gene product or gene deletion could result in tumorigenesis. The transforming ability of overexpressed PTPα indicates that certain PTPases may instead function as oncogenes, perhaps when overexpressed as a consequence of gene translocation or amplification, or of other defects affecting normal regulatory mechanisms. □

Received 21 May; accepted 14 August 1992.

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ACKNOWLEDGEMENTS. We thank J. H. Xiao and P. Chambon for their gift of the vector pXJ41, Y. H. Tan and W. Chia for critical reading of the manuscript, F. Leong for photography and L. T. Seet for secretarial assistance. Financial assistance was provided by the IMCB.

The candidate oncoprotein Bcl-3 is an antagonist of p50/NF-κB-mediated inhibition

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THE candidate oncogene *bcl-3* was discovered as a translocation into the immunoglobulin alpha-locus in some cases of B-cell chronic lymphocytic leukaemias[1]. The protein Bcl-3 contains seven so-called ankyrin repeats. Similar repeat motifs are found in a number of diverse regulatory proteins but the motifs of Bcl-3 are most closely related to those found in IκB proteins in which the ankyrin repeat domain is thought to be directly involved in inhibition of NF-κB activity. No biological function has yet been described for Bcl-3, but it was noted recently[2] that Bcl-3 interferes with DNA-binding of the p50 subunit of NF-κB *in vitro*. Here we demonstrate that Bcl-3 can aid κB site-dependent transcription *in vivo* by counteracting the inhibitory effects of p50/NF-κB homodimers. Bcl-3 may therefore aid activation of select NF-κB-regulated genes, including those of the human immunodeficiency virus.

The structural similarity of the ankyrin repeat domains of Bcl-3 (ref. 1), IκB (Mad-3) (ref. 3), pp40 (ref. 4), and the carboxy-terminal regions of the precursors for the p50 (IκB-γ, pdI) and p50B subunits of NF-κB[2,5–12] prompted us to investigate the potential role of Bcl-3 in the regulation of NF-κB activity. We have developed a transient transfection system with the human embryonic carcinoma cell line NTera-2, which has no detectable NF-κB activity in uninduced cells[11]. Expression of a CAT gene driven by the κB sites of the human immunodeficiency virus (HIV-κB) was entirely dependent on cotransfection with plasmid constructs encoding NF-κB proteins (Fig. 1). The p65 subunit of the biochemically defined p50/p65 NF-κB heterodimer[13–15] could transactivate the reporter to generate very high CAT activity even when transfected alone, as noted previously[10,16,17] (Fig. 1*a*); lower levels of p65, however, only weakly transactivated the reporter and required synergy with cotransfected p50 for high activity (Fig. 1*b*). We observed dramatic concentration-dependent effects on transactivation with p65/p50 cotransfections. Although good synergy of p65 and p50 occurred with amounts of transfected p50 equivalent to or slightly higher than those of p65, more p50 dramatically decreased expression of the CAT reporter. These results agree with a previous report[17] and they indicate that an excess of p50 led to the formation of p50 homodimers which inhibited the transactivation mediated by p65, presumably by competing with p50/p65 heterodimers for κB sites. This interpretation is supported by mobility-shift experiments with nuclear extracts from cells that expressed increasing amounts of exogenously introduced p50 and a constant amount of exogenous p65 (Fig. 1*c*). The resulting increase in p50 homodimers progressively replaced the p50/p65 heterodimers from the κB probe. As previously noted[10,11,16–18], p50 homodimers alone could not transactivate, regardless of the amount transfected, although a potentially different picture emerges from *in vitro* transcription experiments[19,20]. Our data indicate that p50 homodimers could act as physiological inhibitors of NF-κB-like activity, a conclusion also reached in a different experimental setting[21].

As Bcl-3 is a potential IκB-like factor[2], we investigated whether Bcl-3 regulated κB-dependent transactivation *in vivo*. Bcl-3 inhibited transactivation mediated by p50/p65 in NTera-2-based transient transfections, but only at very high concentrations compared with IκB (Fig. 2*a*). Lower concentrations of Bcl-3 actually resulted in a small but consistent increase in transactivation. This increase became very dramatic when inhibiting conditions of high molar ratios of p50 compared with p65, were used (Fig. 2*b*, *c*): increasing levels of Bcl-3 completely reversed the inhibitory effects exerted by excess amounts of p50 in a dose-dependent manner. This effect of Bcl-3 was observed with several κB reporters, including the HIV-κ-B-driven CAT and the entire HIV long terminal repeat (LTR)-driven CAT; this natural promoter/enhancer responded even more strongly (Fig. 2*b* and *c*, respectively). With intermediate levels of Bcl-3 we observed high transactivation of the HIV-κB-driven reporter, comparable to the highest activity seen when p50 and p65 synergized optimally in the absence of Bcl-3 (compare Figs 1*b* and 2*b*). At higher levels of Bcl-3, even the heterodimeric complexes were inhibited (Fig. 2*a*, *b*). In contrast, the HIV-LTR-driven activity continued to increase with the higher levels of Bcl-3, possibly reflecting additional activity of Bcl-3 in this natural promoter (Fig. 2*c*). We propose that the repressive p50 homodimers were a functional target of Bcl-3, Bcl-3 preferentially acted as an anti-repressor in our assays. IκB, which also contains ankyrin repeats, did not act in this way (data not shown). Transfection of increasing amounts of Bcl-3 alone, or together with either p50 or p65 (concentrations as in Fig. 2) did

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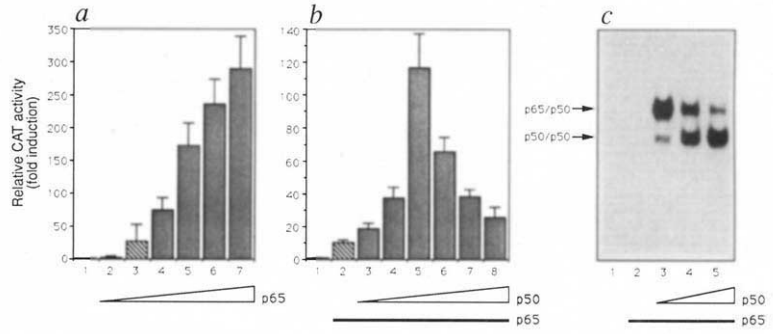

FIG. 1 Transactivation of an HIV-κB-CAT reporter plasmid by transfection of NTera-2 cells with expression vectors encoding *a*, p65 alone or *b*, p65 and p50. *a*, *b*, Show the fold induction of CAT activity over the activity produced by transfection of the reporter alone. The fold-induction values represent the mean of three or more independent transfections after normalizing to the protein concentrations of the cellular extracts. The total concentration of the transfected DNA and of the expression vector were kept constant throughout by adding appropriate amounts of expression vector without insert. Qualitatively similar results were obtained with an HIV-LTR-CAT reporter plasmid. *a*, Column 1, HIV-κB-CAT reporter alone (6 μg); 2–7, *CAT* reporter plus increasing amounts of the p65 vector, 0.01 μg, 0.03 μg, 0.1 μg, 0.3 μg, 1 μg and 3 μg, respectively. The fold induction over the HIV-κB-CAT reporter cotransfected with 0.03 μg of p65 vector (hatched) (see also ref. 16) is the reference point for the subsequent experiments in Fig. 2. *b*, Column 1, HIV-κB-CAT (6 μg); 2, HIV-κB-CAT plus 0.03 μg of p65 vector; 3–8, vectors as used in column 2 plus increasing amounts of the p50 vector, 0.01 μg, 0.03 μg, 0.1 μg, 0.3 μg, 1 μg and 3 μg, respectively. *c*, Increasing amounts of p50 homodimers displace p50/p65 heterodimers from a κB site in electrophoretic mobility shift assays. Nuclear extracts were prepared from NTera-2 cells transfected with: lane 1, the expression vector alone (without insert; 8.5 μg); lane 2, the p65 vector (0.5 μg); lanes 3, 4 and 5, the p65 vector (0.5 μg) and the p50 vector (0.5, 4 and 8 μg, respectively). As before, the total amount of the expression vector was kept constant. Our conditions preclude detection of binding by p65 homodimers. Identification of the complexes was accomplished by transfection of p50 alone and by use of supershifting antibodies (see Fig. 4).

METHODS. The expression of p50 and p65 was directed by the expression vector PMT2T (ref. 31). The p50 construct contains the sequence of the p105 precursor from the ATG start codon to the *Xba*I site[6–9]. The reporter plasmids contain a CAT gene driven by the two κB sites from the HIV virus inserted upstream of a minimal c-*fos* promoter (HIV-κB-CAT)[11]. The transfection procedure and the CAT assays were as described previously[11]. NTera-2 cells were lysed using a Dounce homogenizer and the resulting nuclei were salt-extracted according to standard protocols[32]. For electrophoretic mobility-shift assays 0.5 μl of cell extract was incubated in a total of 10 μl of buffer D (ref. 32) containing 1.3 μg of poly dI-dC (Pharmacia), 1 μg BSA, 0.02% Tween 20, and 0.035 ng of labelled probe (35,000 c.p.m.) for 30 min. The probe was the palindromic κB probe described previously[11].

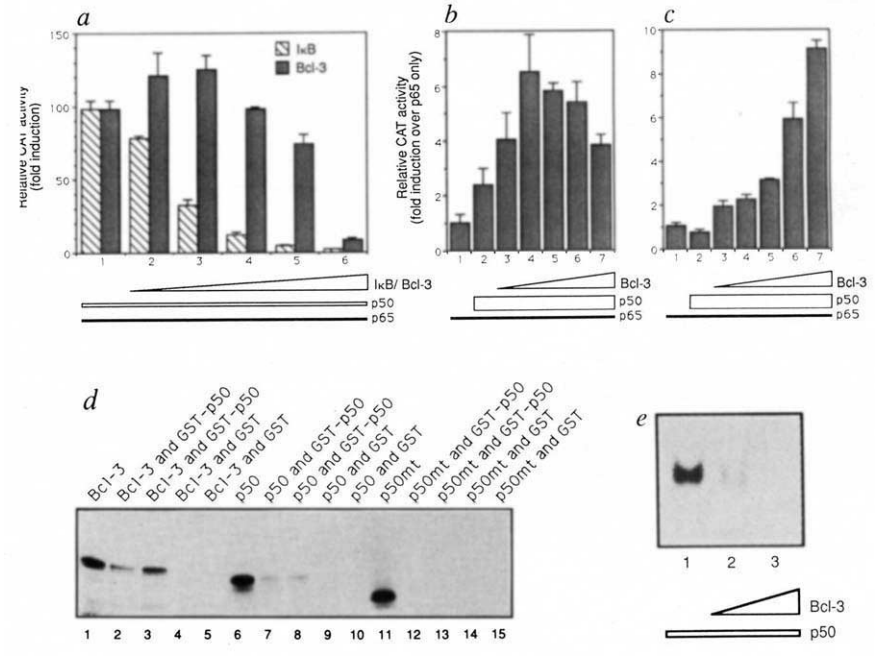

FIG. 2 Bcl-3 regulates NF-κB activity. *a*, Bcl-3 inhibits transactivation mediated by cotransfected p50 and p65 in a dose-dependent manner, although it is much less effective than IκB. Bcl-3 and IκB (Mad-3) (ref. 3) expression was directed by PMT2T. Transfections were performed as outlined in Fig. 1. Columns 1–6, transfections with p50 (0.3 μg), p65 (0.15 μg), HIV-κB-CAT reporter (6 μg) and increasing amounts of cotransfected inhibitors IκB (hatched bars) or Bcl-3 (dark bars); column 1, no inhibitors; 2, 0.03 μg; 3, 0.1 μg; 4, 0.3 μg; 5, 1 μg; 6, 3 μg. *b*, Bcl-3 reverses inhibition mediated by high amounts of p50. The effect of increasing amounts of transfected Bcl-3 was measured under conditions similar to those described in Fig. 1*b* column 8 where high amounts of p50 inhibited transactivation by exogenously introduced p65 and p50/p65 complexes. The fold induction values are relative to the CAT activity obtained with 0.03 μg of p65 vector (column 1). Column 1, HIV-κB-CAT reporter (3 μg) plus 0.03 μg of p65; 2, HIV-κB-CAT plus p65 (0.03 μg) plus p50 (5 μg); 3–7 vectors as used in column 2 plus increasing amounts of Bcl-3 vector, 1.5 μg, 2.5 μg, 3.5 μg, 4.5 μg and 5 μg, respectively. *c*, An HIV-LTR-CAT construct[33] was used here instead of the HIV-κB-CAT. Otherwise the experiments are identical to *b*. Bcl-3 had no effect on the expression of the transfected NF-κB-encoding constructs as determined by western blotting of the p50 protein expressed from the transfected gene (data not shown). *d*, p50 associates with Bcl-3. ^{35}S-labelled Bcl-3 was produced by *in vitro* transcription/translation in reticulocyte lysates and subsequently incubated with bacterially produced glutathione *S*-transferase–p50 fusion proteins (GST–p50) attached to glutathione Sepharose 4B beads (for 1 h at room temperature in 150 mM NaCl, 20 mM Tris–HCl pH 7.4, 0.2% Triton X-100). The beads were precipitated and the attached material was separated on 10% SDS–polyacrylamide gels. The labelled wild-type and mutant p50 molecules were used as the positive and negative controls. The mutant p50 (p50mt) represents a deletion of the C-terminal end of the Rel-homology domain (deleted from the *Spe*I site)[6] incapable of homodimerizing (unpublished observation) whereas the wild type will dimerize with a presumably limited number of monomers on the beads. Lanes 1, 6 and 11 represent 2 μl of the *in vitro* translated material; 5 μl were used for all other lanes. The bacterially produced GST protein alone (5 μg) was used as a negative control for the GST–p50 fusion protein (1.6 μg). Lanes 2/3, 4/5, 7/8, 9/10, 12/13, 14/15 are duplicates of each other, except that the second of each pair contained beads that were pre-incubated with BSA before GST proteins were bound. *e*, Bcl-3 inhibits DNA-binding of p50 homodimers. Nuclear extracts (0.4 μl) from NTera-2 cells transfected with 5 μg of the p50 expression vector were mixed with increasing amounts of whole-cell extracts of NTera-2 cells transfected with 8 μg of the Bcl-3 expression vector (0, 1.25 and 2.5 μl, lanes 1, 2, and 3, respectively). The total amount of whole-cell extract was kept constant by adding appropriate compensating amounts of extract from NTera-2 cells transfected with 8 μg of the insert-less expression vector alone. The probe was the IL-2 Rec κB probe described previously[28]. Extracts were prepared as described in Fig. 1, except that cells were freeze–thawed for whole-cell extractions.

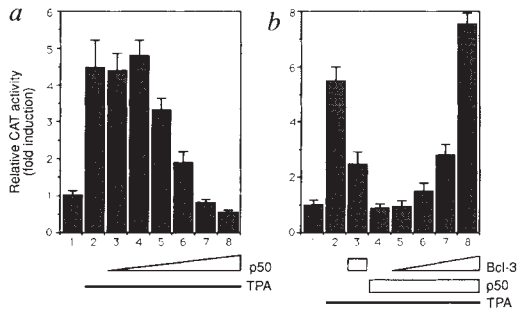

FIG. 3 p50 inhibits transactivation of the HIV-LTR-CAT reporter by endogenous NF-κB, and Bcl-3 reverses this inhibition. NTera-2 cells were stimulated with TPA after transfection with the HIV-LTR-CAT reporter plasmid alone, or together with *a*, increasing amounts of p50 or *b*, a high amount of p50 and increasing amounts of Bcl-3. *a*, Column 1, HIV-LTR-CAT reporter plasmid (6 μg), no stimulation; 2, HIV-LTR-CAT, TPA stimulation; 3–8, as 2, plus increasing amounts of p50, 0.05 μg, 0.15 μg, 0.5 μg, 1.5 μg, 5 μg and 10 μg, respectively. *b*, Column 1, HIV-LTR-CAT (6 μg), no stimulation; 2, HIV-LTR-CAT, TPA stimulated; 3, HIV-LTR-CAT plus Bcl-3 (5 μg), TPA stimulated; 4, HIV-LTR-CAT plus p50 (5 μg), TPA-stimulated; 5–8, as 4, plus increasing amounts of Bcl-3, 0.15 μg, 0.5 μg, 1.5 μg and 5 μg, respectively. Transfected N Tera-2 cells were stimulated with 10 ng ml^{-1} of TPA for 6 h before collection.

not increase transactivation of the HIV-κB-CAT reporter. Furthermore, the results were entirely dependent on the κB sites, as a mutant κB construct was never transactivated (data not shown).

Bcl-3 has been reported to decrease DNA-binding of p50 (ref. 2). We provide evidence for a physical association between Bcl-3 and p50. A glutathione *S*-transferase (GST) fusion protein of p50 was used to coprecipitate radiolabelled Bcl-3 on glutathione-coated beads, whereas a negative control was not precipitated (Fig. 2*d*). Furthermore, antibodies against Bcl-3 were able to coprecipitate p50 but not p65 out of cell extracts (V.B., G.F. & V.S. manuscript in preparation). Finally we demonstrated that binding of p50 homodimers to DNA was progressively inhibited by increasing amounts of extracts from Bcl-3 transfected cells but not from untransfected cells (Fig. 2*e*). The individual ankyrin repeat domains present in the various IκB-related proteins (described previously) may be responsible for their physical association with distinct but related proteins of the NF-κB family; they may inhibit complex formation by shielding functionally relevant domains[2,5,22–27].

The inhibition by p50 and the subsequent reversal of this inhibition by Bcl-3 was observed also in activated cells. NTera-2 cells contain limited but detectable NF-κB-like activity when stimulated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA), possibly because of new synthesis of NF-κB. Exogenously introduced p50 effectively inhibited the TPA-induced endogenous activity seen with the HIV-LTR in a dose-dependent manner (Fig. 3*a*). We observed a small amount of synergy at very low levels of p50 and an increasing suppression at higher levels of p50, as might be expected from the results discussed above. Increasing amounts of cotransfected Bcl-3 reversed the inhibition seen with high p50 levels in these activated cells (Fig. 3*b*). Qualitatively similar results were obtained with the HIV-κB constructs (data not shown). Transfection of Bcl-3 alone (Fig. 3*b*) did not increase the TPA-induced transactivation; rather, it was suppressed (see Fig. 2*a*), confirming that the transfected p50 homodimers must be a functional target for Bcl-3 to cause transactivation.

The potential inhibitory role of p50 homodimers led us to investigate the natural occurrence of such complexes in primary cells. Previous data indicated that p50 homodimers may exist[28,29], but no direct evidence has been documented. We demonstrated an abundance of p50 homodimeric complexes in resting cells. Two distinct κB-specific complexes were present in resting peripheral blood T cells (Fig. 4*a*, lane 1; competition with wild-type and mutant κB sites, lanes 2 and 3, respectively). Use of supershifting antibodies against p50 (lane 4) and p65 (lane 5) allowed us to identify the faster migrating complex as containing predominantly p50 homodimers and the slower one

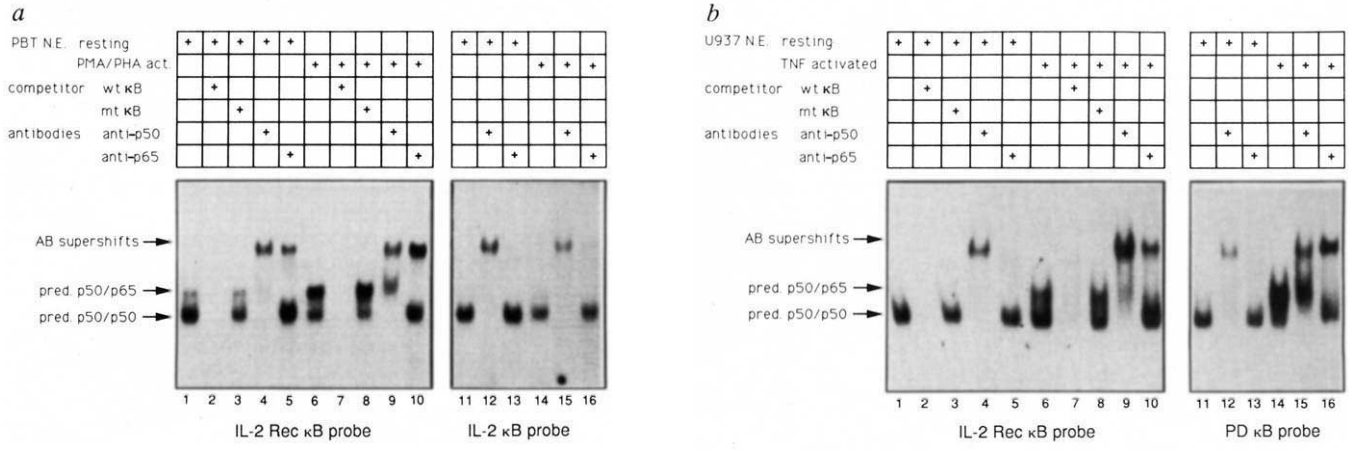

FIG. 4 Electrophoretic mobility-shift assays of nuclear extracts (NE) from resting or phytohaemagglutinin (PHA)/TPA activated (act.) peripheral blood T (PBT) cells (*a*) and from resting or tumour-necrosis factor (TNF)-α activated U937 cells (*b*). Supershifts were performed with antibodies directed against the N-terminal 13 amino acids of p50 (ref. 6) and the N-terminal 14 amino acids following the initiator methionine of p65 (ref. 14). All complexes are marked by arrows (pred., predominant; AB, antibodies). The κB probes used are indicated below the panels. Competitions were performed with a 90-fold excess of cold probes (wt, wild-type; mt, mutant; see later). The indicated assignment of the two complexes was supported further by the fact that they comigrated almost with known p50 homodimeric and p50/p65 heterodimeric complexes obtained using extracts from appropriately transfected NTera-2 cells (p50 truncation was close to the presumed natural processing region)[29]. Use of these transfected cells revealed that the p50 antibody was less efficient in supershifting the p50/p65 heterodimeric complex than the homodimeric complex, indicating that the anti-p50 antibody-mediated supershift here underestimated the p50 content of the upper complex. We note the presence of some heterodimeric complexes in the resting peripheral blood T cells but not in several cell lines. On the basis of fluorescence-activated cell sorting analysis, we think it likely that blood obtained from different volunteers contained sufficient activated cells for detection with this assay (data not shown).

METHODS. Peripheral blood T cells were purified as described previously[34]. T cells were stimulated with PHA(1 μg ml^{-1}) and TPA (10 ng ml^{-1}) for 2.5 h and U937 cells were stimulated with TNF-α (Genzyme, 100 U ml^{-1}) for 2.5 h. To obtain nuclear extracts, T cells were lysed with NP-40 (0.2%) and U937 cells were lysed in a Dounce homogenizer. Preparation of nuclear extracts and mobility shifts were as Fig. 1, except that 0.2 ng of probe were used. For antibody supershifts 0.9 μl of antisera were included as well. The probes have been described previously (PD κB and mutant PD κB[11], IL-2 Rec κB[28] and IL-2 κB and mutant IL-2 κB[6]).

as containing predominantly p50/p65 heterodimers. Specific antibodies against p50B, RelB and c-Rel confirmed that these other proteins were either undetectable or only weakly present in the two complexes (data not shown; see legend to Fig. 4 for additional information). The relative amount of p50 and p50/p65 complexes visualized on gels depended partly on the actual κB sequence used; the interleukin-2 (IL-2) κB site, for example, strongly favoured p50 binding (lanes 11–16), which may have physiological consequences for IL-2 expression[21]. The immunoglobulin κB site (core sequence identical to each HIV κB site) gave results comparable to the IL-2 receptor site. Once cells were stimulated (Fig. 4*a*, lanes 6–10) the ratio of p50/p65 over p50/p50 dramatically increased (compare lanes 1 and 6). We also noted a decrease of the p50 homodimeric complex on activation of T cells which may be due to Bcl-3. A similar activation-dependent decrease in p50 homodimers has recently been reported for mouse T cells[21]. Figure 4*b* demonstrates the presence of p50 homodimeric complexes in U937 cells, in an analysis similar to the one shown for T cells. These data are consistent with, but do not prove, a role of p50 homodimers in inhibiting κB-dependent transcription in resting cells. Activation of the resting cells thus shifts the balance in favour of the transactivating NF-κB complexes, the degree of the shift depending on the actual κB sequence.

Our data reveal an unexpected function for Bcl-3. This putative oncoprotein may aid the activation of certain NF-κB-regulated genes and may play a role in the pathophysiology of HIV. The translocated and consequently inappropriately expressed Bcl-3 gene may contribute to chronic lymphocytic leukaemias by deregulating NF-κB activity, as may the c-Rel and p50B genes when chromosomally rearranged[12,30]. Bcl-3 may shift the balance between inhibition and activation of certain κB-site-regulated target genes.

Note added in proof: A recent report[35] also demonstrates in detail the physical interaction of Bcl-3 with p50/NF-κB. □

Received 26 May; accepted 11 August 1992.

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ACKNOWLEDGEMENTS. We thank A. S. Fauci for support and encouragement; K. Brown, M. Lenardo and A. S. Fauci for comments on the manuscript; C. Rosen for the human p65 plasmid, T. W. McKeithan for the *bcl-3* gene and S. Haskill for the IκB gene; M. Rust for help with manuscript preparation. Vincent Bours is a Senior Research Assistant of the Belgian National Fund for Scientific Research.