

The Rel/NF- κ B Family Directly Activates Expression of the Apoptosis Inhibitor Bcl-x_L

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The transcription factors of the Rel/NF- κ B family are key regulators of immune and inflammatory responses and contribute to lymphocyte proliferation, survival, and oncogenesis. The absolute correlation between the antiapoptotic and oncogenic activities of the Rel/NF- κ B oncoprotein ν -Rel emphasizes the importance of characterizing the death antagonists under NF- κ B control. Our recent finding that the prosurvival Bcl-2 homolog Bfl-1 (also called A1) is a direct transcriptional target of NF- κ B raised the issue of whether NF- κ B is a specific or global regulator of death antagonists in the Bcl-2 family. Here, we demonstrate that NF- κ B differentially regulates the expression of particular Bcl-2-related death inhibitors and that it directly activates the expression of Bcl-x_L. While Bcl-x_L was significantly upregulated by c-Rel and RelA, Bcl-2 was not. Importantly, stimuli that activate endogenous NF- κ B factors also upregulated *bcl-x* gene expression and this effect was antagonized by an inhibitor of NF- κ B activity. The expression of *bcl-x* suppressed apoptosis in the presence or absence of NF- κ B activity. Functional analysis of the *bcl-x* promoter demonstrated that it is directly controlled by c-Rel. These results establish that NF- κ B directly regulates the expression of distinct prosurvival factors in the Bcl-2 family, such as Bcl-x_L and Bfl-1/A1. These findings raise the possibility that some of these factors may contribute to oncogenesis associated with aberrant Rel/NF- κ B activity.

Apoptosis is an inducible suicide program that occurs at all stages of multicellular life. It is required for normal development, immune system function, tissue remodeling, and prevention of inappropriate cellular proliferation. Dysregulated cell death is associated with various pathological conditions, including neurodegenerative disorders, autoimmune diseases, and cancer (reviewed in reference 17). Experimental evidence suggests a proactive role for the Rel/NF- κ B family of transcription factors in the inhibition of programmed cell death, as shown by the following observations. (i) Homozygous inactivation of RelA in mice led to extensive apoptosis in the liver (6). (ii) Inactivation of endogenous Rel/NF- κ B factors by superrepressor forms of the inhibitory protein I κ B α sensitized cells to stimulus-induced apoptosis (32, 54, 57, 62). (iii) Lymphoid cells transformed by the Rel/NF- κ B oncoprotein ν -Rel required continuous expression of ν -Rel for survival. ν -Rel inactivation by a temperature-sensitive mutation or through tetracycline-regulated control resulted in the rapid onset of apoptosis (59, 68). (iv) Transcriptionally competent Rel/NF- κ B factors (ν -Rel, c-Rel, and RelA) blocked apoptosis induced by tumor necrosis factor alpha (TNF- α), whereas those defective for transactivation did not (5, 13, 32, 67).

Consistent with the notion that NF- κ B regulates the expression of genes that antagonize cell death, its protective activity is dependent on RNA and protein synthesis (reviewed in reference 55). Candidate target genes recently identified include those encoding the caspase inhibitors c-IAP1, c-IAP2, and X-IAP, the TNF receptor-associated factors TRAF1 and

TRAF2, and the zinc finger protein A20 and the immediately response gene *IEX-1L* (15, 30, 50, 58, 63, 66). Thus, in addition to regulating the expression of genes important for immune and inflammatory responses, Rel/NF- κ B also controls the transcription of genes that confer resistance to death-inducing signals.

The Bcl-2 family of cell death regulators is critical for determining cell fate in the apoptotic pathway. Bcl-2 and its mammalian homologs Bcl-x_L, Bfl-1 (also called A1), and Mcl-1 block cell death, while Bax, Bcl-x_S, Nbk (also called Bik), Bak, and Bad promote apoptosis (reviewed in reference 1). Each of these factors influences the cleavage-mediated activation of caspases, which act as the ultimate downstream effectors of the suicide program. While little is known about the signaling pathways that control the expression of Bcl-2-related factors and of the transcription factors involved in their regulation, exogenous expression of prosurvival Bcl-2-related proteins was shown to block apoptosis in lymphoid cells under conditions in which NF- κ B activity was inhibited (62). This raised the possibility that some of these factors may lie downstream of NF- κ B in the survival cascade. The recent demonstration that the prosurvival Bcl-2 homolog Bfl-1 is transcriptionally controlled by Rel/NF- κ B is consistent with these results (23, 31, 56, 69). It is therefore important to determine whether NF- κ B is a general or specific regulator of death antagonists in the Bcl-2 family.

Here, we show that NF- κ B can differentially regulate the expression of prosurvival Bcl-2 family proteins. Whereas c-Rel and RelA had no effect on Bcl-2 expression in HT1080 and HtTA cells, they strongly upregulated the expression of Bcl-x_L. *bcl-x* promoter activation was dependent on an NF- κ B DNA site, and its protective activity was correlated with that of c-Rel. These results indicate that NF- κ B activates distinct prosurvival Bcl-2 family proteins and suggest a role for these factors in the inhibition of cell death by Rel/NF- κ B.

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MATERIALS AND METHODS

Plasmids. The human *c-rel* gene (*hc-rel*, a gift from N. Rice, ABL-NCI, Frederick, Md.) was stably expressed in a Tet-OFF system, under the control of a minimal cytomegalovirus (CMV) promoter and seven tetracycline operator sites from plasmid pUHD10-3-hygro (13) (pUHD10-3-hygro-*hc-rel*). In transient transfection assays, *hc-rel* was expressed from the CMV promoter of pJDCMV19SV (pCMV-*hc-rel*). Vectors expressing the death antagonists Bcl-2 and Bcl-x_L from the CMV promoter of pcDNA3.0 (Invitrogen) were a gift from C. Labrie (CHUL, Québec, Canada). pCMV-β-gal expressed the β-galactosidase gene (β-gal) from a CMV promoter (69).

Cell culture and endogenous NF-κB activation. Human HT1080 fibrosarcoma cells, HeLa cervical carcinoma cells, Jurkat T lymphocytic leukemia cells, CEM T cells, and 293 embryonal kidney cells were obtained from the American Type Culture Collection. Jurkat T cell lines expressing wild-type IκBα (IκBα-wt) or a constitutive IκBα inhibitor (IκBαΔN) were a gift from D. W. Ballard (Vanderbilt University, Nashville, Tenn.) (15). The cell line RC-K8, derived from a diffused large cell lymphoma, was a gift from N. Zeleznik-Le (University of Chicago, Chicago, Ill.) (33).

Endogenous NF-κB activity was induced upon treatment with TNF-α (1,000 U per ml; Sigma) for 2 h (CEM) or with phorbol 12-myristate 13-acetate (PMA) (50 ng per ml) plus ionomycin (concentration, 1 μM in 0.05% dimethylsulfoxide) for 2 h (Jurkat).

HeLa HtTA-derived cell clones conditionally expressing p65 (also known as RelA) or p50 (also known as NF-κB1) under tetracycline-regulated control were previously described (67, 69). Human HT1080-tTA fibrosarcoma cells stably expressing the tTA (tetracycline-regulated transactivator) protein were generated by cotransfecting HT1080 cells with pUHD15-1 (21) and pSV2Neo, using SuperFect (Qiagen). Neomycin-resistant HT1080-tTA cell clones were selected with G418 (200-μg potency units) in the presence of tetracycline-HCl (2 μg per ml) and screened for luciferase gene activation following transient transfection of the pUHD13-3 luciferase expression plasmid (21). The HT1080-*hc-rel* cell line that conditionally expressed *hc-rel* under tetracycline control was obtained by transfecting HT1080-tTA cells with pUHD10-3-hygro-*hc-rel*. Cell clones were selected in the presence of hygromycin B (400 μg per ml; Calbiochem) and screened for inducible expression of hc-Rel by immunoblotting. HT1080-*hc-rel* cells were maintained in minimum essential medium supplemented with 10% fetal bovine serum, 2 mM glutamine, Earle's balanced salt solution containing sodium bicarbonate (1.5 g per liter), 1× nonessential amino acids, 0.1 mM sodium pyruvate, and antibiotics (100 U of penicillin per ml and 100 μg of streptomycin per ml). Cells were maintained at 37°C in an atmosphere of 5% CO₂. All cell clones were maintained in the presence of tetracycline (2 μg per ml) and refed every other day.

Immunoblotting. Cells were induced to express the c-Rel, RelA, or p50 protein upon removal of tetracycline from the cell culture medium. Extracts were prepared in lysis buffer (50 mM Tris HCl [pH 7.5], 150 mM sodium chloride, 1% sodium deoxycholate, 1% Triton X-100, 10 μg of leupeptin per ml, 10 μg of pepstatin per ml, 20 μg of aprotinin per ml, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, and 0.5 mM sodium orthovanadate) (42) and quantitated by the method of Bradford (10). Proteins (20 μg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Immunoblotting was performed by enhanced chemiluminescence (ECL; Amersham). Processing of procaspase 3 and procaspase 8 in HT1080-*hc-rel* cells was induced by treatment with TNF-α (5 ng per ml) plus cycloheximide (CHX; 5 μg per ml) before or after a 48-h induction of c-Rel expression. The hc-Rel protein was detected with rabbit polyclonal antibody SC-272 (Santa Cruz Biotechnology). Antibodies to caspase 3, Bcl-x_L, and Bcl-2 were purchased from PharMingen. The anti-caspase 8 antibody was from Zymed Laboratories. An antiactin antibody (Sigma) was used as a control.

Northern blot analysis. Total RNA (20 μg) extracted with RNazol B (TEL-TEST) was fractionated in 1% agarose-formaldehyde gels and transferred onto Hybond-NX membranes (Amersham). Membranes were baked for 10 min at 80°C under vacuum and UV cross-linked with a Stratilinker (Stratagene). Probes were generated by random priming with Klenow DNA polymerase in the presence of [α-³²P] dCTP and [α-³²P] dGTP (19). A 28S oligonucleotide probe (5'-AAGGATCAGAGTAGTGGTATTTCACC-3') was labeled with [γ-³²P]ATP and T4 polynucleotide kinase. Membranes were hybridized in ExpressHyb (Clontech) according to the manufacturer's recommended procedure, or in 5× SSC (0.75 M NaCl plus 75 mM Na citrate [pH 7.0]), 5× Denhardt's solution, 0.5% SDS, and sheared salmon sperm DNA (100 μg per ml) at 65°C overnight. Membranes were washed twice in 2× SSC-0.1% SDS and twice in 1× SSC-0.1% SDS at 65°C, followed by autoradiography.

Apoptosis assays. Cell resistance to TNF-α or anti-Fas antibody-induced apoptosis in transient assays was examined as described previously (60). HT1080 cells (2 × 10⁵) were cotransfected with pCMV-β-gal (0.5 μg) together with pCMV vectors expressing Bcl-x_L or hc-Rel (1.5 μg), using SuperFect reagent (Qiagen). An empty pCMV vector was used as a control. After 30 h, the cells were treated with CHX either alone (30 μg per ml) or together with TNF-α (10 ng per ml; Sigma) or with anti-Fas antibodies (1 μg per ml; Calbiochem) for 14 h. After fixation and staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), cells were counted from a minimum of 10 fields chosen at random. Quantitation of cell survival represents the ratio of the number of cells express-

ing β-gal in wells treated with TNF-α or anti-Fas plus CHX over that in wells treated with CHX alone. In assays of cell death performed in the absence of cycloheximide, HeLa cells (3 × 10⁶) were coelectroporated using a Bio-Rad Gene Pulser (220 V, 960 μF) with pCMV-β-gal (3 μg), an empty CMV vector, or pCMV-IκBαM (12 μg) to constitutively repress NF-κB, alone or together with pCMV-*bcl-xl* (6 μg). Cells were then distributed equally into two 35-mm wells and treated 24 h later with TNF-α (10 ng per ml) for 16 h. Cells were counted from a minimum of 10 fields chosen at random.

Cloning of the human *bcl-x* promoter region and transient CAT assays. The human *bcl-x* promoter region was isolated by nested PCR amplification using a Genome Walker PromoterFinder kit (Clontech) and cloned in a promoterless vector expressing a chloramphenicol acetyltransferase (CAT) reporter gene (pCAT-basic; Promega). *bcl-x* promoter activity was analyzed by transient transfection of HT1080 cells with *bcl-x*-CAT reporter plasmids (0.8 μg) in the presence of a CMV-*hc-rel* expression vector (1.2 μg) or an empty pCMV vector as a control. Assays were performed with 20 μg of protein for 1.5 h. The relative CAT activity represents the average of three independent experiments. The *bcl-x* promoter region (from positions -298 to +22) cloned in pAlter-1 was subjected to site-directed mutagenesis to inactivate the NF-κB motif (mutated bases are underlined) at position -232 (TTTACTGCCC; -298/+22 mκB; Altered Sites Mutagenesis System [Promega]). Mutation of the NF-κB site was confirmed by sequencing.

DNA-binding assays. The binding of Rel/NF-κB factors to the κB DNA site found in the *bcl-x* promoter was assayed in human 293 cells transiently transfected with CMV expression vectors for p50, p65, or c-Rel. Nuclear extracts (3 μg) were incubated with a double-stranded ³²P-labeled NF-κB oligonucleotide probe derived from the *bcl-x* promoter region (5'-AGTGGGGCGGGGGG ACTGCCCCCTCTCCTT-3') or a control interleukin 6 (IL-6)-κB oligonucleotide probe (4 × 10⁴ cpm) (64) in 12.5 mM HEPES (pH 7.9), 12% glycerol, 5 mM MgCl₂, 60 mM KCl, 0.2 mM EDTA, 1 mM dithiothreitol, bovine serum albumin (1 μg per μl), poly(dI-dC) (2 μg) and analyzed on 5% native polyacrylamide gels as described previously (64). Where indicated, nuclear extracts were prepared from HT1080-*hc-rel* cells induced to express c-Rel for 0, 24, 48, 72, or 96 h following the removal of tetracycline. Extracts (3 μg) were analyzed for binding to the *bcl-x* κB DNA oligonucleotide probe in gel retardation assays, as described above.

RESULTS

c-Rel and RelA promote the expression of Bcl-x_L in HT1080 and HtTA cells but have no effect on Bcl-2. To investigate whether NF-κB is a specific or global regulator of death antagonists in the Bcl-2 family, we examined the effect of c-Rel on the steady-state levels of endogenous Bcl-2 and Bcl-x_L proteins in human HT1080-*hc-rel* cells that conditionally expressed the human *c-rel* gene under tetracycline-regulated control. Similar to the HeLa-derived HtTA-CCR43 cell line that we previously characterized (67), HT1080-*hc-rel* cells showed significant protection from TNF-α- or anti-Fas antibody-induced apoptosis following induction of c-Rel expression upon withdrawal of tetracycline (data not shown). Interestingly, expression of c-Rel in these cells was accompanied by a four- to sixfold increase in Bcl-x_L protein levels (Fig. 1A, lanes 1 to 5). The 29-kDa protein doublet that was detected with anti-Bcl-x antibodies is typical of Bcl-x_L (28). In contrast to the strong induction of Bcl-x_L, the steady-state levels of Bcl-2 remained unchanged following c-Rel expression (Fig. 1A, lanes 1 to 5).

Bcl-x_L protein levels were also significantly enhanced in cells expressing the c-Rel-related protein p65 (Fig. 1B, compare lanes 1 to 5). As with c-Rel-expressing cells, the accumulation of Bcl-x_L paralleled that of p65. In contrast to these findings, cells expressing the p50 subunit that is devoid of transcriptional activity on most κB site-containing promoters failed to show any increase in Bcl-x_L protein levels (Fig. 1B, lanes 6 to 9). Together, these results suggested a correlation between the expression of transcriptionally competent Rel/NF-κB proteins and the specific accumulation of the prosurvival factor Bcl-x_L.

The increased accumulation of Bcl-x_L protein correlated with an increase in the steady-state levels of *bcl-xl* transcripts in c-Rel-expressing cells. Northern blots showed a strong enhancement in *bcl-x* gene expression in HT1080-*hc-rel* cells induced to express c-Rel (14-fold increase) (Fig. 1C, compare lanes 2 and 3). On the contrary, *bcl-2* mRNAs were virtually

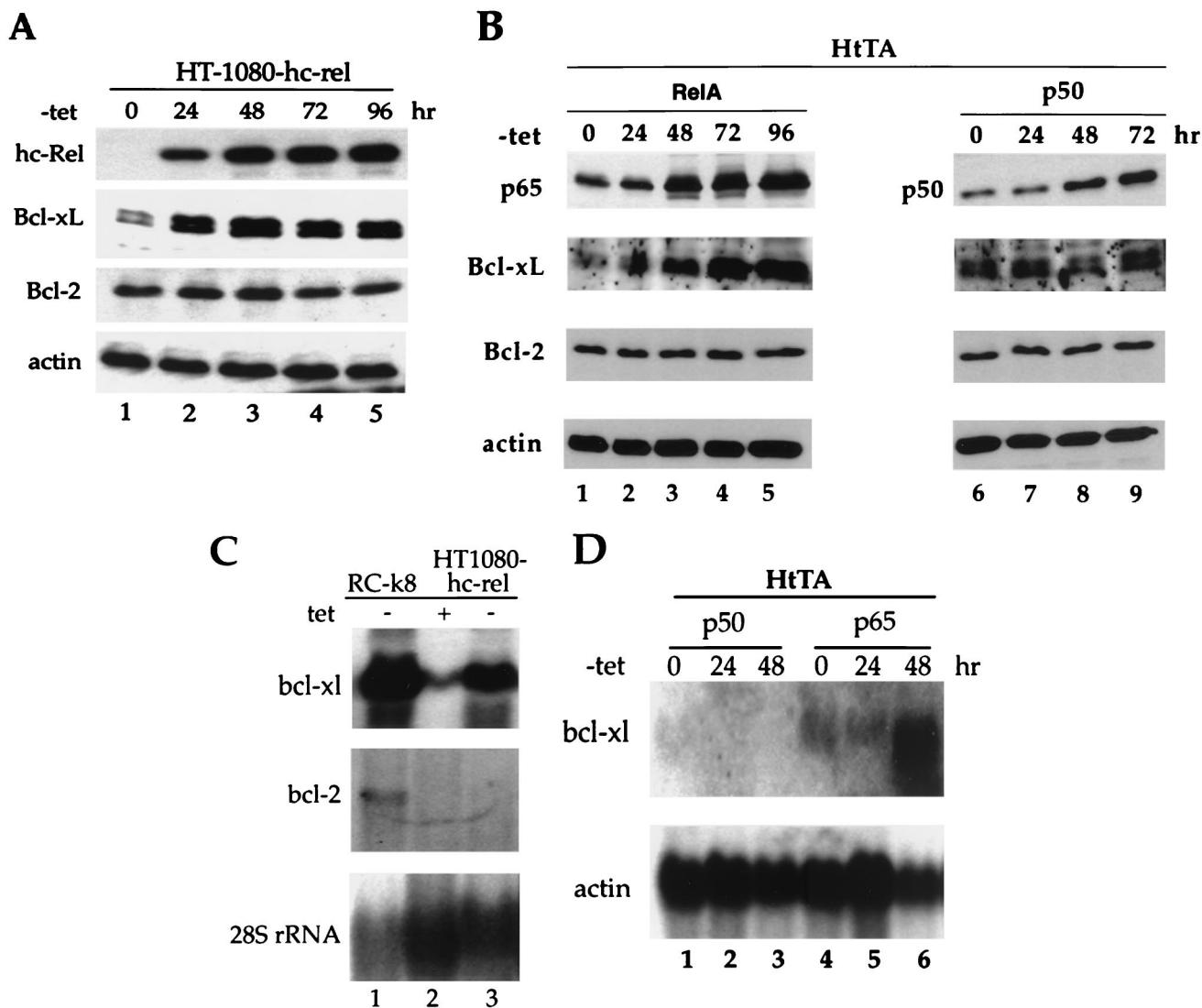


FIG. 1. Rel/NF-κB factors lead to a specific increase in Bcl-x_L expression. (A) Induction of c-Rel expression correlates with increased levels of Bcl-x_L protein but not Bcl-2. Cell extracts (30 μg) from HT1080-*hc-rel* cells maintained in the presence (lane 1) or absence (lanes 2 to 5) of tetracycline were resolved by SDS-PAGE and analyzed by immunoblotting with antibodies specific for c-Rel (hc-Rel), Bcl-x_L, Bcl-2, or actin. Proteins were visualized by ECL. (B) Expression of p65 but not p50 correlates with increased levels of Bcl-x_L protein but not Bcl-2. Cell extracts (30 μg) from HtTA-RelA (lanes 1 to 5) or HtTA-p50 cells (lanes 6 to 9) maintained in the presence (lanes 1 and 6) or absence (lanes 2 to 5 and 7 to 9) of tetracycline were resolved by SDS-PAGE and analyzed by immunoblotting with antibodies specific for c-Rel, Bcl-x_L, Bcl-2, or actin. Proteins were visualized by ECL. (C) *bcl-x* gene expression is induced by ectopic expression of c-Rel. Expression of *bcl-x* transcripts in human RC-K8 cells (lane 1) or HT1080-*hc-rel* cells (lanes 2 and 3) maintained in the presence (lane 2) or absence (lanes 1 and 3) of tetracycline for 72 h. The blot was successively hybridized to *bcl-x*, *bcl-2*, and 28S rRNA probes. (D) The p65 subunit of NF-κB but not the p50 subunit upregulates *bcl-xl* transcripts. *bcl-x* gene expression in HtTA-p50 (lanes 1 to 3) or HtTA-RelA cells (lanes 4 to 6) maintained in the presence (lanes 1 and 4) or absence (lanes 2, 3, 5, and 6) of tetracycline for 24 (lanes 2 and 5) or 48 h (lanes 3 and 6). The blot was hybridized to *bcl-x* and actin probes. tet, tetracycline.

undetectable in HT1080-*hc-rel* cells and remained unaffected by the expression of c-Rel (Fig. 1C, lanes 2 and 3). Similarly, *bcl-xl* was found to be highly expressed in human RC-K8 cells in comparison to the low levels of *bcl-2* transcripts (Fig. 1C, lane 1). These cells are derived from a human diffuse large cell lymphoma associated with overexpression of a rearranged *c-rel* gene (33).

Consistent with these results, *bcl-x* gene expression was also strongly induced by p65. Northern blot analysis showed a ninefold increase in *bcl-x* transcript levels 48 h after the removal of tetracycline from the HeLa-derived HtTA-RelA cell line to induce expression of p65 (Fig. 1D, compare lanes 4, 5, and 6). As anticipated, p50 failed to promote *bcl-x* gene expression (Fig. 1D, lanes 1 to 3). These data indicated a selective effect

of transcriptionally competent Rel/NF-κB subunits on *bcl-xl* expression but not on *bcl-2* expression.

Stimuli that activate NF-κB also upregulate *bcl-x* gene expression. To further investigate the functional regulation of Bcl-x_L, we verified the effects of endogenous NF-κB activation on *bcl-x* gene expression. The basal levels of *bcl-x* transcripts were significantly increased upon stimulation of human CEM T cells with TNF-α (Fig. 2A, lanes 1 and 2). The participation of NF-κB in *bcl-x* gene expression was further substantiated by the analysis of human Jurkat T cells stimulated with PMA plus ionomycin to activate endogenous NF-κB factors. Basal levels of *bcl-x* transcripts were increased threefold following PMA-plus-ionomycin treatment of both parental Jurkat T cells and Jurkat T cells expressing IκBα-wt in comparison to untreated

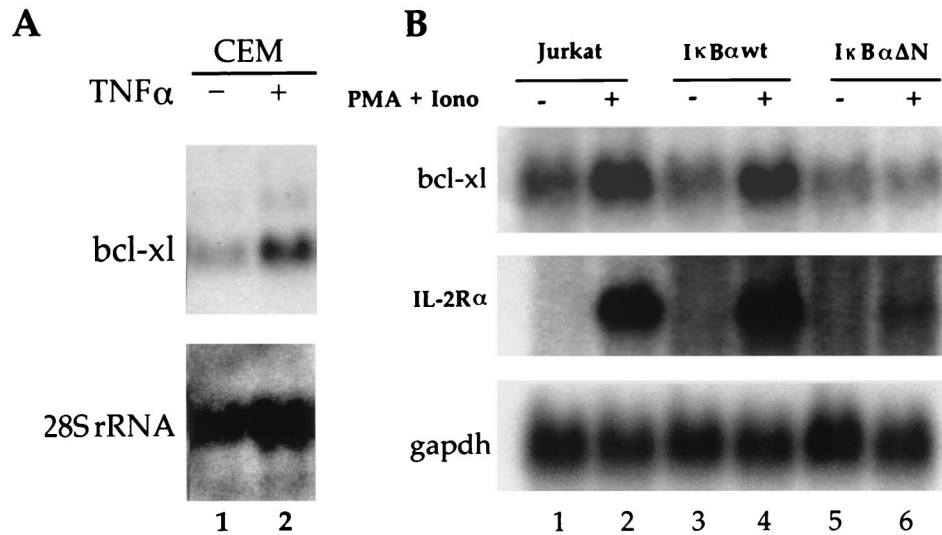


FIG. 2. Expression of *bcl-x* is dependent on endogenous Rel/NF- κ B activity. (A) *bcl-x* gene expression is induced by endogenous NF- κ B activation. Human CEM T cells were left untreated (lane 1) or were treated with TNF- α for 2 h (lane 2). Total RNA (20 μ g) was hybridized with 32 P-labeled probes for *bcl-x* or 28S rRNA. (B) Induction of *bcl-x* gene expression is blocked by I κ B α Δ N, a dominant inhibitor of NF- κ B. Parental Jurkat T cells (lanes 1 and 2) and Jurkat cells expressing I κ B α -wt (lanes 3 and 4) or I κ B α Δ N (lanes 5 and 6) were treated with PMA plus ionomycin (Iono) for 2 h to induce endogenous NF- κ B activity (lanes 2, 4, and 6) or with dimethyl sulfoxide alone as a control (lanes 1, 3, and 5). Total RNA (20 μ g) was hybridized to 32 P-labeled probes for *bcl-x* or the gene encoding IL2R α or glyceraldehyde-3-phosphate dehydrogenase (gapdh).

cells (Fig. 2B, lanes 1 to 4). This enhanced expression correlated with the induction of mRNAs for IL-2 receptor alpha (IL2R α), a known target gene for NF- κ B. Importantly, the constitutive repression of NF- κ B in Jurkat-I κ B α Δ N cells by a mutant of I κ B α that is resistant to proteolytic degradation (15) markedly decreased the response of *bcl-x* (Fig. 2B, compare lanes 5 and 6). Together, these data indicated that the activation of NF- κ B enhances *bcl-x* gene expression.

The protective activity of Bcl-x_L is correlated with that of c-Rel and can substitute for NF- κ B to block TNF- α -induced cell death. We investigated the protective activities of Bcl-x_L and c-Rel in transient transfection assays. HT1080 cells were cotransfected with a CMV- β -gal reporter plasmid together with pCMV vectors expressing either Bcl-x_L or c-Rel or an empty vector as a control. Cells were treated with CHX either alone or together with TNF- α or anti-Fas antibodies. Cell survival represents the ratio of the number of cells expressing β -gal in wells treated with TNF- α plus CHX or anti-Fas plus CHX to that in wells treated with CHX alone. The cotransfection of pCMV-*hc-rel* with CMV- β -gal significantly suppressed cell killing induced either by TNF- α or by Fas receptor cross-linking with an anti-Fas antibody (Fig. 3A). c-Rel increased cell survival 10-fold following addition of TNF- α and 5.5-fold following treatment with anti-Fas antibodies. Consistent with the protective activity of c-Rel in this assay, *bcl-xl* also efficiently repressed apoptosis induced by either stimulus (eightfold for TNF- α and fivefold for anti-Fas [Fig. 3A]). The correlation between the protective activities of c-Rel and Bcl-x_L is consistent with the possibility of a functional relationship between these factors.

Prior work indicated that Bcl-x_L efficiently blocked the cleavage-mediated activation of procaspase 3 (CPP32), a critical downstream effector caspase in the apoptotic pathway (14, 18, 46). Likewise, the induction of c-Rel expression in HT1080-*hc-rel* cells blocked cleavage-mediated activation of procaspase 3 in response to TNF- α -plus-CHX treatment (Fig. 3B). HT1080-*hc-rel* cells maintained in the presence of tetracycline to block c-Rel expression underwent significant processing of

procaspase 3 within 6 h of TNF- α -plus-CHX treatment, as shown by the disappearance of the procaspase 3 band (Fig. 3B, compare lanes 7 and 9 to lane 1). The 17-kDa cleavage product of procaspase 3 processing was detected only after long exposure times and is shown in the middle panel. In contrast, the caspase 3 proenzyme was significantly more resistant to cleavage in cells induced to express c-Rel following the removal of tetracycline (Fig. 3B, lanes 8 and 10).

Interestingly, the induction of c-Rel expression also suppressed the cleavage-mediated activation of FADD-like IL-1 β convertase enzyme (FLICE; also known as procaspase 8), the apical caspase in the death receptor caspase cascade (Fig. 3C, compare lanes 8 and 10 to lanes 7 and 9). While Bcl-x_L was reported to function downstream of FLICE (8), others showed that it can prevent the oligomerization of FADD and the recruitment of FLICE coincident with the inhibition of apoptosis (38). Although more studies are needed to establish the extent to which Bcl-x_L contributed to this activity, the conditional expression of c-Rel in HT1080-*hc-rel* cells conferred a protective activity similar to that provided by Bcl-x_L, consistent with the upregulation of *bcl-xl* transcripts by Rel/NF- κ B factors.

Importantly, Bcl-x_L also suppressed TNF- α -induced killing in cells in which endogenous NF- κ B activity was suppressed by a serine-to-alanine mutant of I κ B α that is resistant to signal-induced degradation (I κ B α M) (54). While the expression of I κ B α M sensitized HeLa cells to killing induced by TNF- α in the absence of CHX, the cotransfection of Bcl-x_L rescued the cells from apoptosis (Fig. 3D). Together, the results support a role for Bcl-x_L as one of several death antagonists that function in the NF- κ B pathway for cell survival.

c-Rel directly regulates the *bcl-x* promoter. To clarify the nature of the response of *bcl-xl* to Rel/NF- κ B, we isolated and characterized the regulatory sequences that lie upstream of the *bcl-x* cDNA. Nested PCR amplification of an adapter-ligated human genomic library generated a product of 435 bp with a 3' end derived from the 5' end of the *bcl-xl* cDNA and extending 5' into adjacent genomic sequences (Genome Walker Pro-

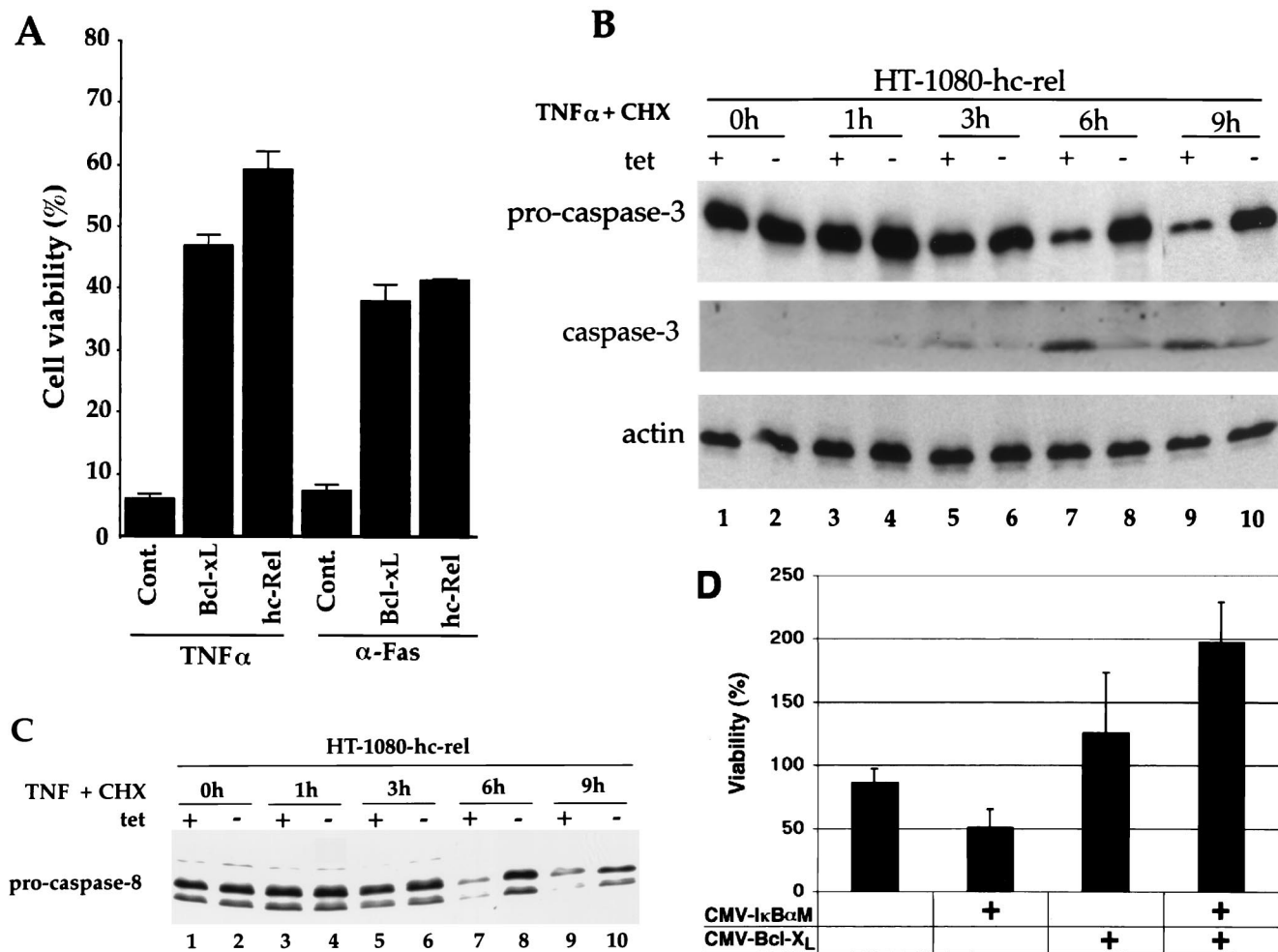


FIG. 3. The protective activity of Bcl-x_L is correlated with that of c-Rel. (A) Both Bcl-x_L and c-Rel suppress apoptosis induced by TNF- α or Fas receptor cross-linking. HT1080 cells were cotransfected with pCMV- β -gal together with an empty pCMV vector control (Cont.), pCMV-*bcl-xl*, or pCMV-*hc-rel*. The cells were treated with CHX alone or together with TNF- α or anti-Fas antibodies for 14 h and stained with X-Gal to quantitate cell survival. Cell viability represents the ratio of the number of cells expressing β -gal in wells treated with TNF- α or anti-Fas antibodies together with CHX to that in wells treated with CHX alone. Cells were counted from a minimum of 10 fields chosen at random. The average survival from three independent experiments is shown. Error bars show standard deviations. (B) Similarly to Bcl-x_L, c-Rel prevents the processing of procaspase 3. HT1080-*hc-rel* cells were maintained in the presence (lanes 1, 3, 5, 7, and 9) or absence (lanes 2, 4, 6, 8, and 10) of tetracycline for 48 h. Cells were left untreated (lanes 1 and 2) or treated with TNF- α plus CHX for 1, 3, 6, or 9 h (lanes 3 to 10). Cell extracts (30 μ g) were resolved by SDS-PAGE and analyzed by immunoblotting with antibodies specific for caspase 3 or actin. The 17-kDa cleavage product of procaspase 3 processing was detected only after long exposure times and is shown in the blot of the middle row. (C) c-Rel suppresses the processing of procaspase 8. HT1080-*hc-rel* cells were maintained in the presence (lanes 1, 3, 5, 7, and 9) or absence (lanes 2, 4, 6, 8, and 10) of tetracycline for 48 h. Cells were left untreated (lanes 1 and 2) or treated with TNF- α plus CHX for 1, 3, 6 or 9 h (lanes 3 to 10). Cell extracts (30 μ g) were resolved by SDS-PAGE and analyzed by immunoblotting with an antibody specific for caspase 8. The 55-kDa doublet is characteristic of procaspase 8. (D) Bcl-x_L can suppress TNF- α -induced cell death under conditions in which NF- κ B activity is suppressed. HeLa cells were cotransfected with pCMV- β -gal and an empty pCMV vector or pCMV-I κ B α M, alone or together with pCMV-*bcl-xl*. The cells were treated with TNF- α alone for 16 h and stained with X-Gal. Cell survival represents the ratio of the number of cells expressing β -gal in wells treated with TNF- α to that in wells left untreated. The average survival from three experiments is shown. Error bars show standard deviations. tet, tetracycline.

moterFinder kit; Clontech). The sequence was identical to that previously reported (DDBJ, EMBL, and GenBank accession no. D30746). A putative NF- κ B DNA binding site was identified at position -232 relative to the first nucleotide in the human *bcl-xl* cDNA sequence (DDBJ, EMBL, and GenBank accession no. L20121; GGGACTGCCC; position -77 relative to the transcription start site) and is conserved in the mouse *bcl-x* promoter region (22).

Gel retardation assays confirmed the binding of c-Rel, p65, and p50 to an oligonucleotide probe containing this site (Fig. 4A, lanes 8 to 10). A consensus IL-6 κ B oligonucleotide probe was used as a control for Rel/NF- κ B protein expression (Fig. 4A, lanes 3 to 5). A time course gel shift analysis was then used to monitor the binding of Rel factors to this site in induced

HT1080-*hc-rel* cells and to see its relationship to endogenous Bcl-x_L expression (Fig. 4B). In this assay, Rel factor binding to the *bcl-x* κ B DNA site was correlated with the time course for the induction of Bcl-x_L in these cells (Fig. 1A).

After directional cloning into a promoterless CAT reporter plasmid (Fig. 5A), *bcl-x* promoter activity was assayed by transient transfection of HT1080 cells in the presence or absence of CMV-*hc-rel*. The -298/+22 *bcl-x*-CAT reporter construct showed little activity on its own, but the cotransfection of CMV-*hc-rel* increased the construct's expression 16-fold (Fig. 5B). Progressive deletions removing 5' sequences from the *bcl-x* promoter region rapidly interfered with its response to c-Rel. Importantly, a mutant promoter with a deletion endpoint mapping only 10 nucleotides downstream of the NF- κ B

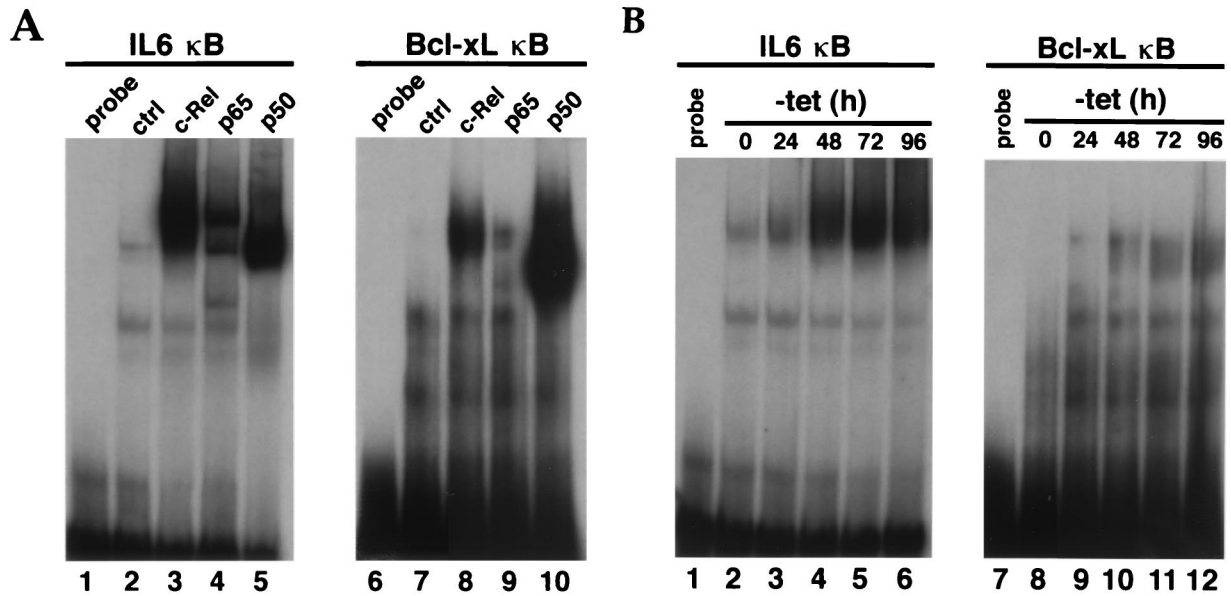


FIG. 4. Rel/NF- κ B factors bind to a κ B DNA site derived from the human *bcl-x* promoter region. (A) Binding of Rel/NF- κ B factors to the *bcl-x* κ B DNA site. Nuclear extracts from human 293 cells transiently transfected with CMV expression vectors for c-Rel (lanes 3 and 8), p65 (lanes 4 and 9), or p50 (lanes 5 and 10) were incubated with double-stranded 32 P-labeled oligonucleotides containing the *bcl-x* κ B DNA site at position -232 (lanes 6 to 10) or an IL-6- κ B DNA-binding motif as a control (ctrl) for NF- κ B protein expression (lanes 1 to 5). Mock transfected cells (lanes 2 and 7) and probes alone (lanes 1 and 6) were used as controls. DNA-protein complexes were resolved from unbound probes in a 5% native polyacrylamide gel. (B) Binding of induced Rel factors to the *bcl-x* κ B DNA site is correlated with induction of endogenous Bcl- x_L expression. Nuclear extracts from HT1080-*hc-rel* cells induced to express c-Rel for 0, 24, 48, 72, or 96 h upon removal of tetracycline (tet) were incubated with double-stranded 32 P-labeled oligonucleotides containing the *bcl-x* κ B DNA site at position -232 (lanes 8 to 12) or a control IL-6- κ B DNA-binding motif (lanes 2 to 6). The probes alone (lanes 1 and 7) were used as controls. DNA-protein complexes were resolved from unbound probes in a 5% native polyacrylamide gel.

motif showed a 65% reduction in activity in comparison to the wild-type promoter construct ($-222/+22$ *bcl-x*-CAT) (Fig. 5B). These results are consistent with our data showing increased expression of Bcl- x_L in response to Rel/NF- κ B factors. Site-directed mutagenesis was used to inactivate the NF- κ B site at position -232 to confirm its role in the Rel-mediated induction of the *bcl-x* promoter (TTTACTGCC; $-298/+22$ m κ B). While this mutation slightly decreased the basal activity of the *bcl-x* promoter, it greatly reduced its responsiveness to c-Rel (Fig. 5C). The response of the $-298/+22$ m κ B *bcl-x*-CAT mutant promoter construct to c-Rel was decreased down to 3.5-fold, in comparison to the 16-fold induction observed with the wild-type $-298/+22$ *bcl-x*-CAT reporter plasmid. Together, these results demonstrate a direct role for Rel/NF- κ B in the regulation of *bcl-xl* expression.

DISCUSSION

The strong correlation that exists between the antiapoptotic and oncogenic activities of Rel/NF- κ B factors has elicited a fervent search for the target genes that participate in the NF- κ B survival pathway. Recent work from members of our group and others demonstrated that NF- κ B directly regulates expression of the prosurvival Bcl-2 family protein Bfl-1 and its mouse homolog A1 (23, 31, 56, 69). The positioning of one member of the important Bcl-2 family of death antagonists in the NF- κ B survival pathway raised the question of whether NF- κ B also controls other death inhibitors from the Bcl-2 family. Here, we demonstrate that Bcl- x_L is also a transcriptional target of NF- κ B. We show that ectopically expressed and endogenous Rel/NF- κ B factors specifically upregulated the expression of *bcl-xl*. This effect was dependent on NF- κ B activity, as a mutant form of I κ B α that can physiologically inhibit

NF- κ B antagonized *bcl-x* gene induction. Functionally, both c-Rel and Bcl- x_L exhibited similar antiapoptotic activities toward cell death induced by TNF- α or by Fas receptor cross-linking. This agrees with studies indicating that expression of either Bcl- x_L or Rel/NF- κ B factors antagonizes apoptosis in response to various death-inducing stimuli (2, 8, 14, 32, 43, 49, 54, 57, 62, 67). The mapping of a functional κ B DNA site in the human *bcl-x* promoter region confirmed its Rel-dependent response. Together, these data demonstrate the direct participation of NF- κ B in the transcriptional control of the prosurvival factor Bcl- x_L .

Our finding that NF- κ B is an important inducer of *bcl-xl* is consistent with recent reports showing the NF- κ B-dependent upregulation of *bcl-xl* upon CD40-mediated activation of B lymphocytes, in primary neurons stimulated with TNF, and in mouse T cells expressing human T-cell leukemia virus type 1 (HTLV-1) Tax (31, 51, 52). However, the regulation of *bcl-xl* expression appears to be quite complex and the contribution of NF- κ B to this process may depend on cell type, differentiation stage and/or activating stimuli. For instance, while the studies described above point to a proactive role for NF- κ B in inducing *bcl-xl*, other analyses looking for death antagonists under NF- κ B control failed to reveal alterations in *bcl-xl* transcript levels (23, 58, 63). Similarly, no change in *bcl-xl* expression was observed in IL-3-dependent mouse B cells expressing HTLV-1 Tax (34). Understanding of the relationship between NF- κ B and Bcl- x_L is also complicated by data implicating NF- κ B as a negative regulator of Bcl- x_L in double-positive thymocytes (24) and by reports pointing to the ability of Bcl- x_L to interfere with NF- κ B activation upstream of I κ B α degradation (3). Additional work is thus needed to sort out the functional and regulatory relationships between NF- κ B and Bcl- x_L and the conditions under which they take place.

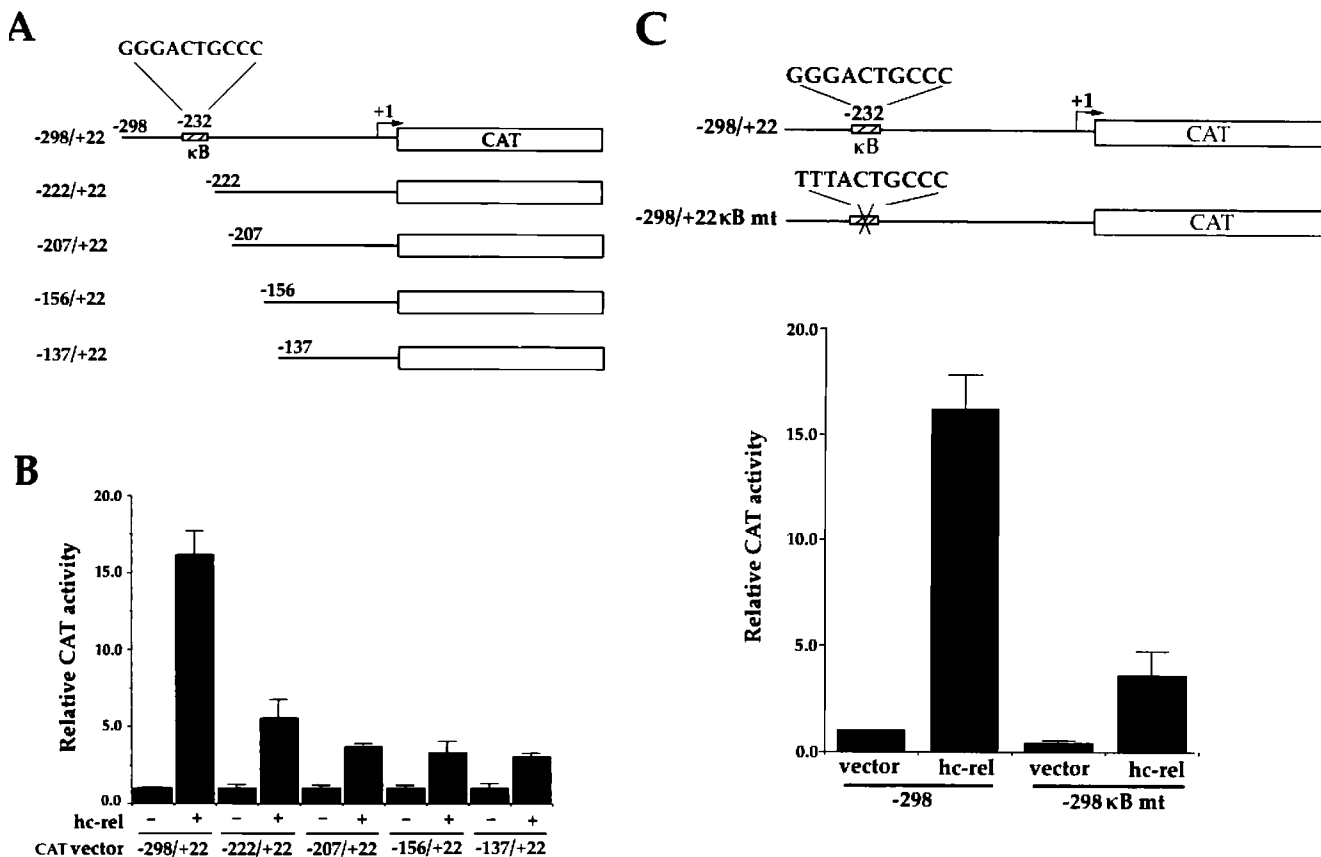


FIG. 5. The human *bcl-x* promoter contains a κB DNA site responsible for its Rel-dependent induction. (A) Schematic representation of *bcl-x* CAT reporter gene constructs. CAT reporter plasmids driven by various regions of the human *bcl-x* promoter are shown. The NF-κB site at position -232 relative to the first nucleotide in the *bcl-x* cDNA is indicated. (B) c-Rel-dependent transactivation of the *bcl-x* promoter. HT1080 cells were cotransfected with *bcl-x*-CAT reporter plasmids together with pCMV-*c-rel* or an empty pCMV vector as a control. The average CAT activity from three independent experiments is shown. (C) NF-κB site-dependent activation of the *bcl-x* promoter. HT1080 cells were cotransfected with *bcl-x*-CAT reporter plasmid (-298/+22) or the κB site mutant (mt) plasmid (-298/+22 mt) together with pCMV-*hc-rel*, or an empty pCMV vector as a control. The average CAT activity from three independent experiments is shown. The arrow indicates the transcription start site for *bcl-x*. Error bars show standard deviations.

Our findings do not exclude the possibility that NF-κB may function in conjunction with other transcription factors to regulate *bcl-x* gene expression. Similarly to NF-κB, transcription factors Stat1, Stat5, and Ets2 were also recently implicated in the regulation of *bcl-xl* expression (20, 45, 47) and multiple binding sites for other transcription factors were identified in the *bcl-x* promoter (22). The regulation of *bcl-xl* induction by multiple transcription factors may thus explain why significant levels of *bcl-xl* transcripts have been observed in the absence of NF-κB activation. In this respect, it is noteworthy that the response of *bcl-x* to endogenous NF-κB activation differed somewhat from the response that our group and others previously described for its homolog *bfl-1/a1* (31, 69). Whereas cells that lack nuclear NF-κB activity failed to show any expression of *bfl-1/a1*, a basal level of endogenous *bcl-x* gene expression was observed in several cell lines, which was further induced upon NF-κB activation. These data are consistent with a model in which NF-κB may act in concert with other transcription factors to control the expression of *bcl-xl* depending on cell type and/or activating stimuli. Nevertheless, the results described herein indicate that NF-κB is one of several important inducers of *bcl-xl*.

As a result of alternative splicing, *bcl-x* encodes two death regulators that exhibit opposing activities (7). Bcl-x_L exerts a protective effect, whereas the shorter form, Bcl-x_S, is proapoptotic.

The 29-kDa protein doublet detected in our immunoblots with anti-Bcl-x antibodies is typical of Bcl-x_L (Fig. 1A) (28). No change was observed at the position for its proapoptotic isoform, Bcl-x_S. Our results therefore highlight the participation of NF-κB in the control of *bcl-x* gene activation to block cell death through upregulation of the prosurvival Bcl-x_L factor. In a recent study, Tsukahara et al. also observed NF-κB-mediated induction of *bcl-xl* but not *bcl-xs* in CTLL-2 cells expressing Tax (52). It will be interesting to investigate the mechanism underlying the selective induction of the *bcl-x* promoter by NF-κB that leads to production of the antiapoptotic long form rather than the proapoptotic short form.

The ability of c-Rel to transcriptionally regulate *bcl-x* and its homolog *bfl-1/a1* is in contrast with its failure to induce *bcl-2* gene expression in our cells. While NF-κB has been implicated in the induction of *bcl-2* in certain cells (51) but not others (52), the absence of a *bcl-2* response in our system implies that NF-κB is able to selectively regulate particular prosurvival genes in the Bcl-2 family in defined cellular environments. This differential regulation is consistent with the fact that Bcl-x_L and Bcl-2 can display different patterns of expression in various hematopoietic cells and tissues. For instance, *bcl-2* is intensely expressed in naive and memory B cells but is mostly absent from germinal center B cells. In contrast, *bcl-xl* is weakly expressed in naive and memory B cells but is abundant in ger-

minal center B cells where *c-rel* is constitutively expressed (12, 28, 35). Thus, despite the sequence and functional homology between Bcl-x_L and Bcl-2, their different modes of regulation enable them to exhibit complementary protective activities in hemolymphoid tissues by allowing them to participate in distinct survival programs in different cells and at different stages of differentiation (reviewed in references 41 and 65).

In experiments not shown here, we observed that transcripts for the Bcl-2-related protein Mcl-1 were also upregulated by c-Rel and NF-κB activation and that *mcl-1* mRNAs were abundantly expressed in immune tissue where NF-κB is active (data not shown). However, we have so far been unable to demonstrate that NF-κB activity is required for *mcl-1* gene expression. While NF-κB activation in Jurkat T cells treated with PMA plus ionomycin efficiently promoted *mcl-1* gene expression, a similar degree of induction was observed in Jurkat-IκBαΔN cells in which endogenous NF-κB activity was constitutively repressed (data not shown). This is in contrast to the results that members of our group obtained with *bcl-xl* and *bfl-1/al* (Fig. 2B) (69). Further work is therefore needed to characterize the *mcl-1* promoter region and to assess the role of NF-κB in its regulation. Taken together, our findings indicate that NF-κB can specifically regulate particular cell death inhibitors in the Bcl-2 family.

Dysregulated Rel/NF-κB activity has been implicated in several hematopoietic and solid tumors (reviewed in references 34 and 40). For example, *c-rel* is amplified in human extranodal diffuse large cell lymphomas and constitutive NF-κB/Rel activity was observed in Hodgkin's lymphoma and in breast, colon, and prostate carcinoma (4, 9, 11, 16, 25, 29, 39, 48, 61; F. Payvandi, W. X. Zong, C. Gélinas, P. Amenta, and A. B. Rabson, unpublished data). Although the mechanisms involved remain to be clarified, the ability of NF-κB to promote cell survival is likely to contribute to malignancy. Interestingly, *bcl-xl* is overexpressed in lymphoid and hematopoietic tumors as well as in a high proportion of colorectal, prostate, and primary breast carcinomas (26, 27, 37, 44, 53). While Bcl-x_L is unlikely to be the only antiapoptotic factor responsible for the protective activity of NF-κB, these observations suggest that the dysregulated expression of Bcl-x_L, Bfl-1, and other prosurvival factors under NF-κB control may be important for the oncogenic process. Future challenges will be to elucidate the pathways responsible for the differential regulation of death antagonists controlled by NF-κB and to establish their respective contribution to tumors associated with aberrant Rel/NF-κB activity.

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