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Human T-Cell Leukemia Virus Type I Tax Induces Expression of the Rel-Related Family of κB Enhancer-Binding Proteins: Evidence for a Pretranslational Component of Regulation

NAOMICHI ARIMA,[1] JERRY A. MOLITOR,[1] MATTHEW R. SMITH,[1] JEROME H. KIM,[1] YASUHISA DAITOKU,[2] AND WARNER C. GREENE[1]*

Department of Medicine and Department of Microbiology and Immunology, Howard Hughes Medical Institute, Duke University Medical Center, Durham, North Carolina 27710,[1] and Kagoshima University, Faculty of Medicine, The First Department of Medicine, 8-35-1 Sakuraga-oka, Kagoshima 890, Japan[2]

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The Tax protein of the human T-cell leukemia virus type I (HTLV-I) serves as a potent transcriptional activator of its own long terminal repeat as well as select cellular genes, including interleukin-2 and the alpha subunit of the interleukin-2 receptor. Tax activation of these two growth-related genes appears to involve the induced nuclear expression of DNA-binding proteins that specifically engage related κB enhancer elements present in the 5′ regulatory regions of these genes. In human T cells, κB enhancer-binding activity has been discerned as an unexpectedly large family of UV cross-linked nucleoprotein adducts, termed p50, p55, p75, and p85. The protein components of each of these DNA-protein adducts have been shown to share structural similarity with the v-*rel* oncogene product. The p55 adduct is composed of the 50-kDa subunit of NF-κB derived from a 105-kDa precursor polypeptide, while the p50 adduct contains a smaller protein that is closely related to NF-κB p50. The p75 adduct contains the 65-kDa subunit of NF-κB, while the p85 adduct is composed of the human c-*rel* proto-oncogene product. We now demonstrate that HTLV-I Tax, in the absence of other viral pX gene products, is capable of inducing the nuclear expression of all four of these κB-binding proteins in human T cells, with most marked effects involving c-Rel and NF-κB p65. Tax induction of the nuclear expression of c-Rel and NF-κB p50 is regulated, at least in part, at a pretranslational level involving increases in c-*rel* and NF-κB p105 mRNA expression. To study the pattern of expression of these κB-specific proteins in cells infected with the whole HTLV-I, seven cloned HTLV-I-infected T-cell lines were established from the peripheral blood of patients with adult T-cell leukemia. Of note, only three of these seven cell lines produced Tax, and c-*rel* mRNA and nuclear protein expression was confined to these three cell lines. In contrast, NF-κB p50 and NF-κB p65 were constitutively expressed in the nuclei of all seven of the HTLV-I-infected cell lines, even in the absence of detectable Tax or other viral gene expression. These findings raise the possibility of an alternate, Tax-independent pathway for the induced nuclear expression of NF-κB p50 and NF-κB p65 following HTLV-I infection.

The human T-cell leukemia virus type I (HTLV-I) has been etiologically linked with both the adult T-cell leukemia (ATL), an aggressive and often fatal neoplasm of $CD4^+$ T lymphocytes (44, 45, 58), and tropical spastic paraparesis or HTLV-I-associated myelopathy, a progressive demyelinating disease resembling but distinct from multiple sclerosis (21, 42). The HTLV-I provirus encodes two *trans*-acting regulatory gene products, Rex and Tax, that are both essential for viral replication and likely involved in viral pathogenesis (14, 49). The 27-kDa Rex protein regulates HTLV-I structural gene expression (*gag*, *pol*, and *env*) at a posttranscriptional level by specifically binding to a complex RNA stem-loop structure, termed the Rex response element (1, 8, 9, 24, 26, 27, 31, 50, 57). This protein-RNA interaction promotes cytoplasmic expression of the unspliced or singly spliced viral mRNAs that uniquely encode the HTLV-I structural and enzymatic proteins and thereby promote virion assembly (26, 27). In the absence of Rex, these viral mRNAs remain sequestered in the nucleus where they are either completely spliced or degraded (26).

In contrast to Rex, the HTLV-I Tax protein functions as a potent transcriptional activator of the retroviral long terminal repeat (LTR) leading to high level expression of all viral genes (14, 31, 54). In addition, Tax stimulates the expression of various cellular genes, including interleukin-2 (IL-2), IL-2 receptor-alpha (IL-2Rα), granulocyte macrophage colony-stimulating factor, and c-*fos* (16, 20, 30, 38, 52, 55). Tax induction of these cellular transcription units does not involve the direct interaction of this viral *trans*-activator with DNA. Rather, Tax acts in a more indirect fashion by altering the expression or activity of select host transcription factors (6, 10, 11, 28, 32, 35, 48, 53). Specifically, Tax activation of the HTLV-I LTR and c-*fos* proto-oncogene appears to involve altered activity of one or more members of the cyclic AMP response element binding protein/activating transcription factor (CREB/ATF) family of transcription factors (32, 53). In contrast, Tax activation of the IL-2Rα and IL-2 genes involves the induced nuclear expression of a family of κB enhancer-binding proteins (6, 10–12, 23, 28, 33, 35, 39, 40, 41, 47, 48, 53).

Early studies suggested that NF-κB-binding activity corresponded to a single 50-kDa DNA-binding subunit (NF-κB p50) noncovalently associated with a larger 65-kDa subunit (NF-κB p65) originally thought to lack DNA-binding activity (2, 51). Although these proteins are constitutively expressed in virtually every cell type studied, the DNA-binding activity of the NF-κB complex is uniquely regulated at a posttrans-

* Corresponding author.

lational level by its association with one or more cytoplasmic inhibitors, termed IκB (2, 3). The interaction of IκB with the NF-κB complex appears to be mediated through the NF-κB p65 subunit (4). NF-κB-binding activity can be induced in many cells by an array of stimuli, including phorbol esters, select growth factors, and various cytokines (10, 11, 36, 39, 43). These agents promote the rapid dissociation of IκB from the cytoplasmic complex (2–4), perhaps involving phosphorylation of this inhibitor (22). In turn, this disassembly process allows nuclear translocation of NF-κB p50 and NF-κB p65. Both the human (KBF1) (33) and murine (23) equivalents of NF-κB p50 have now been molecularly cloned and found to encode a 105-kDa precursor. This precursor lacks DNA-binding activity but is apparently posttranslationally cleaved to yield NF-κB p50, which does bind DNA. In addition, NF-κB p105 was noted to share striking N-terminal homology with the v-*rel* oncogene product and *dorsal* protein of *Drosophila melanogaster* (12, 23, 33, 39). Similarly, cDNAs encoding human and murine NF-κB p65 have been isolated and found to encode proteins containing both intrinsic κB-specific DNA-binding activity and N-terminal homology with v-*rel* (41, 47). In this regard, the v-Rel oncoprotein also corresponds to a specific κB enhancer-binding protein; however, this protein functions as a dominant repressor of NF-κB-directed transcription (7, 29, 46).

Other investigations have revealed the presence of an even larger family of inducible and Rel-related κB enhancer-binding proteins in human T cells (6, 40). Specifically, by in situ UV cross-linking techniques, four DNA protein adducts, termed p50, p55, p75, and p85, have been identified with an array of different κB enhancer elements and nuclear extracts isolated from phorbol ester-stimulated Jurkat T cells (40). The protein components of these adducts appear related to the Rel oncoprotein as each is specifically immunoprecipitated by anti-v-Rel antibodies (7). Of note, these four proteins are expressed in the nucleus in pairs with distinctly different kinetics after T-cell activation (40). Specifically, p55 and p75 appear in the nucleus of Jurkat T cells within minutes after phorbol ester induction, while p50 and p85 are not expressed in the nucleus until 4 to 16 h later. Recent studies have shown that the p55 and p75 cross-linked adducts contain the NF-κB p50 and NF-κB p65 subunits, respectively (4a, 7). Other studies (7) have shown that p85 is composed of the human c-*rel* proto-oncogene product (13). At present, the protein component of the p50 adduct remains less well defined. However, proteolytic cleavage of p50 and p55 with four different agents has revealed remarkably similar fragmentation patterns, suggesting that these proteins are closely related (40). The p50 DNA-protein adduct may represent a proteolyzed form of NF-κB p50, a differently processed form of the NF-κB p105 precursor polypeptide, or an alternately spliced form of the NF-κB p105 transcript. Alternatively, p50 could correspond to the product of a distinct but closely related gene.

In the present report, we have investigated the biological effects of the HTLV-I Tax protein on the activation of this Rel-related family of κB enhancer-binding proteins in human T cells. We now demonstrate that Tax induces the expression of all four of these DNA-binding proteins; however, it exerts its most prominent effects on the nuclear expression of p75 (NF-κB p65) and p85 (c-Rel). We further demonstrate that Tax activation of c-Rel and NF-κB p50 nuclear expression is not solely regulated at a posttranslational level but also involves Tax-induced increases in NF-κB p105 and c-*rel* mRNA expression. Finally, using clonal HTLV-I-infected T-cell lines isolated from patients with ATL who are either productively or latently infected with HTLV-I, we have unexpectedly found that p55 (NF-κB p50) and p75 (NF-κB p65) are expressed in the nuclei of these cells in a manner apparently independent of Tax production. In contrast, c-Rel nuclear expression is restricted to those HTLV-I-infected cell lines that produce detectable levels of Tax. These findings demonstrate that Tax activates the nuclear expression of c-Rel and suggest an additional Tax-independent mechanism for the induction of nuclear NF-κB p50 and NF-κB p65 in these virally infected T cells.

MATERIALS AND METHODS

Cell lines and preparation of nuclear extracts. The cloned HTLV-I-infected cell lines K3T, F6T, Su9T01, Oh13T, C8ID and S1T were established from the peripheral blood mononuclear cells of patients with ATL by culture in IL-2. These cell lines and HTLV-I-infected MT-2 cells, as well as control HTLV-I-negative Jurkat and CEM leukemic T-cell lines, were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin, and streptomycin. Southern blot analysis confirmed the presence of integrated HTLV-I proviruses in the genomes of each of the cloned cell lines established from the ATL patients (data not shown). Jurkat T cells were also stably transfected with the HTLV-I pX cDNA expression vector, spXHF/82C, and antisense pX cDNA expression vector, spXHF/82-NC, as previously described (55). Transient transfection of Jurkat T cells with wild-type or mutant Tax expression plasmids was performed by using DEAE dextran, as previously described (53). Nuclear extracts were prepared by the method of Dignam et al. (17), except that the addition of buffer B (17) was omitted. Alternatively, certain nuclear extracts were prepared by the method of Dynan (18).

Oligonucleotide probes and competitors. Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer and cleaved with ammonia. Oligonucleotides were recovered by using purification cartridges (Applied Biosystems) and further purified by preparative polyacrylamide gel electrophoresis (PAGE) (37).

In vitro DNA–protein-binding and UV cross-linking studies. Short, photoreactive κB duplexes were prepared by annealing 27 base templates from the human IL-2Rα gene (5, 6, 40) with 10 base primers and then by extension with the Klenow fragment of DNA polymerase I in the presence of 5-bromo-2′-deoxyuridine 3′-triphosphate and [α-^{32}P]deoxynucleoside triphosphates (56). Electrophoretic mobility shift assays (EMSAs) were performed as previously described (5, 40) with 5 to 10 μg of extracted nuclear proteins in the presence and absence of a 100-fold molar excess of unlabeled oligonucleotide competitors to determine the specificity of shifted nucleoprotein complexes. DNA–protein-cross-linking studies were also performed as previously described (5). Briefly, these photoreactive probes were incubated with ~5 μg of nuclear extracts and irradiated in solution with UV light (300-nm source at 5 cm for 30 min). Alternatively, in situ cross-linking was performed within the nondenaturing gel after EMSA, as previously described (40). Cross-linked samples were analyzed directly on sodium dodecyl sulfate (SDS)–7.5% polyacrylamide gels electrophoresed under reducing conditions.

Northern (RNA) blot analysis. Total RNA was prepared from each cell line by the method of Chirgwin et al. (15), and 20 μg of each sample was size fractionated on formaldehyde-agarose gels (37). After being transferred to nitrocellulose filters, the blots were hybridized with ^{32}P-labeled DNA

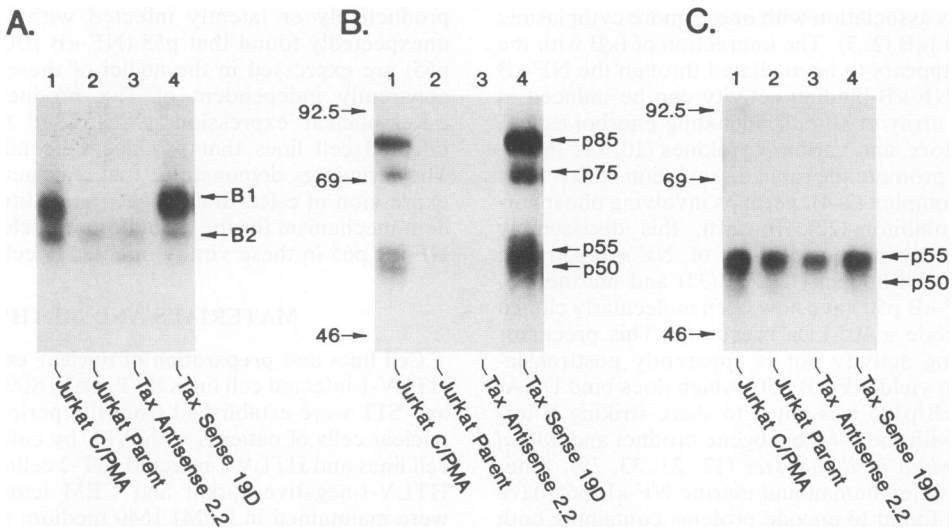

FIG. 1. The indicated cell lines were cultured in 7.5% fetal calf serum–RPMI, and nuclear extracts were prepared as described previously (17). The control Jurkat C cell line was stimulated for 16 h with PMA (50 ng/ml) prior to extract preparation (18). Nuclear extracts (10 μg) were incubated with 5-bromo-2′-deoxyuridine-substituted radiolabeled IL-2Rα κB probe, and the protein-DNA complexes were resolved from excess unbound probe by EMSA with nondenaturing 6.5% PAGE. The positions of the resultant B1 and B2 κB-specific shifted complexes are indicated (A). After EMSA, the wet polyacrylamide slab was exposed to 300 nM UV light (15 min; 7,000 μW/cm^2) to covalently cross-link B1- and B2-specific proteins to the probe in situ (40). After exposure of film to the wet polyacrylamide, gel slices from the B1 and B2 positions were excised individually and analyzed by discontinuous SDS–7.5% PAGE under reducing conditions (B and C, respectively). Positions of molecular weight markers are indicated on the left; the molecular weights of the specific DNA-protein adducts p50, p55 (NF-κB p50), p75 (NF-κB p65), and p85 (c-Rel) are indicated on the right.

fragments prepared by the random-priming method of Feinberg and Vogelstein (19) and washed as previously described (34). All filters were also probed with either γ-actin (a gift from Peter Gunning) (25) or glyceraldehyde 3′-phosphate dehydrogenase (GAPDH, obtained from the American Type Culture Collection) to assess any variations in mRNA loading.

Western blot analysis. Western blotting (immunoblotting) of nuclear lysates prepared from ATL cell lines was performed as previously described (53). Briefly, these lysates were subjected to electrophoresis on discontinuous SDS-10% polyacrylamide gels, and then the proteins were transferred to nitrocellulose membranes. Membranes were then probed with rabbit anti-Tax antisera directed against amino acid residues 321 to 353 of the Tax protein (anti-Tax; 1:2,000 dilution) and ^{125}I-protein A (10^4 cpm per cm^2 of nitrocellulose membrane). Immunoreactive proteins were visualized by autoradiography.

RESULTS

We have previously described human Jurkat T-cell lines stably transfected with HTLV-I pX cDNA expression plasmids (55). These cells constitutively express low but functionally significant amounts of the HTLV-I Tax protein. These cells also have the potential to encode other pX-derived gene products, notably the 27-kDa Rex protein. We have employed these cell lines to study the possibility that Tax may differentially induce the nuclear expression of select members of the Rel-related family of κB enhancer-binding proteins identified in the UV cross-linking assays.

By using the κB enhancer element from the IL-2Rα gene, the NF-κB-binding activity present in phorbol ester-activated Jurkat T cells can be resolved as two distinct nucleoprotein complexes, designated B1 and B2 (11, 40). Consistent with prior studies (6), expression of both the B1 and B2 nucleoprotein complexes was detected in the Tax-expressing Jurkat 19D cell line (Fig. 1A, lane 4) or control Jurkat T cells activated with phorbol-12-myristate-13-acetate (PMA) (lane 1). In contrast, only small amounts of the B2 complex were detected in the nuclei of the parental Jurkat T cells (lane 2) or in the nuclei of control Jurkat 2.2 T cells stably transfected with an antisense *tax* cDNA expression plasmid (lane 3). The formation of both the B1 and B2 nucleoprotein complexes was blocked by the addition of a 100-fold molar excess of unlabeled wild-type, but not mutant, IL-2Rα κB oligonucleotides, indicating the sequence-specific nature of these DNA-protein interactions (data not shown).

Prior UV cross-linking studies performed with PMA-induced nuclear T-cell extracts have revealed that the more rapidly migrating B2 complex is primarily composed of the p55 (NF-κB p50) and p50 adducts (40). In contrast, the more slowly migrating B1 complex contains not only these smaller proteins but also NF-κB p65 and c-Rel, which give rise to the two larger κB enhancer adducts, p75 and p85, respectively. To assess the pattern of induction of these specific κB-binding proteins by the HTLV-I Tax protein, in situ UV cross-linking studies were performed on the individual B1 and B2 complexes shown in Fig. 1A. The parental Jurkat cells and antisense Tax 2.2 cell line contained no detectable cross-linked proteins in the B1 position (Fig. 1B, lanes 2 and 3), while the Tax-expressing Jurkat 19 D cells (Fig. 1B, lane 4) and control Jurkat cells induced with PMA (Fig. 1B, lane 1) expressed all four cross-linked species, p50, p55, p75, and p85. In contrast, cross-linking analyses of the B2 complex revealed nuclear expression of p50 and p55 in all of the cell lines, which was unaltered by the expression of Tax (Fig. 1C, lanes 1 through 4).

As noted, the HTLV-I Tax protein does not appear to bind directly to DNA but rather acts by activating or inducing

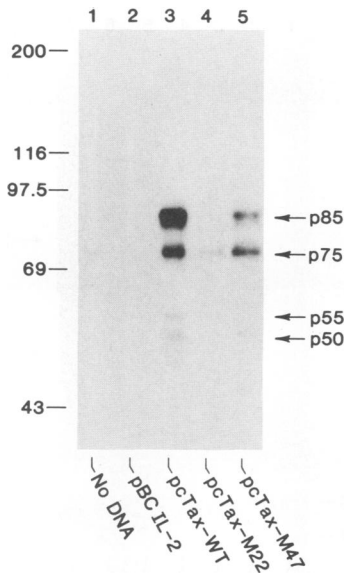

FIG. 2. Transient transfection of Jurkat T cells with HTLV-I Tax cDNA preferentially induces nuclear expression of the p75 (NF-κB p65) and p85 (c-Rel) κB-binding proteins. Wild-type or mutant Tax expression plasmids or the control pBC IL-2 vector was transiently expressed in Jurkat T cells with DEAE dextran, as previously described (53). After 48 h of culture, nuclear extracts were prepared followed by in situ UV cross-linking (40). The resultant B1 complex was excised and analyzed on denaturing SDS–7.5% polyacrylamide gels under reducing conditions. Positions of molecular weight markers and the molecular weights of the specific DNA-protein adducts are indicated.

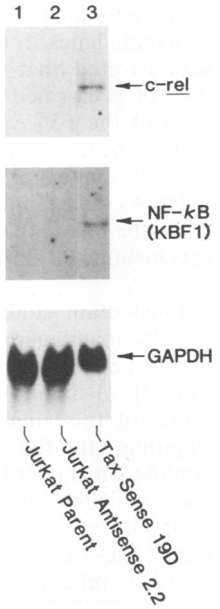

FIG. 3. Jurkat T cells stably producing HTLV-I pX cDNA constitutively express higher levels of c-*rel* and NF-κB p105 (KBF1) mRNA than found in control Jurkat cells. Total RNA was isolated, and 20-μg aliquots per lane were size fractionated on 1% agarose–2.2 M formaldehyde gels and then transferred to nitrocellulose. The filter was serially hybridized with three radiolabeled DNA probes, including a 1.85-kb *Hin*dIII-*Eco*RI fragment of c-*rel* from the pGEM85 plasmid (upper panel) (7); a *Hin*dIII-*Dra*I fragment of KBF1 from the pSK KBF1 plasmid (middle panel) (a gift from Alain Israel) (33) or a 0.78-kb *Xba*I-*Pst* fragment from the human 3-GAPDH plasmid (obtained from the American Type Culture Collection) (lower panel).

various host transcription factors, including the NF-κB and CREB/ATF families of enhancer-binding proteins (6, 10, 11, 28, 32, 35, 48, 53). Recently, mutants of the HTLV-I Tax protein that selectively interfere with Tax activation of these two transcription factor pathways have been identified (53). Specifically, the M22 Tax mutant fails to activate κB-enhancer-dependent promoters but almost normally activates cyclic AMP-response-element-dependent promoters. In contrast, the M47 Tax mutant fails to activate cyclic AMP-response-element-dependent gene expression but induces nearly normal κB-dependent gene expression. To conclusively demonstrate that Tax is the biologically active pX-derived gene product mediating the induction of this family of κB-binding proteins, Jurkat T cells were transiently transfected with expression plasmids encoding either the wild-type Tax protein or the mutated M22 or M47 versions of this viral *trans*-activator. Importantly, all of these expression vectors are, by design, incapable of directing the synthesis of the HTLV-I Rex protein because of the introduction of a mutation within the initiator codon for Rex. Nuclear extracts were prepared (18) from these transiently transfected Jurkat cells, and in situ cross-linking (40) was performed on the κB-specific B1 EMSA complexes. As shown in Fig. 2, neither mock-transfected nor control pBC IL-2-transfected Jurkat T cells displayed any detectable κB-binding proteins (Fig. 2, lanes 1 and 2). In contrast, transfection of the wild-type *tax* expression plasmid, pcTax, induced prominent nuclear expression of p75 (NF-κB p65) and p85 (c-Rel) and small but significant amounts of p50 and p55 (NF-κB p50) (lane 3). In contrast, transfection of the M22 Tax mutant failed to induce any of these κB-binding species (lane 4), while the M47 Tax mutant induced increases in all four species, with particularly prominent effects on p75 (NF-κB p65) and p85 (c-Rel) expression (lane 5).

Prior studies have suggested that NF-κB-binding activity is predominantly regulated at a posttranslational level through its sequestration in the cytoplasm by an inhibitor(s), termed IκB (2, 3). In this regard, it has remained unclear how the nuclear HTLV-I Tax protein activates expression of the latent cytoplasmic stores of these κB enhancer-binding proteins. Direct addition of partially purified Tax to cytoplasmic extracts has failed to induce the release of NF-κB-binding activity (52a). In view of these results, we considered the possibility that Tax might act at a pretranslational level by increasing mRNA expression for this Rel-related family of gene products. Indeed, mitogen- and cytokine-induced alterations in NF-κB p105 mRNA levels have recently been described (12, 39). The availability of human NF-κB p105 (KBF1, a generous gift from Alain Israel) (33) and c-*rel* cDNA (7, 13) probes permitted testing of this hypothesis. As shown in Fig. 3, c-*rel* mRNA was undetectable in both the unstimulated parental Jurkat and antisense Tax Jurkat 2.2 cell lines (upper panel, lanes 1 and 2) but was readily detected in the Tax-expressing Jurkat 19D cells (upper panel, lane 3). NF-κB p105 (KBF1) mRNA was also present in the Tax-expressing Jurkat 19D cells (middle panel, lane 3) but proved undetectable in the Jurkat parent and control antisense Tax Jurkat 2.2 cells (middle panel, lanes 1 and 2). Hybridization of this same RNA blot with a control glyceraldehyde 3′-phosphate dehydrogenase probe confirmed the

presence of nearly comparable quantities of mRNA in each of the lanes (bottom panel, lanes 1 through 3). In prior studies, we have demonstrated that each of these cells contain small quantities of preformed cytoplasmic protein mediating the formation of the p50-, p55-, p75-, and p85-cross-linked adducts (40). Thus, we predict that these cells must, in fact, constitutively express a low level of each of these corresponding mRNAs. Our failure to detect c-*rel* and NF-κB (KBF1) mRNA in the unstimulated control cell lines thus presumably reflects insufficient sensitivity in this Northern blotting assay.

Although the Tax transfection studies described above have permitted a relatively unambiguous assessment of the effects of this viral *trans*-activator on the expression of various members of the NF-κB family of enhancer-binding proteins, the transfection of this single viral gene almost certainly does not recapitulate the full spectrum of intracellular events accompanying the infection of cells with the whole HTLV-I. To assess the effects of HTLV-I infection on the expression of this family of κB enhancer-binding proteins, we have analyzed a series of seven cloned $CD4^+$ T-cell lines established from the peripheral blood of patients with HTLV-I-induced ATL. Each of these cell lines has been shown to contain integrated HTLV-I provirus at the time of analysis (data not shown). We first analyzed the expression of any HTLV-I mRNA in these cloned T-cell lines with a pX-derived viral probe that detects all known viral transcripts. Surprisingly, only three of the seven ATL cell lines expressed detectable viral mRNA (Fig. 4A). Consistent with these results at the RNA level, Tax protein production was only detected in the three viral RNA-positive cell lines by immunoblotting with a peptide-specific rabbit anti-Tax antibody (Fig. 4B). Thus, four of these cell lines appear latently infected with HTLV-I, while three are productively infected and express Tax.

The availability of these different cell lines allowed us to assess whether c-*rel* or NF-κB p105 (KBF1) mRNA expression was correlated with Tax production. The same RNA blot shown in Fig. 4 was rehybridized with cDNA probes specific for these κB-specific DNA-binding proteins. As shown in the upper panel of Fig. 5A, c-*rel* mRNA expression was only detected in the three Tax-expressing cell lines (lanes 5 through 7). In contrast, NF-κB p105 (KBF1) mRNA expression was present in all seven of the ATL cell lines as well as in the control Jurkat and CEM T-cell lines (Fig. 5A, middle panel, lanes 1 through 9). Probing with γ-actin confirmed nearly comparable quantities of hybridizing mRNA in each lane of the blot (Fig. 5A, lower panel, lanes 1 through 9). Thus, c-*rel* but not NF-κB p105 mRNA expression is correlated with viral gene expression and Tax production in these HTLV-I-infected cell lines.

Nuclear expression of each of the κB-specific protein components of the p50, p55, p75, and p85 adducts was next assessed by UV cross-linking of nuclear extracts prepared from each of these cell lines (Fig. 5B). As found at the mRNA level, nuclear expression of p85 (c-Rel) was confined to the three HTLV-I-infected cell lines producing Tax. In contrast, nuclear expression of p50, p55 (NF-κB p50), and p75 (NF-κB p65) was present in all of the ATL cell lines independent of the production of Tax; however, none of these proteins was detected in the nuclei of either the control Jurkat or CEM T-cell lines (lanes 8 and 9). These findings thus support a Tax-independent mechanism for the induced nuclear expression of NF-κB p50 and NF-κB p65 in the HTLV-I-infected T cells. In contrast, high-level nuclear

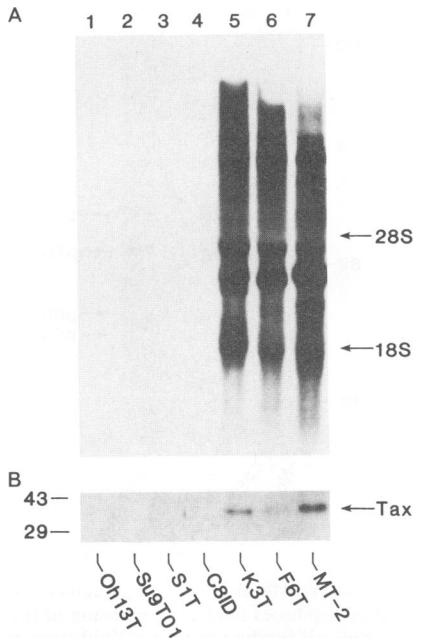

FIG. 4. Analysis of HTLV-I mRNA expression and Tax protein production in cloned HTLV-I-infected T-cell lines derived from patients with ATL. (A) Total RNA (20 μg per lane) from seven cloned ATL cell lines was size fractionated, blotted, and probed with a 0.19-kb *Sma*I-*Acc*I Tax fragment from the pcTax plasmid (53). The positions of 28S and 18S rRNA are indicated at the right. Predominant 2.1-, 4.2-, and 8.5-kb viral mRNA species (49) were detected in the K3T, F6T, and MT-2 cell lines (lanes 5 through 7) but not in the Oh13T, Su9T01, SIT, and C8ID cell lines (lanes 1 through 4). (B) Western blot analysis of Tax protein expression in nuclear extracts of these HTLV-I-infected T-cell lines. Five micrograms of each indicated nuclear extract was electrophoresed on discontinuous SDS–10% polyacrylamide gels and transferred to nitrocellulose membranes. The filter was probed sequentially with rabbit anti-Tax antisera and ^{125}I-radiolabeled protein A. The relevant region of the SDS-polyacrylamide gel containing the 40-kDa Tax protein is shown.

expression of c-Rel appears dependent on Tax production in the ATL cells.

DISCUSSION

In the present study, we have explored the inductive effects of the HTLV-I Tax *trans*-activator on each of the four recognized members of the Rel-related family of κB enhancer-binding proteins identified by UV cross-linking, including p50 (NF-κB p50 related), p55 (NF-κB p50), p75 (NF-κB p65), and p85 (c-Rel). Analyses of stable Jurkat T-cell transfectants constitutively expressing Tax (and perhaps other pX gene products) revealed the induced nuclear expression of all four members of this transcription factor family. Tax was confirmed as the active HTLV-I pX-derived activity in this response by transient transfection of Jurkat T cells with vectors that encoded Tax but not Rex. Marked induction of the nuclear expression of c-Rel and p75 (NF-κB p65) was observed in these transient transfection experiments, with more modest increases in the nuclear expression of p50 and p55 (NF-κB p50). Transfection of mutated versions of the HTLV-I *tax* gene (53), which functionally segregate the biological effects of Tax on the NF-κB and CREB/ATF families of transcription factors, produced

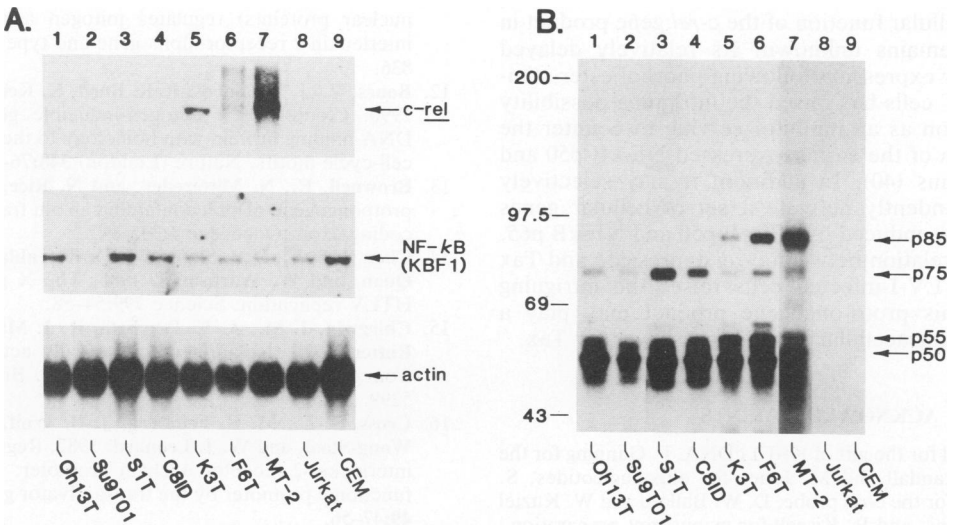

FIG. 5. (A) Analysis of c-*rel* and NF-κB p105 (KBF1) mRNA expression in the cloned HTLV-I-infected T-cell lines. Total RNA (20 μg) from each of the indicated cell lines was size fractionated, blotted, and hybridized with radiolabeled c-*rel* (upper panel), NF-κB p105 (KBF1) (middle panel), or γ-actin (lower panel) cDNA probes. (B) UV cross-linking analyses of κB-specific DNA-binding proteins expressed in the nuclei of the HTLV-I-infected T-cell lines captured with a photoreactive ^{32}P-IL-2Rα κB enhancer probe. The migration of the p50-, p55 (NF-κB p50)-, p75 (NF-κB p65)-, and p85 (c-Rel)-cross-linked adducts and known molecular weight standards are indicated.

sharply different effects on the nuclear induction of these κB enhancer-binding proteins. Specifically, the Tax-M22 mutant (Gly-137Leu→AlaSer), which is selectively deficient in its ability to activate κB-dependent transcription units (human immunodeficiency virus type I LTR and IL-2Rα promoter), failed to induce nuclear expression of any of the four κB enhancer-binding proteins. In contrast, the Tax-M47 mutant (Leu-319Leu→AlaSer), which lacks the ability to activate CREB/ATF-responsive promoters, such as the HTLV-I LTR, induced the nuclear expression of the κB enhancer-binding proteins, with the most prominent effects again involving p75 (NF-κB p65) and p85 (c-Rel).

It remained unclear how Tax activated the expression of these κB enhancer-binding proteins, particularly since all of these DNA proteins exist sequestered to some degree in the cytoplasm by an inhibitor (40) while Tax is predominantly expressed in the nucleus (31, 53). As noted, the addition of partially purified Tax protein to the cytoplasmic extracts of either resting Jurkat T cells or unstimulated HeLa cells has not revealed any release of NF-κB p50 or the other Rel-related proteins, suggesting that Tax may not act by directly disrupting these cytoplasmic complexes (52a). In view of the well-recognized transcriptional activating effects of Tax, we studied the possibility that Tax might act at a pretranslational level, serving to augment the expression of NF-κB p105 (KBF1) and c-*rel* mRNA. Indeed, analysis of the Tax-expressing Jurkat cell line 19D revealed significant expression of both c-*rel* and NF-κB p105 (KBF1) mRNA. In contrast, the basal expression of these mRNA species occurring in the control parental and Tax antisense Jurkat T-cell lines was below the level of detection of this assay. These findings support a pretranslational component for Tax induction of this family of enhancer-binding proteins. Similar increases in NF-κB p105 mRNA levels have been reported after mitogen, phorbol ester, or cytokine stimulation of cells (12, 39). Thus, it seems clear that the regulation of this family of κB enhancer-binding proteins is not exclusively controlled at a posttranslational level via their interactions with one or more cytoplasmic inhibitors. At present, it remains unclear whether these Tax-induced increases in c-*rel* and NF-κB p105 (KBF1) mRNA levels reflect enhanced transcription of the respective genes, or alternatively, stabilization of the preexistent mRNAs.

We have also studied the pattern of activation of these κB enhancer-binding proteins in T cells infected with the whole HTLV-I. This experimental situation may more closely resemble the intracellular events occurring in ATL than that produced by transfection of the isolated *tax* gene. A series of seven cloned HTLV-I-infected T-cell lines derived from patients with ATL were studied. Each of these cell lines contained integrated HTLV-I provirus; however, only three of the cell lines expressed detectable viral mRNA and Tax protein. The apparent latent nature of the HTLV-I infection in the remaining four cell lines permitted important comparisons in the potential Tax-dependent versus Tax-independent nature of nuclear expression of these various κB enhancer-binding proteins. Analysis of RNA in these ATL cell lines revealed that Tax production was correlated with increased c-*rel* mRNA expression. In contrast, NF-κB p105 (KBF1) mRNA was detected in all seven of the HTLV-I-infected cell lines as well as in two control leukemic T-cell lines not infected with this virus. At the protein level, nuclear expression of the p85 adduct containing c-Rel was also detected only in those cells expressing Tax. In contrast p50, p55 (NF-κB p50), and p75 (NF-κB p65) were expressed in the nuclei of all seven HTLV-I-infected cells, apparently independent of the production of Tax. These proteins, however, were not continuously expressed in the nuclei of the two control T-cell lines. Together, these findings raise the possibility of a Tax-independent pathway for HTLV-I induction of the nuclear expression of NF-κB p50 and NF-κB p65 proteins. Alternatively, it is possible that very small amounts of Tax are, in fact, present in the four negative cell lines and that nuclear NF-κB p50 and NF-κB p65 are more readily induced than c-Rel. However, this possibility is made less likely by our transient transfection results in which nuclear c-Rel and NF-κB p65 are preferentially induced by Tax compared with NF-κB p50. At present,

the precise intracellular function of the c-*rel* gene product in T lymphocytes remains unknown. Its relatively delayed kinetics of nuclear expression following phorbol ester stimulation in Jurkat T cells has raised the intriguing possibility that it may function as an inhibitor serving to counter the activating function of the earlier expressed NF-κB p50 and NF-κB p65 proteins (40). In addition, it may selectively target and independently activate a set of cellular genes different from those induced by NF-κB p50 and NF-κB p65. Certainly, the correlation between c-*rel* expression and Tax production in HTLV-I-infected cells raises the intriguing possibility that this proto-oncogene product may play a central role as an intracellular second messenger for Tax.

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