

Induction of Bcl-x_L Expression by Human T-Cell Leukemia Virus Type 1 Tax through NF-κB in Apoptosis-Resistant T-Cell Transfectants with Tax

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Human T-cell leukemia virus type 1 (HTLV-1) Tax is thought to play a pivotal role in immortalization of T cells. We have recently shown that the expression of Tax protected the mouse T-cell line CTLL-2 against apoptosis induced by interleukin-2 (IL-2) deprivation and converted its growth from being IL-2 dependent to being IL-2 independent. In this study, we demonstrate that constitutive expression of *bcl-xl* but not *bcl-2*, *bcl-xs*, *bak*, *bad*, or *bax* was associated with apoptosis resistance after IL-2 deprivation in CTLL-2 cells that expressed Tax. Transient-transfection assays showed that *bcl-x* promoter was transactivated by wild-type Tax. Similar effects were observed in mutant Tax retaining transactivating ability through NF-κB. Deletion or substitution of a putative NF-κB binding site identified in the *bcl-x* promoter significantly decreased Tax-induced transactivation. This NF-κB-like element was able to form a complex with NF-κB family proteins in vitro. Furthermore, Tax-induced transactivation of the *bcl-x* promoter was also diminished by the mutant IκBα, which specifically inhibits NF-κB activity. Our findings suggest that constitutive expression of Bcl-x_L induced by Tax through the NF-κB pathway contributes to the inhibition of apoptosis in CTLL-2 cells after IL-2 deprivation.

Human T-cell leukemia virus type 1 (HTLV-1) is the etiological agent of adult T-cell leukemia (22, 34). HTLV-1 encodes a 40-kDa regulatory protein, Tax, that accelerates viral gene expression (6, 13, 15, 39, 41). This protein is thought to be responsible for the transforming features of HTLV-1. This conclusion is supported by several lines of evidence, such as in vitro transformation of rodent fibroblasts and human T cells (1, 27, 46) and in vivo induction of a variety of mesenchymal tumors and leukemia in transgenic mice carrying the *tax* gene (20, 29).

Various mechanisms of T-cell leukemogenesis by HTLV-1 Tax have been proposed. Some of them relate to the transcriptional activator function of this protein. Tax activates the transcription of not only HTLV-1 itself but also a number of cellular genes *in trans*. This transactivation by Tax is mediated through interaction with transcriptional factors CREB, NF-κB, and serum response factor (14, 42–44, 56). Thus, the expression of a variety of target genes related to cellular activation and growth is enhanced by Tax. These include genes for interleukin-2 (IL-2), IL-6, granulocyte-macrophage colony-stimulating factor, the α chain of IL-2 receptor, Fra-1, c-Myc, c-Fos, and c-Jun (36, 55). Aberrant expression of these proteins could be involved in the dysregulated proliferation of HTLV-1-infected cells.

Recent studies support another hypothesis; that Tax may induce cell cycle promotion through protein-protein interaction. Suzuki et al. (45) demonstrated that Tax binds and inactivates p16^{INK4a}, a negative regulatory molecule of the cell

cycle. Tax may also directly associate with cyclin D, which is important in cell cycle transition from G₁ to S phase (30).

Tax also influences cell apoptosis. We and others have previously shown that human T-cell lines expressing Tax were resistant to apoptosis signals triggered through Fas receptors (2, 9). A similar resistance to apoptosis was observed in T cells derived from transgenic mice carrying the *tax* gene (25). Resistance to cell apoptosis is one of the mechanisms of autoimmunity and is also an important requirement for the immortalization of T cells (7, 33). Other investigators, however, have reported opposite observations; Tax induces apoptosis of rat fibroblasts and a human T-cell line under certain conditions (7, 53). The precise mechanisms of the Tax effect on apoptosis are not well understood.

We have recently shown that the expression of Tax in a mouse T-cell line CTLL-2 converted its growth from being IL-2 dependent to being IL-2 independent. Not only promotion of cell cycle but also prevention of apoptosis occurred in these cells in the absence of IL-2 (23). In the present study, we investigated the mechanisms by which Tax prevents apoptosis. We demonstrated (i) that constitutive expression of Bcl-x_L, one of the molecules protecting cells against apoptosis, is associated with resistance of CTLL-2/Tax transfectants to apoptosis induced by IL-2 deprivation and (ii) that Tax transactivates *bcl-xl* through the NF-κB pathway. This is the first report that indicates the involvement of Bcl-x_L in HTLV-1 Tax-induced inhibition of apoptosis in T cells.

MATERIALS AND METHODS

Plasmids. Plasmids containing wild-type *tax* (pBMT-2Tax), mutant *tax* genes (pBTax703 and pBTaxM22) driven by a β-actin promoter, and its control plasmid (pHβAPr-1-neo) were used (21, 26). Wild-type Tax encoded in pBMT-2Tax is effective in the activating NF-κB, CARγ, and CRE sites. On the other hand, the

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Tax mutant encoded by p β Tax703 can activate the NF- κ B site but not the CArG or the CRE site, while the Tax mutant encoded by p β TaxM22 can activate the CArG and CRE site but hardly the NF- κ B site (40, 44). We also used the mouse *bcl-x* promoter-driven luciferase (Luc) reporter plasmid pGL2-3.2, which contained a 3.2-kbp fragment upstream of the *bcl-x* gene (19). pGL2(1160), pGL2(848), pGL2(822), and pGL2(631) were newly generated by insertion of DNA fragments of the *bcl-x* promoter at the base positions of -1160 to -75, -848 to -75, -822 to -75, and -631 to -75 from the translation initiation ATG of the *bcl-x* gene, respectively, into the basic pGL2 vector containing a Luc reporter gene (Promega, Madison, Wis.). These DNA fragments were amplified by PCR with DNA templates extracted from CTLL-2 cells and four sense primers, (5'-CCGCTCGAGGAGGTCTCCACTGTGGGAGC-3', 5'-CCGCTCGAGGAGGATCCCTTTAGGGTTTC-3', 5'-CTCATCTAGGGCTGGTACTT-3', and 5'-CCGCTCGAGTGGTGCATGGAGGAACCAGG-3') and one antisense primer (5'-GAAGATCTAGCGATTCTCTTCCAGGATC-3'). pGL κ BM is a mutant of pGL2(848), carrying CC-to-GG mutations at positions of -841 and -840 within the NF- κ B binding site, which were generated by PCR by using primers with the mutation. Plasmid integrity was confirmed by DNA sequencing. Another Luc reporter plasmid, WT-Luc, containing five tandem repeats of 21-bp Tax-inducible CREs of HTLV-1 LTR originated from 21WT-CAT (16), was kindly provided by J. Fujisawa (Kansai Medical University, Osaka, Japan). pcDNA3-*I κ B α M* was constructed by cloning an *I κ B α M*-coding region in pBlue-script SK(-)-*I κ B α* S32A/S36A, a kind gift from J. Inoue (Tokyo University, Tokyo, Japan), into pcDNA3 (Invitrogen, Carlsbad, Calif.) with the cytomegalovirus promoter. pSV- β -Galactosidase Vector (Promega) was also used.

Cell lines and transfections. The mouse IL-2-dependent T-cell line CTLL-2 (18) was maintained in RPMI 1640 medium with 10% fetal calf serum (FCS) (RPMI-10% FCS), 50 μ M 2-mercaptoethanol and 1 nM human recombinant IL-2 (hrIL-2; Ajinomoto, Yokohama, Japan) at 37°C and 5% CO₂. CTLL-2 cell clones permanently expressing wild-type and mutant HTLV-1 Tax were established by neomycin-selection after gene transfection as described below. CTLL-2 cells (10⁷) were resuspended in 0.7 ml of FCS-free RPMI containing 10 μ g of wild-type or mutant Tax expression plasmids and electroporated by use of a Gene Pulser (Bio-Rad, Hercules, Calif.) at 320 V and 950 μ F. Then, the cells were resuspended in 20 ml of RPMI-10% FCS and 1 nM hrIL-2. After 48 h, the cells were selected in 24-well plates in G418-containing medium (0.4 mg/ml) for 3 weeks. Wells were screened for Tax expression by Western blot analysis, and cells in the wells positive for Tax were further cloned by the limiting-dilution method. In this study, we used two or three randomly selected clones for each group of CTLL-2/Tax transfectants. We also used an IL-2-independent T-cell line, Jurkat, which originated from human acute lymphocytic leukemia (38).

Western blot analysis. Expressions of HTLV-1 Tax, Bcl-x_L, and α -tubulin in CTLL-2 cell clones were analyzed by Western blot assays as described previously (23). Briefly, cell lysates prepared from CTLL-2 (5 \times 10⁶ cells) were subjected to electrophoresis under reduced conditions on a discontinuous 12.5% polyacrylamide gel with sodium dodecyl sulfate, and the proteins in the gel were transferred to polyvinylidene difluoride membranes. The blots were first incubated with a human serum containing anti-Tax antibodies, rabbit antiserum specific for Bcl-x_L (Transduction Laboratories, Lexington, Ky.), or mouse monoclonal antibody to α -tubulin (Oncogene, Cambridge, Mass.), then rinsed, and finally incubated with second antibodies conjugated with horseradish peroxidase. Sites of antibody binding were visualized by using ECL Western blotting detection system (Amersham International Plc, Little Chalfont, Buckinghamshire, United Kingdom).

RPA. We analyzed the levels of *bcl-xl*, *bcl-xs*, *bak*, *bax*, *bcl-2*, and *bad* gene mRNAs in CTLL-2 cells cultured with or without IL-2 for 48 h by RNase protection assay (RPA) with the RPA II kit (Ambion, Austin, Tex.) as described previously (24). Cells were lysed in Isogen (Nippon Gene, Toyama, Japan) solution, and total cellular RNA was extracted by separation over CHCl₄ and precipitation with isopropanol. The total RNA (20 μ g) was hybridized with a mixture of ³²P-labeled antisense RNA probes (3 \times 10⁴ cpm) overnight at 42°C in a hybridization buffer containing 80% formamide and then treated with RNase A/T1. The resultant protected RNAs were separated on a 5% acrylamide gel with 8 M urea and visualized by autoradiography. As internal controls, the mRNA levels of a ribosomal protein (*L32*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were analyzed. RNA probes used for this assay were synthesized by in vitro transcription with templates included in an mAPO-2 multiprobe template set (PharMingen, San Diego, Calif.).

Transient-transfection assay. To evaluate the ability of Tax to activate transcription of *bcl-x*, 10⁶ Jurkat cells were cotransfected by the DEAE-dextran method with 4 μ g of a reporter plasmid pGL2-3.2 or control WT-Luc, 1 μ g of pSV- β -galactosidase containing the bacterial β -galactosidase gene driven by the simian virus 40 (SV40) promoter, and an effector plasmid containing wild-type or mutant Tax at a 1:2 molar ratio with the reporter plasmids. After 48 h of incubation, cells were harvested, and Luc activity in the cellular lysates was measured with a luminometer (Lumat; Berthold & Co.). All Luc assays were performed in triplicates. At the same time, the activity of β -galactosidase in the lysates was measured as described previously (37). Luc activity was normalized with β -galactosidase activity.

EMSA. Binding activity of NF- κ B proteins to NF- κ B elements was examined by electrophoretic mobility shift assay (EMSA) as described previously (23). In

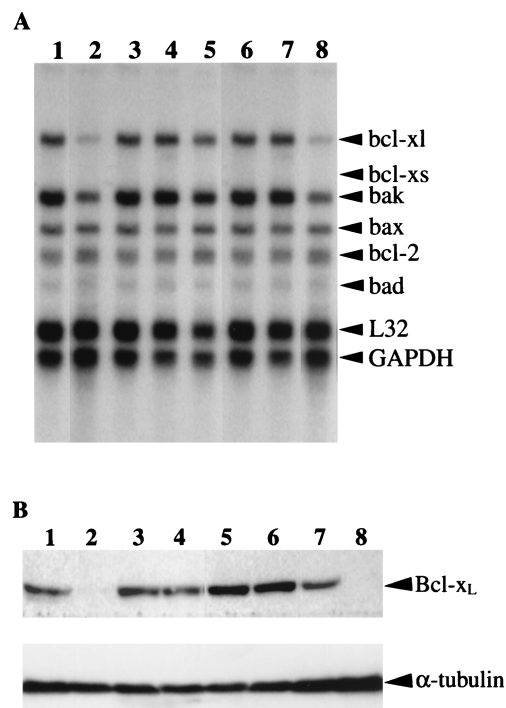


FIG. 1. Constitutive expression of *bcl-xl* mRNA and Bcl-x_L protein in CTLL-2/Tax transfectants in the absence of IL-2. (A) RNase protection assays with specific probes for *bcl-xl*, *bcl-xs*, *bak*, *bax*, *bcl-2*, and *bad* genes were performed by using 20 μ g of total RNA samples extracted from CTLL-2/Vec-#22 (lanes 1 and 2), CTLL-2/WT-#34 (lanes 3 and 4), CTLL-2/703-#23 (lanes 5 and 6), and CTLL-2/M22-#23 (lanes 7 and 8) after 48 h of culture in the presence (lanes 1, 3, 5, and 7) or absence (lanes 2, 4, 6, and 8) of 1 nM IL-2. Similar results were obtained with another set of independent clones. (B) Western blot analysis of Bcl-x_L (top) and α -tubulin (bottom) expression in CTLL-2/Tax transfectants. In each lane, we used 50 μ g of total cell lysates prepared from a similar set of CTLL-2 clones as described in panel A. The arrangement of lanes is similar to that of panel A. Specific protein bands are indicated by arrows. Similar results were obtained with another set of independent clones.

brief, 10 μ g of nuclear extracts prepared from 10⁷ of CTLL-2/Tax transfectants cultured with or without IL-2 for 48 h were preincubated with a binding buffer containing 1 μ g of poly(dI-dC). They were then incubated with approximately 20,000 cpm of ³²P-labeled oligonucleotide probes containing NF- κ B like elements for 20 min at room temperature. The DNA-protein complex was separated on a 5% polyacrylamide gel and visualized by autoradiography. To examine the specificity of the NF- κ B-like element of the probes, unlabeled competitor oligonucleotides were preincubated with nuclear extracts for 20 min on ice prior to incubation with probes. The probes or competitors utilized were prepared by annealing the sense and antisense synthetic oligonucleotides as follows: NF- κ B-like element in the *bcl-x* promoter, 5'-cgatAAAGGGACTTCCAAGat-3' and 3'-taTTTCCCTGAAGGTTctagc-5'; NF- κ B-like mutant element, 5'-cgatAAACCGACTTCCAAGat-3' and 3'-taTTTGGCTGAAGGTTctagc-5'; typical NF- κ B element of SV40 early promoter, 5'-cgatAGAGGGGACTTCCGat-3' and 3'-taTCTCCCTGAAAGGctagc-5'; and typical CRE sequence of T-cell receptor α promoter, 5'-cgatCCATGACGTCATGGat-3' and 3'-taGGTACTG CAGTACctagc-5'. (Lowercase letters signify nonsense nucleotides inserted to make cohesive ends, which increases the radiolabeling efficiency.) To identify NF- κ B proteins in the DNA-protein complex by EMSA, antibodies specific for various NF- κ B family proteins, including NF- κ B p65, NF- κ B p50, c-Rel, RelB, and NF- κ B p52 (Santa Cruz Biotechnology, Santa Cruz, Calif.), were used to elicit supershift and/or inhibition of the bands. These antibodies were incubated with the nuclear extracts for 1 h on ice prior to incubation with radiolabeled probes in EMSA.

RESULTS

Constitutive expression of *bcl-x* mRNA and Bcl-x_L protein in CTLL-2 cells stably transfected with HTLV-1 Tax. We previously established CTLL-2 clones stably transfected with various Tax plasmids such as wild-type Tax (WT), mutant Tax retaining ability to activate NF- κ B (703), or mutant Tax defi-

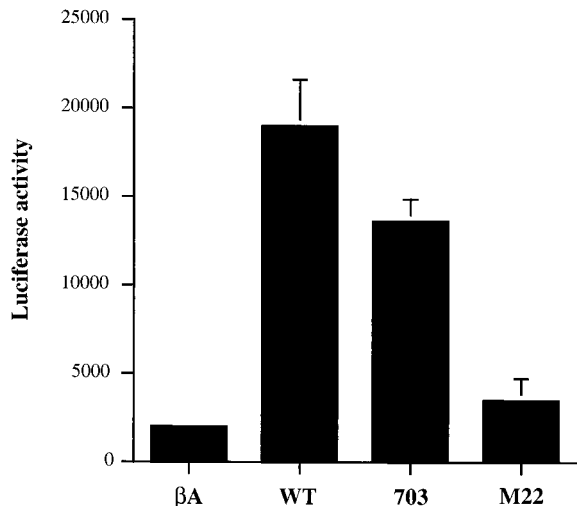


FIG. 2. Transactivation of *bcl-x* promoter by Tax. Jurkat cells (10⁶) were cotransfected with 2 μg of the Luc reporter plasmid pGL2-3.2 driven by the *bcl-x* promoter, 1 μg of pSV-β-galactosidase, and 2 μg of Tax-expressing plasmids or its control vector pHβAPr-1-neo (βA). The Tax-expressing plasmids utilized were pBMT-2Tax (WT), pβTax703 (703), and pβTaxM22 (M22). Luciferase activities were normalized with β-galactosidase activity. Data represent the mean ± the standard deviation (SD) of three independent experiments.

cient in activating the NF-κB site (M22) (23). By using these clones, we demonstrated that CTLL-2 clones transfected with WT Tax (CTLL-2/WT) and mutant Tax 703 (CTLL-2/703) but not the parental CTLL-2 or CTLL-2 clones transfected with Tax M22 (CTLL-2/M22) or control vector (CTLL-2/Vec) were resistant to apoptosis induced by IL-2 deprivation (23).

To dissect the molecular mechanisms of Tax-induced inhibition of apoptosis in CTLL-2 cells, we determined the

mRNA-levels of several apoptosis-related genes, including *bcl-xl*, *bcl-xs*, *bak*, *bax*, *bcl-2*, and *bad* in CTLL-2/Tax transfectants, by using RNase protection assays. A representative result is shown in Fig. 1A. In a clone of CTLL-2/Vec (CTLL-2/Vec-#22), the mRNA level of *bcl-xl*, which is known to inhibit apoptosis, was markedly decreased after 48 h of IL-2 deprivation. Expression of *bak* slightly decreased by IL-2 deprivation, but none of the other members of the *bcl* family tested was significantly affected by IL-2 deprivation. *Bcl-xs* mRNA, a spliced variant of *bcl-xl* (19), was not detected in any of these CTLL-2 cells regardless of the presence of IL-2. In contrast, clones of CTLL-2/WT and CTLL-2/703 (CTLL-2/WT-#34 and CTLL-2/703-#23, respectively) showed a constitutive expression of *bcl-xl* even in the absence of IL-2. However, the induction of *bcl-xl* mRNA in a CTLL-2/M22 clone (CTLL-2/M22-#23) was dependent on the presence of IL-2. Similar results were obtained by using another set of independent clones of CTLL-2/Tax transfectants.

Constitutive expression of the Bcl-x_L protein in stable CTLL-2 transfectants with Tax was also confirmed by Western blot analysis. As shown in Fig. 1B, CTLL-2/WT-#34 and CTLL-2/703-#23 but not CTLL-2/Vec or CTLL-2/M22-#23 expressed detectable levels of Bcl-x_L protein after 48 h of IL-2 deprivation. The levels of α-tubulin, a cytoskeletal protein as an internal control, was comparable among these clones. The protein levels of Bak in CTLL-2/WT clones did not differ from those of CTLL-2/Vec clones in the absence of IL-2 (data not shown). These results suggested that the constitutive expression of Bcl-x_L in CTLL-2/WT and CTLL-2/703 clones in the absence of IL-2 may contribute to Tax-induced resistance to apoptosis. We then explored the mechanisms of Bcl-x_L induction by HTLV-1 Tax.

Transactivation of *bcl-x* promoter by HTLV-1 Tax. Using transient-transfection assays, we then assessed whether Tax can enhance *bcl-x* promoter activity. In these assays, HTLV-1-

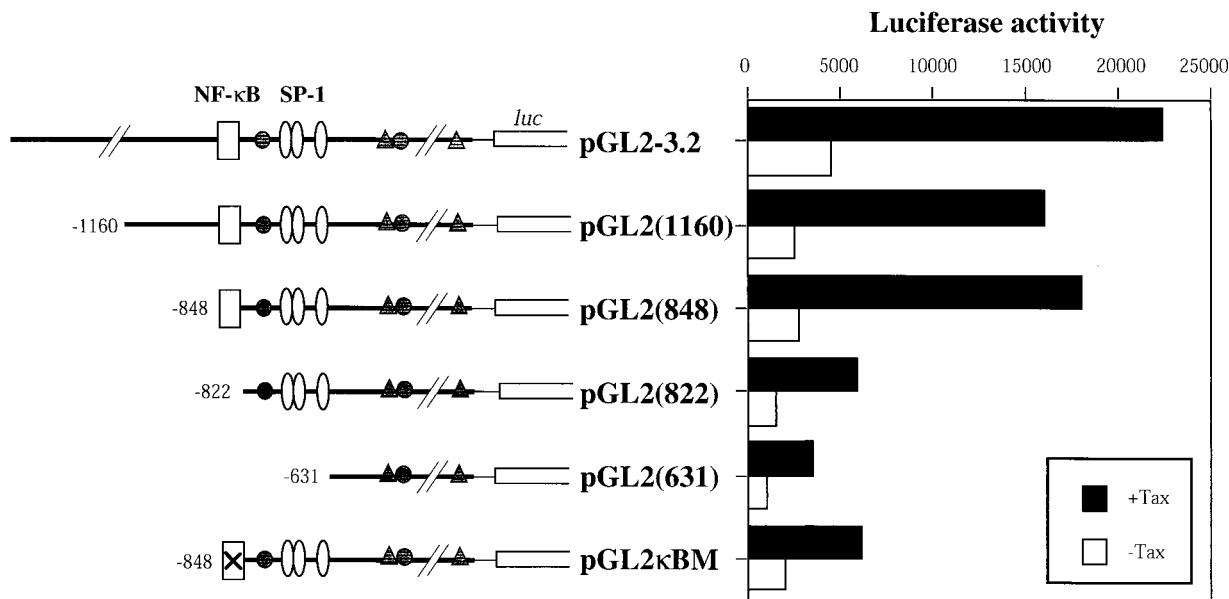


FIG. 3. Mapping of HTLV-1 Tax-responsive region in the *bcl-x* promoter. A series of Luc reporter plasmids based on pGL2 vectors containing fragments of 5' flanking region of the *bcl-x* gene were cotransfected into Jurkat cells with WT Tax plasmid pBMT-2Tax (solid bars) or its control plasmid (open bars), together with reference plasmid pSV-β-galactosidase. Luciferase activities normalized with β-galactosidase activities after 48 h of incubation are indicated. Constructs of the reporter plasmids utilized are schematically illustrated in the left panel. Fragments of the 5' flanking region of *bcl-x* gene are represented by heavy lines, and the number at the 5' end of each construct indicate the 5'-most end base positions from the translation initiation codon of the *bcl-x* gene. The locations of the binding sites for transcription factors NF-κB (□), SP-1 (○), Ets-1 (●) and GATA-1 (△), identified by using a transcription factor database (1a), are also indicated. pGL2κBM contained mutations within the NF-κB site (⊗) as described in Materials and Methods. Similar results were obtained in two independent experiments.

negative IL-2-independent human T-cell line Jurkat but not CTLL-2 cells were used since CTLL-2 cells cannot survive in the absence of IL-2 after transfection with plasmids. Jurkat cells were cotransfected with Tax effector plasmids and a Luc reporter plasmid (pGL2-3.2) containing a 3.2-kbp segment of 5' flanking region of the mouse *bcl-x* gene in the absence of IL-2 (19). As shown in Fig. 2, the Luc activities of Jurkat cells transfected with WT Tax or Tax 703 were seven to nine times higher than those of cells transfected with the control vector. However, Luc activity in cells transfected with Tax M22 plasmid was almost equivalent to that in control cells. These results indicated that both WT Tax and Tax 703 transactivates *bcl-x* promoter activity, presumably through the NF- κ B pathway.

Mapping of HTLV-1 Tax-responsive regions in the *bcl-x* promoter. We next mapped Tax-responsive elements in the mouse *bcl-x* promoter. Several consensus motifs for the binding of transcription factors such as SP-1, Ets-1, and GATA-1 have been identified (19). We also identified an NF- κ B binding motif (GGGACTTCC) similar to the one in the immunoglobulin promoter, which was located at positions -848 to -840 of the *bcl-x* promoter in an inverted direction. To examine the contributions of these sites for Tax-induced transactivation, a series of Luc reporter constructs with several fragments of different lengths of 5' deleted *bcl-x* promoters were prepared and transiently transfected into Jurkat cells along with Tax expression plasmid or control plasmid (Fig. 3). The highest level of Tax-induced Luc activity was observed in cells transfected with the longest construct, pGL2-3.2. The two deletion constructs, pGL2(1160) and pGL2(848), also responded to Tax significantly. All three constructs contained both SP-1 and NF- κ B binding site-like elements. However, the Tax-induced Luc activity was markedly reduced in cells transfected with pGL2(822), which had the SP-1 but not the NF- κ B site. The shortest construct pGL2(631), which lacked both the NF- κ B and the SP-1 sites, elicited minimal responses to Tax. Furthermore, a mutated NF- κ B-Luc plasmid, pGL κ BM, which had the same length as pGL2(848) but had a CC-to-GG mutation at positions -841 and -840 within the NF- κ B motif, exhibited markedly reduced levels of Tax-induced transactivation as observed with pGL2(822). These results suggest that the NF- κ B site located at positions -848 to -840 in the *bcl-x* gene promoter region is one of the major responsive elements of Tax-induced transactivation of *bcl-x*.

Binding of NF- κ B family proteins to the NF- κ B-like element in *bcl-x* promoter. We further examined whether NF- κ B family proteins can actually bind to the putative NF- κ B element identified in the *bcl-x* promoter by EMSA. The synthetic oligonucleotide containing the NF- κ B-site at positions of -848 to -840 in the *bcl-x* promoter was used as a probe. As shown in Fig. 4A, a clear shifted band was observed when the probes were incubated with nuclear extracts of CTLL-2/WT-#34 cultured in the presence or the absence of IL-2. However, the binding activity of nuclear extracts of CTLL-2/*Vec*-#22 was dependent on the presence of IL-2. Similar results were observed with parental CTLL-2 cells (data not shown). Complex formation was competed by 100-fold excess cold probe or unlabeled oligonucleotides with a typical NF- κ B consensus sequence of SV40 early promoter but not by oligonucleotides with a mutant NF- κ B-like sequence of the *bcl-x* promoter or other unrelated competitors such as CRE sequence. When the mutant NF- κ B-like sequence was used as a probe, complex formation was not observed with nuclear extracts from CTLL-2/WT-#34 cultured in the absence of IL-2. When anti-NF- κ B p50 antibody was preincubated with nuclear factors of CTLL-2/WT-#34, the bands formed with the probes supershifted in the EMSA, whereas anti-NF- κ B p65 and anti-RelB partially

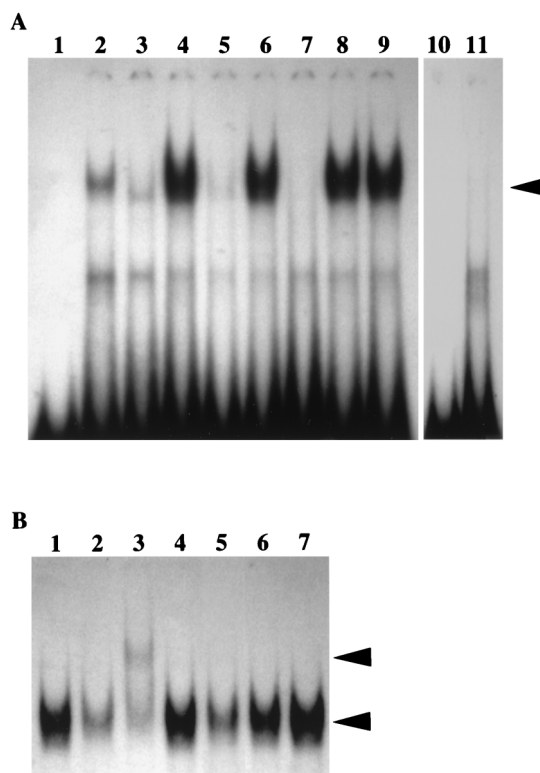


FIG. 4. Binding of NF- κ B family proteins to 32 P-labeled oligonucleotide probes in EMSA. (A) Nuclear extracts (10 μ g) from CTLL-2/*Vec*-#22 (lanes 2 and 3) and CTLL-2/WT (lanes 4 to 9) cultured in the presence (lanes 2 and 9) or absence (lanes 3 to 8) of IL-2 were incubated with (lanes 5 to 8) or without (lanes 2 to 4 and 9) a 100-fold excess of competitors and then incubated with probes containing the NF- κ B-like element in the *bcl-x* promoter. The DNA-protein complexes were separated on a 5% polyacrylamide gel and visualized by autoradiography. Competitors used included unlabeled probe (lane 5), mutant NF- κ B like element (lane 6), a typical NF- κ B sequence (lane 7), and a typical CRE sequence (lane 8). Lane 1 represents the band of probes alone. Radiolabeled probes containing a mutant NF- κ B-like element were also incubated without (lane 10) or with (lane 11) nuclear extracts from CTLL-2/WT cultured in the absence of IL-2. Specific bands are indicated by arrows. (B) Nuclear extracts (10 μ g) from IL-2-untreated CTLL-2/WT were preincubated without (lane 1) or with antibodies specific for NF- κ B family proteins, including NF- κ B p65 (lane 2), NF- κ B p50 (lane 3), c-Rel (lane 4), RelB (lane 5), NF- κ B p52 (lane 6), and control rabbit antiserum (lane 7), and then incubated with radiolabeled probe containing the NF- κ B-like element in the *bcl-x* promoter. The lower arrow indicates NF- κ B-DNA complex alone, while the upper arrow indicates a supershifted band with an anti-NF- κ B p50 antibody. Similar results were obtained with at least three independent CTLL-2/WT clones.

inhibited the complex formation (Fig. 4B). A similar pattern of supershift was also observed when a typical NF- κ B consensus sequence was used as a probe (data not shown). These results indicated that p65 and p50 subunits of NF- κ B and RelB can bind the NF- κ B like element identified in the *bcl-x* promoter.

Suppression of Tax-induced enhancement of *bcl-x* promoter activity by blocking NF- κ B pathway. We then examined whether blocking of the NF- κ B pathway can affect Tax-induced transactivation of *bcl-xl* by transient assays by using a super-repressor form of I κ B α mutant (I κ B α M) that specifically suppresses NF- κ B activity. I κ B α M contains serine to alanine mutations at residues 32 and 36, which abolish phosphorylation sites by I κ B kinase. The I κ B α M expression vector was cotransfected into Jurkat cells along with the WT Tax plasmid and the pGL2-3.2. As shown in Fig. 5A, the elevated Luc activity in response to WT Tax was markedly suppressed by cotransfection with I κ B α M but not with the control plasmid. In contrast,

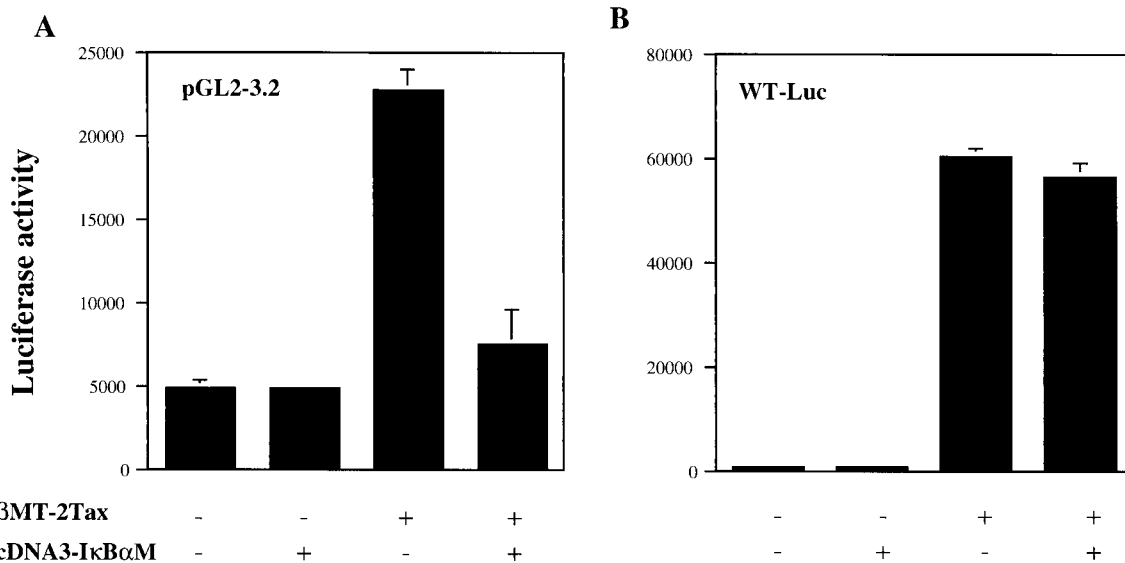


FIG. 5. Involvement of NF-κB pathway in Tax-induced transactivation of *bcl-x* promoter. (A) Jurkat cells were cotransfected with 4 μg of *bcl-x* promoter-Luc reporter plasmid pGL2-3.2, 1 μg of pSV-β-galactosidase, and various combinations of 2 μg of pβMT-2Tax and 2 μg of pcDNA3-IκBαM as indicated. The total plasmid amount for transfection was always adjusted up to 9 μg by adding control plasmids pβBAPR-1-neo or pcDNA3. The Luc activity of the samples after 48 h of incubation was normalized to the β-galactosidase activity. Data represent the mean ± the SD of three independent experiments. (B) Control experiments with reporter plasmids without potential NF-κB binding sites. Jurkat cells were transfected with 4 μg of HTLV-LTR (21 bp)-Luc reporter plasmid WT-Luc, 2 μg of pβMT-2Tax, 1 μg of pSV-β-galactosidase, and 2 μg of pcDNA3-IκBαM or pcDNA3. The luciferase activity was indicated as described in panel A.

the Luc activity of another Luc reporter plasmid, WT-Luc, which can be activated by Tax through the CRE pathway but not through the NF-κB pathway (16), was not affected by IκBαM (Fig. 5B). These results confirmed that the NF-κB pathway was involved in Tax-induced transactivation of the *bcl-x* promoter in these cells.

Involvement of NF-κB pathway in constitutive *bcl-x* promoter activity in CTLL-2/Tax transfectants. Finally, we examined involvement of the NF-κB pathway in Tax-induced constitutive expression of *bcl-x* in CTLL-2 cells. CTLL-2/WT-#34 were transfected with the pGL2-3.2 in the presence or absence of IκBαM in IL-2-free conditions. As shown in Fig. 6A, the *bcl-x* promoter activity in CTLL-2/WT-#34 was partially inhibited by transfection with IκBαM but not with control plasmid. The Luc activity of WT-Luc lacking potential NF-κB sites was not affected by IκBαM in these cells (Fig. 6B). Similar results were obtained with at least three independent CTLL-2/WT clones. These results confirmed the involvement of the NF-κB pathway in Tax-induced transactivation of *bcl-x* in CTLL-2 cells, as well as in Jurkat cells.

DISCUSSION

In this study, we demonstrated that HTLV-1 Tax transactivates the *bcl-x* promoter through NF-κB. Tax-mediated constitutive expression of Bcl-x_L in CTLL-2 cells expressing Tax is presumed to prevent these cells from apoptosis induced by IL-2 deprivation. This can be one of the molecular mechanisms of apoptosis resistance shown in our previous study, which demonstrated that Tax converted IL-2-dependent growth of CTLL-2 to IL-2-independent growth (23).

RNA protection assays showed that *bcl-x*_L, but not other members of *bcl* family tested, was constitutively expressed in CTLL-2/WT and CTLL-2/703 clones in the absence of IL-2 (Fig. 3A). Constitutive expression of Bcl-x_L protein was also observed in these cells (Fig. 3B). Bcl-x_L has been shown to inhibit apoptosis of several types of cells including T lympho-

cytes (4, 32), and overexpression of Bcl-x_L protects CTLL-2 cells against apoptosis after IL-2 deprivation (3). Therefore, Tax-induced constitutive expression of Bcl-x_L can be the underlying molecular mechanism of resistance of CTLL-2 cells expressing Tax to apoptosis after IL-2 deprivation. Other investigators, however, reported that the otherwise IL-3-dependent B cells could grow independently of IL-3 when they expressed both Tax and c-Myc but not Tax or c-Myc alone (28). *bcl-x* induction by Tax was not observed in these cells. Such variation in the ability of Tax to induce *bcl-x* among different

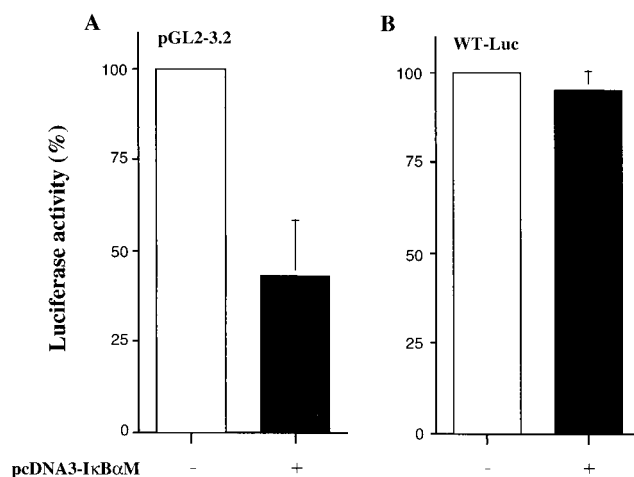


FIG. 6. Inhibition of constitutive activation of *bcl-x* promoter in CTLL-2/Tax transfectants by IκBαM. (A) CTLL-2/WT clone cells were transfected with pGL2-3.2, pSV-β-galactosidase, and either pcDNA3-IκBαM (solid bar) or pcDNA3 (open bar). Luc activity after 48 h of incubation in the absence of IL-2 was normalized to β-galactosidase activity and indicated as the percentage of activity in the IκBαM(-) sample. Data represent the mean ± the SD of three independent experiments. (B) Control experiments with the WT-Luc reporter plasmid instead of pGL2-3.2.

cell types implies a requirement of cellular mediators in this phenomenon.

Although Bcl-2 is also known to protect cells against apoptosis (10, 11), the expression of *bcl-2* did not correlate with Tax-induced resistance to apoptosis of CTLL-2 in the present system. This finding is consistent with that of previous reports demonstrating that Tax did not induce *bcl-2* expression in human endothelial cells and a pro-B cell line (28, 31). Expression of *bax*, an accelerator of apoptosis, was not significantly affected by Tax in the present study. However, other investigators reported that Tax repressed *bax* transcription (5). In general, the Bcl-2/Bax and Bcl-x_L/Bax ratios are thought to be important determinants of apoptosis (35). Our results of Tax-induced activation of *bcl-xl*, together with the finding of Tax-induced repression of *bax* by others, suggest that Tax inhibits apoptosis by increasing the Bcl-x_L/Bax ratio in HTLV-1-infected cells.

Tax is known to activate NF-κB by activating IκB kinases and other related molecules (8, 17, 47, 54). Involvement of the NF-κB pathway in Tax-induced transactivation of *bcl-xl* was strongly suggested by our results that the *bcl-x* promoter was activated by mutant Tax preferentially activating NF-κB (Fig. 3) and that an NF-κB-like element at positions -848 to -840 from the translation initiation codon of *bcl-x* gene was responsive for Tax-induced activation of the *bcl-x* promoter (Fig. 4). The binding of NF-κB family proteins, including NF-κB p65, NF-κB p50, and RelB, to the NF-κB-like element that we identified in the mouse *bcl-x* promoter was also confirmed in vitro (Fig. 4B). Similar NF-κB binding sequences were also found in the human *bcl-x* promoter (12). Furthermore, the Tax-mediated *bcl-x* promoter activation was profoundly inhibited by a super-repressor mutant of IκBα (Fig. 5 and 6) or by introduction of mutations in the NF-κB-like element in the mouse *bcl-x* promoter constructs. These findings suggest that the NF-κB-like element is one of the major responsive regions of Tax-induced *bcl-x* promoter activation.

NF-κB activation is known to inhibit apoptosis induced by tumor necrosis factor alpha or immunoglobulin G cross-linking in several types of cells unrelated to HTLV-1 (48, 49, 51). A few reports suggested the involvement of inhibitor of apoptosis protein (50) and *IEX-1L* (52), although these could be a part of multiple mechanisms and may also vary among different cell types. The molecular mechanisms of NF-κB-induced inhibition of apoptosis, apart from the *bcl-xl* induction demonstrated in the present study, remain to be clarified.

In conclusion, we demonstrated in the present study that Tax enhanced the expression of Bcl-x_L at least through the NF-κB pathway, and that this was associated with cellular resistance to apoptosis. Tax-mediated Bcl-x_L induction may be one of the underlying mechanisms of inhibition of apoptosis of HTLV-1 infected cells, which might contribute to the pathogenesis of HTLV-1 infection.

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