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Extracellular Tax_1 Protein Stimulates Tumor Necrosis Factor-β and Immunoglobulin Kappa Light Chain Expression in Lymphoid Cells

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The human T-cell leukemia virus type I *tax*$_1$ gene product is responsible for the increased expression of several cytokine and cellular genes that contain NF-κB regulatory sequences. Our laboratory has previously demonstrated that purified, extracellular Tax_1 protein induced the nuclear accumulation of NF-κB binding activity in lymphoid cells. Since HTLV-I infection causes increased levels of lymphotoxin tumor necrosis factor-β [TNF-β] and immunoglobulin secretion, we have studied the interaction of NF-κB proteins from Tax_1-stimulated cells with the TNF-β and immunoglobulin kappa (Igκ) light chain genes. Tax_1 induction of NF-κB occurred in the presence of cycloheximide, and Tax_1 stimulation did not result in increased levels of NF-κB or c-*rel* RNA. These results indicate that new synthesis of NF-κB proteins was not required for induction of NF-κB-binding activity. With use of the Igκ NF-κB-binding site as a probe, two distinct NF-κB gel shift complexes were induced by the Tax_1 protein. A slower-migrating complex, C1, was inhibited by the addition of purified IκB. In contrast, the faster-migrating C2 complex was not inhibited by IκB, but C2 was increased by detergent treatment of cytoplasmic extracts, suggesting that its binding activity was also regulated by an inhibitor. The Tax_1-stimulated proteins that interacted with the NF-κB-binding sites in the Igκ and TNF-β promoters were distinct. A 75-kDa protein preferentially associated with the Igκ NF-κB-binding site. In contrast, a 59-kDa protein associated with the TNF-β NF-κB-binding site. Tax_1 stimulation led to increased levels of TNF-β and Igκ mRNA, as measured by reverse transcription and polymerase chain reaction analysis. These results represent the first experimental evidence that extracellular Tax_1 can regulate the expression of endogenous cellular genes.

Human T-cell leukemia virus type I (HTLV-I) infection is associated with adult T-cell leukemia (ATL) and tropical spastic paraparesis/HTLV-I-associated myelopathy (TSP/HAM). The HTLV-I genome encodes a 40-kDa protein, Tax_1, that functions as a transcriptional transactivator of the viral long terminal repeat and cellular genes. In addition to, or as a result of, its transcriptional regulation of cellular genes, Tax_1, plays a critical role in cellular transformation. T-cell proliferation and immunologic defects observed during HTLV-1 infection are apparently due to the effect of the HTLV-I Tax_1 transactivator on viral and cellular gene expression (6, 10, 18, 21). Several immunologic and metabolic alterations are observed during HTLV-I infection. These include increased expression of interleukin-2 receptor alpha chain (IL-2Rα; Tac antigen), increased T-cell proliferation, loss of cytotoxic T-lymphocyte function, altered regulation of immunoglobulin synthesis, and increased expression of lymphotoxin (tumor necrosis factor-β [TNF-β]) (31, 33, 43, 44, 47). Tax_1 inductions of IL-2 and IL-2Rα are thought to be important early events in the transformation of T-cells by the HTLV-I virus (8, 10, 18, 21, 34, 39, 40, 50). TNF-β may have important cytolytic effects and contribute to bone resorption and hypercalcemia (31, 43), which frequently complicate ATL (44, 47). The neurological degeneration observed in TSP/HAM patients may also result from immunologic alterations resulting from HTLV-I infection (19). Antibody and cytotoxic lymphocyte responses frequently occur not only to viral surface determinants such as the Gag and envelope proteins but also p40 Tax_1 antigen (9, 12, 19, 48), suggesting that Tax_1 protein escapes from cells and is recognized by the immune system. Consistent with this hypothesis, we have shown that Tax_1 protein is present in the culture media of HTLV-I-transformed cells (24). Thus, Tax_1 protein may regulate the expression of immunoregulatory cytokine genes, including IL-2Rα, TNF-β, and immunoglobulin kappa (Igκ) light chain genes, in both infected and uninfected cells.

Several cytokine and cellular genes, including IL-2, granulocyte-macrophage colony-stimulating factor, TNF-β, vimentin, HLA class I and class II, IL-6, gamma interferon, and Igκ light chain genes, are known to contain NF-κB-binding domains in their promoter/enhancer regions (1, 22, 23, 28, 29, 31, 32, 39). Several of these genes are activated by Tax_1 through the NF-κB transcription factor. For example, Tax_1 expression in virally infected and transfected cells induces IL-2Rα gene expression through induction of the NF-κB family of DNA-binding proteins, including NF-κB, HIVEN 86, and c-Rel (6, 8, 21). TNF-β is also likely stimulated in HTLV-I-infected cells via NF-κB (31, 43).

The NF-κB family of DNA-binding proteins consists of several members which positively and negatively regulate gene expression (7, 17, 27). The activation of the members of the NF-κB/c-*rel* family is complex and may result from distinct pathways. Stimulation of HIVEN 86 and c-*rel* require several hours and may be under transcriptional control (27). The p50 NF-κB derives from the proteolytic processing of a 105-kDa precursor which is sequestered in the cytoplasm via C-terminal ankyrin repeats (14, 20, 35). p50 is further regulated in the cytoplasm through interaction with the 65-kDa transmodulator protein and the cytoplasmic inhibitor IκB, which is released by phosphorylation of IκB (3, 4, 5, 13, 38, 45, 51). Several extracellular signals, including Tax_1, IL-1α, TNF-α, phorbol myristate acetate, and lipopolysaccharide (LPS), have been shown to induce NF-κB DNA-binding activity. One active DNA-binding

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form of the NF-κB complex is composed of a heterotetramer of the 65- and 50-kDa subunits (5, 45, 46). Additional DNA-binding NF-κB complexes have been reported. Inducible NF-κB DNA-binding proteins identified by preparative gel shift assay and UV cross-linking include 50-, 55-, 75-, and 85-kDa proteins (7, 27).

We have previously shown that purified, recombinant extracellular Tax_1 protein introduced into cell culture induced the nuclear accumulation of NF-κB DNA-binding proteins in pre-B lymphocytes (24). The present study shows that Tax_1 protein can stimulate Igκ light chain and TNF-β genes, both of which contain the NF-κB DNA element. The Tax_1 protein stimulates the NF-κB DNA-binding activity in the absence of protein synthesis and does not significantly alter the levels of NF-κB or c-*rel* RNA expression. Finally, Tax_1 stimulation induces several NF-κB proteins which bind differentially to the Igκ light chain and TNF-β promoter elements. The differences in NF-κB-binding proteins bound to the two distinct promoter/enhancer elements may provide insight into the patterns of regulation of these genes and the functions of the various NF-κB DNA-binding proteins.

MATERIALS AND METHODS

Cells. 70Z/3 cells are mouse pre-B lymphocytes derived from a methyl nitrosourea-induced tumor in a (C57BL/6 × DBA/2)F_1 mouse (ATCC T1B 158; originated by P. Kincade, Sloan-Kettering Institute, Rye, N.Y.). Cells were cultured according to American Type Culture Collection specifications.

Purification of Tax_1. Tax_1 protein was expressed in *Escherichia coli* (15) and purified as described by Lindholm et al. (25). Mock bacterial extracts were prepared from *E. coli* not expressing the Tax_1 gene. For some experiments, the purified Tax_1 protein was extracted with an equal volume of chloroform at 4°C for 1 h with periodic vortexing. The aqueous layer was collected after centrifugation at 10,000 rpm for 5 min in a Beckman microfuge. Chloroform-extracted supernatants were analyzed for residual protein by silver stain and for the ability to stimulate NF-κB gel shift activity in 70Z/3 cells.

Assay of NF-κB induction. Murine 70Z/3 pre-B cells were plated at 10^6 cells per ml in 15 ml of RPMI medium and cultured as indicated above. The cells were cultured overnight prior to stimulation with 25 nM purified Tax_1, an equal volume of bacterial extract, chloroform-extracted Tax_1, 50 ng of tetradecanoylphorbol acetate (TPA) per ml, or 5 μg of *Salmonella enteritidis* LPS (Sigma) per ml. Cells were harvested at 3 h unless otherwise indicated. For assay of Tax_1 clearance by immunoprecipitation, the cells were incubated for 3 h with Tax_1 protein (2.5 nM, final concentration), diluted Tax_1 protein, or immunoprecipitation supernatant which would contain an equivalent amount of Tax_1 protein (see below). Nuclear and cytoplasmic extracts were prepared by the method of Osborn et al. (30). For gel shift assays, NF-κB oligonucleotides containing the murine Igκ enhancer 5′-GATCCAGAGGGGACTTTCCGAGAG-3′(49) or TNF-β sequence 5′-GATCCAGAGGGGCTTCCCCGAG AG-3′ were Klenow labelled with [^{32}P]dGTP (Amersham). The labelled oligonucleotides were desalted with G-25 spin columns (Boehringer Mannheim) and ethanol precipitated. The probe was resuspended in 10 mM Tris (pH 7.5)–1 mM EDTA. The gel shift reactions were performed in a volume of 20 μl in gel shift reaction buffer (10 mM Tris HCl [pH 7.5], 40 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol) with 6 μg of nuclear or cytoplasmic extract, 0.5 to 2 ng (~50,000 cpm) of labelled NF-κB oligonucleotide, and 3 μg of poly (dI-dC · dI-dC) (Pharmacia) at room temperature for 20 min. Some reaction mixtures were incubated with a 100-fold excess of unlabelled mutant or wild-type Igκ NF-κB oligonucleotides to identify the specific NF-κB gel shift complex (49). Gel shift reactions were also performed with 70Z/3 cytoplasmic extracts prepared during lysis of cells for nuclear extracts (11, 30). Cytoplasmic extracts were dialyzed against buffer D (30) prior to use. Gel shifts with 70Z/3 cytoplasmic extracts were performed in the presence or absence of the detergents deoxycholic acid (DOC) and Nonidet P-40 (NP-40) to allow NF-κB binding in these extracts (3, 4, 51). Purified Tax_1 protein was added as indicated to the reaction mix to determine whether it could directly cause dissociation of the IκB–NF-κB complex and allow NF-κB binding to the murine Igκ probe. The samples were loaded onto a 5.0% neutral polyacrylamide gel and electrophoresed in 0.5× Tris-borate-EDTA at 10 V/cm for 3 h. The gels were then dried, and autoradiography was performed.

Immunoprecipitation of Tax_1 protein. Four micrograms of Tax_1 protein in 100 mM Tris (pH 8.0)–100 mM NaCl (TSB) was diluted in 100 μl of normal rabbit serum (NRS; GIBCO) and cleared twice by centrifugation with 200 μl of 20% Pansorbin (Calbiochem) at 4°C. The resulting supernatant was divided in half and incubated with either 400 μl of rabbit anti-Tax_1 serum or 400 μl of NRS for 2 h at 4°C. The immune complexes were precipitated with 800 μl of 20% Pansorbin. The supernatants from the immunoprecipitation reactions were analyzed by Western immunoblot and used in NF-κB induction assays (see above). Tax_1 protein was diluted in TSB or NRS for controls.

Preparative gel shift assay and UV cross-linking. Preparative gel shift reactions (4×) were performed at 25°C for 20 min. The gel shift reaction mixtures were loaded in a large well of a 5% polyacrylamide gel. Separate reaction mixtures with 100-fold excesses of wild-type and mutant competitor oligonucleotides were included in separate wells to facilitate identification of the specific inducible gel shift complexes. The gel was electrophoresed at 8 to 10 V/cm for 2.5 h. The gel was then exposed for 35 to 40 min to 300 nm of UV light at a total energy of 19 J/cm^2 in a Bioslinker (Bios Corp., New Haven, Conn.) An autoradiogram was performed to locate the gel shift complexes. The specific complexes were identified and excised. The gel pieces were soaked in 4× sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) buffer for 30 min prior to heat denaturation at 100°C for 5 min. The gel pieces and SDS-PAGE buffer were loaded on a 7.5% SDS-polyacrylamide gel. After electrophoresis, the gels were fixed and autoradiographed. The UV cross-linking reaction was optimized by annealing an 8-base sense oligonucleotide with the 24-base antisense oligonucleotide template for Klenow labelling. The Klenow reaction was performed in the presence of excess deoxynucleotide, including a 50:50 mix of dTTP and bromodeoxyuridine (BrdU) as described by Ballard et al. (7).

Reverse transcription and PCR of cellular RNA. Total cellular RNA was harvested after cells received stimulation with purified recombinant Tax_1 protein or control bacterial extracts. The cells were lysed by treatment with 4 M guanidinium isothiocyanate, and the lysate was overlaid on 5.7 M CsCl solution. The resulting gradients were centrifuged for 18 h at 36,000 rpm in a Beckman SW 55Ti rotor at 18°C. The RNA was harvested, ethanol precipitated, and quantitated by UV absorbance at 260 nm. The RNA was stored in ethanol until use in reverse transcription and polymerase chain reaction (PCR) assays. Aliquots of cellular

RNAs were then reverse transcribed with 3′ antisense primers appropriate for the genes being studied. The reverse transcription reaction was performed with 400 mM deoxynucleotides and 400 U of mouse mammary tumor virus reverse transcriptase for 1 h at 37°C as previously described by Tendler et al. (41). In preliminary experiments, a standard dilution of samples using 1, 3, and 10 μg of total cellular RNA was used for the analysis of Tax_1 stimulation of endogenous gene activity; 3 μg of total cellular RNA provided the most sensitive and consistent differentiation of TNF-β and Igκ, i.e., gene activation by extracellular Tax_1 in this system (data not shown). The reverse-transcribed products were then subjected to PCR in the presence of 1 pmol of 5′ sense and 3′ antisense primers, 200 mM deoxynucleoside triphosphates, and 2.5 U of *Taq* polymerase in a buffer containing 50 mM KCl and 1.5 mM $MgCl_2$ (Perkin-Elmer/Cetus). The reactions were subjected to 30 cycles of amplification with annealing at 50°C, polymerization at 72°C, and denaturation at 94°C. Aliquots (5 μl) of the PCR reactions were loaded onto 1.5% agarose gels and electrophoresed. The gels were stained with ethidium bromide and photographed prior to Southern blot. The gels were soaked in 1 M NaOH–1.5 M NaCl for 15 min at 25°C and then in 1.0 M Tris HCl (pH 7.5)–1.5 M NaCl for 15 min prior to Southern blotting. The gels were blotted overnight in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The nitrocellulose membranes were baked at 80°C for 2 h and placed in prehybridization buffer for 6 to 8 h. The blots were probed overnight in 6× SSC at 65°C with 10 ng of ^{32}P-labelled sense probe per ml for each of the genes being studied in the presence of 100 μg of herring sperm DNA per ml. The blots were washed two times with 2× SSC for 5 min each time at room temperature, two times with 2× SSC–1% SDS at 55°C for 30 min each time, and two times with 0.1× SSC at 37°C for 30 min each time. The blots were autoradiographed with Kodak XAR film. Based on the sequence positions of the PCR primers, the products detected by internal probes on the Southern blot were 513 bp for TNF-β, 320 bp for Igκ light chain, 683 bp for c-*rel*, 1502 bp for NF-κB, and 636 bp for actin.

RESULTS

Tax_1 induction of NF-κB is specific and occurs in the absence of protein synthesis. We have previously shown that soluble purified Tax_1 protein could stimulate 70Z/3 cells in a transient, dose-dependent manner (24). Induction of NF-κB DNA-binding activity occurred at 1 to 7 h following the exposure of cells to extracellular Tax_1. Mock bacterial extract and chloroform-extracted Tax_1 preparations failed to yield stimulation of NF-κB DNA-binding activity. To further demonstrate the specificity of NF-κB induction by the Tax_1 protein, Tax_1 was cleared by immunoprecipitation with anti-Tax_1 antibodies. We found that 2.5 nM Tax_1 protein added to 70Z/3 cells caused a significant induction of NF-κB DNA-binding activity at 3 h (Fig. 1A; compare lanes 1 and 2 with lanes 6 and 7). The supernatant from an immunoprecipitation containing Tax_1 protein and rabbit anti-Tax_1 serum showed no significant induction of NF-κB above controls (compare lane 5 with lanes 6 and 7). In contrast, the supernatant from an immunoprecipitation containing Tax_1 protein and NRS caused significant induction of NF-κB-binding activity when added to the 70Z/3 cells (lane 4). By Western blot analysis, anti-Tax_1 serum, but not NRS, completely cleared the samples of Tax_1 protein (data not shown). The NF-κB induction in 70Z/3 cells by extracellular Tax_1 protein was also blocked by anti-Tax_1 $F(ab')_2$ antibodies but not by preimmune $F(ab')_2$ antibodies (data not shown).

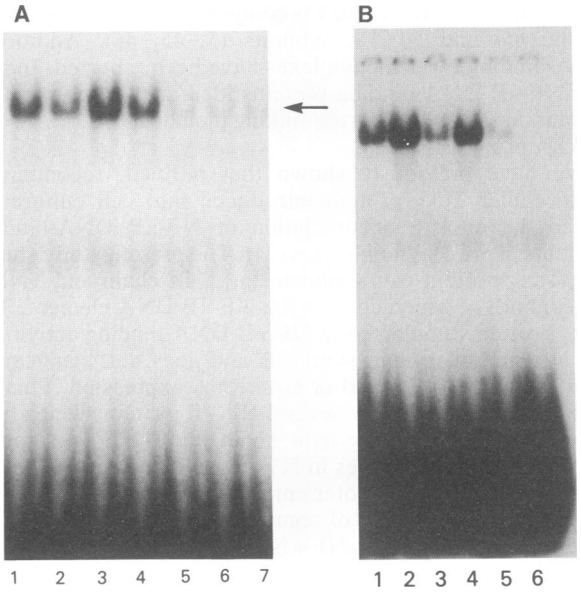

FIG. 1. (A) Gel shift assay for Tax_1 clearance effect by immunoprecipitation with anti-Tax_1 antibodies. Gel shift assays were performed with the Igκ NF-κB probe and nuclear extracts of 70Z/3 cells treated with Tax_1 protein (lane 1), Tax_1 preincubated with buffer (lane 2), Tax_1 preincubated with NRS (lane 3), or supernatants from immunoprecipitation reactions with Tax_1 protein and either NRS (lane 4) or rabbit anti-Tax_1 serum (lane 5). Chloroform-extracted Tax_1-treated cells (lane 6) and control, untreated cells (lane 7) were also assayed for NF-κB gel shift activity. The specific NF-κB gel shift band is indicated by the arrow. (B) Gel shift assay for cycloheximide effect on Tax_1 induction of nuclear NF-κB DNA binding. 70Z/3 cells received 3-h treatments with Tax_1 protein (lane 1), Tax_1 and cycloheximide (lane 2), TPA (lane 3), TPA and cycloheximide (lane 4), cycloheximide (lane 5), or chloroform-extracted Tax_1 (lane 6). The gel shift assay was performed with 2 ng of Igκ NF-κB probe and 6 μg of nuclear protein.

To determine whether Tax_1 could stimulate NF-κB-binding activity in the absence of protein synthesis, 70Z/3 cells were incubated with TPA, which is known to stimulate NF-κB (38), or Tax_1 in the presence or absence of cycloheximide. Compared with the control chloroform-extracted Tax_1-treated cells, Tax_1 stimulation for 3 h yields a strong gel shift complex with the Igκ light chain NF-κB probe (Fig. 1B; compare lanes 1 and 6). The level of NF-κB induction was similar to that with TPA stimulation (lane 3). Cotreatment of cells with Tax_1 protein and cycloheximide for 3 h caused increased gel shift activity above that of Tax_1-stimulated cells (compare lanes 1 and 2). Consistent with previously reported results (38), TPA and cycloheximide treatment for 3 h also caused increased gel shift activity above the level obtained with TPA stimulation alone (compare lanes 3 and 4). Cycloheximide treatment of cells alone gave little or no increase in gel shift complexes compared with the control-treated cells (compare lanes 5 and 6). These results indicate that Tax_1 protein was able to stimulate NF-κB gel shift complexes in the absence of protein synthesis and therefore did not require the de novo synthesis of NF-κB.

To determine whether the NF-κB or c-*rel* gene was induced at the transcriptional level, reverse transcription and PCR analysis of total cellular RNA from Tax_1-stimulated and control 70Z/3 cells was performed, using primers for

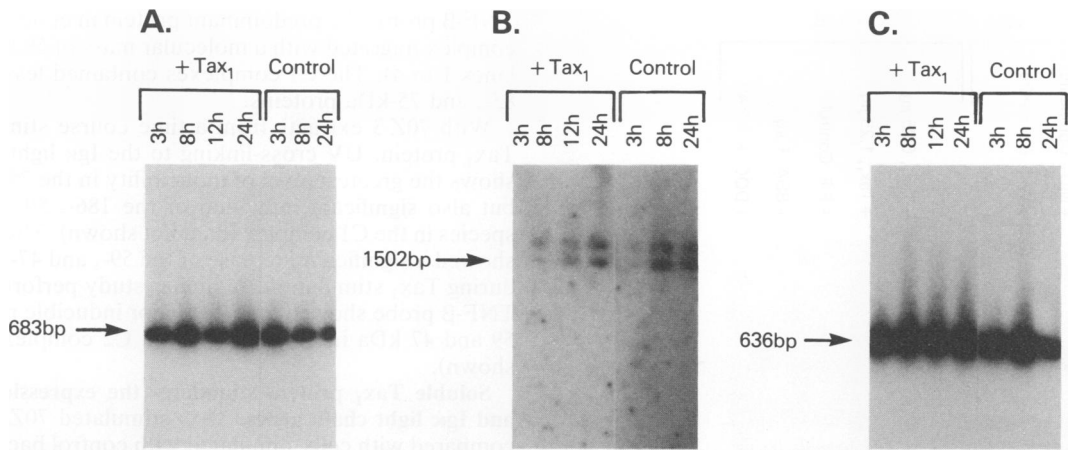

FIG. 2. Southern blot analysis of reverse-transcribed and PCR-amplified DNA from c-*rel* and NF-κB cellular RNA. 70Z/3 cells received treatment with Tax_1 protein or control bacterial extract for the times indicated. Total cellular RNA was harvested by guanidine isothiocyanate lysis and CsCl centrifugation; 3 μg of cellular RNA was reverse transcribed with 3′ antisense primers for the c-*rel*, NF-κB, or actin gene. The cDNA was amplified by PCR, using 5′ sense and 3′ antisense primers for the genes being studied. The amplified products were analyzed by Southern blot, using probes specific for the c-*rel* (A), NF-κB (B), and actin (C) genes. The probes hybridized with amplified DNA fragments appropriate for the genes studied and the placement of primers (arrows). Actin was included as a noninducible control.

c-*rel* and NF-κB. Similar to the actin control (636 bp) (Fig. 2C), c-*rel* (683 bp) and NF-κB (1502 bp) (Fig. 2A and B, respectively) gene products were not increased in Tax_1-treated cells above the level for controls. The ability of reverse transcription PCR analysis to detect quantitative changes in the level of mRNA synthesis is demonstrated below (see Fig. 7).

Tax_1 protein does not directly inhibit activity of IκB. We considered the possibility that purified Tax_1 protein could prevent the reassociation of the IκB inhibitor with NF-κB, resulting in an increase in NF-κB-binding activity in the absence of protein synthesis. To test this, purified IκB was preincubated with dilutions of a stimulated nuclear extract known to contain NF-κB activity. We first determined that 2 μl of the purified IκB would completely inhibit the NF-κB gel shift activity from 3 μg of stimulated nuclear extracts (Fig. 3; compare lanes 3 and 4). When 0.25 or 1.25 μg of the purified Tax_1 protein was added to the incubation mix containing nuclear extract prior to the addition of IκB, no change in IκB activity was observed (lanes 5 and 6). In contrast, when the nuclear extract was incubated with purified IκB in the presence of DOC and NP-40, NF-κB binding was detected (lane 9). These results suggest that purified Tax_1 protein was not able to directly prevent the reassociation of IκB and NF-κB in vitro.

Tax_1 stimulation of NF-κB complexes shows a different pattern of binding to the Igκ light chain and TNF-β NF-κB DNA elements. While both the Igκ light chain- and TNF-β NF-κB-binding sites conform to the NF-κB consensus sequence, there are significant nucleotide differences between the two binding sites. In all of our previous studies, we had utilized the Igκ light chain NF-κB sequence to analyze DNA-binding activity. We therefore compared the ability of the Igκ light chain and TNF-β NF-κB probes to form gel shift complexes with Tax_1-stimulated nuclear extracts. The Igκ light chain probe formed predominately a slowly migrating complex, designated C1, which showed rapid and transient induction during the time course of Tax_1 stimulation (Fig. 4A, lanes 1 to 5). The maximal gel shift activity occurred at 1 to 3 h of Tax_1 stimulation. The TNF-β probe formed two complexes: one which comigrated with the C1 band of the Igκ light chain probe, and a faster-migrating complex, designated C2, which was present in higher proportion and persisted at a increased level through the course of Tax_1 stimulation (lanes 8 to 12). The control bacterial extract-treated cells did not show significant induction of NF-κB gel shift complexes with either the Igκ (lanes 6 and 7) or TNF-β (data not shown) probe.

Igκ- and TNF-β NF-κB-binding proteins are regulated by distinct cytoplasmic inhibitors. The major cytoplasmic inhibitor of NF-κB-binding activity, IκBα, has been purified and has a molecular mass of 37 kDa (51). A second form of the inhibitor, IκBβ, has a molecular mass of 43 kDa. Both IκBα and -β inactivate NF-κB complexes containing the 65-kDa transmodulator protein. We next tested whether the C1 and C2 gel shift complexes could both be inhibited by IκB. The purified IκB fraction was capable of inhibiting the C1 complexes of either the Igκ light chain or TNF-β NF-κB probe with Tax_1- or TPA-stimulated nuclear extracts (Fig. 4B; compare lanes 4 and 5, 6 and 7, 12 and 13, and 14 and 15). In contrast, no decrease in the level of C2 complex formation was observed. The C2 complexes slightly increased in quantity after preincubation of the extracts with purified IκB. The fact that IκB was able to inhibit the C1 complex but not the C2 complex suggests that these complexes were composed of different NF-κB DNA-binding proteins.

It has previously been shown that DOC and NP-40 treatment of cytoplasmic NF-κB complexes releases proteins which normally function to inhibit NF-κB-binding activity. Cytoplasmic extracts from unstimulated 70Z/3 cells were studied for the presence of NF-κB DNA-binding proteins. Unstimulated 70Z/3 cytoplasmic extracts showed no gel shift activity with either the TNF-β NF-κB probe (Fig. 5, lane 4) or the Igκ light chain NF-κB probe (data not shown). Treatment of the unstimulated cytoplasmic extracts (lane 4) with DOC and NP-40 (lane 5) or DOC and CHAPSO (27) (lanes 6 and 7) prior to the gel shift reaction resulted in a marked increase in both C1 and C2 gel shift activity. This result suggests that the NF-κB-binding factors in both the C1 and C2 complexes were sequestered in the cytoplasm in an inactive form prior to Tax_1 treatment, requiring either the

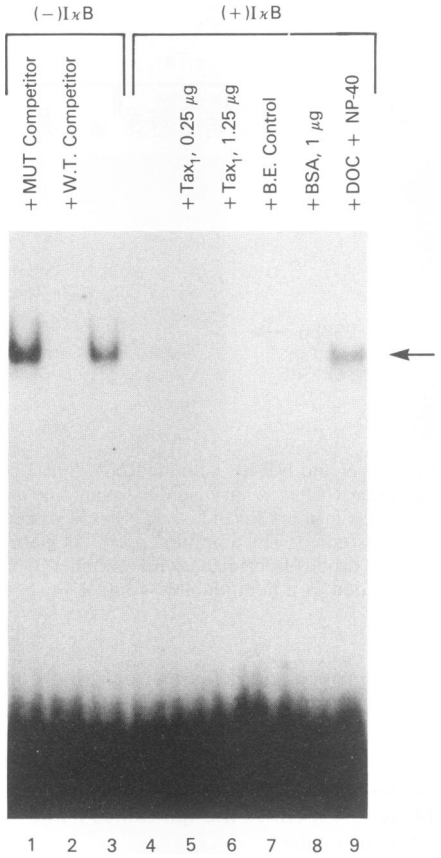

FIG. 3. Gel shift assays showing IκB reassociation with NF-κB. A stimulated 70Z/3 nuclear extract containing NF-κB DNA-binding activity (lanes 1 to 4) was titrated with purified IκB to achieve complete inhibition of NF-κB gel shift activity with the Igκ NF-κB probe (lane 4). Two concentrations of Tax_1 protein (lanes 5 and 6), control bacterial extract (B.E.; lane 7), bovine serum albumin (BSA; lane 8), or the detergents DOC and NP-40 (lane 9) were preincubated with the NF-κB-containing nuclear extracts for 1 min prior to addition of the purified IκB. Only the detergent mixture was able to prevent the inhibition of gel shift activity by the purified IκB. A 100-fold excess of mutant (MUT; lane 1) or wild-type (W.T.; lane 2) competitor oligonucleotide was added to show the specificity of the gel shift complex (arrow).

release of an inhibitory protein or a posttranslational modification for activation.

UV cross-linking of NF-κB DNA-binding proteins. The results of the IκB inhibition studies suggested that the protein compositions of the C1 and C2 complexes were distinct. To examine the protein composition of the gel shift complexes, preparative reactions were performed with BrdU-labelled probes for UV cross-linking studies. Following incubation with the nuclear extracts, the BrdU-labelled probes yielded gel shift complexes similar to those produced by probes labelled without BrdU (Fig. 6A, insert). The DNA protein complexes were UV cross-linked in the gel and were subsequently analyzed by 7.5% SDS-PAGE. The major protein in the C1 complex with the Igκ light chain probe at 3 h of stimulation with Tax_1 or TPA had a molecular mass of 75 kDa (Fig. 6A, lanes 1 and 3). The C1 complex also contained minor proteins migrating at 186, 85, 59, and 47 kDa. In contrast, the Igκ C2 complex contained predominantly a 59-kDa protein (lanes 2 and 4). With use of the TNF-β probe, the predominant protein in either the C1 or C2 complex migrated with a molecular mass of 59 kDa (Fig. 6B, lanes 1 to 4). The C1 complexes contained less of the 186-, 85-, and 75-kDa proteins.

With 70Z/3 extracts from a time course stimulation with Tax_1 protein, UV cross-linking to the Igκ light chain probe shows the greatest level of inducibility in the 75-kDa protein but also significant induction of the 186-, 59-, and 47-kDa species in the C1 complex (data not shown). The C2 complex showed a significant increase of the 59-, and 47-kDa proteins during Tax_1 stimulation. A similar study performed with the TNF-β probe showed that the major inducible proteins were 59 and 47 kDa in both the C1 and C2 complexes (data not shown).

Soluble Tax_1 protein stimulates the expression of TNF-β and Igκ light chain genes. Tax_1-stimulated 70Z/3 cells were compared with cells stimulated with control bacterial extract for expression of endogenous TNF-β or Igκ light chain genes. Total cellular RNA harvested at the times indicated was reverse transcribed with antisense primers specific to TNF-β, Igκ light chain, and actin genes followed by PCR amplification with appropriate 5′ sense and 3′ antisense primers. For these experiments, 3 μg of cellular RNA provided consistent differentiation in levels of these inducible gene products. Based on the sequence position of the PCR primers, the products detected by internal probes on the Southern blot were 513 bp for TNF-β, 320 bp for Igκ light chain, and 636 bp for actin. The TNF-β amplified gene product showed a significant increase above control by 3 h of Tax_1 stimulation with increasing levels of TNF-β product at 8, 12, and 24 h of stimulation (Fig. 7A). The hybridization probe for TNF-β detected three bands on the Southern blot. The 513-bp product corresponds to the expected product from spliced TNF-β mRNA. The upper two bands likely come from partially spliced mRNA. The Igκ light chain gene product was not significantly increased by 3 h of Tax_1 stimulation; however, increased levels were observed after 8 h of Tax_1 stimulation and remained at the same increased level at 12 and 24 h of stimulation (Fig. 7B). The actin control 636-bp product did not show an increase during Tax_1 stimulation (Fig. 7C).

DISCUSSION

To demonstrate induction of Igκ light chain and TNF-β genes by Tax_1 protein, we have used reverse transcription and PCR amplification of cellular RNA as described by Tendler et al. (41). The actin gene served as a constitutively active, noninducible control with several extracellular stimuli, including Tax_1, IL-1α, or LPS (data not shown). We observed differences in the time course of stimulation of Igκ light chain and TNF-β genes. The Igκ light chain gene product increased at 8 h after Tax_1 addition and did not change further at 12 or 24 h. The TNF-β amplified gene product showed increased levels above controls as early as 3 h and increased further at 8, 12, and 24 h of stimulation. These results indicate that the Igκ and TNF-β genes show temporal differences in expression in response to Tax_1 induction. Since both genes contain distinct NF-κB binding promoter/enhancer elements, it is possible that the regulation of these genes may be due to differences in the NF-κB proteins interacting with these elements.

It has recently been shown that the NF-κB DNA-binding proteins consist of a family of related transcription factors (7, 27). At least four NF-κB DNA-binding proteins have been identified in phorbol myristate acetate-induced human

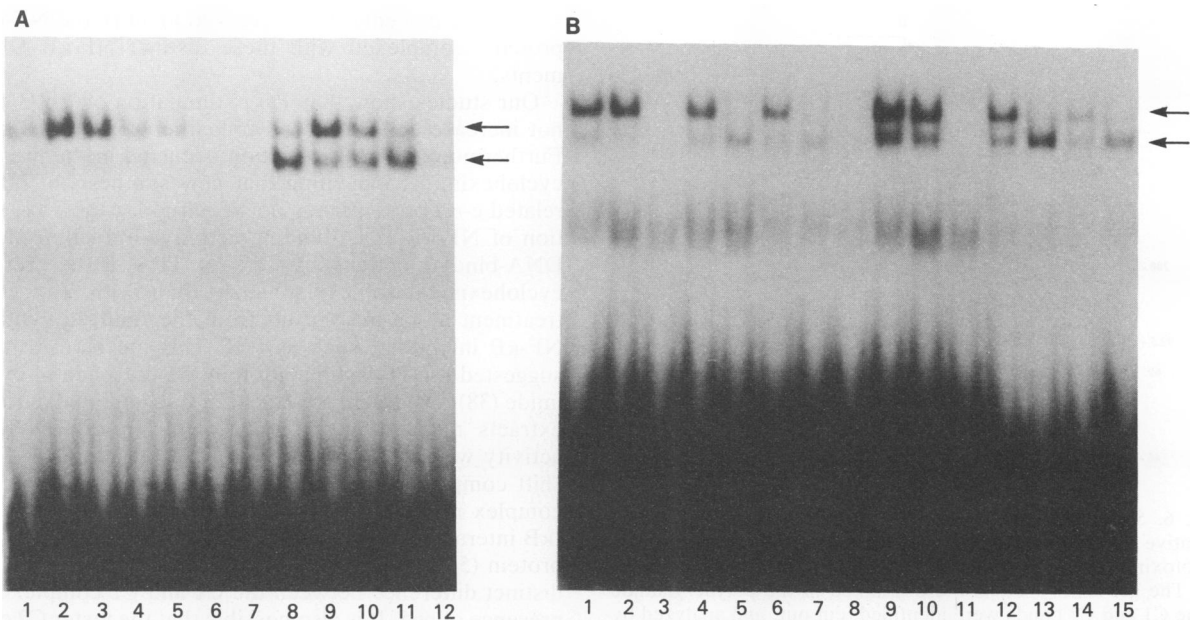

FIG. 4. (A) Time course assay comparing Igκ light chain and lymphotoxin NF-κB DNA gel shift activity during Tax_1 stimulation. 70Z/3 cells received stimulation with purified recombinant Tax_1 protein for 15 min (lanes 1 and 8), 1 h (lanes 2 and 9), 3 h (lanes 3 and 10), 7 h (lanes 4 and 11), or 24 h (lanes 5 and 12) or treatment with control bacterial extract for 7 h (lane 6) or 24 h (lane 7). Gel shift assays were performed with the Igκ light chain (lanes 1 to 7) or lymphotoxin (TNF-β) NF-κB (lanes 8 to 12) probe from the prepared nuclear extracts. The specific gel shift complexes identified with competitor DNAs are indicated as a slower-moving (C1) and a faster-moving (C2) complex. (B) Assay of NF-κB gel shift activity in the presence or absence of purified IκB. 70Z/3 cells received 3 h of stimulation with either TPA at 50 ng/ml (lanes 1 to 5 and 9 to 13), Tax_1 protein at 1 μg/ml (lanes 6, 7, 14, and 15), or chloroform-extracted Tax_1 (lane 8). Gel shifts were performed with 6 μg of nuclear extract with −2 ng of either the Igκ light chain (lanes 1 to 8) or lymphotoxin (TNF-β) NF-κB (lanes 9 to 15) probe. Purified IκB was added to some reactions (lanes 5, 7, 13, and 15). The specific slower-migrating C1 and the faster-migrating C2 complexes (arrows) were identified by incubation of the reactions in the presence of a 100-fold excess of mutant (lanes 2 and 10) or wild-type (lanes 3 and 11) competitor DNA.

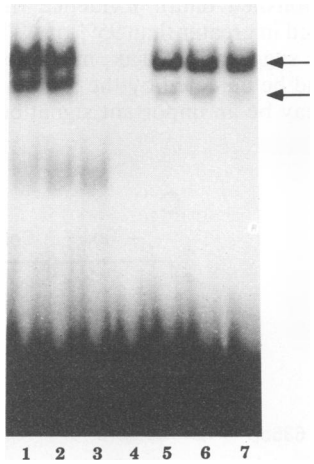

FIG. 5. Gel shift assay showing effect of detergent on cytoplasmic NF-κB binding to the TNF-β DNA probe. Gel shift assays were performed with TPA-stimulated nuclear extracts (6 μg each) (lanes 1 to 3) or unstimulated cytoplasmic extracts (6 μg each) (lanes 4 to 7), using 2 ng of labelled TNF-β probe. The cytoplasmic extracts received either no additional treatment (lane 4), DOC and NP-40 (lane 5), or DOC and CHAPSO (lane 6 and 7). The migration of specific NF-κB gel shift complexes (arrows) was obtained by incubation of the nuclear extract gel shift reactions with a 100-fold excess of mutant (lane 2) or wild-type (lane 3) competitor DNA.

T cells, including p50 (NF-κB), p55, p75, and p85 (c-*rel*). Although p55 and p75 were increased as little as 20 min after phorbol myristate acetate stimulation, p50 and p85 appeared only after several hours, implying that these factors may be regulated by different mechanisms (27). Several studies have shown that p50 NF-κB exists as a cytoplasmic complex with p65 and the inhibitor IκB which is released during stimulation of cells by a signal transduction mechanism (4, 5, 13, 38, 45). Recently, the gene for p50 has been cloned. The full-length cDNA encodes a 105-kDa protein which exists as a precursor, likely bound to the cytoskeleton via ankyrin repeats (14, 20). It is postulated that the 105-kDa precursor is proteolytically processed to the DNA-binding protein, p50 NF-κB. NF-κB complexes may consist of a p50-p50 homodimer or a more transcriptionally active p50-p65 heterodimer (14, 36). How the p65 is able to confer transcriptional activation to the complex is not certain, but it may stabilize the NF-κB DNA-binding complex or present an activating domain not present in the p50 protein (2). Recent evidence shows that p65 possesses weak DNA-binding activity and in the presence of p50 the DNA-binding activity is greatly increased (2, 36). Homodimers consisting of p50 show a greater tendency to bind palindromic NF-κB sites, while p50-p65 heterodimers bind to NF-κB sites with less symmetry, and possibly extend the range of NF-κB binding sites (46). In addition, the more palindromic TNF-β NF-κB site contains one less nucleotide for incorporation of BrdU, perhaps causing p65 to be less efficiently cross-linked to this probe by UV radiation. The identities of the protein-DNA complexes seen in our UV cross-linking analyses are not yet

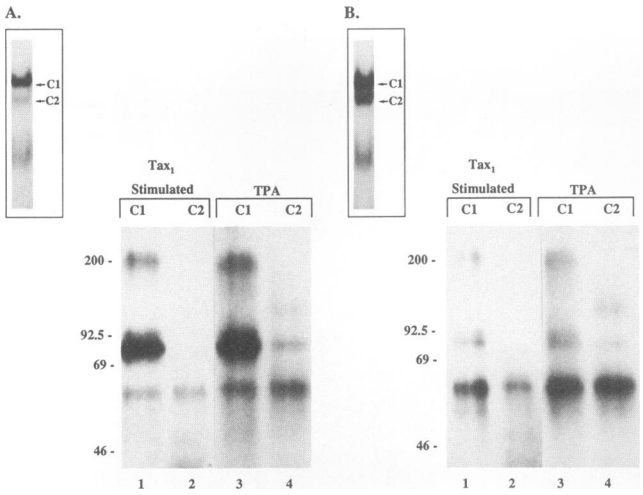

FIG. 6. SDS-PAGE analysis of DNA-protein complexes from preparative gel shift assays. A BrdU-labelled Igκ light chain (A) or lymphotoxin (TNF-β) (B) NF-κB DNA was used for preparative gel shifts. The gel shifts were UV cross-linked in the polyacrylamide gel. The C1 and C2 bands were identified, cut out, and analyzed by 7.5% SDS-PAGE. Tax_1 stimulated 70Z/3 nuclear extracts (lanes 1 and 2) were analyzed in parallel with TPA-stimulated 70Z/3 nuclear extracts (lanes 3 and 4). The slower-migrating C1 complexes contain predominantly 75-, 186-, and 59-kDa proteins, while the faster-migrating C2 complexes are composed predominantly of the 59-kDa complex.

determined. Given the Tax_1 induction of NF-κB gel shift complexes in the presence of cycloheximide and the sensitivity of only the C1 complex to IκB, we suggest that the p59 protein is likely p50 and the p75 protein is p65. The difference in molecular weight is likely due to the DNA cross-linked to the protein. Our observation that the more palindromic TNF-β probe shows a greater proportion of C2 complexes which contain the p59 protein, and the less palindromic Igκ light chain NF-κB probe shows a greater proportion of p75 in the NF-κB gel shift complex, is consistent with this model. Alternatively, the differences in molecular weight could be due to the differences of NF-κB present in human versus mouse cells. Using immunologic reagents, studies are currently in progress to identify the NF-κB/c-*rel* proteins complexed with these distinct NF-κB DNA elements.

Our studies show that Tax_1 stimulation of 70Z/3 cells did not increase cellular RNAs for either p50 NF-κB or c-*rel*. Furthermore, NF-κB induction occurred in the presence of cycloheximide, indicating that new synthesis of NF-κB or related c-*rel* proteins was not required for the Tax_1 stimulation of NF-κB DNA-binding activity. Induction of NF-κB DNA-binding activity by Tax_1 or TPA in the presence of cycloheximide to levels higher than with Tax_1 or TPA treatment alone may result from the reduced synthesis of NF-κB inhibitors, such as IκB. This mechanism has been suggested for TPA stimulation in the presence of cycloheximide (38). Detergent treatment of unstimulated cytoplasmic extracts resulted in the release of NF-κB DNA-binding activity which comigrated with both C1 and C2 NF-κB gel shift complexes. Purified IκB inhibited only the slower C1 complex in our assays. It has been previously shown that IκB interacts with NF-κB through a 65-kDa transmodulator protein (5, 45, 46). Our results suggest, therefore, that one distinct difference between the C1 and C2 complexes is the presence of p65. It is also possible that the faster C2 complex was released from a second inhibitor distinct from IκB. Tax_1 protein was unable to prevent the reassociation of IκB with NF-κB in vitro. These and our previous studies (24) suggest that Tax_1 does not directly dissociate or regulate the binding of IκB.

In addition to its effects on the regulation of viral and cellular gene expression in HTLV-I-infected cells, the Tax_1 protein is capable of stimulating expression of cellular genes such as Igκ and TNF-β in uninfected cells. In addition, extracellular Tax_1 protein has been shown to stimulate proliferation of human peripheral blood lymphocytes (26). The Tax_1-stimulated lymphocytes also show increased expression of IL-2Rα (26a). Using several immunologic assays, we have demonstrated that the Tax_1 protein is present as an extracellular protein in HTLV-I-transformed cells (24; unpublished data). Evidence that antibodies to Tax_1 are detected in greater than 95% of ATL and TSP/HAM patients further suggests that Tax_1 may exist as an extracellular protein and be detected by the immune system. Extracellular Tax_1 may be an important signal between HTLV-I-

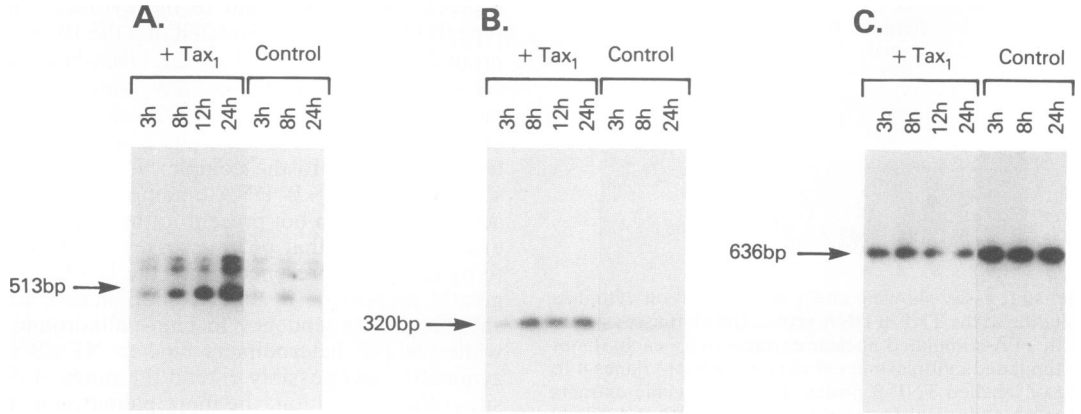

FIG. 7. Southern blot analysis of reverse-transcribed and PCR-amplified DNA from TNF-β and Igκ cellular RNA. 70Z/3 cells received treatment with the recombinant Tax_1 protein or control bacterial extract for the times indicated. Total cellular RNA was harvested, reverse transcribed, and PCR amplified (see legend to Fig. 2). The amplified products from the lymphotoxin (TNF-β) (A), Igκ light chain (B), and actin (C) genes were identified by Southern blot with probes specific for these genes. Actin was included as a noninducible control.

infected T cells and uninfected cells, leading to the altered regulation of immunoglobulin and TNF-β synthesis. Of considerable interest is the fact that the cytotoxicity factor, TNF-β, also functions as an osteoclast-activating factor stimulating bone resorption (42). Hypercalcemia, which frequently complicates HTLV-I-associated ATL, may result from stimulation of an osteoclast-activating factor, such as TNF-β, via transcellular stimulation by the Tax_1 protein. The role of extracellular Tax_1 in the stimulation of these and other cytokine genes containing the NF-κB enhancer element should provide important insight to the pathogenesis of HTLV-I infection.

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