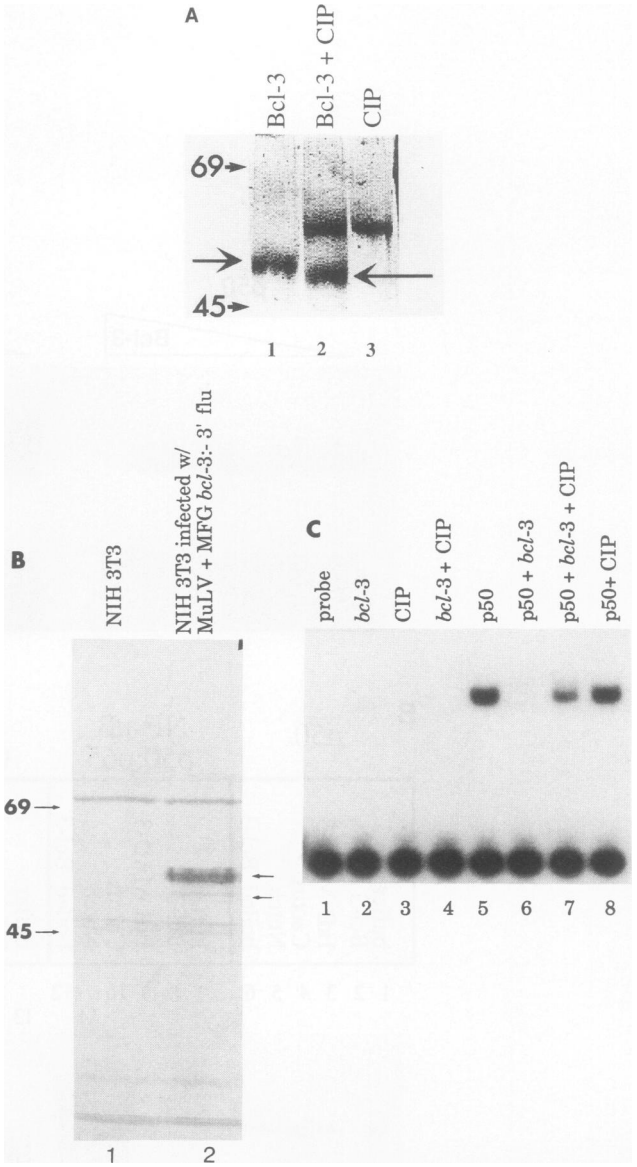


FIG. 2. Evidence that phosphorylation regulates the inhibitory activity of Bcl-3. (A) Bcl-3 protein was mixed with equivalent amounts of CIP as indicated. The arrows indicate the apparent sizes (in kilodaltons) of baculovirus Bcl-3 prior to and after CIP treatment. Proteins were visualized by Coomassie blue staining. (B) NIH 3T3 cells were transfected with a mixture of the full-length helper virus murine leukemia virus (MuLV) in pZAP (35) and the MFG retrovirus containing full-length influenza virus (flu) epitope-tagged murine *bcl*-3, as indicated. Two days later, supernatants containing helper and *bcl*-3 viruses were harvested and used to infect fresh NIH 3T3 cells (7). After 48 h, cells were harvested, lysed, and subjected to SDS-PAGE. Western blot analysis of transferred proteins was carried out with the monoclonal antibody to the influenza virus HA epitope. Multiple bands are visible and are indicated by arrows. (C) Phosphatase-treated Bcl-3 prepared as for panel B was used in EMSA (see Fig. 2) to test the role of phosphorylation in the activity of Bcl-3. Lanes: 1, ^{32}P-radiolabeled Igκ DNA motif alone; 2, Bcl-3; 3, CIP; 4, CIP-treated Bcl-3; 5, p50; 6, p50 plus Bcl-3; 7, CIP-treated Bcl-3 plus p50; 8, CIP-treated p50.

determine the localization of Bcl-3, we constructed another retrovirus vector with the HA epitope tag at the carboxyl termini of Bcl-3 (MFG-*bcl*-3-HA). Immunofluorescent staining of cells transduced with this construct showed that epitope-tagged Bcl-3 localized predominantly to the nucleus, although some slight staining was observed in the cytoplasm (Fig. 3A, panels 1 and 2).

To determine whether Bcl-3 could localize to the nucleus in the presence of p50, we conducted double immunofluorescence staining of cells after coinfection with viruses that expressed HA-p50 (pZip-HA-p50) and wild-type Bcl-3 (MFG-*bcl*-3). We first confirmed by immunolocalization the previous studies showing that p50 is localized constitutively to the nucleus of cells (5, 18). For this experiment, NIH 3T3 cells were infected with a recombinant retrovirus expressing an amino-terminal HA epitope-tagged p50 molecule (pZIP-HA-p50). Two days after infection, cells were stained for HA-specific expression, using a monoclonal anti-HA antibody. Cells expressing HA-p50 showed clear immunolocalization of HA-p50 protein to the nucleus (Fig. 3A, panels 3 and 4). Cells were then coinfected with retroviruses expressing either HA-p50 or Bcl-3. When Bcl-3 was expressed by itself in cells, it was present in the nucleus, as was observed for epitope-tagged Bcl-3–HA (panel 5). When cells coexpressed HA-p50 and Bcl-3, specific staining for both molecules was observed in the nucleus (compare panels 5 and 6). To show that a nonspecific effect of the HA tag on one of the two proteins does not lead to artifactual results, infections were also carried out and imaged by using wild-type p50 (pZip-p50) and HA-Bcl-3 (MFG-*bcl*-3-HA). The results were identical with use of either pairing (data not shown). This finding is a strong indication that in vivo Bcl-3 plays some role in the nucleus, in sharp contrast to the role of classically defined IκB, which is proposed to maintain regulated cytoplasmic residence of NF-κB.

We examined whether Bcl-3 could influence the subcellular localization of a p50 protein mutant at its nuclear-localizing signal (NLS) sequence. We first confirmed that the p50-ΔNLS protein localized primarily to the cytoplasm. Infection of NIH 3T3 cells with the pZip-p50-ΔNLS-HA retrovirus and immunofluorescence staining showed that, as previously reported (5, 18), removal of the NLS results in a localization of this mutant p50 to the cytoplasm (compare p50 in Fig. 3A, panel 4, with p50-ΔNLS in Fig. 3B, panel 1), although some slight staining is observed in the nucleus. Surprisingly, coexpression of Bcl-3 and p50-ΔNLS-HA resulted in the redistribution of much of the Bcl-3 to the cytoplasm of cells (Fig. 3B, panels 2 and 3; compare Bcl-3 protein in cells coexpressing p50 and those not coexpressing p50). Therefore, p50-ΔNLS seems capable of redirecting the localization of Bcl-3, probably including a physical association between p50 and Bcl-3 in cells. This finding suggests that the subcellular location of p50 might influence the localization of Bcl-3.

Because baculovirus-produced Bcl-3 is capable of actively inhibiting the DNA binding of p50 in vitro, and the proteins colocalize to the nucleus, we examined whether antibodies directed against either Bcl-3 or p50 could immunoprecipitate the other component from cell lysates. In cells transfected with p50, antibodies to p50 immunoprecipitated a band of ~47-kDa apparent molecular mass that is the p50 product (Fig. 4, lane 1). This 47-kDa band corresponds in size to the p50-specific bands observed in Western blots of lysates of these cells (not shown). Antibodies directed against Bcl-3 immunoprecipitated a pair of 52-kDa bands from *bcl*-3-transfected lysates (lane 6). Again, these bands correspond in size to Bcl-3-specific bands observed in Western blots of lysates of these cells and to proteins observed by Western blots of infected epitope-tagged Bcl-3 in NIH 3T3 cells (Fig. 2A). By Western blot and Coomassie blue stain analysis, the apparent amount of p50 produced in transfected 293 cells is in great excess over the amount of Bcl-3 produced (data not shown). When p50 and *bcl*-3 were cotransfected, antibodies to Bcl-3 coimmunoprecipitated p50 bands (lane 10). Antibodies to p50 coprecipitated Bcl-3 bands (lane 9). The addition of 500 ng of unlabeled Bcl-3 resulted in the specific blocking of immunoprecipitated labeled Bcl-3 (lane 7) but resulted in the coimmunoprecipitation of a level of free labeled p50 (lane 11) equivalent to that observed in lane 10. The ability to precipitate labeled p50 with cold Bcl-3 protein is likely due to excess production of transfected p50 and its association with unlabeled Bcl-3 protein-antibody complex.

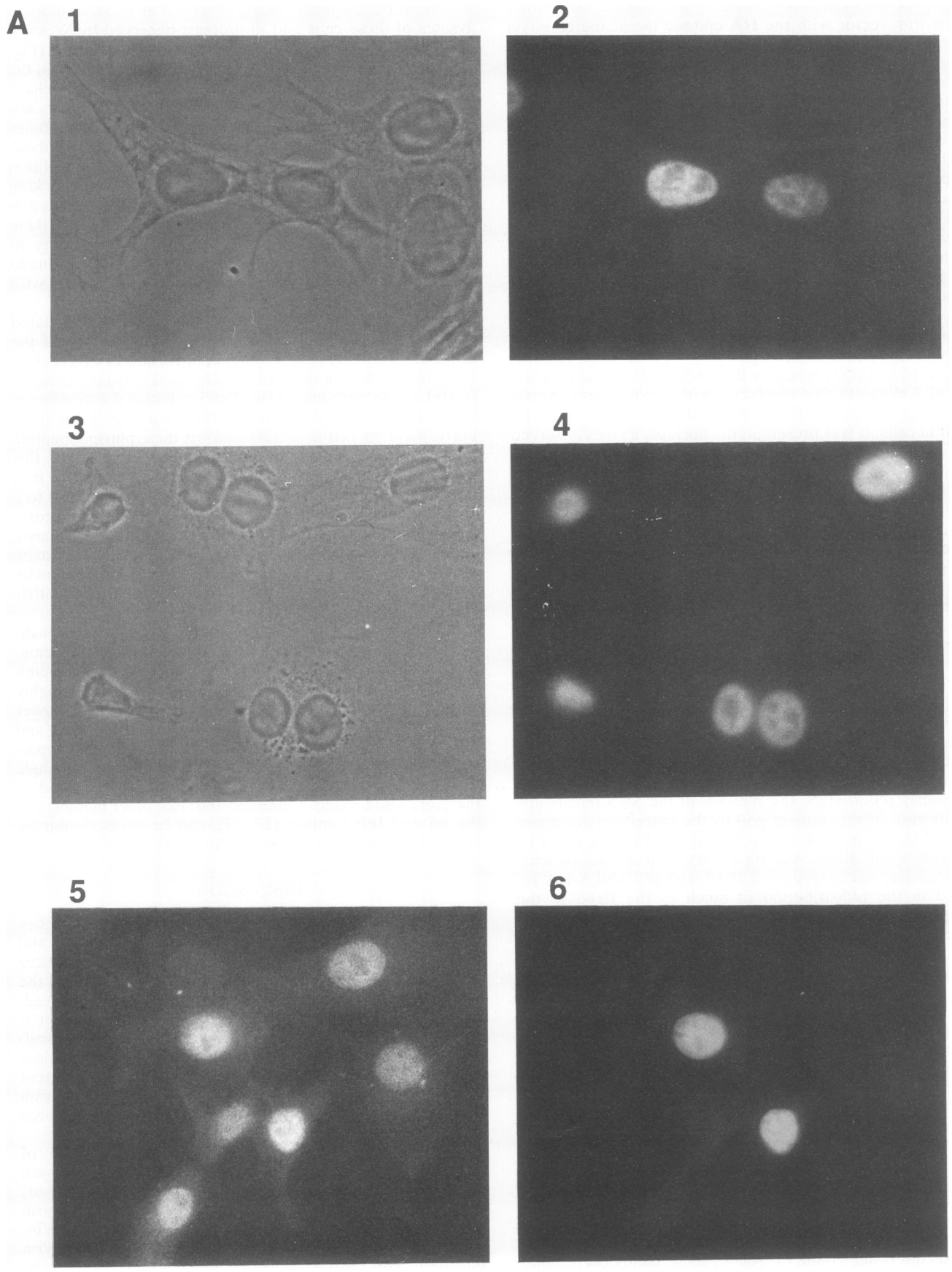
We also observed a 37-kDa band that immunoprecipitated in transfected and untransfected 293 cells with use of the anti-Bcl-3 antibody (Fig. 4, lanes 2, 6, 10, and 14). Kerr et al. (22a) report that an induced 37-kDa band that they observe in HeLa cells with use of anti-Bcl-3 polyclonal antibodies is a probable proteolytic product of full-length Bcl-3 and might correspond to IκB-β as revealed by their phosphopeptide analysis. Since we do not observe any increase of this 37-kDa band in 293 cells after transfection of Bcl-3, it is unlikely that full-length Bcl-3 is significantly processed to a smaller 37-kDa form, and we believe that it is a cross-reactive protein. Additionally, IκB-β has been previously shown to be a 45-kDa protein (45). Thus, Bcl-3 is likely to be a novel protein with some similarities to the originally defined IκB polypeptides but is clearly distinct from either IκB-α or IκB-β.

Bcl-3 mRNA is widely expressed. p50 is expressed constitutively in many mammalian cells, including all tested hematopoietically derived cells (13). *bcl*-3 mRNA shows a similarly broad expression pattern. *bcl*-3 mRNA is highly expressed in the spleen and liver (Fig. 5). There was lower expression in the heart, skeletal muscle, lung, kidney, and testes, with no apparent expression in the brain. The apparent size of *bcl*-3 mRNA is 2.8 kb, and there is only a single resolvable species of poly(A)-containing mRNA. *bcl*-3 is therefore also broadly expressed and, like other members of the *rel* and IκB families (17, 37), can be transcriptionally induced following stimuli that induce NF-κB (32).

DISCUSSION

We find that the baculovirus-produced Bcl-3 protein can interact specifically with $(p50)_2$ and $(p52)_2$ complexes and inhibit their binding to the κB motif present in the enhancer of the Igκ gene. This inhibitory activity is modulated by the phosphorylation state of the protein. When overexpressed, Bcl-3 localizes by itself to the cell nucleus, and when it is coexpressed with p50 in cells, the two simultaneously localize to the nucleus and form a complex evident in nuclear extracts. By contrast, IκB-α is a protein whose apparent function is to regulate NF-κB localization in the cytoplasm of cells. Thus, Bcl-3 is a new type of regulator of Rel-related transcriptional activators.

Structurally, Bcl-3 protein has a distinct pattern of ankyrin-related repeats that is also found in IκB-α, avian pp40, and IκB-γ (the C-terminal region of the p50 precursor, p105). This pattern is different from that in other ankyrin repeat-containing proteins such as Notch, SWI4, SWI6, ankyrin, or Glp-1 (30). Furthermore, there are seven ankyrin repeats in Bcl-3 just as in IκB-γ, making them structurally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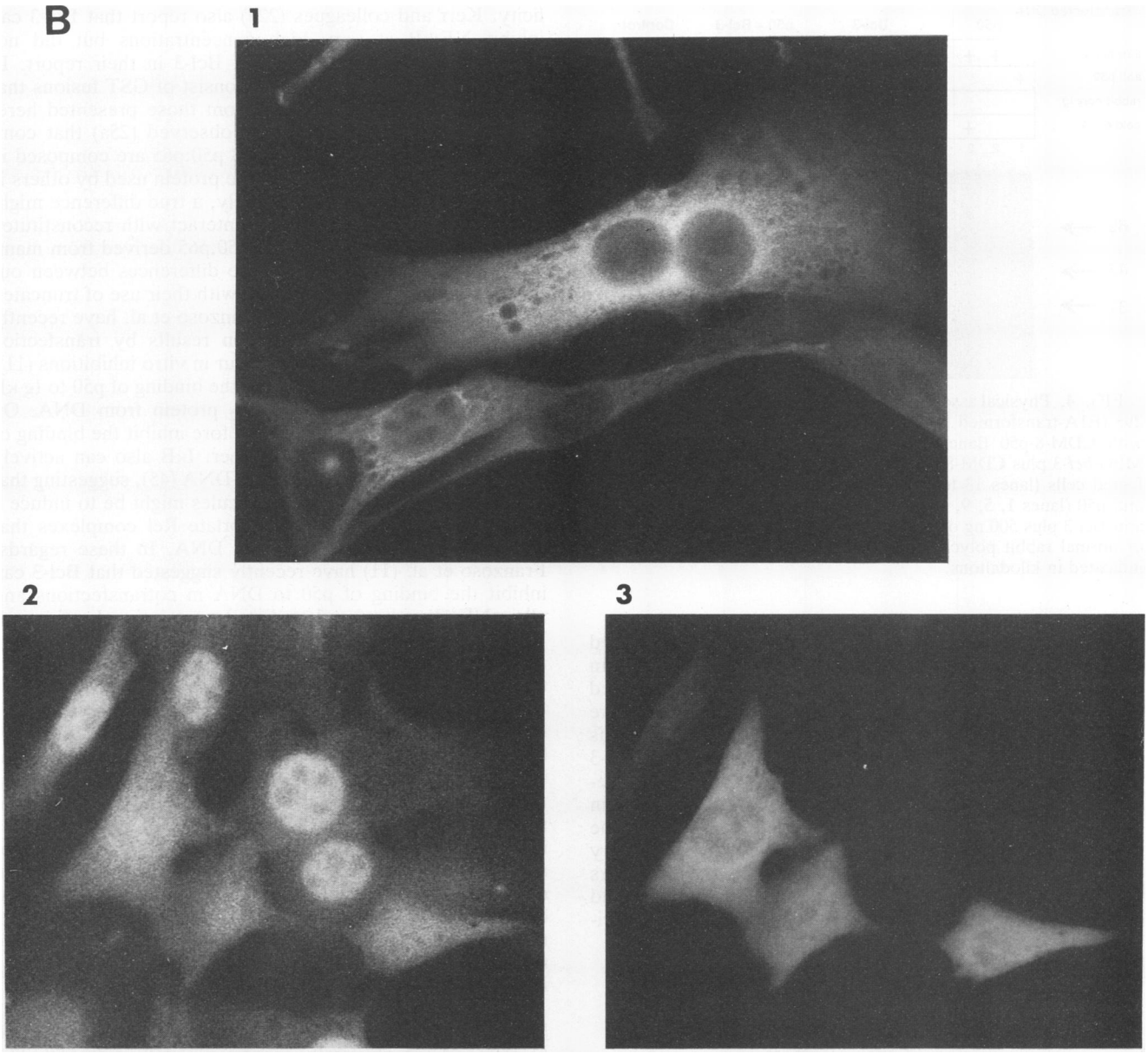

FIG. 3. Localization of Bcl-3 to the nucleus alone and in the presence of p50. Cells were infected with epitope-tagged p50 and *bcl*-3 retroviruses and stained as described in Materials and Methods. (A) Colocalization of p50 and Bcl-3. Panels: 1, phase-contrast image of panel 2; 2, infection with *bcl*-3 (epitope-tagged) retrovirus and immunofluorescence stained with anti-HA-epitope monoclonal antibody; 3, phase-contrast image of panel 4; 4, infection with 5′-HA epitope-tagged p50 retrovirus, immunolocalized with anti-HA epitope monoclonal antibody; 5 and 6, coinfection of cells with pZip-HA-p50 and MFG-*bcl*-3 imaged with anti-Bcl-3 polyclonal antibody (panel 5) and anti-HA (panel 6). (B) Evidence that a nuclear-localizing mutant of p50 can redirect Bcl-3 to the cytoplasm. Panels: 1, p50-ΔNLS-HA stained with anti-HA; 2, pZIP-p50-ΔNLS-HA- and MFG-*bcl*-3-coinfected cells stained with anti-Bcl-3 (panel 2) and anti-HA (panel 3).

distinguishable from the five-repeat IκB-α family. Functionally, IκB-γ and Bcl-3 are also most related because each is focused on $(p50)_2$ (19, 26), while IκB-α and IκB-β are focused on p65 and heterodimeric NF-κB. Bcl-3 is different from IκB-γ, however, in being nuclear and regulated by phosphorylation. Furthermore, Bcl-3 under certain circumstances can be an activator of transcription (11a). There is an as yet uncharacterized form of IκB called β that is also regulated by phosphorylation but binds well to p65 and c-Rel. (These authors consider this form of IκB to be related to pp40, although our protein sequence data clearly established the homology of IκB-α and pp40 [9, 30].)

While this report was in preparation, Wulczyn et al. (43) reported that bacterially produced Bcl-3 showed a preference for inhibiting p50 rather than p65 binding to DNA, but they found that the activities of Bcl-3 and IκB-α were comparable against the p50:p65 heterodimer. This observation is in distinct contrast to our results. We found that Bcl-3 efficiently inhibited eukaryotically expressed p50 and p52 binding at concentrations that did not inhibit reconstituted

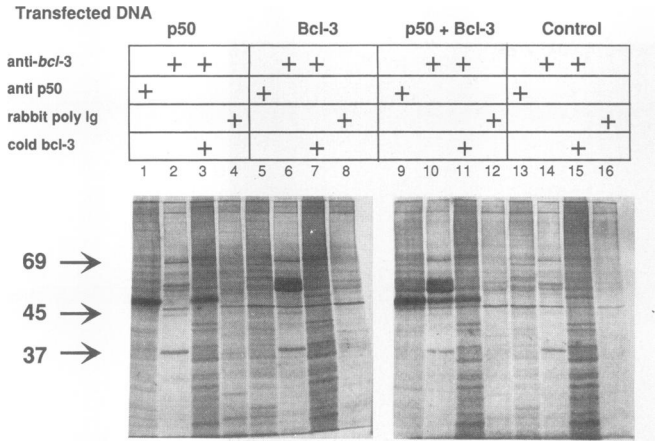

FIG. 4. Physical association of Bcl-3 with p50 in cells. Lysates of 293 (E1A-transformed human embryonic kidney) cells transfected with CDM-8-p50 (lanes 1 to 4), MFG-*bcl*-3 (lanes 5 to 8), or MFG-*bcl*-3 plus CDM-8-p50 (lanes 9 to 12) and of control untransfected cells (lanes 13 to 16) were immunoprecipitated with either anti-p50 (lanes 1, 5, 9, and 13), anti-Bcl-3 (lanes 2, 6, 10, and 14), anti-Bcl-3 plus 500 ng of cold Bcl-3 protein (lanes 3, 7, 11, and 15), or normal rabbit polyclonal Ig (lanes 4, 8, 12, and 16). Sizes are indicated in kilodaltons.

NF-κB p50 and p65 binding. They used bacterially produced and therefore probably poorly phosphorylated protein. In our experiments, bacterially and baculovirus-generated Bcl-3 proteins have indistinguishable specificities. A more critical difference is probably that their Bcl-3 protein was truncated at both ends. Of a full-length 454-amino-acid Bcl-3 protein, these authors reported using a 289-amino-acid deletion mutant that corresponded to primarily the ankyrin domains from 94 to 383 amino acids, removing most of the flanking region that potentially contain important regulatory domains. In addition, the proteins used by these authors underwent denaturation-renaturation. Such truncation and denaturation might have caused their protein to lose specificity. Kerr and colleagues (22a) also report that Bcl-3 can inhibit NF-κB at very high concentrations but did not present titration of the levels of Bcl-3 in their report. In addition, their Bcl-3 constructs consist of GST fusions that might lead to results different from those presented here. Finally, since we have recently observed (25a) that complexes once thought to be NF-κB p50:p65 are composed in part of c-Rel, it is possible that the protein used by others is not authentic NF-κB. Alternatively, a true difference might exist between Bcl-3's ability to interact with reconstituted p50:p65 compared with NF-κB p50:p65 derived from mammalian cells. We believe that the differences between our data and those of others may lay with their use of truncated or improperly modified Bcl-3. Franzoso et al. have recently reported transcriptional inhibition results by transfection that are in good agreement with our in vitro inhibitions (11).

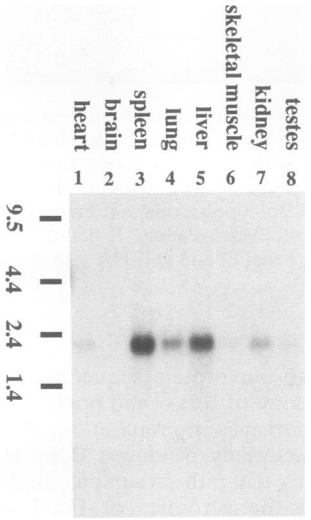

FIG. 5. Evidence that *bcl*-3 mRNA is widely expressed. *bcl*-3 cDNA was radiolabeled and hybridized against a nitrocellulose filter to which were bound poly(A)-enriched fractions of total RNA from the indicated tissues. Sizes are indicated in kilobases.

Bcl-3 was capable of inhibiting the binding of p50 to Ig κB sites and removing bound $(p50)_2$ protein from DNA. On certain κB sites, Bcl-3 might therefore inhibit the binding of p50 to DNA in a regulated manner. IκB also can actively dissociate its target, NF-κB, from DNA (45), suggesting that a general ability of IκB-like molecules might be to induce a conformational change in appropriate Rel complexes that catalyzes their dissociation from DNA. In these regards, Franzoso et al. (11) have recently suggested that Bcl-3 can inhibit the binding of p50 to DNA in cotransfections and allow NF-κB to bind and mediate transcriptional activation. Thus, Bcl-3 might act to remove p50 (a transcriptional activator in its own right [12]) from certain κB sites to allow the binding of repressors or activators. Interestingly, we have observed that Bcl-3 does not inhibit the binding of p50 to H-2 κB sites (11a). The ability of Bcl-3 to inhibit $(p50)_2$ binding to certain sites is reminiscent of the site-dependent transcriptional induction previously observed for p50 (12). In that study, it was shown that p50 assumes a protease-resistant structure on H-2 κB motifs but not upon Ig or beta interferon κB motifs. With H-2 κB motifs placed upstream of a minimal promoter, p50 was a strong in vitro transcriptional activator, whereas it acted weakly as an activator on an Ig or beta interferon κB site. Thus, in vivo, the possibility exists that p50 bound to the H-2 motif acts as a constitutive transcriptional inducer in a structural conformation that is refractory to inhibition by Bcl-3.

Bcl-3 differs from IκB in it subcellular localization. Bcl-3 localizes to the nuclei of cells (Fig. 3C), even in the presence of p50, and can physically associate in cells with p50. The cellular association with p50 is supported by the striking finding that a p50 mutant deleted for the NLS can redirect Bcl-3 subcellular localization to the cytoplasm. Therefore, although Bcl-3 has a capacity to intrinsically localize to the nucleus by an unknown mechanism, the location of p50 can dominate the localization of Bcl-3, perhaps by masking the as yet undefined NLS of Bcl-3. This is in contrast to previous models in which IκB regulates NF-κB activity by its sequestration in the cytoplasm, presumably by masking of the NLS of p65 (4). Zabel and Baeuerle (45) have postulated that IκB may have a role in the nucleus of cells as a result of the ability of IκB to actively dissociate NF-κB bound to DNA in vitro. Because baculovirus-produced Bcl-3 also actively dissociates p50 bound to DNA (Fig. 1B, lanes 1 and 2), a role of Bcl-3 in cells might be to sequester p50 in the nucleus in a non-DNA-binding complex. It is also possible that certain modified forms of Bcl-3 associate with p50 while p50 is bound to DNA (11a). It might be relevant that Kang et al. have recently reported that a deoxycholate-releasable factor that inhibits the binding of p50 to DNA can be found in the

nucleus of activated T-cell clones (21). This activity might represent Bcl-3 or the other described specific inhibitor of p50, IκB-γ.

Interestingly, *bcl*-3 mRNA levels increase after stimuli that activate NF-κB (32), suggesting that NF-κB can play a direct role in the transcriptional induction of this mRNA. This is similar to the increase in IκB-α mRNA levels in monocytes after activation (17) and during the acute-phase response in liver (38). Such synthesis might represent a feedback mechanism to produce new Bcl-3 or IκB, replacing that which has been modified or degraded after activation (33a). We note that the promoter region of human *bcl*-3 (32) contains three potential NF-κB-binding motifs. Two of these motifs, (GGGACACCCC) and (GGGAAGTCCC), are κB motifs that would be predicted to bind p50 homodimers or NF-κB. Interestingly, both sites, when extended into 11 nucleotides by using flanking sequence, also share resemblance with the 11-bp KBF-1 site that binds p50 homodimers in the K^b promoter (20). Therefore, it is possible that p50 participates directly in the regulation of Bcl-3.

Bcl-3 and IκB-γ are both directed at p50 homodimers, contain seven ankyrin domains apiece, and share considerable homology (reference 30) and references therein). It is possible that these two soluble inhibitors target p50 for different regulatory reasons. Thus, the question arises as to where the specificity resides in Bcl-3 and the CTR of p105 and IκB-α for their cognate Rel proteins and how this specificity difference translates to functional differences. Structure predictions of the four classes of inhibitory ankyrin proteins (Bcl-3, IκB-α, pp40, p105, and p100) strongly predict a helix-turn-helix-turn topology for the ankyrin domain (30a). In this model, the relatively conserved TPLH motif constitutes the turn just before the beginning of the helix; helix-ending residues are found near the end of each ankyrin motif. Thus, it is possible that the ankyrin domains fold upon themselves in a series of helices. In support of this view, another protein family, the snap-helix proteins of the TPR family, are composed of repeated 35-amino-acid motifs that are predicted to form helix-turn structure (16). Like the ankyrin motif, the TPR snap-helix motif was originally identified in the cell division control genes of *Saccharomyces cerevisiae*, and both were later both found in similar settings such as transcription, protein-protein association, and matrix structure. The TPR motif has little apparent overall sequence homology to ankyrin domains. However, ankyrin motifs (30) contain groups of conserved small side chain residues (alanines and glycines) as well as conserved bulky hydrophobic residues (leucines and histidines) at appropriate spacing that might well serve as knobs and holes of helix-associating domains, analogous to the model proposed for the TPR structure (16). Examination of these structural and functional similarities of ankyrin and TPR motifs might help in an understanding of how proteins containing such internally repeated domains carry out their heterogeneous functions.

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