

The candidate proto-oncogene *bcl-3* encodes a transcriptional coactivator that activates through NF- κ B p50 homodimers

Takashi Fujita,¹ Garry P. Nolan,² Hsiou-Chi Liou, Martin L. Scott, and David Baltimore³

The Rockefeller University, New York, New York 10021 USA

The candidate proto-oncogene *bcl-3* encodes a protein that shares structural features with I κ B- α and other proteins that bind to members of the Rel protein family. Here, we show that in contrast to the inhibitory activity of I κ B- α , the *bcl-3* gene product superactivates NF- κ B p50 homodimer-mediated gene expression both in vivo and in vitro. BCL-3 protein can, as well, selectively associate with p50 homodimers in the presence of DNA containing a κ B motif. These results strongly suggest that BCL-3 can act as a transcriptional coactivator, acting through DNA-bound p50 homodimers.

[Key Words: NF- κ B; p50; I κ B; transcription; coactivator]

Received January 8, 1993; revised version accepted May 21, 1993.

The candidate proto-oncogene *bcl-3* was identified by the cloning of chromosomal breakpoints from chronic lymphocytic leukemia cells containing a t(14;19) translocation (Ohno et al. 1990). *bcl-3* expression is activated by translocation without apparent alteration of the encoded protein structure. The deduced primary structure of BCL-3 contains seven repeats of an ~30-amino-acid motif (ankyrin repeat) found in several other proteins (Nolan and Baltimore 1992). These include erythrocyte ankyrin, a cytoskeletal protein; yeast cell cycle regulatory proteins, such as *cdc10* and *SWI6*; and the transmembrane receptors *notch*, *TAN1*, and *int-3*.

The closest relatives of BCL-3 are proteins that affect the activity and intracellular localization of NF- κ B. I κ B- α , a protein encoded by MAD-3 cDNA (Haskill et al. 1991), has five full copies of the ankyrin motif in a pattern very similar to that found in BCL-3 (Nolan and Baltimore 1992). It inhibits the DNA binding of NF- κ B and sequesters it in the cytoplasm (Baeuerle and Baltimore 1988a). NF- κ B is a protein of two subunits, p50 and p65, both of which are related in structure to the *rel* oncogene protein (Nolan and Baltimore 1992). I κ B has the highest affinity for the p65 subunit (Baeuerle and Baltimore 1989). Like BCL-3, the I κ B- γ protein has seven ankyrin motifs (Inoue et al. 1992a; Liou et al. 1992). It is the carboxyl half of the p105 NF- κ B-1 precursor of the p50

subunit, as well as an independent protein in some lymphoid cells (Liou et al. 1992). A close relative of NF- κ B-1, NF- κ B-2 p100, also has an I κ B- γ -related carboxyl terminus (Neri et al. 1991; Schmid et al. 1991).

BCL-3 is functionally related to, but distinct from, I κ B- α in that it preferentially interacts with the p50 subunit of NF- κ B (Wulczyn et al. 1992) and both proteins colocalize to the nucleus (Nolan et al. 1993), whereas the primary target for I κ B- α is the p65 subunit of NF- κ B and the complex is found in the cytoplasm. Also, the amino acids flanking the ankyrin repeats of I κ B- α and BCL-3 differ markedly. The latter, but not the former, contains proline-rich regions in its amino and carboxyl termini and a serine-rich carboxy-proximal region. Because the ankyrin repeat domain is implicated in protein-protein interactions, these unique regions might have effector roles in the nucleus. In previous work, however, it was found that BCL-3 protein produced by bacteria (Wulczyn et al. 1992) or insect cells (Nolan et al. 1993) inhibits the DNA-binding activity of NF- κ B p50, suggesting that it has an inhibitory role.

p50 homodimers [KBF-1, (p50)₂] are detected in the nucleus of some cells as a binding activity specific for a site within the major histocompatibility complex (MHC) class I (H-2) gene enhancer. The detection of KBF-1 and the expression of the H-2 gene is highly correlated (Burke et al. 1989; Israël et al. 1989). In vivo, competition experiments have demonstrated that the factor interacting with the H-2 κ B site activates MHC gene expression (Baldwin and Sharp 1987; Israël et al. 1987). However, recent transient transfection experiments using chloramphenicol acetyltransferase (CAT) as a reporter failed

Present addresses: ¹Department of Tumor Cell Biology, Tokyo Metropolitan Institute of Medical Science, Bunkyo-ku, Tokyo 113, Japan; ²Department of Pharmacology, Stanford University, Stanford, California 94305-6907 USA.

³Corresponding author.

to show significant *trans*-activation by cDNA-mediated p50 expression (Schmid et al. 1991; Schmitz and Baeuerle 1991; Beg et al. 1992). More recently, while our study was in progress, Franzoso et al. (1992) reported that BCL-3 acts as an antagonist of p50 that strongly inhibits κ B motif-dependent CAT gene expression.

In the following experiments, we employed a transient transfection system using luciferase as reporter of κ B-regulated transcription. Contrary to the previous results (Schmitz and Baeuerle 1991; Franzoso et al. 1992), we observed that p50 activates gene expression both *in vivo* and *in vitro* and that BCL-3 can superactivate in a p50-dependent manner. Furthermore, BCL-3 protein produced in mammalian cells associated with p50 which was bound to a κ B oligonucleotide *in vitro*. These results strongly suggest that BCL-3 does not function solely as an inhibitor but, rather, that it can act as a transcriptional coactivator for (p50)₂.

Results

BCL-3 synergistically activates gene expression with p50 in vivo

Various laboratories have reported that in cotransfection experiments, the p50 subunit of NF- κ B activates transcription poorly from κ B-driven reporter constructs while p65 activates well (Schmitz and Baeuerle 1991; Beg et al. 1992). However, we found that *in vitro* (p50)₂ is an excellent transcriptional activator on certain κ B sites and that (p65)₂ is not an especially good activator (Fujita et al. 1992). We have therefore reinvestigated the activity of these regulators in a cotransfection system using a luciferase reporter system rather than the previously utilized CAT system. Luciferase protein is detectable at very low levels (De Wet et al. 1987) and is intrinsically unstable, whereas the CAT assay is less sensitive and the protein is more stable. Thus, luciferase activity should reflect the instantaneous concentration of its mRNA rather than measuring the protein that accumulates over many hours as is assayed by CAT.

We transfected into mouse L929 cells a luciferase reporter gene along with effector constructs encoding various NF- κ B-related proteins. The reporter contained a simple promoter from the β interferon (IFN- β) gene (Fujita et al. 1987), along with three upstream κ B sites. The κ B motif studied first was that from the immunoglobulin κ gene (Ig κ), which responds *in vitro* about equally to p50 and p65. With increasing concentration of either effector plasmid, there was an increasing synthesis of luciferase (see Fig. 1A, bars 4 and 7; other data not shown). If the reporter lacked κ B sites, p50 was unable to stimulate. It is evident that p50 is an activator of transcription on an Ig κ κ B motif.

We then investigated whether BCL-3 would affect the response to p50. We compared it with I κ B- α , an inhibitor known to interact with p65. Expression of BCL-3 or I κ B alone had little effect on reporter gene expression (Fig. 1A). Coexpression of p50 with the reporter gave the expected stimulation, and BCL-3 augmented that simula-

tion further (Fig. 1A). Because Franzoso et al. (1992) had previously reported quite different results with the NTera-2 cell line—a human embryonic carcinoma cell that lacks endogenous NF- κ B-related proteins—we examined the behavior of our reporter and expression plasmids in that cell line. A similar activation of the luciferase reporter gene by p50 was observed in these cells, as well as perhaps a greater BCL-3 augmentation (Fig. 1B). In L929 cells, p65 also stimulated, but BCL-3 gave no augmentation and I κ B repressed the activation by either p50 or p65 (Fig. 1A). The opposing effects of BCL-3 and I κ B on p50-mediated activation were particularly clearly demonstrated in titrations of the effector plasmid (Fig. 1C). The inhibitory effect of I κ B *in vitro* and *in vivo* to (p65)₂ or p50/p65 is well documented (Baeuerle and Baltimore 1989; Nolan et al. 1991; Beg et al. 1992), but this is the first demonstration that gene activation mediated by p50 can be inhibited by I κ B under certain conditions. Whereas the p50–I κ B interaction is not evident by mobility shift assay unless a large excess of I κ B is added (Liou et al. 1992), this result is consistent with the finding that p50 homodimer can be coimmunoprecipitated (Inoue et al. 1992b) and sequestered in cytoplasm by ectopic expression of I κ B (Beg et al. 1992).

Franzoso et al. (1992) used a reporter containing natural HIV LTR and showed that this construct is activated by BCL-3 in the presence of p50 and p65. We tested a similar HIV LTR luciferase construct as reporter (Fig. 1D). The reporter is little activated either by p50 or BCL-3 alone; however, it is dramatically activated by coexpression of both. Thus, the synergism seen with synthetic κ B constructs can be reproduced using a more physiological regulatory DNA sequence.

Physiologically, p50 is processed from precursor p105 by proteolytic removal of its carboxyl region (Blank et al. 1991). Transfection of a p105 expression plasmid in human kidney cell line 293 and mouse L929 cells resulted in an accumulation of p50 as well as its precursor, as evidenced by Western blotting (data not shown). Transfection of a p105 expression plasmid into L929 cells gave no detectable stimulation of luciferase, but BCL-3 was able to activate transcription just as it could with direct p50 expression (Fig. 2A). This shows that physiologically processed p50 can participate in gene activation synergistically with BCL-3. The lack of direct activation by p105 could be the result of the level of p50 derived by cleavage.

Our findings contrast with those reported by Franzoso et al. (1992). They found that coexpression of p65 is prerequisite for BCL-3 to activate gene expression and interpreted the result as indicating that p65 is the true activator and BCL-3 acts by removing inhibitory (p50)₂. However, in the system described here, ectopic p65 expression is not required and expression of both p50 and BCL-3 are required for maximal gene expression. The p50 protein is not encoded as such but derives by proteolysis from a p105 precursor (Blank et al. 1991), and different laboratories have made different p50 constructs by truncating a p105-encoding plasmid at particular places. In certain reports (Beg et al. 1992; Franzoso et al. 1992),

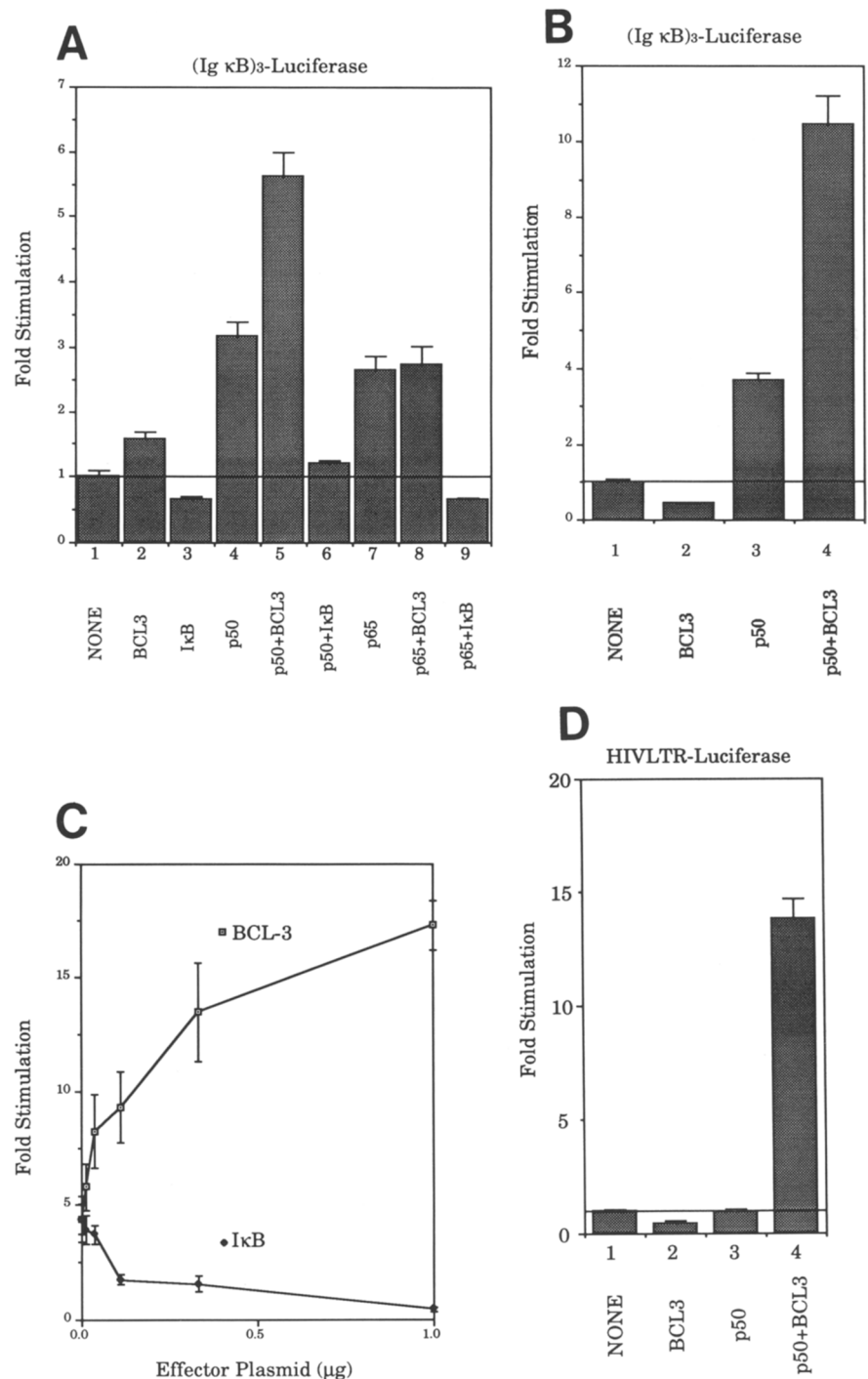


Figure 1. Effect of BCL-3 and I κ B on κ B motif-dependent gene activation in vivo. (A) L929 cells were transfected with a reporter plasmid (p-55Ig κ Luc, 0.5 μ g) and effector plasmids (each 0.05 μ g). (1) No effector; (2) pCDMBCL3; (3) pCDMI κ B; (4) pCDM50; (5) pCDM50 + pCDMBCL3; (6) pCDM50 + pCDMI κ B; (7) pCDM65; (8) pCDM65 + pCDMBCL3; (9) pCDM65 + pCDMI κ B. In all of the cotransfection experiments, total amounts of plasmid were kept constant by adding the vector without insert. (B) Ntera2 cells were transfected with reporter plasmid (p-55Ig κ Luc, 3 μ g) and effector plasmids (1 μ g each). Error bars represent s.e. from triplicate transfection results. (1) No effector; (2) pCDMBCL3; (3) pCDM50; (4) pCDM50 + pCDMBCL3. (C) L929 cells were transfected with p-55Ig κ Luc (0.5 μ g) and indicated amounts of pCDMBCL3 (\square) or pCDMI κ B (\blacklozenge). (D) L929 cells were transfected with pHIVLTRLuc (0.5 μ g) and effector plasmids (0.05 μ g each). (1) No effector; (2) pCDMBCL3; (3) pCDM50; (4) pCDMBCL3 + pCDM50. Error bars in A, C, and D represent s.e. from quadruplicate transfection trials.

p50 was expressed from a cDNA truncated by digestion with the restriction enzyme *Xba*I to give a 503-amino-acid protein [p50XbaI, which directs synthesis of an apparently 60-kD polypeptide in L929 cells (T. Fujita et al., unpubl.)]. In our laboratory p50 was produced from a plasmid truncated at an *Fsp*I site (Fujita et al. 1992) to produce a protein of 401 amino acids that migrates coincidentally with authentic p50. We compared as activators our p50, p50XbaI, and p52, a protein closely related

to p50 (Fig. 2B). p50, p50XbaI, and p52, respectively, activated the reporter gene moderately; however, only p50 and p52 superactivated with BCL-3. Because p50 and p50Xba are accumulated in similar amounts in transfected L929 cells (data not shown), the failure of p50Xba to cooperate with BCL-3 is likely attributable to its extended length, compared with that of p50. The magnitude of activation by p52 and p50 was comparable in repeated experiments (data not shown). Thus, it is clear

why Franzoso et al. (1992) were unable to see synergism of their p50 and BCL-3, but it is not apparent why they did not see activation with p50.

An expression plasmid for a mutant BCL-3, lacking the unique amino- and carboxy-terminal regions (Δ BCL-3) was constructed. Δ BCL-3 was expressed in the nucleus in L929 cells (data not shown) but failed to superactivate with p50 and was even inhibitory to the activation by p50 (Fig. 2C). Thus, the amino- and carboxy-terminal regions of BCL-3 are needed for coactivation with p50 and might contain transcriptional activation sequences. Without these sequences, BCL-3 acts as an inhibitor.

Association of BCL-3 with p50, which is bound to κ B motif DNA

It was reported previously that a bacterially expressed polypeptide corresponding to a truncated human BCL-3 (289 amino acids of the full-length 454 amino acids) (Wulczyn et al. 1992), as well as murine BCL-3 produced by a baculovirus system (Nolan et al. 1993), inhibited binding of p50 to κ B motif DNA. These results appear inconsistent with the *trans*-activation as described here. The amino acid sequences flanking the ankyrin repeat are unique, highly conserved between human and mouse, and contain multiple potential phosphorylation sites that may modify the function of the wild-type protein. Therefore, we expressed the full-length mouse

BCL-3 and Δ BCL-3 protein in human kidney cell 293 cells and further tested their properties. Transfection of a BCL-3 expression plasmid into 293 cells caused an accumulation of BCL-3 protein detectable by Western blotting with anti-BCL-3 rabbit serum (Fig. 3, lane 3). Cells transfected with either empty vector or a p50 expression plasmid did not accumulate BCL-3 (Fig. 3, lanes 1,2). This is in contrast to the observation that overexpressed p65 induces an accumulation of its target molecule, I κ B- α (Scott et al. 1993). The BCL-3 protein was produced as a broad band consisting of multiple species (Fig. 3, lanes 3,7). This is likely the result of phosphorylation, because phosphatase treatment converted the pattern into a nearly homogeneous, faster-migrating species (Fig. 3, lane 8). In contrast, Δ BCL-3 was expressed as sharp band (\sim 35 kD), and its mobility was unchanged after phosphatase treatment (Fig. 3D). Thus, it is likely that the clustered serine and threonine residues in BCL-3, which were removed by truncation, are sites of phosphorylation.

The stimulation by BCL-3 in the transfection experiments suggested that it might bind to p50 to form a ternary complex with DNA. To test this idea, we incubated NF- κ B p50 or p65 proteins with BCL-3 protein and a biotinylated κ B motif DNA. Resulting complexes were purified by streptavidin-agarose column chromatography (Materials and methods). After washing the column with five bed volumes of binding buffer, the bound pro-

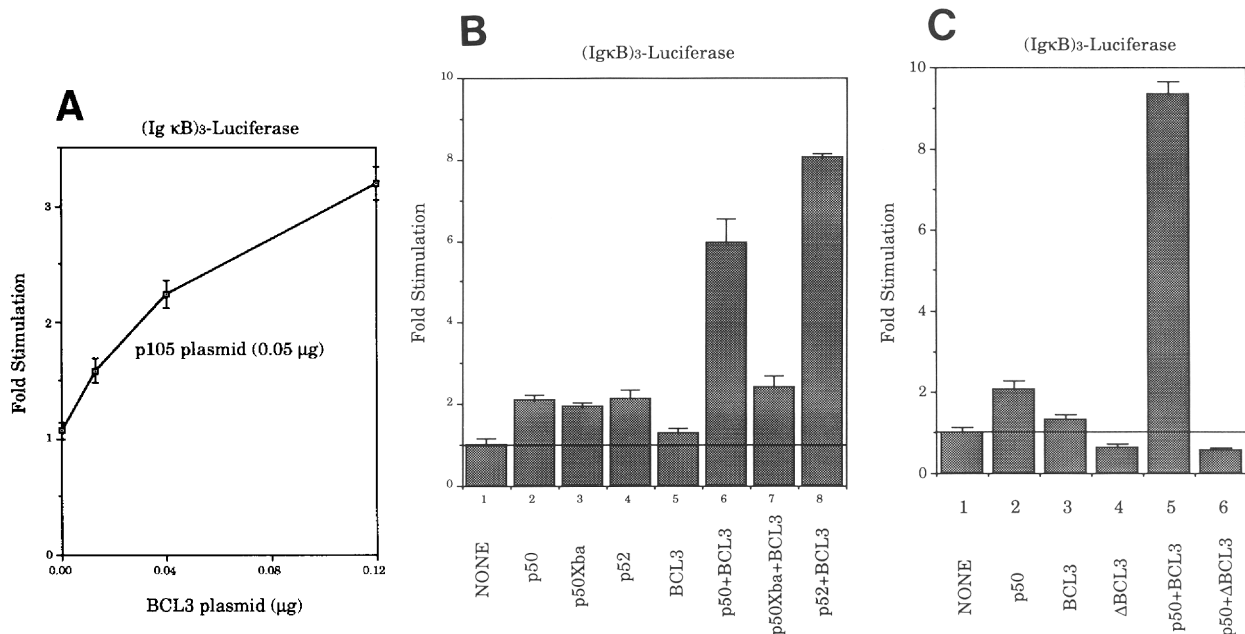


Figure 2. Effect of mutated p50 and BCL-3 on coactivation. (A) Naturally processed p50 can coactivate with BCL-3. L929 cells were transfected with 0.5 μ g of p-55Ig κ Luc, 0.05 μ g of pEVRF105 (a p105 expression vector), and indicated amounts of pCDMBCL3. Stimulation by BCL-3 expression plasmid alone (0.12 μ g) was 1.2-fold. (B) An artificially processed long p50 (p50Xba) failed to coactivate with BCL-3. L929 cells were transfected with 0.5 μ g of p-55Ig κ Luc and effectors (0.05 μ g). (1) No effector; (2) pCDM50; (3) pCMV50Xba; (4) pRSVp52; (5) pCDMBCL3; (6) pCDM50 + pCDMBCL3; (7) pCMV50Xba + pCDMBCL3; (8) pRSVp52 + pCDMBCL3. (C) A mutant BCL-3 (Δ BCL-3) failed to coactivate with p50. L929 cells were transfected with 0.5 μ g of p-55Ig κ Luc and effectors (0.05 μ g). (1) No effector; (2) pCDM50; (3) pCDMBCL3; (4) pCDM Δ BCL3; (5) pCDM50 + pCDM Δ BCL3; (6) pCDM50 + pCDM Δ BCL3. Error bars represent 2.E. from quadruplicated transfection trials.

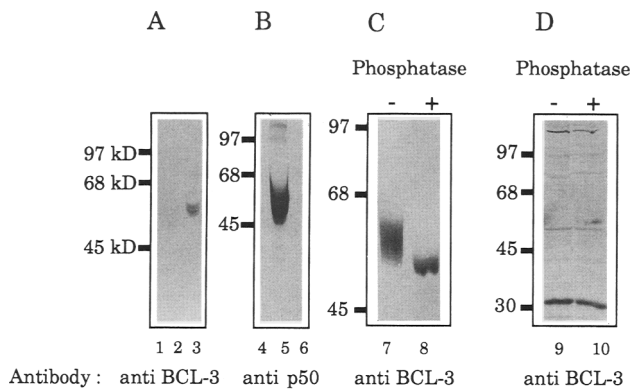
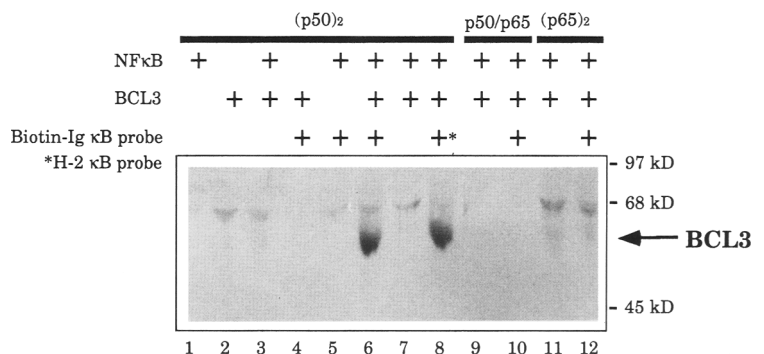


Figure 3. Expression of mouse BCL-3 in human 293 cells. 293 cells (3×10^6 cells) were transfected with expression plasmids (10 μ g): pCDM8 (1,4); pCDM50 (2,5); pCDMBCL3 (3,6,7,8); and pCDM Δ BCL3 (9,10). Cell lysates corresponding to 10^5 cells were analyzed by SDS-PAGE and Western blotting using anti-BCL-3 rabbit serum (A,C,D) or anti-p50 rabbit serum (B). Lysates containing BCL-3 and Δ BCL-3 (7,9) were treated with acid phosphatase (1.0 units) prior to SDS-PAGE (8,10).

teins were eluted, resolved by SDS-PAGE, and analyzed by immunoblotting using an anti-BCL-3 antibody (Fig. 4). BCL-3 did not bind to the κ B motif DNA by itself (lane 4), but in the presence of $(p50)_2$ it was recovered in a complex associated with DNA (lane 6). Ternary complex formation was observed using both Ig κ and H-2 κ B motifs. p65 homodimers or p50/65 heterodimers tested under the same conditions failed to interact with BCL-3, although these proteins were efficiently retained on immunoglobulin κ B motif DNA, or H-2 κ B motif DNA, as shown by immunoblotting using anti-p50 and anti-p65 antibodies (data not shown). These results demonstrate that BCL-3 can specifically associate with $(p50)_2$ while the homodimer is bound to a κ B motif in DNA.

We attempted to demonstrate the ternary complex by mobility shift assay using the same protein preparations as in the previous experiment. However, the mobility of the p50–DNA complex was identical in the presence or absence of BCL-3. Also, our anti-BCL-3 antibody failed to affect the mobility of the p50–DNA complex when BCL-3 was added. Presumably BCL-3 dissociates from the complex during the electrophoretic analysis. Re-

Figure 4. BCL-3 specifically associates with p50 bound to κ B motif DNA. Insect cell-derived NF- κ B subunits (1 pmole) were mixed with 293 cell-derived BCL-3 (1.2 pmole), and biotinylated DNA probe containing three copies of the κ B motif from the Ig κ gene (1.2-pmole sites) was incubated in different combinations. Proteins bound to DNA were separated out by streptavidin–agarose column chromatography. The bound fractions were size fractionated by electrophoresis in SDS, and the presence of BCL-3 was determined by Western blotting using a rabbit anti-BCL-3 serum. The additions to individual samples are indicated.



cently, Bours et al. (1993) demonstrated significant ternary complex formation composed of p50, BCL-3, and κ B motif DNA under their EMSA conditions. Interestingly, under the same conditions, BCL-3 does not inhibit binding of p50 to DNA. This is consistent with our observation that mammalian BCL-3 exhibited a moderate inhibitory effect on p50 DNA binding only when $>10\times$ molar excess of BCL-3 was added, whereas insect cell-derived BCL-3 was clearly highly inhibitory to p50 DNA binding (Nolan et al. 1993; T. Fujita et al., unpubl.). This is presumably attributable to the altered modification of proteins in insect versus mammalian cells (Höss et al. 1990; also see Discussion).

BCL-3 activates transcription by p50 in vitro

The above results prompted us to test the function of BCL-3 as a transcriptional activator in vitro. The 293 cell-derived BCL-3 and a control extract from mock-transfected 293 cells were partially purified by mono Q chromatography (Materials and methods). HeLa cell nuclear extract, which is devoid of detectable amounts of p50, p65 (Fujita et al. 1992), and BCL-3 proteins (determined by Western blotting; data not shown), was supplemented with partially purified BCL-3 or a control preparation and used for transcription reactions (also free of p50 and p65 as shown by immunoblotting; data not included). A mixture of a reference template (containing only a TATA box) and a test template (containing three tandem repeats of a κ B motif) were first reacted with varying amounts of the NF- κ B subunits on ice and then transcribed in HeLa cell nuclear extract with or without added BCL-3 (Materials and methods). Transcripts were detected by primer extension and quantitated using a PhosphorImager: Their values were normalized to the reference transcript. The IFN- β κ B motif, shown previously to be activated poorly by p50 homodimer in vitro, was significantly further activated by the addition of BCL-3 (Fig. 5B). Addition of BCL-3 alone (Fig. 5B, ordinate) did not increase transcription. Transcriptional activation by p65 homodimers was not affected by BCL-3 (Fig. 5A), consistent with the finding that BCL-3 does not associate with $(p65)_2$ (Fig. 4). Similarly, activation by p50/65 heterodimers was not affected by BCL-3 (Fig. 5C), which also failed to interact with p50/65 in vitro (Fig. 4).

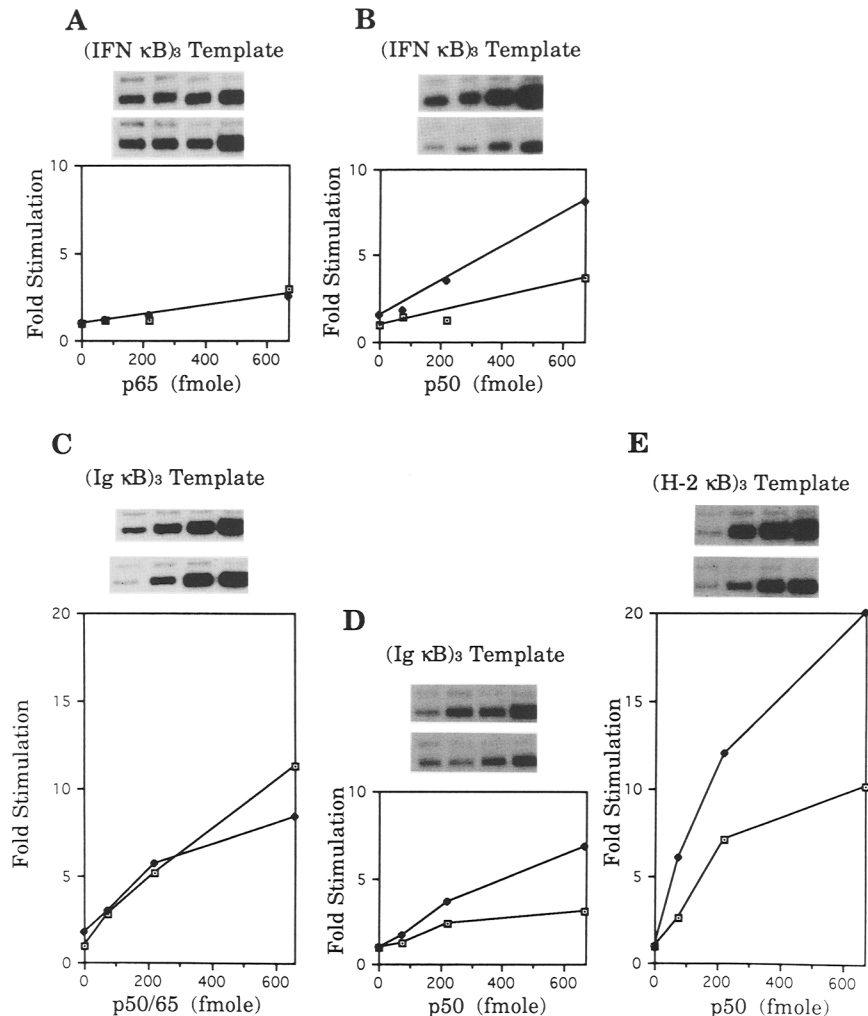


Figure 5. In vitro, BCL-3 activates a κ B motif-containing promoter synergistically with p50. Templates containing three tandem repeats of the IFN- β κ B motif (A,B), the Ig κ B motif (C,D), or the H-2 κ B motif (E) were first reacted with the indicated amounts of purified recombinant p65 (A), p50 (B,D,E) or p50/65 (C). The templates were then transcribed using HeLa cell nuclear extract supplemented with partially purified control fraction (\square) or BCL-3 fraction (\blacklozenge). Original autoradiographs are shown above each panel: Control (bottom set) or plus BCL-3 (top set). Primer extension products for test transcripts (bottom band) and reference transcripts (top band) are seen as described previously (Fujita et al. 1992). The amounts of NF- κ B subunits added were 0, 74, 220, and 670 fmoles (from left to right).

BCL-3 also augmented p50-dependent transcription on immunoglobulin and H-2 κ B sites (Fig. 5D,E). Because the H-2 κ B site is the one activated most efficiently by (p50)₂, the further stimulation by BCL-3 produced the highest level of in vitro activity that we have observed using NF- κ B-related proteins (Fig. 5E). The two- to threefold activation by BCL-3 was approximately that observed in vivo (Fig. 2). These in vitro results again confirm that BCL-3 has properties distinct from those of I κ B- α , which acts as a strong inhibitor of transcription in vitro (Kretzschmar et al. 1992; T. Fujita, G.P. Nolan, H.-C. Liou, M.L. Scott, and D. Baltimore, unpubl.).

Discussion

BCL-3, a transcriptional coactivator

We find that BCL-3 can be a transcriptional coactivator, in direct contrast to the inhibitory function of the related I κ B- α protein. In the presence of (p50)₂, but not by itself or with p50/p65 or (p65)₂, BCL-3 forms a ternary complex with DNA and stimulates transcription. We also find that (p50)₂ is itself an activator; BCL-3 superstimu-

lates its activity by two- to threefold. Transfection experiments and in vitro analyses give congruent results. Recently, Bours et al. (1993) reported similar findings that BCL-3 can directly coactivate with p52.

Our findings with BCL-3 are reminiscent of earlier work on the herpes virus coactivator, VP16. That protein, too, is inactive alone but binds in a ternary complex with Oct-1 and stimulates transcription as a consequence of a highly acidic *trans*-activation domain (Tanaka et al. 1988). VP16 interacts with both Oct-1 and the DNA in the complex; we have no indication that BCL-3 interacts with DNA.

BCL-3 might increase the magnitude of transcription by (p50)₂ through two mechanisms. First, the proline-rich regions in BCL-3 could provide activation domains as do those in the CTF/NF-1 and AP-2 factors (Williams et al. 1988; Mermod et al. 1989). Our finding that Δ BCL-3, which lacks the proline domains, failed to activate may be relevant to this. Second, p50, whose transcriptional activity is correlated to a specific protease-resistant conformation in vitro (Fujita et al. 1992), may be put into its active conformation more efficiently as a result

of association with BCL-3. These are not mutually exclusive notions.

The observation that excess mammalian BCL-3 inhibits DNA binding by $(p50)_2$ (Nolan et al. 1993) suggests that the ternary complex is less stable than the p50–DNA complex. In vitro transcription results suggest that although less stable, this unit can participate in forming an active initiation complex resulting in a higher rate of transcription.

Gene regulation by p50 and BCL-3

Biologically, the individual NF- κ B subunits have multiple specific functions. The classical NF- κ B, p50/65, in most cell types is a dormant activator, held in the cytoplasm, and in a non-DNA-binding state, by I κ B- α (Baeuerle and Baltimore 1988a,b, 1989; Nolan and Baltimore 1992). Upon cellular stimulation, the heterodimers are released from inhibition and move to the nucleus to actively promote specific gene expression (Nolan and Baltimore 1992). The $(p50)_2$ form, and perhaps $(p52)_2$ in some cells, detected in the nuclei of many cell types as KBF-1 (Israël et al. 1987), activates certain κ B sites constitutively and should be subject to coactivation by BCL-3. Because the p50 homodimer has an extremely high affinity for DNA binding ($K_d \sim 5$ pM) (Zabel et al. 1991; Fujita et al. 1992), as compared with other eukaryotic DNA-binding proteins ($K_d \sim 10$ – 100 nM) (Chodosh et al. 1986; Meisterernst et al. 1988; Harada et al. 1989; Cao et al. 1991), it might bind effectively to chromosomal DNA but not function as a strong activator. The coactivator BCL-3 could then easily recognize the bound $(p50)_2$ and interact with it. It remains to be demonstrated whether BCL-3 can change DNA-binding sequence specificity of $(p50)_2$. If this is the case, BCL-3 would participate in a more complex form of regulation. I κ B- γ (Liou et al. 1992) can bind to $(p50)_2$ and act as an inhibitor; how its regulatory properties interact with those of BCL-3 remains to be determined. A $(p65)_2$ form can readily be made in vitro and may exist in cells. Free $(p65)_2$ can apparently up-regulate the synthesis of I κ B- α , thus ensuring that it and NF- κ B are maintained in their inactive state (Scott et al. 1993). Finally, there are other Rel-related subunits—Rel itself, RelB, and the p52 derived from p100 (NFKB-2)—each of which can interact with the other Rel proteins, producing a panoply of potential regulatory interactions.

Phosphorylation of BCL-3 and regulation

The activities of BCL-3 differ from those of I κ B- α . The interaction of I κ B- α with $(p65)_2$ and p50/65 heterodimers [and even $(p50)_2$, albeit weakly] has two effects: It strongly impairs their DNA-binding ability, and it sequesters them in the cytoplasm. In short, the activity of I κ B- α appears to be inhibitory. This may not be the whole story, however, because Morin and Gilmore (1992) reported that I κ B and the carboxy-terminal segment of p105 have activation domains as assayed in yeast cells. BCL-3 has two activities: one inhibitory and the other stimulatory. The inhibitory activity has been

documented extensively (Franzoso et al. 1992; Wulczyn et al. 1992; Kerr et al. 1993; Naumann et al. 1993; Nolan et al. 1993): It involves a specific interaction with $(p50)_2$ or $(p52)_2$ that prevents binding to DNA and even removes bound $(p50)_2$ from DNA. Franzoso et al. (1992), however, have shown that the consequences can be stimulatory if the bound $(p50)_2$ is removed from DNA and is replaced by p50/p65, which is a stronger activator. BCL-3 protein expressed in bacteria or insect cells shows the strong DNA-binding inhibitory effect specific to $(p50)_2$.

In vitro, however, mammalian cell-derived BCL-3 binds to its target molecule, $(p50)_2$, without dissociating it from DNA, and the BCL-3/p50 interaction activates transcription. The difference between the mammalian cell-derived BCL-3 and the inhibitory proteins from bacteria and insect cells could be a result of post-translational modification. Höss et al. (1990) reported that SV40 large T antigen produced in insect cells is altered in its phosphorylation as well as DNA-binding pattern. The critical serine residues responsible for binding to site II in the SV40 replication origin and down-regulation are underphosphorylated. In the case of insect cell-derived BCL-3, artificial removal of phosphate residues by phosphatase treatment results in an inactivation of BCL-3 (Nolan et al. 1993), suggesting that some phosphorylation is required for interaction with $(p50)_2$. It is likely that the function of BCL-3 protein can be regulated through phosphorylation of certain amino acid residues, making it a direct activator, an indirect activator, or even an inhibitor. If this is so, BCL-3 can play the sophisticated role of a double agent, depending on the needs of developmental processes or environmental conditions.

In vivo function of BCL-3

We have limited information about the expression of BCL-3 protein in vivo. Its mRNA is expressed in a wide variety of tissues and is particularly evident in spleen, liver, and lung. In the regenerating liver, a κ B site-binding factor called posthepatectomy factor (PHF) is highly induced without p50/65 induction (Tewari et al. 1992). Because PHF is reactive with anti-p50 antibody, BCL-3 might be involved in the formation of PHF.

Results that differ from other reports

Our results differ from those of others, including Bours et al. (1992, 1993) and Franzoso et al. (1992), who failed to observe gene activation by p50 or synergism by p50 and BCL-3. One possible explanation for this difference is the nature of the p50 proteins used. Our expression vector was designed to make 50-kD protein (amino acids 1–401; Fujita et al. 1992), with an apparent molecular mass (by electrophoretic analysis) very similar to the endogenously processed p50 derived from overexpressed p105 in 293 and L929 cells (data not shown). Also, transfection of a p105 expression plasmid to L929 cells, which results in the expression of endogenously processed mature p50, showed synergism with BCL-3 (Fig. 2A). Other

studies have utilized a construct that should produce a significantly longer p50 (*XbaI* truncation, amino acids 1–503; expected molecular mass, >60 kD), including those studies that failed to see activation by p50 and BCL-3 (Beg et al. 1992; Franzoso et al. 1992). The longer p50 is not processed to a proper size in vivo (T. Fujita et al., unpubl.) and failed to cooperate with BCL-3 efficiently (Fig. 2B). Cell line differences or peculiarities of the transfection method are unlikely explanations for the different results because we found a similar synergism using the NTera-2 cell line that was used by Franzoso et al. (1992) and following their protocol (Fig. 2B). Because we showed that a reporter gene regulated by the HIV LTR was also coactivated by p50 and BCL-3, it is unlikely that the reporter used in our other experiments is peculiar. In some of the published cotransfections, the level of BCL-3 could be inappropriate to reproduce synergism with p50 because mammalian BCL-3 added in excess over p50 can be inhibitory for DNA binding (Franzoso et al. 1992; T. Fujita et al., unpubl.).

Materials and methods

Construction of plasmids

Reporter constructs with or without three tandemly repeated κ B motifs upstream of a minimal IFN- β promoter [–55 to +19 (Fujita et al. 1992)] were prepared from pBL, a plasmid containing *Photinus pyralis* luciferase cDNA (De Wet et al. 1987) and the polyadenylation site from pSV2 (between *HindIII* and *HincII* sites of pBluescript KS+). pBL was digested with *SmaI* and *HindIII*, and the larger fragment was isolated. This fragment and a *HindIII*–*SaII* fragment (whose *SaII* end had been blunted) from p-55Igkcat (Fujita et al. 1992) were ligated to generate p-55IgkLuc. pHIVLTRLuc (originally p22Luc), containing the HIV-1 LTR upstream of the luciferase structural gene, was a kind gift of Dr. K. Saksela (The Rockefeller University, New York). pEVRF105 was constructed by cloning the p105 cDNA (Ghosh et al. 1990) into pEVRF1 (Matthias et al. 1989). pCDM50, pCDM65, pCDMBCL3, pCDM Δ BCL3, and pCDMI κ B were constructed by cloning the p50 cDNA (from pVLP50; Fujita et al. 1992), the p65 cDNA (from pVLP65; Fujita et al. 1992), the mouse BCL-3 cDNA (Nolan et al. 1993), a portion of mouse BCL-3 (Δ BCL-3; spanning amino acids 104–352) and the κ B- α cDNA (Liou et al. 1992) into CDM8 (Seed 1987) downstream of the cytomegalovirus promoter. The p50Xba expression vector, pCMV50Xba, was a kind gift of Dr. S. Akira (Tokyo University, Japan). pRSVp49 was obtained from C.S. Duckett.

Luciferase activity assay

L929 cells were seeded 1 day before transfection (5×10^5 cells/well in 24-well dishes). Transfection was performed by the DEAE-dextran protocol (Seed 1987). Total amounts of plasmid were kept constant by adding empty vector. After 48 hr, the culture medium was removed and cell lysis buffer was added (200 μ l; Luciferase Assay System, Promega). A portion of the extract (3 μ l) was reacted with luciferase substrate (27 μ l). The luminescence was quantitated by a scintillation counter (Beckman) in the single photon mode. Backgrounds from the substrate solution and the counter were \sim 30,000 cpm. L929 cells transfected with reporter construct alone usually gave 100,000–1,000,000 cpm, which was taken as basal activity (onefold).

NTera cells were transfected as described elsewhere (Bours et al. 1992). Extracts (500 μ l) were prepared from 3×10^6 cells, and a portion (3 μ l) was subjected to luciferase reaction (above).

Western blotting

Western blotting was performed using 1 : 1000 diluted rabbit polyclonal antisera as primary antibody (Liou et al. 1992) and alkaline phosphatase-conjugated goat anti-rabbit antibody as secondary reagent. Phosphatase treatment was performed in 0.1 M MES buffer (pH 6.0).

BCL-3 production and purification

293T cells were seeded 24 hr before transfection (3×10^6 cells/5-cm dish). Cells were transfected with pCDMBCL3 by the calcium phosphate method. Fifteen hours after addition of DNA, the cells were washed and incubated further for 33 hr. Whole-cell extract was prepared with buffer D' (20 mM HEPES at pH 7.9, 1 mM EDTA, 1 mM DTT, 10% glycerol, 0.1% NP-40), containing 400 mM NaCl, by vortexing briefly. The suspension was clarified by centrifugation (100,000g for 30 min), and the supernatant was diluted with buffer D' to a final NaCl concentration of 50 mM. After removal of insoluble materials by centrifugation (100,000g for 30 min), the lysate was applied to an FPLC mono Q column. The column was washed with buffer D' containing 50 mM NaCl, and proteins were eluted by NaCl gradient (50–1000 mM, in the same buffer). BCL-3 was reproducibly eluted at \sim 100 mM NaCl as detected by Western blotting using anti-BCL-3 antiserum. Control fractions were prepared identically, except pCDMBCL3 DNA was omitted. The control and BCL-3 fractions were adjusted to 50 mM NaCl by addition of buffer D'. The control lysate was negative for BCL-3 by Western blotting (data not shown). Protein species contained in the partially purified control and BCL-3 fractions were indistinguishable after SDS-PAGE and Coomassie brilliant blue staining.

Test for association of κ B motif DNA, NF- κ B subunits and BCL-3

The probes used were double-stranded DNA oligonucleotides containing three tandem repeats of either the κ B or H-2 κ B motifs (Fujita et al. 1992). The oligonucleotides were annealed, and their ends were filled in with Klenow DNA polymerase in the presence of a trace amount of [32 P]dCTP, together with cold dCTP, dTTP, dGTP, and biotin-dATP (each 0.2 mM). About 65% of the probe molecules were biotinylated (32.5% of the ends). Reaction mixtures (50 μ l) containing combinations of biotinylated κ B motif DNA (1.2 pmole), NF- κ B subunits (1 pmole), and 293-cell derived BCL-3 (crude, 1.2 pmole) in buffer D' were incubated at 4°C for 10 min and applied to a streptavidin-agarose column (20- μ l bed). The column was washed with 100 μ l of buffer D' containing 50 mM NaCl. The bound proteins were eluted with SDS-PAGE sample buffer and subjected to Western blotting analysis. p50 and p65 homodimers and p50/65 heterodimers were quantitated by Scatchard analysis using known amounts of κ B motif DNA as probe. BCL-3 was quantitated by Western blotting using known amounts of insect cell-derived BCL-3 as standards.

In vitro transcription

In vitro transcription was performed as described previously, except that partially purified control or BCL-3 fraction (the equivalent of 400 fmoles of BCL-3) was added. Test and reference transcripts were quantitated as described previously (Fujita

et al. 1992). Transcription of test template by unsupplemented HeLa cell extract was taken as basal activity (onefold).

Acknowledgments

This research was supported by fellowships from the Human Frontier Science Program (T.F.), the Leukemia Society (G.P.N.), the Cancer Research Institute (H.-C.L.), the National Institutes of Health (NIH) (CA51462) (M.L.S.), and NIH grant GM39458-04 (D.B.) T.F. is grateful for encouragement by workers in the Baltimore laboratory, the Taniguchi laboratory, and the Department of Tumor Cell Biology (Tokyo).

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

- Bauerle, P.A. and D. Baltimore. 1988a. Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF- κ B transcription factor. *Cell* **53**: 211–217.
- . 1988b. I κ B: A specific inhibitor of the NF- κ B transcription factor. *Science* **242**: 540–546.
- . 1989. A 65-kD subunit of active NF- κ B is required for inhibition of NF- κ B by I κ B. *Genes & Dev.* **3**: 1689–1698.
- Baldwin, A.S. and P.A. Sharp. 1987. Binding of a nuclear factor to a regulatory sequence in the promoter of the mouse H-2K^b class I major histocompatibility gene. *Mol. Cell. Biol.* **7**: 305–313.
- Beg, A.A., S.M. Ruben, R.I. Scheinman, S. Haskill, C.A. Rosen, and A.S. Baldwin. 1992. I κ B interacts with the nuclear localization sequences of the subunits of NF- κ B: A mechanism for cytoplasmic retention. *Genes & Dev.* **6**: 1899–1913.
- Blank, V., P. Kourilsky, and A. Israël. 1991. Cytoplasmic retention, DNA binding and processing of the NF- κ B p50 precursor is controlled by a small region in its C-terminus. *EMBO J.* **10**: 4159–4167.
- Bours, V., P.R. Burd, K. Brown, J. Villalobos, S. Park, R.-P. Ryseck, R. Bravo, K. Kelly, and U. Siebenlist. 1992. A novel mitogen-inducible gene product related to p50/p105-NF- κ B participates in transactivation through a κ B site. *Mol. Cell. Biol.* **12**: 685–695.
- Bours, V., G. Franzoso, V. Azarenko, S. Park, T. Kanno, T. Brown, and U. Siebenlist. 1993. The oncoprotein bcl-3 directly transactivates through κ B motifs via association with DNA-binding p50B homodimers. *Cell* **72**: 729–739.
- Burke, P.A., S. Hirschfeld, Y. Shirayoshi, J.W. Kasik, K. Hamada, E. Appella, and K. Ozato. 1989. Developmental and tissue-specific expression of nuclear proteins that bind the regulatory elements of the major histocompatibility complex class I gene. *J. Exp. Med.* **169**: 1309–1321.
- Cao, Z., R.M. Umek, and S.L. McKnight. 1991. Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. *Genes & Dev.* **5**: 1538–1552.
- Chodosh, L.A., R.W. Carthew, and P.A. Sharp. 1986. A single polypeptide possesses the binding and transcription activities of the adenovirus major late transcription factor. *Mol. Cell. Biol.* **6**: 4723–4733.
- De Wet, J.R., K.V. Wood, M. DeLuca, D.R. Helinski, and S. Subramani. 1987. Firefly luciferase gene: Structure and expression in mammalian cells. *Mol. Cell. Biol.* **7**: 725–737.
- Franzoso, G., V. Bours, S. Park, M. Tomita-Yamaguchi, K. Kelly, and U. Siebenlist. 1992. The candidate oncoprotein Bcl-3 is an antagonist of p50/NF- κ B-mediated inhibition. *Nature* **359**: 339–342.
- Fujita, T., H. Shibuya, H. Hotta, K. Yamanishi, and T. Taniguchi. 1987. Interferon- β gene regulation: Tandemly repeated sequences of a synthetic 6 bp oligomer function as a virus-inducible enhancer. *Cell* **49**: 357–367.
- Fujita, T., G.P. Nolan, S. Ghosh, and D. Baltimore. 1992. Independent modes of transcriptional activation by the p50 and p65 subunits of NF- κ B. *Genes & Dev.* **6**: 775–787.
- Ghosh, S., A.M. Gifford, L.R. Rivere, P. Tempst, G.P. Nolan, and D. Baltimore. 1990. Cloning of the p50 DNA binding subunit of NF- κ B: Homology to *rel* and *dorsal*. *Cell* **62**: 1019–1029.
- Harada, H., T. Fujita, M. Miyamoto, Y. Kimura, A. Furia, T. Miyata, and T. Taniguchi. 1989. Structurally similar but functionally distinct factors, IRF-1 and IRF-2, bind to the same regulatory elements of IFN and IFN-inducible genes. *Cell* **58**: 729–739.
- Haskill, S., A.A. Beg, S.M. Tompkins, J.S. Morris, A.D. Yurochko, A. Sampson-Johannes, K. Mondal, P. Ralph, and A.S. Baldwin. 1991. Characterization of an immediate-early gene induced in adherent monocytes that codes I κ B-like activity. *Cell* **65**: 1281–1289.
- Höss, A., I. Moarefi, K.-H. Scheidtmann, L.J. Cisek, J.L. Corden, I. Dornreiter, A.K. Arthur, and E. Fanning. 1990. Altered phosphorylation pattern of simian virus 40 T antigen expressed in insect cells by using a baculovirus vector. *J. Virol.* **64**: 4799–4807.
- Inoue, J., L.D. Kerr, A. Kakizuka, and I.M. Verma. 1992a. I κ B γ , a 70 kd protein identical to the C-terminal half of p110 NF- κ B: A new member of the I κ B family. *Cell* **68**: 1109–1120.
- Inoue, J.-I., L.D. Kerr, N. Rashid, H.R. Davis, H.R.J. Bose, and I.M. Verma. 1992b. Direct association of pp40/I κ B β with rel/NF- κ B proteins: Role of ankyrin repeats in the inhibition of DNA binding activity. *Proc. Natl. Acad. Sci.* **89**: 4333–4337.
- Israël, A., A. Kimura, M. Kieran, O. Yano, J. Kanellopoulos, O. Le Bail, and P. Kourilsky. 1987. A common positive trans-acting factor binds to enhancer sequences in the promoters of mouse H-2 and β_2 -microglobulin genes. *Proc. Natl. Acad. Sci.* **84**: 2653–2657.
- Israël, A., O. Le Bail, D. Hatat, J. Pielt, M. Kieran, F. Logeat, D. Wallach, M. Fellous, and P. Kourilsky. 1989. TNF stimulates expression of mouse MHC class I genes by inducing an NF- κ B-like enhancer binding activity which displaces constitutive factors. *EMBO J.* **8**: 3793–3800.
- Kerr, L.D., C.S. Duckett, P. Wamsley, Q. Zhang, P. Chiao, G. Nabel, T.W. McKeithan, P.A. Bauerle, and I.M. Verma. 1993. The proto-oncogene BCL-3 encodes an I κ B protein. *Genes & Dev.* **6**: 2352–2363.
- Kretzschmar, M., M. Meisterernst, C. Scheidereit, G. Li, and R.G. Roeder. 1992. Transcriptional regulation of the HIV-1 promoter by NF- κ B in vitro. *Genes & Dev.* **6**: 761–774.
- Liou, H., G.P. Nolan, S. Ghosh, T. Fujita, and D. Baltimore. 1992. The NF- κ B p50 precursor, p105, contains an internal I κ B-like inhibitor that preferentially inhibits p50. *EMBO J.* **11**: 3003–3009.
- Matthias, P., M. M. Müller, E. Schreiber, S. Rusconi, and W. Schaffner. 1989. Eukaryotic expression vectors for the analysis of mutant proteins. *Nucleic Acids Res.* **17**: 6418.
- Meisterernst, M., I. Gander, L. Rogge, and E.-L. Winnacker. 1988. A quantitative analysis of nuclear factor I/DNA interactions. *Nucleic Acids Res.* **16**: 4419–4435.
- Mermoud, N., E.A. O'Neill, T.J. Kelly, and R. Tjian. 1989. The proline-rich transcriptional activator of CTF/NF-1 is distinct from the replication and DNA binding domain. *Cell* **58**: 741–753.
- Morin, P.J. and T.D. Gilmore. 1992. The C terminus of the

- NF- κ B p50 precursor and an I κ B isoform contain transcription activation domains. *Nucleic Acids Res.* **20**: 2453–2458.
- Naumann, M., F.G. Wulczyn, and C. Scheidereit. 1993. The NF- κ B precursor p105 and the proto-oncogene product Bcl-3 are I κ B molecules and control nuclear translocation of NF- κ B. *EMBO J.* **12**: 213–222.
- Neri, A., L. Lombardi, C.-C. Chang, M. Salina, P. Corradini, A.T. Maiolo, R.S.K. Chaganti, and R. Dalla-Favera. 1991. B cell lymphoma-associated chromosomal translocation involves candidate oncogene *lyt-10*, homologous to NF- κ B p50. *Cell* **67**: 1075–1087.
- Nolan, G.P. and D. Baltimore. 1992. The inhibitory ankyrin and activator Rel proteins. *Curr. Opin. Genet. Dev.* **2**: 211–220.
- Nolan, G.P., S. Ghosh, H.C. Liou, P. Tempst, and D. Baltimore. 1991. DNA binding and I κ B inhibition of the cloned p65 subunit of NF- κ B, a *rel*-related polypeptide. *Cell* **64**: 961–969.
- Nolan, G., T. Fujita, K. Bhatia, C. Huppi, H.-C. Liou, M. Scott, and D. Baltimore. 1993. The *bcl-3* proto-oncogene encodes a nuclear I κ B-like molecule that preferentially interacts with NF- κ B p50 and p52 in a phosphorylation-dependent manner. *Mol. Cell. Biol.* **13**: 3557–3566.
- Ohno, H., G. Takimoto, and T.W. McKeithan. 1990. The candidate proto-oncogene *bcl-3* is related to genes implicated in cell lineage determination and cell cycle control. *Cell* **60**: 991–997.
- Schmid, R.M., N.D. Perkins, C.S. Duckett, P.C. Andrews, and G.J. Nabel. 1991. Cloning of an NF- κ B subunit which stimulates HIV transcription in synergy with p65. *Nature* **352**: 734–736.
- Schmitz, M.L. and P.A. Baeuerle. 1991. The p65 subunit is responsible for the strong transcription activating potential of NF- κ B. *EMBO J.* **10**: 3805–3817.
- Scott, M.L., T. Fujita, H.-C. Liou, G.P. Nolan, and D. Baltimore. 1993. The p65 subunit of NF- κ B regulates I κ B by two distinct mechanisms. *Genes & Dev.* (in press).
- Seed, B. 1987. An LFA-3 cDNA encodes a phospholipid-linked membrane protein homologous to its receptor CD2. *Nature* **329**: 840–842.
- Tanaka, M., U. Grossniklaus, W. Herr, and N. Hernandez. 1988. Activation of the U2 snRNA promoter by the octamer motif defines a new class of RNA polymerase II enhancer elements. *Genes & Dev.* **2**: 1746–1778.
- Tewari, M., P. Dobrzanski, K.L. Mohn, D.E. Cressman, J.-C. Hsu, R. Bravo, and R. Taud. 1992. Rapid induction in regenerating liver of RL/IF-1 (an I κ B that inhibits NF- κ B, RelB-p50, and c-Rel-p50) and PHF, a novel κ B site-binding complex. *Mol. Cell. Bio.* **12**: 2898–2908.
- Williams, T., A. Admon, B. Lüscher, and R. Tjian. 1988. Cloning and expression of AP-2, a cell type-specific transcription factor that activates inducible enhancer elements. *Genes & Dev.* **2**: 1557–1569.
- Wulczyn, F.G., M. Naumann, and C. Scheidereit. 1992. Candidate proto-oncogene *bcl-3* encodes a subunit-specific inhibitor of transcription factor NF- κ B. *Nature* **358**: 597–599.
- Zabel, U., R. Schreck, and P.A. Baeuerle. 1991. DNA binding of purified transcription factor NF- κ B: Affinity, specificity, Zn⁺⁺-dependence and differential half site recognition. *J. Biol. Chem.* **266**: 252–260.



The candidate proto-oncogene *bcl-3* encodes a transcriptional coactivator that activates through NF-kappa B p50 homodimers.

T Fujita, G P Nolan, H C Liou, et al.

Genes Dev. 1993, 7:

Access the most recent version at doi:[10.1101/gad.7.7b.1354](https://doi.org/10.1101/gad.7.7b.1354)

References

This article cites 44 articles, 18 of which can be accessed free at:
<http://genesdev.cshlp.org/content/7/7b/1354.full.html#ref-list-1>

License

Email Alerting Service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](#).
