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Differential Expression of Rel Family Members in Human T-Cell Leukemia Virus Type I-Infected Cells: Transcriptional Activation of c-*rel* by Tax Protein

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The Tax protein of the human T-cell leukemia virus type I (HTLV-I) has been shown to induce nuclear expression of Rel family NF-κB-binding proteins. However, under different experimental conditions, different members of the Rel family were induced (N. Arima, J. A. Molitor, M. R. Smith, J. H. Kim, Y. Daitoku, and W. G. Greene, J. Virol. 65:6892–6899, 1991). In this study, using specific immunological reagents capable of distinguishing individual members of the Rel family proteins, we show that only c-Rel, not NF-κB p50 or p65, is induced in HTLV-I-infected cells. Preferential c-*rel* induction by HTLV-I infection was detected at the protein and RNA levels as well as in the nuclear NF-κB-binding form. Induced c-*rel* expression was also detected in cells stably transfected with *tax* cDNA, further correlating the c-*rel* induction with viral Tax expression. An increase in c-*rel* mRNA was detected within 3 h after induction of Tax expression, suggesting that this effect is at least partially regulated at the level of transcription. Furthermore, using a particle bombardment method for gene cotransfection, we show that Tax can transcriptionally activate the c-*rel* promoter in a T-cell line, Jurkat.

Human T-cell leukemia virus type I (HTLV-I) causes adult T-cell leukemia (43, 61) and tropical spastic paraparesis (17, 26), which is a progressive neurological disease resembling but distinct from multiple sclerosis. The HTLV-I provirus encodes two *trans*-acting proteins, Rex and Tax (10, 12, 50). Rex is a 27-kDa protein that regulates splicing and transport of HTLV-I mRNAs. The 40-kDa Tax protein has been shown to be a strong transcriptional activator that induces the expression of viral genes as well as a number of cellular genes, including those encoding various cytokines, the AP-1 family proto-oncogenes, nerve growth factor, vimentin, class I major histocompatibility complex, and others (4, 15, 20, 25, 34, 37, 42, 44, 49, 53, 57, 58).

Although Tax is capable of inducing expression of many genes, the mechanism of this transactivation is not clear. Tax appears to activate most of the known target genes, not through direct binding to their promoters but through the NF-κB or cyclic AMP-responsive element-binding protein or activating transcription factor and their binding sites (4, 27, 32, 35, 36, 44, 56). It has been proposed that Tax protein physically associates with cellular DNA-binding proteins to activate the expression of cellular or viral genes (7, 27, 36, 56, 63, 64). NF-κB was originally characterized as a multisubunit nuclear factor; its specific binding to immunoglobulin κ light-chain enhancer results in activation of κ-chain expression in B-cell development (51, 52). Recently, it was shown that the consensus κB enhancer-binding motif is present in many viral and cellular genes, suggesting that gene activation by NF-κB is likely involved in the transcriptional regulation of diverse genes. The best-characterized form of the NF-κB factor is a dimeric complex consisting of p50 and p65 subunits; both subunits were found to have DNA-binding and transcriptional activation capabilities (for a review, see reference 3; 32).

The recent molecular cloning of p50, p65, and other NF-κB-related genes revealed that all the NF-κB family genes share sequence homology with the v-*rel* oncogene, the c-*rel* proto-oncogene, and the *Drosophila melanogaster* dorsal gene which is involved in ventral-dorsal development (18, 19, 29, 40, 46, 47). These gene products also share functional similarities. All are transcriptional regulators that specifically bind to the consensus NF-κB motif. These proteins are located in both nuclear and cytoplasmic compartments of the cell, and their ability to activate transcription is tightly associated with their physical translocation into the nucleus. On the basis of the structural and functional similarities of these proteins, these proteins are now referred to as Rel family proteins (19).

HTLV-I infection induces nuclear expression of κB enhancer-binding proteins. Using an electrophoretic mobility shift assay (EMSA) and UV cross-linking, Ballard et al. (6) showed that these DNA-protein adducts, with molecular sizes of 85, 75, 55, and 50 kDa, contain different members of the Rel family proteins. The 85-kDa adduct contains the c-Rel protein p80, the 75- and 55-kDa adducts contain the NF-κB subunits p65 and p50, respectively, and the 50-kDa adduct contains a protein smaller than but closely related to the p50 NF-κB subunit (5, 6, 28). Arima et al. (1) recently showed that all four of these Rel family proteins were induced in a nuclear DNA-binding form by the HTLV-I Tax

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protein, in the absence of other viral gene products. However, various members of this family were induced under different experimental conditions. In Jurkat T cells transiently transfected with *tax* cDNA, the c-Rel and p65 nuclear κB enhancer-binding proteins were preferentially induced (1). In Jurkat cells stably transfected with Tax, the mRNA levels of c-*rel* and p105 (the p50 precursor) were augmented; however, in Tax-expressing cell lines established from HTLV-I-infected patients, only induction of c-*rel*, but not p50, correlated with Tax expression (1). These researchers suggested a pretranslational regulation of c-*rel* and p105 expression by Tax but did not distinguish whether enhanced transcription of the genes or enhanced stabilization of the mRNAs is involved.

In this study, we used specific antisera capable of distinguishing individual members of the Rel family proteins to show that p80 and a potentially novel p60 form of c-Rel, but not p50 or p65 κB, were induced in Tax-expressing cells. Furthermore, we used a c-*rel* promoter construct to demonstrate that Tax protein can transcriptionally activate the c-*rel* promoter.

MATERIALS AND METHODS

Cell lines and culture conditions. MT-2 (38), HUT102 (16), and 81-66 (48) are HTLV-I-infected T-cell lines. 81-66 is infected with a defective HTLV-I, and the only viral gene product it expresses is Tax protein (48). Hamilton (55) is a mature T-cell line derived from a T-cell lymphoma. Jurkat is a $CD4^+$ T-cell line, not infected with HTLV-I, established from a patient with T-cell leukemia (American Type Tissue Culture). J-tax is a Jurkat line stably transfected with the Tax expression plasmid MAX *neo* (41), which is under the control of a methallothionein promoter that can be induced by $ZnCl_2$ (10 μM) or phorbol-12-myristate-13-acetate (PMA) (50 ng/ml). All lymphoid cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 U of penicillin per ml, and 50 μg of streptomycin per ml.

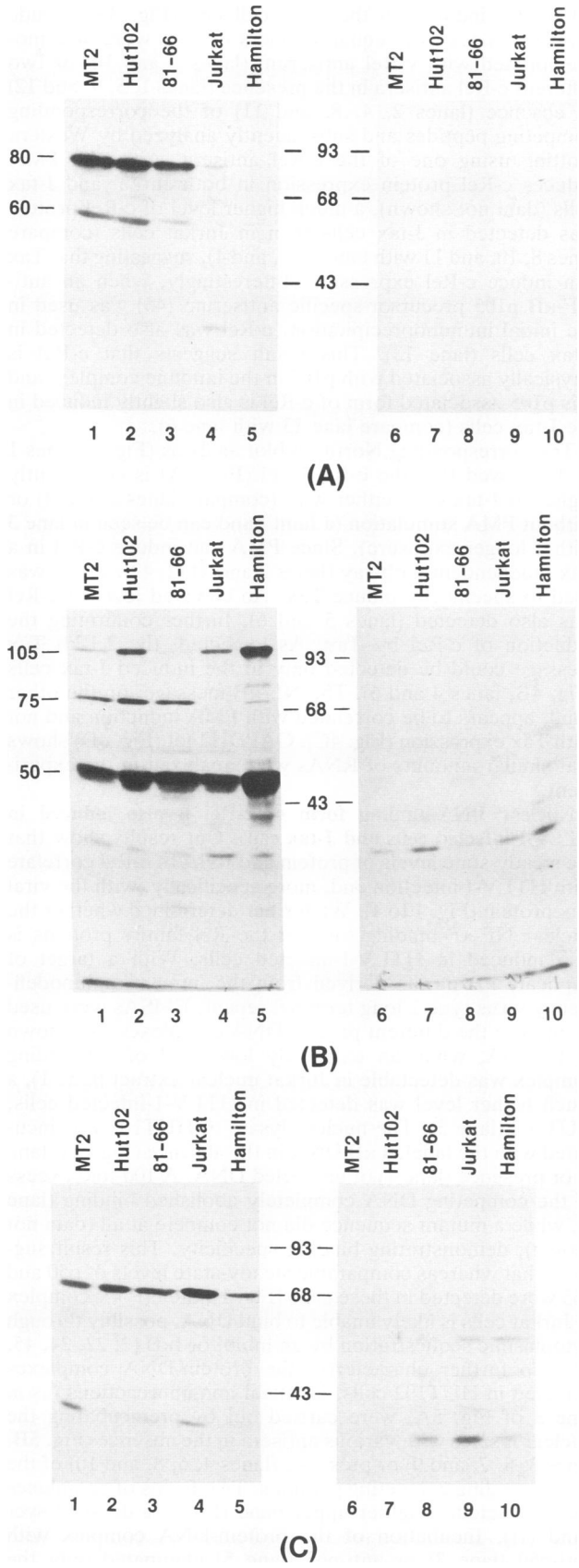
Western blot (immunoblot) and immunoprecipitation analysis of Rel family proteins. Crude protein (80 μg) (assayed with BCA Kit; Pierce) from each cell line was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and electrophoretically transferred onto polyvinylpyrrolidone membranes (Schleicher and Schuell) (33). After the membrane was blocked and washed, it was incubated with rabbit antiserum at a dilution (typically 1:5,000) and subsequently reacted with peroxidase-conjugated anti-rabbit immunoglobulin serum (Boehringer Mannheim, Corp.). Immunoreactivity was visualized by an enhanced chemiluminescence detection system (Amersham Corp.). In some experiments, equal numbers of cells were lysed in RIPA buffer (20 mM Tris hydrochloride [pH 7.6], 2 mM EDTA, 150 mM NaCl, 1% deoxycholate, 1% Triton X-100, 1% aprotinin, 70 μg of phenylmethylsulfonyl fluoride per ml, 40 μg of *N-p*-tosyl-L-phenylalanine chloromethyl ketone per ml, 5 μg of *N*-α-*p*-tosyl-L-lysine chloromethyl ketone, 5 μg of leupeptin per ml), and the cleared lysate was incubated with antisera with or without the presence of the competing peptide. The immune complexes were collected with protein A-Sepharose, washed with RIPA buffer six times, resolved by SDS-PAGE, and subjected to Western blotting with antisera as described above.

Antisera. All antisera are polyclonal rabbit antisera raised against either v-Rel fusion protein(s) (54) or synthetic peptides (45). Anti-c-Rel 265 and anti-c-Rel 1135 were raised against human c-Rel peptides corresponding to amino acids 573 to 587 and 493 to 509, respectively. Tax antisera (S182 and S183), generously provided by S. Oroszlan, were raised in two rabbits against Tax peptide corresponding to the C-terminal 15 amino acids. Anti-p105N 1141, anti-p105C 1140, and anti-p65C 1226 were made against synthetic peptides corresponding to amino acids 2 to 15, 955 to 969, and 537 to 550 of the corresponding NF-κB protein sequences, respectively (45).

Northern (RNA) blot analysis. Total RNA (30 μg) from each sample was used in Northern blot analysis (33). α-^{32}P-labeled (Random-Priming DNA Labeling Kit; Boehringer-Mannheim Corp.) probes that had specific activities greater than 10^9 cpm/μg were used. The membrane was washed with a solution containing 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% SDS at 55 to 65°C for 20 min and then autoradiographed with a double intensifying screen from 1 h to 7 days. The c-*rel* probe was an *Eco*RV-*Nde*I fragment containing the exons 5 to 7 of human cDNA (8, 9). The NF-κB p105 probe was a purified fragment containing the entire p50 cDNA (29). The Tax probe has been previously described (11), and the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe is a polymerase chain reaction-amplified 194-bp fragment as described previously (33).

EMSA. Nuclear extracts were prepared (13) from Jurkat, HUT102 and J-tax cells, and 10 μg was used in each binding reaction. The NF-κB-binding probe used was a double-stranded oligonucleotide (5′-ACAAGGGACTTTCCGCTGG GGACTTTCCAG-3′ was one of the strands) containing two κB motifs derived from the human immunodeficiency virus type 1 long terminal repeat. The probe was labeled with [γ-^{32}P]ATP (7,000 Ci/mmol) (Amersham Corp.) with T4 polynucleotide kinase, and 5×10^5 cpm was used in each reaction. The nuclear extract was preincubated with carrier DNA (10 μg of denatured salmon sperm DNA) for 5 min, followed by the addition of antiserum (1 μl) with or without the competing peptides (1 μg) for further incubation for 5 min before the addition of DNA probe. After a 40-min incubation at 20 to 25°C, the DNA-protein complex was resolved by 6% polyacrylamide gel electrophoresis and autoradiographed.

Luciferase assay and gene transfer by particle bombardment method. Gene transfer into Jurkat cells was performed by the Accell particle bombardment method as described previously (60). The reporter construct, chicken c-*rel* promoter linked to a luciferase gene (21), and the RSV-Tax expression plasmid (11) have been described elsewhere. Each plasmid DNA preparation was precipitated onto 0.95-μm-diameter gold particles at a dosage of 1.25 μg of DNA per mg of gold beads. In cotransfection experiments, the plasmid DNAs, each at 1.25 μg of DNA per mg of gold bead, were mixed in solution and coprecipitated onto the same bead preparation. DNA-coated beads were evenly distributed onto a Mylar sheet (surface area 3.24 cm^2) at a density of 0.05 mg of beads per cm^2. One million Jurkat cells in 25 μl of culture medium were spread onto a 35-mm-diameter petri dish, covering a surface area of approximately 3.24 cm^2, and bombarded for gene transfer at an optimized discharge voltage (7 kV). Immediately following bombardment, the cells were resuspended in 1.5 ml of fresh culture medium to establish continuous cultures. At 18 h postbombardment, cells in suspension were harvested and lysed in extraction buffer, and the supernatant was assayed for luciferase activity (60).



RESULTS

HTLV-I-infected cells express high levels of c-Rel, but not NF-κB p50 or p65. c-Rel protein expression was analyzed in a variety of HTLV-I-infected and noninfected cell lines by Western blot analysis using two antisera made against different regions of the carboxyl-terminal (C-terminal) domain of the human c-Rel protein. With both antisera, c-Rel reactivity was detected in proteins with molecular sizes of 80 (p80) and 60 (p60) kDa (result from one antiserum shown in Fig. 1A) which was not detected with preimmune sera (data not shown). The reactivities were completely abolished when the immune sera were preincubated with the corresponding competing synthetic peptides, demonstrating the c-Rel specificity of these proteins (Fig. 1A, lanes 6 to 10). Significantly higher levels of both p80 and p60 were detected in HTLV-I-infected cells, MT2, 81-66, and HUT102 (Fig. 1A, lanes 1 to 3), than in the control T cells, Jurkat and Hamilton (lanes 4 and 5). This result showed that in addition to the p80 c-Rel protein, a potentially novel p60, highly related to c-Rel, was overexpressed in HTLV-I-infected cells. Since HTLV-I infection has been shown to induce the nuclear expression of the Rel family κB-binding activity, we then evaluated whether the viral infection correlates with the levels of the NF-κB proteins p50 and p65. In contrast to the c-Rel overexpression, p50 (Fig. 1B) and p65 (Fig. 1C) were not expressed at higher levels in HTLV-I-infected cells (lanes 1 to 3) than in the control cells (lanes 4 and 5). Interestingly, a 75-kDa protein was concomitantly detected with p50 and p105 precursor proteins (Fig. 1B). While the identity of this protein remains unclear, it reproducibly exhibited specific immunoreactivity (compare Fig. 1B, lanes 1 to 5 and 6 to 10). The virus-negative line Hamilton contains the highest level of p50 and p105 NF-κB proteins, yet expresses almost undetectable levels of c-Rel. This experiment suggests that HTLV-I infection is correlated only with an elevated steady-state level of c-Rel protein, not with a change in the steady-state level of p50 or p65.

To evaluate whether the observed differential protein expression was the result of differential RNA expression, total cellular RNAs from the same panel of cells were analyzed by Northern blot analysis (Fig. 2). High levels of the 8-kb c-Rel message were detected only in HTLV-I-infected cells (Fig. 2A, lanes 1 to 3) and not in the control cells (lanes 4 and 5). Correlating with viral expression, an HTLV-I *tax* gene probe detected reactivity only in the virus-infected cells (Fig. 2B, lanes 1 to 3). On the other hand, the level of 3.8-kb NF-κB p105 message was low and was detected at a similar level among all tested cell lines except for Hamilton (Fig. 2C). A cellular gene, GAPDH, was used

FIG. 1. c-Rel proteins are induced in HTLV-I-infected cells. An aliquot containing 80 μg of crude protein from each cell line was resolved by SDS-PAGE (10% polyacrylamide), electrophoretically transferred to membrane, and reacted with immune serum at a dilution of 1:5,000 (lanes 1 to 5), or the immune serum was preincubated with the cognate synthetic peptide (lanes 6 to 10), Anti-c-Rel 1135 (A), anti-p105N 1141 (B), or anti-p65C 1226 (C), and the immunoreactivity was revealed by the enhanced chemiluminescence detection system. Identical results were obtained using the two c-Rel antisera, 1135 and 265. MT2 (lanes 1 and 6), 81-66 (lanes 2 and 7), and HUT102 (lanes 3 and 8) are HTLV-I-infected cell lines, and Jurkat (lanes 4 and 9) and Hamilton (lanes 5 and 10) are control T-cell lines that do not contain virus. The numbers at the sides of the gels are the molecular sizes (in kilodaltons).

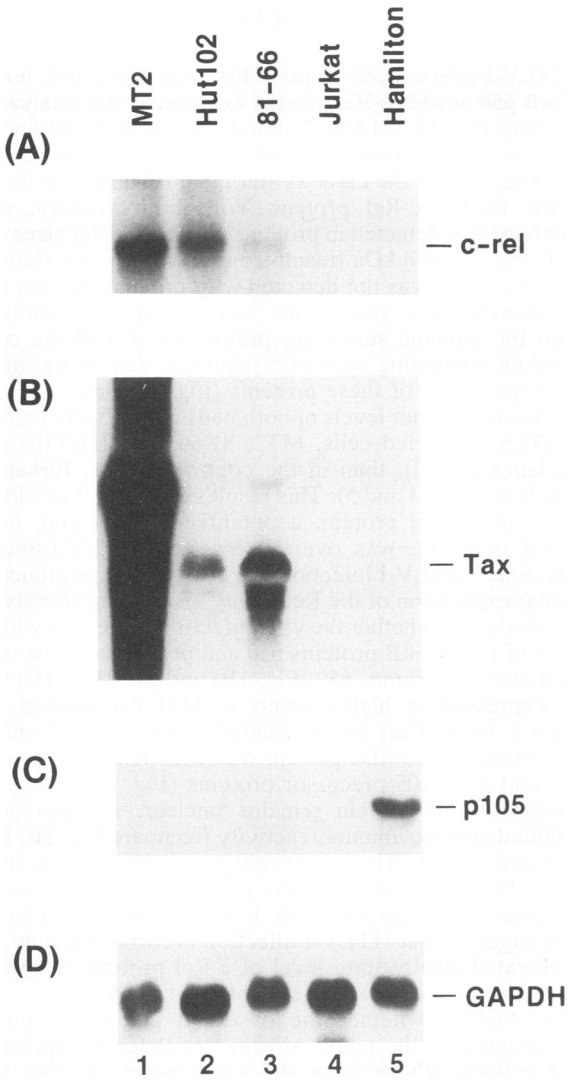


FIG. 2. c-*rel* RNA is elevated in HTLV-I-infected cell lines. Total RNA (30 μg) was extracted from each cell line and subjected to sequential Northern blot analyses using c-Rel (A), HTLV-I Tax (B), NF-κB p105 (C), and GAPDH (D) probes. The exposure times of the individual blots were 3 days (A), 1 day (B), 3 days (C), and 2 h (D).

as a control for normalizing the amounts of RNA analyzed in the Northern blot analyses (Fig. 2D).

c-Rel expression is induced in J-tax cell line. It is of interest that c-Rel is induced in the 81-66 cell line, which is infected with a defective HTLV-I that produces only one viral protein, the Tax gene product (55). The overexpression of c-Rel protein and RNA in this cell line suggested that the viral Tax protein alone might be sufficient to induce c-Rel expression. To evaluate this possibility, we performed further analyses on the J-tax cell line. Immunoprecipitation using anti-Tax C-terminal antisera followed by Western blot analysis showed that the Tax protein was readily detected in PMA-induced J-tax cells (Fig. 3A, lanes 5 and 7) but not in the control Jurkat cells (lanes 1 to 4). Preincubation of the antisera with the competing peptide completely abolished Tax reactivity, confirming the immunological specificity (lanes 6 and 8).

Similar experiments were performed to determine whether c-Rel was induced in the J-tax cell line (Fig. 3B). Crude cellular lysates from equal numbers of cells were immunoprecipitated with v-Rel antiserum (lanes 3 and 10) or two different c-Rel antisera in the presence (lanes 1, 5, 9, and 12) or absence (lanes 2, 4, 8, and 11) of the corresponding competing peptides and subsequently analyzed by Western blotting using one of the c-Rel antisera. Although PMA induces c-Rel protein expression in both Jurkat and J-tax cells (data not shown), a much higher level of c-Rel protein was detected in J-tax cells than in Jurkat cells (compare lanes 8, 10, and 11 with lanes 2, 3, and 4), suggesting that Tax can induce c-Rel expression. Interestingly, when an anti-NF-κB p105 precursor-specific antiserum (45) was used in the initial immunoprecipitation, c-Rel was also detected in J-tax cells (lane 13). This result suggests that c-Rel is physically associated with p105 in the immune complex, and this p105-associated form of c-Rel is also slightly induced in the J-tax cells (compare lane 13 with lane 6).

The corresponding Northern blot analysis (Fig. 4, lanes 1 to 4) showed that the c-Rel level (Fig. 4A) is consistently higher in J-tax cells either with (compare lanes 2 and 4) or without PMA stimulation (a faint band can be seen in lane 3 with a longer exposure). Since PMA can induce c-Rel in a Tax-independent pathway (lanes 2 and 4) (2, 42), $ZnCl_2$ was used to specifically induce Tax. An elevated level of c-Rel was also detected (lanes 5 and 6), further confirming the induction of c-Rel by Tax. As expected, the 2.1-kb Tax message could be detected only in the induced J-tax cells (Fig. 4B, lanes 4 and 6). The NF-κB message, on the other hand, appears to be correlated with PMA induction and not with Tax expression (Fig. 4C). GAPDH blot (Fig. 4D) shows that similar amounts of RNAs were analyzed in the experiment.

Nuclear DNA-binding form of c-Rel is also induced in HTLV-I-infected cells and J-tax cells. Our results show that the steady-state levels of protein and RNA of c-Rel correlate with HTLV-I infection and, more specifically, with the viral Tax protein (Fig. 1 to 4). We further determined whether the nuclear NF-κB-binding form of the Rel family proteins is also induced in HTLV-I-infected cells. With a target of duplicate κB motifs derived from the human immunodeficiency virus type 1 long terminal repeat, EMSAs were used to analyze the different protein-DNA complexes. As shown in Fig. 5A, while an extremely low level of κB-binding complex was detectable in Jurkat nuclear extract (lane 1), a much higher level was detected in HTLV-1-infected cells, HUT102 (lane 3). The nuclear lysate of HUT102 was incubated with the labeled κB DNA in the absence (Fig. 5A, lane 3) or presence (lane 4) of unlabeled DNA. A 100-fold excess of the competing DNA completely abolished binding (lane 4), while a mutant sequence did not compete at all (data not shown), demonstrating binding specificity. This result suggests that whereas comparable steady-state levels of p50 and p65 were detected in these cells (Fig. 1), the NF-κB complex in Jurkat cells is likely unable to bind DNA, possibly through cytoplasmic sequestration by an inhibitor IκB (2, 22, 24, 45, 62). To further characterize the protein-DNA complexes detected in HUT102 cells, identical immunoreactions (as in lane 3 of Fig. 5A) were carried out by preincubating the nuclear lysates with various antisera in the absence (Fig. 5B, lanes 3, 5, 7, and 9) or presence (lanes 4, 6, 8, and 10) of the corresponding competing peptides. Two forms of complexes were detected, a lighter upper band (I) and a darker lower band (II). Incubation of the protein-DNA complex with anti-p50 (lane 3) or anti-p65 (lane 5) eliminated only the

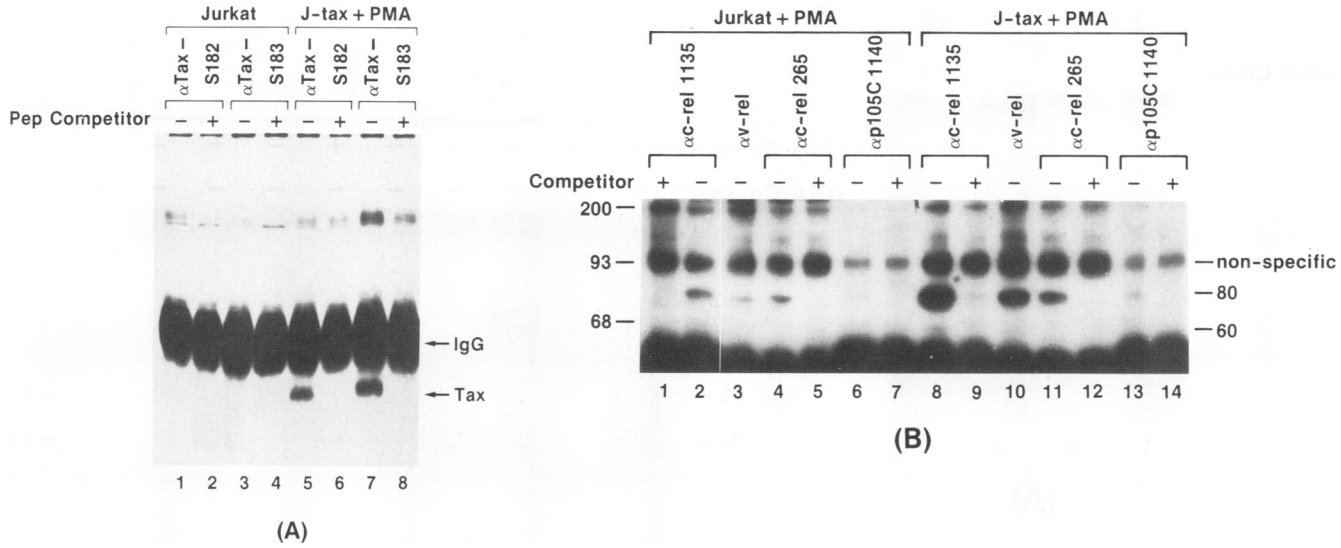
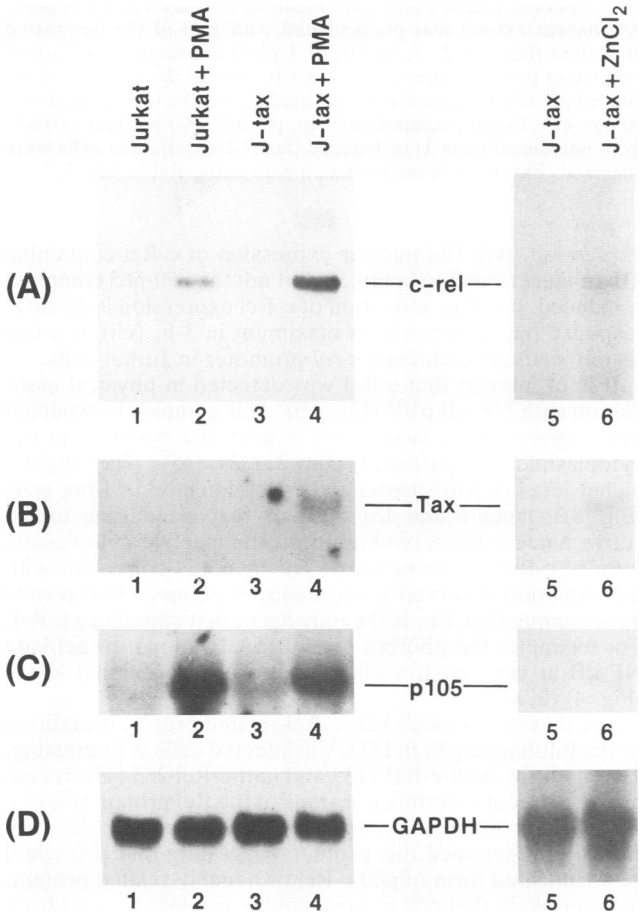


FIG. 3. Tax (A) and c-Rel (B) proteins are induced in J-tax cell line. Both Jurkat and J-tax cells were stimulated with PMA (40 ng/ml) for 16 h. (A) Equal numbers of cells were collected, lysed, immunoprecipitated with the designated anti-Tax antisera (S182 and S183) with (+) (lanes 2, 4, 6, and 8) or without (–) (lanes 1, 3, 5, and 7) the competing peptide (Pep Competitor). The washed immunoprecipitates were resolved by SDS-PAGE (10% polyacrylamide), transferred to membrane, immunoblotted with S182, and visualized by the enhanced chemiluminescence detection system. The heavy bands above the indicated Tax bands are rabbit immunoglobulin molecules. (B) Lysates from equal numbers of cells were immunoprecipitated with the indicated serum, anti-c-Rel 1135 (lanes 1, 2, 8, and 9), anti-c-Rel 265 (lanes 4, 5, 11, and 12), anti-v-Rel (lanes 3 and 10), or anti-p105C 1140. Immunoprecipitation was also performed in the presence (+) of the competing peptide (Competitor) (lanes 1, 5, 7, 9, 12, and 14). The immune complexes were processed as stated above for panel A and immunoblotted with anti-c-Rel 1135. The p80 and p60 c-Rel proteins are indicated. The p60 band in lane 11 could be detected after longer exposure (not shown). The numbers at the sides of the gel are molecular sizes (in kilodaltons).



upper band, not the lower band. On the other hand, incubation with anti-c-Rel antisera eliminated only the lower band (lanes 7 and 9). As a control, the competing synthetic peptides were added to the reaction mixtures, which resulted in complete restoration of the original complexes (lanes 4, 6, 8, and 10). This result suggests that the form I complex predominantly contains the conventional NF-κB complex, p50 and p65, and the form II complex contains the c-Rel protein. The c-Rel-containing form (band II) is much stronger than the κB-containing form (band I) in terms of the radioactive intensity contained in each complex. EMSA performed in J-tax cells (Fig. 5C, lane 1) and $ZnCl_2$-stimulated J-tax cells in the absence (lane 2) or presence (lane 3) of anti-c-Rel further showed that the c-Rel-containing NF-κB-binding complex was specifically induced. This result is consistent with our observation of the preferential induction of c-Rel in Tax-expressing HTLV-I cells and J-tax cells.

Kinetics of c-Rel RNA induction in J-tax cells. In order to evaluate the molecular mechanism(s) of c-Rel by Tax, a kinetic study was carried out. J-tax cells were stimulated with $ZnCl_2$, harvested at intervals during treatment, and analyzed by Northern blot analyses. As shown in Fig. 6, the RNA levels for both c-*rel* (Fig. 6A) and Tax (Fig. 6B) were maximum 3 h after $ZnCl_2$ treatment, indicating that c-Rel

FIG. 4. c-*rel* RNA is elevated in J-tax cells. Total RNA (30 μg) extracted from untreated (lanes 1 and 3), PMA-treated (40 ng/ml, 16 h) or $ZnCl_2$-treated (10 μM, 3 h) cells was analyzed in each lane. The same RNA blot was sequentially hybridized with the c-Rel (A), Tax (B), NF-κB p105 (C), and GAPDH (D) probes. The exposure time for each panel was approximately 3 days (A), 1 day (B), 5 days (C), and 5 h (D). Lanes 1 to 4 and lanes 5 to 6 are results from different experiments.

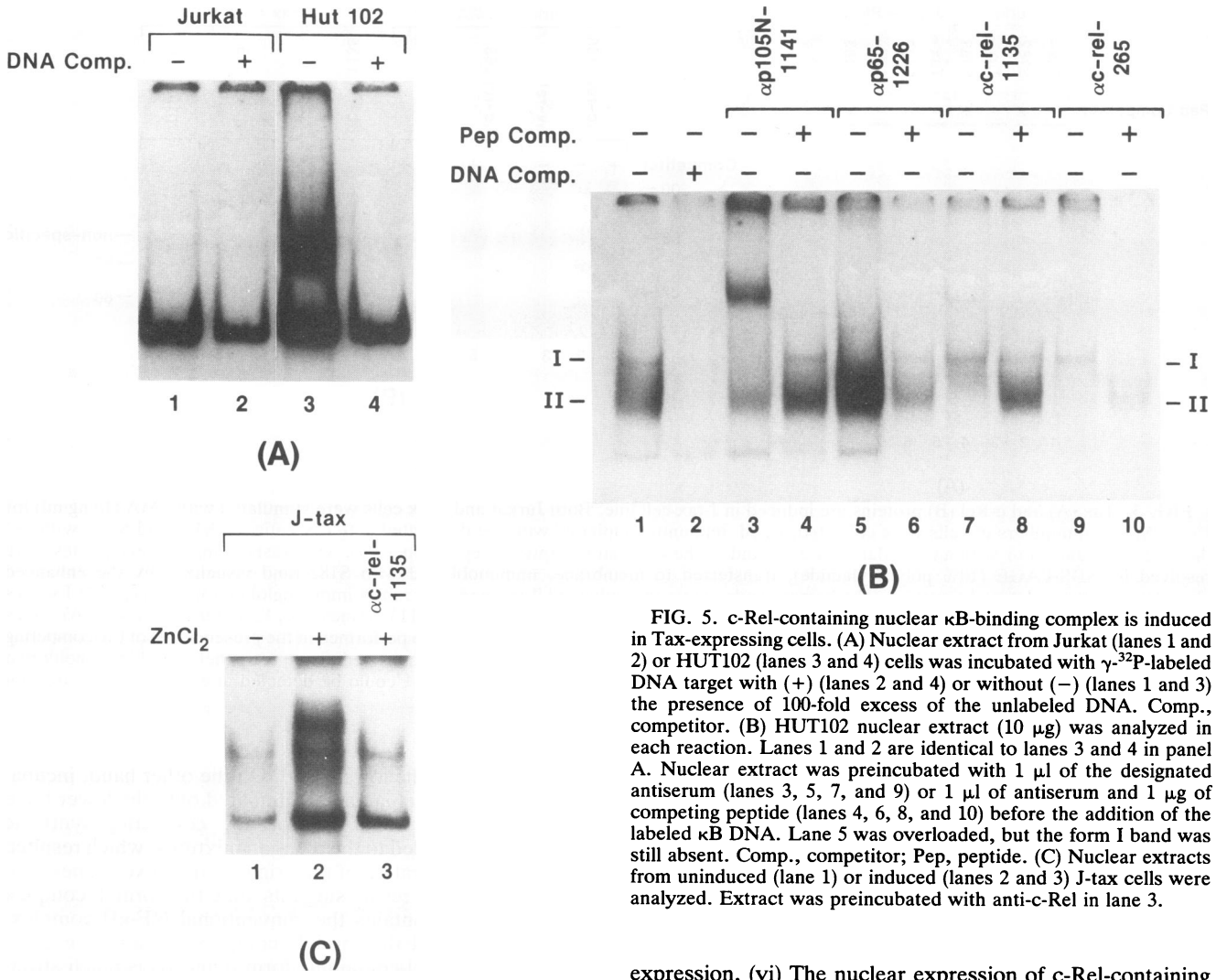


FIG. 5. c-Rel-containing nuclear κB-binding complex is induced in Tax-expressing cells. (A) Nuclear extract from Jurkat (lanes 1 and 2) or HUT102 (lanes 3 and 4) cells was incubated with γ-^{32}P-labeled DNA target with (+) (lanes 2 and 4) or without (−) (lanes 1 and 3) the presence of 100-fold excess of the unlabeled DNA. Comp., competitor. (B) HUT102 nuclear extract (10 μg) was analyzed in each reaction. Lanes 1 and 2 are identical to lanes 3 and 4 in panel A. Nuclear extract was preincubated with 1 μl of the designated antiserum (lanes 3, 5, 7, and 9) or 1 μl of antiserum and 1 μg of competing peptide (lanes 4, 6, 8, and 10) before the addition of the labeled κB DNA. Lane 5 was overloaded, but the form I band was still absent. Comp., competitor; Pep, peptide. (C) Nuclear extracts from uninduced (lane 1) or induced (lanes 2 and 3) J-tax cells were analyzed. Extract was preincubated with anti-c-Rel in lane 3.

induction is an immediate-early response. The control GAPDH expression is constant throughout the experiment (Fig. 4C).

HTLV-I Tax protein transactivates c-*rel* promoter. Since Tax upregulates c-*rel* RNA expression, we tested whether the Tax protein can activate c-*rel* promoter activity. The particle bombardment method (60) was used to cotransfect Jurkat cells. Transient gene expression assays using the luciferase reporter gene, with or without Tax expression vector, showed that Tax readily activated the c-*rel* promoter more than ninefold (Fig. 7). Using a similar cotransfection strategy, Rous sarcoma virus promoter activity was not activated by Tax (data not shown).

DISCUSSION

In this study, we have shown the following. (i) The steady-state levels of c-Rel protein and RNA, but not of p50 or p65, are consistently higher in HTLV-I-infected cells than in control T cells. (ii) A potentially novel form of the 60-kDa c-Rel-related protein is also induced in HTLV-I-infected cells. (iii) The level of c-Rel physically associated with the p50 precursor, p105, is elevated in HTLV-I-infected cells. (iv) The viral Tax protein alone is sufficient to induce c-Rel expression. (vi) The nuclear expression of c-Rel-containing κB-enhancer-binding complex, but not the p50-p65 complex, is induced. (vi) Tax activation of c-Rel expression is an early response that can reach its maximum in 3 h. (vii) Tax can readily activate a chicken c-*rel* promoter in Jurkat cells.

It is of interest that c-Rel was detected in physical association with NF-κB p105 (Fig. 3B). This complex is expected to be cytoplasmic, since p105 is normally confined in the cytoplasmic compartment (18, 22, 29, 45). The slightly higher level of p105-associated c-Rel detected in J-tax cells (Fig. 3B, lanes 6 and 13) suggests that in addition to the active nuclear DNA-binding form, the inactive cytoplasmic form of c-Rel is also induced. This result is consistent with the induction observed at the steady-state level. Our results do not imply that Tax is the only agent that stimulates c-Rel. For example, the phorbol ester PMA is known to activate NF-κB at both posttranslational and transcriptional levels (Fig. 4) (2, 40).

The detection of a 60-kDa c-Rel-related protein in addition to the full-length p80 in HTLV-I-infected cells is interesting. Two antisera, anti-c-Rel 1135 and anti-c-Rel 265 (45), recognizing different C-terminal regions of the Rel protein (Fig. 1), and an antiserum raised against a fusion protein (data not shown) all identified this protein, suggesting that p60 could be a truncated form of p80 c-Rel or a highly related protein. It is not likely that p60 is a proteolytic product derived from

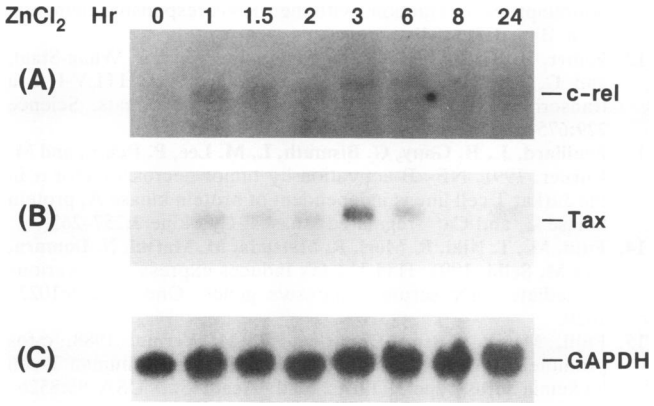


FIG. 6. Kinetics of c-Rel induction in J-tax cells by Northern blot analysis. J-tax cells were induced with 10 μM $ZnCl_2$ for the indicated hours. Total RNA (30 μg) extracted from each time point was subjected to Northern hybridization with c-Rel (A), Tax (B), or GAPDH (C) probe. The exposure time was 3 days (A and B) or 1 h (C).

p80, because virtually undetectable levels of p60 were observed in B-cell lines that constitutively express a high level of p80 analyzed identically (unpublished results). The origin of the p60 protein is currently under investigation.

Two forms of the NF-κB-binding complexes were detected in HTLV-I-infected cells (Fig. 5B). The form II c-Rel-containing complex is apparently present at a much higher level than the form I p50-p65 complex. This result is consistent with our overall finding that c-Rel is preferentially induced in HTLV-I-infected cells. We cannot conclude from this experiment that the form II complex contains only the c-Rel protein. It is conceivable that there may be other Rel family proteins also present in the form II complex for which we do not have an appropriate reagent to assay. Our results are similar but more consistent than that reported by Arima et al. (1). These researchers showed a much lower level of p50 and p105 message than that of c-Rel in HTLV-I cell line MT-2. In contrast, using UV cross-linking to analyze the DNA-binding complexes, they showed a much higher level of p50 than of c-Rel. In this study, consistent with our protein and RNA analyses, we detected higher levels of the DNA-binding form of c-Rel than p50 in similar cell lines including HUT102 (Fig. 5) and others (data not shown).

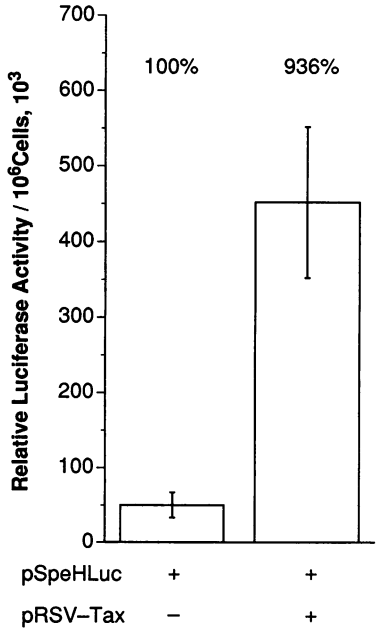


FIG. 7. HTLV-I Tax protein activates chicken c-*rel* promoter. Transfection and luciferase assay were described in Materials and Methods. The relative luciferase activity is presented in an arbitrary unit, and each number represents an average of four replicates.

The kinetic study showed that c-Rel message is elevated concomitantly with the expression of Tax, suggesting transcriptional activation. This induction is probably mediated by Tax through the NF-κB element present in the c-*rel* promoter (21). The quick induction result also agrees with a recent report showing that Tax can induce various immediate-early, serum-responsive genes (14). After 3 h, both Tax and c-Rel messages declined. This result suggests that c-Rel expression is highly regulated by a specific cellular mechanism(s), as has been frequently observed for the immediate-early responses of other proto-oncogenes. It is also consistent with the autoregulation of c-Rel expression proposed by Hannink and Temin (21). We further examined whether the viral Tax protein can activate the c-*rel* promoter. A particle bombardment method was used to efficiently cotransfect Tax and reporter genes into Jurkat cells. In the presence of Tax, the c-*rel* promoter was activated more than ninefold, whereas no significant activation was detected with a Rous sarcoma virus promoter (data not shown).

Tax is a potent transcriptional activator that has been suggested to be critical in tumorigenesis in transgenic mice (23, 39) and the maintenance of the transformed phenotype (59). In HTLV-I-infected patients, the *tax* gene was found to be preferentially retained (30), suggesting an important role in the leukemogenesis of adult T-cell leukemia. Tax strongly stimulates transcription of its own viral genome and many cellular genes. Tax is not a conventional DNA-binding protein; Tax probably indirectly mediates various cellular transcription factor(s) to activate the viral gene. The recent finding (7, 64) of physical association of Tax and the cyclic AMP-responsive element-binding protein supports this notion. The mechanism involved in Tax induction of the nuclear expression of the NF-κB-binding protein is less clear. The mechanism does not appear to be posttranslational, since the addition of partially purified Tax to cytoplasmic extracts failed to induce the release of NF-κB-binding activity (1). Our result points to a possible mechanism that may at least partially explain the induction of NF-κB-binding protein. The c-*rel* promoter contains a consensus NF-κB motif (21). Upon Tax stimulation, a low level of preexisting NF-κB complex (either with or without c-Rel) can turn on c-*rel* transcription. When more c-Rel proteins are available, a c-Rel-containing NF-κB complex will be formed and further activate c-*rel* transcription. By analogy to the mechanism proposed for the activation of the viral promoter, it will be important to determine whether Tax physically associates with any Rel family proteins to activate the c-*rel* promoter.

The preferential induction of c-Rel over the prototypical p50 or p65 NF-κB may play an important role in HTLV infection. Ballard et al. (6) showed that there is a distinct biphasic kinetics present in the nuclear induction of the Rel family proteins in T cells. The nuclear induction of active p50 and p65 upon stimulation takes place within minutes, whereas the nuclear appearance of p80 c-Rel and another

molecule (which is smaller but related to p50) does not take place until hours later. This result suggests that different members of the Rel family proteins, although sharing many structural and functional similarities, can behave differently in subtle ways. The quick induction of the nuclear binding form of p50 or p65 in T cells may represent an effective regulation at the posttranslational level that can respond instantly to certain biochemical or cellular stimuli. The slow induction of other members of the Rel family protein may involve de novo synthesis, as in the case of c-Rel. Late induction may bear subtle differences from early induction such that it provides another level of regulation, such as fine-tuning or surveying the first-level control. It is important to study the differences among the Rel family members in terms of their physical association with one another and with other cellular proteins and their interactions with various target genes (31). Information obtained in such studies will allow us to better understand the mechanisms involved in regulation of Rel family proteins and leukemogenesis by HTLV-I.

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