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HTLV-I TAX INTERACTIONS WITH THE NF-κB/IκB REGULATORS OF TRANSCRIPTION

by

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À Richard ainsi qu'à Nicole, Isabelle et ma famille
pour leurs encouragements de tous les instants

À Annisabelle, pour son sourire

"Vous aimerez les sciences si les gens qui vous les ont enseignées les aimaient et s'ils ont eu du plaisir à vous les enseigner"
Hubert Reeves

Abstract

The human T cell leukemia virus type I (HTLV-I) is the etiological agent of adult T cell leukemia. The oncogenic potential of HTLV-I resides in the viral Tax protein which behaves as a strong transcriptional activator of viral gene expression. Tax is also capable of *trans*-activating numerous growth regulatory genes by an indirect mechanism. One of the targets of Tax protein is the NF-κB/IκB family of transcriptional regulators. The objective of this thesis was to characterize the molecular mechanisms by which Tax interacts with the NF-κB/IκB proteins to initiate aberrant gene expression. HTLV-I infected and Tax expressing T cell models were initially used to demonstrate a constitutive level of NF-κB DNA binding activity and increased NF-κB-dependent transcriptional activity. NFKB2 and c-Rel subunits were overexpressed in these cells and constituted the majority of the NF-κB/Rel heterodimers binding to DNA. A reduction in NF-κB p65 nuclear expression and DNA binding activity was also observed. Importantly, a novel complex composed of NFKB2 p100 and Tax was detected in HTLV-I infected cells, thus demonstrating physical interaction between viral and cellular proteins. These *in vivo* observations were confirmed in Tax-NF-κB co-transfection studies which demonstrated a Tax-dependent correlation between expression of NFKB2 p100 and processing to p52, induction of c-Rel, and *trans*-activation of NF-κB-mediated gene expression. A co-transfection-immunofluorescence assay provided additional support for direct Tax-NF-κB physical interactions by demonstrating the ability of p100, p105 and p52 to modify the intracellular localization of Tax. The possibility that constitutive NF-κB activity resulted from a Tax-mediated effect on IκBα function was also examined. Constitutive phosphorylation and increased turnover of IκBα were observed in HTLV-I infected and Tax expressing T cells. In these cells, *mad*-3 (IκBα) mRNA expression was increased 7 to 20 fold, although the steady state level of the protein was reduced. These results indicated that the viral Tax protein may indirectly mediate phosphorylation of IκB, thus targeting IκBα for degradation, leading to constitutive NF-κB activity. Together these studies demonstrated that Tax has multiple effects on NF-κB/IκB proteins. These protein-protein interactions contribute to alterations in host gene regulation, which ultimately lead to HTLV-I-induced T cell proliferation and leukemogenesis.

Résumé

Le virus HTLV-I (human T cell leukemia virus type I) est l'agent étiologique de la leucémie des cellules T chez l'adulte. Le potentiel oncogénique de ce virus réside dans la protéine virale Tax qui est un puissant transactivateur de transcription des gènes viraux. Tax est également capable de transactiver d'une manière indirecte de nombreux gènes contrôlant la croissance cellulaire. Une des cibles de la protéine Tax est la famille de régulateurs transcriptionnels NF-κB/IκB. L'objectif de cette thèse était de caractériser les mécanismes moléculaires par lesquels Tax interagit avec les protéines NF-κB/IκB pour initier une expression génique aberrante. Les cellules exprimant Tax ou celles qui sont infectées par HTLV-I présentaient une activité de liaision à l'ADN de NF-κB constitutive, ainsi qu'une activité transcriptionnelle dépendante de NF-κB plus élevée. Les sous-unités NFKB2 et c-Rel étaient surexprimées dans ces cellules et représentaient la majorité des hétérodimères se liant à l'ADN. De plus, l'expression nucléaire de la sous-unité p65 ainsi que son activité de liaision à l'ADN étaient réduites. Notamment, un nouveau complexe composé des protéines NFKB2 p100 et Tax fut détecté dans les cellules qui sont infectées par HTLV-I, démontrant ainsi une interaction physique entre des protéines virales et cellulaires. Ces observations *in vivo* furent confirmées par des co-transfections Tax-NF-κB qui démontrèrent une correlation dépendante de Tax entre l'expression de NFKB2 p100, la protéolyse de p100 à p52, l'induction de c-Rel, et la *trans*-activation dépendante de NF-κB. Des essais d'immunofluorescence fait sur des cellules transfectées transitoirement soutiennent l'hypothèse d'une interaction directe entre Tax et les protéines NF-κB en démontrant la capacité des sous-unités p100, p105 et p52 de modifier la localisation intracellulaire de la protéine Tax. La possibilité que l'activité constitutive de NF-κB soit le résultat d'un effet de Tax sur la fonction de l'inhibiteur IκBα fut également examinée. Dans les cellules infectées par HTLV-I ou dans celles qui expriment Tax, IκBα était phosphorylé d'une façon constitutive et son taux de renouvellement était augmenté 3 fois. L'expression du gène codant IκBα (*mad*-3) était également augmentée de 7 à 20 fois, bien que les niveaux protéiniques étaient réduits. Ces résultats indiquent que la protéine virale Tax, en promouvant la phosphorylation d'IκBα, probablement d'une manière indirecte, provoque une dégradation rapide de l'inhibiteur,

conduisant ainsi à une activité NF-κB constitutive. L'ensemble des résultats présentés ici révèle que Tax a des effets multiples sur les membres de la famille de régulateurs transcriptionnels NF-κB/IκB. Ces interactions protéine-protéine contribuent à dérégler l'expression génique dans les cellules infectées par HTLV-I, ce qui mène à une prolifération des cellules T, et au développement éventuel d'une leucémie.

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Preface of the Thesis

Work presented in this thesis represents what is known as the "Tax" project. Over the last five years, the candidate played the most active role in terms of design, execution and coordination of the experiments pertaining to this project. However, other people made significant contributions to the project as well and the candidate chose to present them in this thesis to provide a more complete picture of Tax-NF-κB/IκB interactions.

The candidate was totally responsible for experiments presented in Figures 5 to 10, 17 to 19, and 24, and was assisted by Normand Pépin, Louisa Petropoulos, and Jenny Garoufalis in experiments described in Figures 15, 23, and Table 5. Dr. Jacqueline Lanoix was responsible for experiments presented in Figures 11, 12, 14, 21, 22, and 25. Normand Pépin was responsible for the data presented in Figures 13, 16, and 20, as well as for the construction of the NF-κB/IκB expressing plasmids. Anne Roulston prepared NF-κB/IκB specific polyclonal antibodies, and kindly allowed me to present the "Biochemistry of NF-κB Activation" figure (Figure 4) that she recently prepared.

The candidate is grateful to the NIH AIDS Reagent Repository for providing the Tax polyclonal antibody, and HTLV-I infected cell lines, and to the following persons for contributing reagents and cells that were useful for the completion of this thesis: Dr. A. Bédard, Dr. A. Cochrane, Dr. W.C. Greene, Dr. A. Israël, Dr. G. Nabel, Dr. C. Rosen, and Dr. S. Ruben. Special thanks are given to Dr. N. Rice for helpful advice and discussion, and for providing the laboratory with peptide sequences, NF-κB specific antibodies, and the c-Rel expression plasmid.

The results presented in this thesis are, for the most part, described in the following manuscripts:

Lacoste J, Cohen L, and Hiscott J : NF-κB activity in T cells stably expressing the Tax gene of human T cell lymphotropic virus type I. *Virology* **184**: 553-562, 1991.

Lanoix J, Lacoste J, Pépin N, Rice N, and Hiscott J : Overexpression of NFKB2

and c-Rel : a mechanism for Tax-mediated *trans*-activation via the NF-κB/Rel signalling pathway. *Oncogene* **9**: 841-852, 1994.

Lacoste J, Lanoix J, Pépin N, and Hiscott J : Interactions between HTLV-I Tax and NF-κB/Rel proteins in T cells. *Leukemia* **8** (supp.1): S71-S76,1994.

Pépin N, Roulston A, Lacoste J, Lin R, and Hiscott J : Subcellular redistribution of HTLV-I Tax protein by NF-κB/Rel transcription factors. *Virology* **204**: 706-716, 1994.

Lacoste J, Petropoulos L, Pépin N, and Hiscott J : Constitutive phosphorylation and turnover of IκBα in HTLV-I-infected and Tax-expressing T cells. *Journal of Virology* **69**: 564-569, 1995.

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GENERAL DISCUSSION

CHAPTER 1

GENERAL INTRODUCTION

Adult T cell leukemia (ATL) results from a multi-step process initiated by the infection of T lymphocytes with the human T cell leukemia virus type I (HTLV-I). HTLV-I encodes a strong transcriptional activator, Tax, which acts via 21 base pair (bp) repeats found in the viral long terminal repeat (LTR). Tax also *trans*-activates cellular genes regulated by DNA motifs different from those governing LTR expression. Tax does not bind DNA and accumulating evidence suggests that its *trans*-activation potential is mediated indirectly via pre-existing cellular transcription factors. One of the targets of Tax protein is the NF-κB/IκB complex. In HTLV-I infected cells, the Tax protein promotes T cell proliferation by an unknown mechanism that results in the eventual emergence of a clonal population of leukemic cells. The object of this thesis is to characterize the molecular mechanisms by which Tax interacts with NF-κB/IκB proteins, and to examine the effects of Tax on cellular gene expression. The present chapter is aimed at providing background knowledge necessary for a complete understanding of the thesis subject.

1. The ATL Model of Leukemogenesis

1.1 *Adult T cell leukemia*

In 1977, a Japanese group published the first clinical description of adult T cell leukemia (1), an endemic disease in southwestern Japan (2). ATL is a malignancy of mature CD4-positive T helper lymphocytes (3). Typical ATL cells are morphologically pleiomorphic with highly lobulated nuclei (3), chromosomal breaks and karyotypic abnormalities that are often indicative of the degree of disease severity (4, 5). Another characteristic feature of ATL leukemic cells is

elevated expression of the interleukin 2 receptor (IL-2R) (6). Tumor cell growth however, does not require expression of IL-2R (7). ATL generally occurs in late adulthood (8) and affects both female and male, especially those coming from families with previous histories of ATL (1, 9).

Soon after its isolation as the first human retrovirus, HTLV-I was implicated as the etiological agent of ATL through seroepidemiological studies (10-13). HTLV-I positive individuals are clustered in specific regions such as southwestern Japan, the West Indies, Central Africa, the Caribbean basin, and southeastern USA, where most ATL cases are diagnosed (2, 14-17). Sensitive detection methods revealed that tumor cells from ATL patients contain clonal patterns of proviral integration, while non-leukemic cells from the same patients are not infected (11, 12, 18-21). There is limited HTLV-I gene expression in ATL patients (15, 22, 23), and since the proviral genome is often deleted (11), it appears that continued viral expression is not required for maintenance of the transformed phenotype. The resulting low virus titers in infected individuals explain why transmission of HTLV-I is possible only through the transfer of living infected cells, via milk, blood and sexual contact (9, 24, 25). In some individuals, HTLV-I infection results in development of lymphoma rather than leukemia, hence ATL is also referred to as adult T cell leukemia/lymphoma (ATLL) (26, 27).

HTLV-I has been associated with a number of other pathologies, including tropical spastic paraparesis/HTLV-I-associated myelopathy (TSP/HAM). As with ATL, the geographical distribution of TSP/HAM is the same as that of HTLV-I. TSP/HAM is a chronic neurodegenerative syndrome and affected individuals suffer progressive demyelination of motor neurons of the spinal cord, leading to

myelopathy (28, 29). Although the pathogenesis of this disease has yet to be established, it is believed that HTLV-I infection is important to the development of TSP/HAM, since antibodies against HTLV-I are detected in TSP/HAM patients (29, 30). Interestingly, multiple sclerosis, a similar but more severe pathology, has also been associated with HTLV-I infection, albeit inconclusively (31, 32). Polymyositis, an inflammatory muscle disorder, has also been associated with HTLV-I infection (33-36).

HTLV-I infection and subsequent seroconversion occur early in life and ATL develops 20 to 30 years later (8). Only 1 to 4% of carriers will develop the malignancy (37, 38). However, clinical stages of HTLV-I infection and ATL development have been established. The majority of HTLV-I infected individuals are asymptomatic carriers (2, 16, 39). At this stage, viral sequences are detected as integrated forms and are generally polyclonal with respect to integration sites (19). The next stage , pre-ATL or pre-leukemia, is also an asymptomatic state, diagnosed by incidental detection of leukocytosis and/or morphologically abnormal lymphocytes (40-42). Monoclonal or oligoclonal patterns of provirus integration are present in these abnormal T cells (40). Some of these patients progress to acute ATL (see below). Thirty percent of HTLV-I infected individuals presenting clinical manifestations have the chronic/smoldering form of ATL (8, 43). Features of this stage include normal white cell counts, prominent skin lesions (resulting from cutaneous infiltration by malignant lymphocytes) and modest bone marrow and visceral involvement (8, 43). Chronic/smoldering ATL is a less aggressive form of ATL that eventually progresses to acute ATL, a highly aggressive disease with very poor prognosis. Survival, after diagnosis, is measured in months. This stage is characterized by elevated white blood cell counts, presence of morphologically abnormal

lymphocytes, hypercalcemia, skin lesions as well as important visceropathy (8). Leukemic cells are represented by one dominant clone, carrying a single rearrangement of the T-cell antigen receptor β gene (44). Monoclonal or oligoclonal patterns of proviral insertion are detected in leukemic cells derived from patients with chronic/smoldering or acute ATL (18, 45-47).

1.2 HTLV-I infection in vitro

The cellular receptor used by HTLV-I to penetrate target cells is not well characterized. However, monoclonal antibodies that block infectivity and syncytia formation have been generated, and the putative cellular receptors recognized by these antibodies are located on chromosome 17 and 11 (48-50). HTLV-I can infect several human and mammalian cell types: however only T lymphocytes are immortalized by HTLV-I infection (51-54). HTLV-I infection is achieved efficiently *in vitro* by co-cultivation of CD4-positive (helper/inducer phenotype) primary T lymphocytes with mitomycin-treated or lethally irradiated HTLV-producing cell lines (54-58); cell-free transmission of HTLV-I is possible but inefficient (51, 59, 60).

Cells infected with HTLV-I *in vitro* constitutively express elevated amounts of IL-2Rα (6, 61-64). Initially, infection results in transient IL-2 expression and rapid T cell proliferation, possibly the result of an autocrine stimulatory loop created by the expression of both IL-2 and its receptor. Polyclonal patterns of proviral integration are detected in infected T cells at this stage (61, 65, 66). T cell proliferation and IL-2 production decrease two to four weeks after infection and T cell growth becomes dependent on addition of exogenous IL-2. This stage of

infection is also characterized by the presence of oligo or monoclonal patterns of HTLV-I inserts in infected cells (54, 61, 67). These cells can be cultured for prolonged periods of time without antigenic stimulation (54, 67). Clonal lines that no longer require exogenous IL-2 eventually emerge from these HTLV-I infected T cells (68). Thus, the process of HTLV-I infection *in vitro*, in terms of T cell proliferation, IL-2 dependency, IL-2R expression and proviral integration, is reminiscent of ATL development. Importantly, HTLV-I-mediated transformation, *in vivo* or *in vitro* , implicates an IL-2/IL-2R autocrine stimulatory loop in T cell proliferation.

Classical mechanisms of retrovirally-induced tumorigenesis have been described, based on several decades of study on animal RNA tumor viruses (69, 70). Chronic leukemia viruses (eg. Moloney Murine Leukemia Virus) induce formation of monoclonal tumors and specific proviral integration sites are required for that process. These viruses lack viral oncogenes and transformation occurs by LTR-mediated activation of nearby cellular oncogenes (69). Acute leukemia viruses (eg. Avian Sarcoma Virus) induce polyclonal tumors shortly after viral infection. Viruses of this group are generally replication defective due to the substitution of essential viral genes by cellular-derived oncogenes which are responsible for tumor formation (69). HTLV-I-induced tumorigenesis differs from these established paradigms of retroviral oncogenesis. Monoclonal patterns of proviral integration are detected in ATL leukemic cells (18, 47). However, there is no common integration site among ATL patients (46). HTLV-I carries no genetic information derived from mammalian cells and therefore does not encode a typical cellular oncogene (71). Thus transformation mediated by HTLV-I defines a new mechanism of retroviral oncogenesis. Careful analysis of the integrated provirus provided the

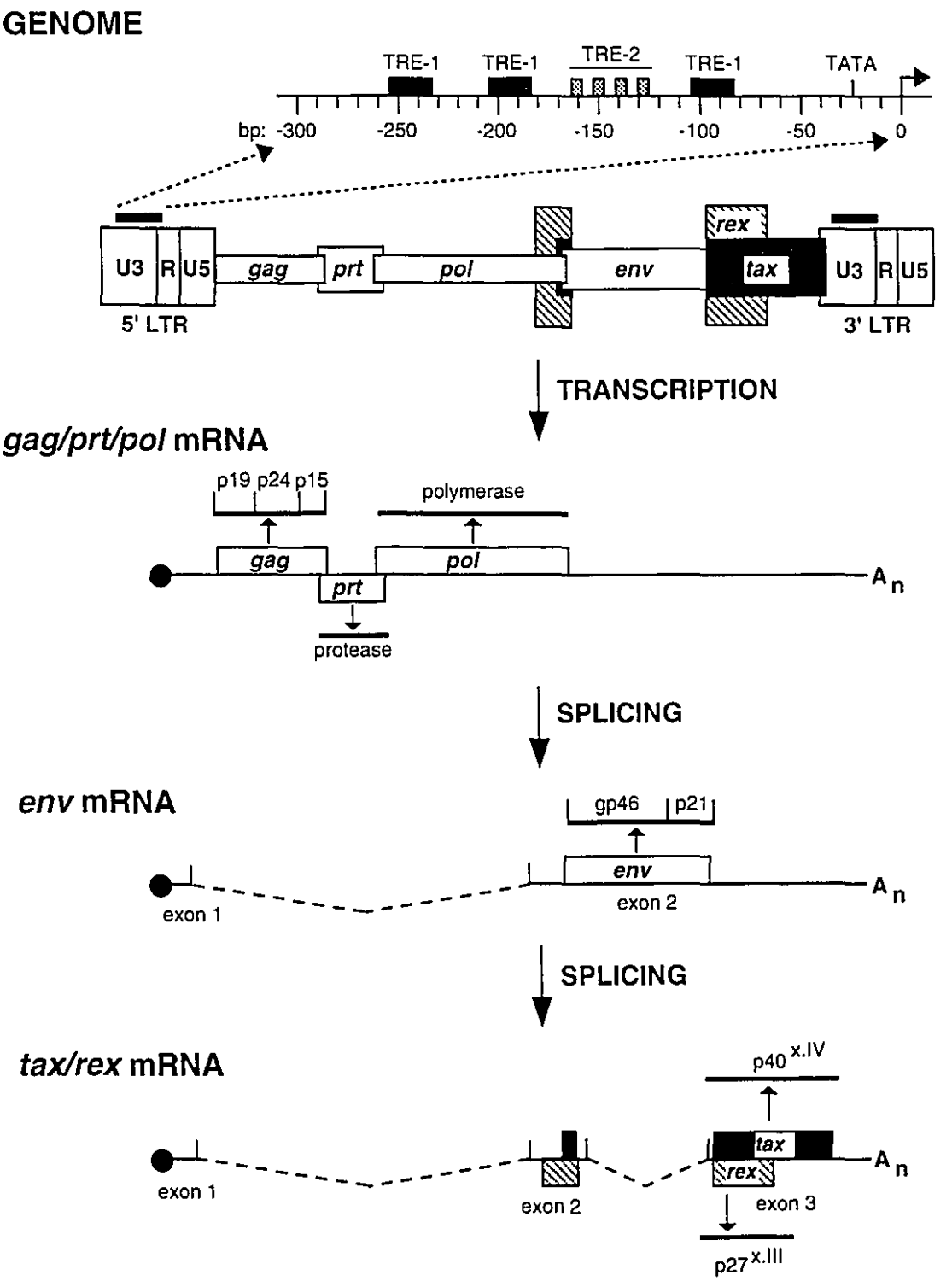
first evidence for this novel mechanism of tumorigenesis.

1.3 *Genetic structure of HTLV-I*

The HTLV-I viral genome is 9 kilobases (kb) and structurally similar to other replication-competent retroviruses (Fig. 1) (71). Viral genes are flanked by 5' and 3' long terminal repeats (LTRs) which possess strong enhancer activity required for viral gene expression. Transcription of the proviral genome produces a primary, unspliced mRNA transcript (*gag/prt/pol* mRNA), from which three polypeptides are generated. The first protein is an immature polypeptide encoded by the *gag* (group-specific antigens) gene. Proteolytic processing results in production of mature proteins that form the matrix and the nucleocapsid of the virion (p19, p24 and p15 proteins) (71). The second polypeptide originates from the *prt* (protease, in a different open reading frame) gene and contains protease activity that is required for proteolytic maturation of the *gag* gene products (71). Finally, reverse transcriptase and integrase functions encoded by the *pol* (polymerase) gene constitute the third polypeptide translated from this primary mRNA. The *env* (envelope) gene encodes the glycoproteins (gp) that form the virion envelope. The precursor polypeptide is translated from a singly spliced mRNA transcript. Exon 1 is non-coding and originates in the 5' LTR. The splicing event joins exon 1 to exon 2 which carries the envelope open reading frame. The precursor (gp62) is processed into gp46, the surface glycoprotein, and p21, the transmembrane protein (72, 73). gp46 is believed to be the viral cell attachment protein (74).

The peculiarity of the HTLV-I genome is the large (1.6 kb) region located

FIGURE 1. Genomic Organization of the HTLV-I Provirus. The overlapping viral genes are flanked by 5' and 3' LTRs (grey boxes; the U3, R and U5 regions are shown). The white boxes indicate the genes coding for the group-specific antigens (*gag*), the protease (*prt*), the polymerase (*pol*) and the envelope proteins (*env*). The hatched and black boxes indicate the genes coding for the Rex and Tax proteins, respectively (*rex* and *tax*). The black bar above the U3 region denotes the regulatory region. This region is also shown enlarged above the proviral genome. The arrow denotes the mRNA start site. The two types of Tax responsive elements are represented by black (TRE-1) and grey (TRE-2) boxes. Viral mRNA transcripts are shown below the proviral genome. The cap structure and the polyadenylation site are represented by black dots and A_n, respectively. The unspliced transcript encodes structural proteins (p19, p24, p15, protease and polymerase). The singly spliced transcript encodes the envelope proteins (gp46 and p21), and the Tax ($p40^{x.IV}$) and Rex ($p27^{x.III}$) proteins are derived from doubly spliced mRNA.


GENOME
TRE-1
TRE-1
TRE-2
TRE-1
TATA
bp: -300
-250
-200
-150
-100
-50
0
U3
R
U5
gag
prt
pol
env
rex
tax
U3
R
U5
5' LTR
3' LTR
TRANSCRIPTION
gag/prt/pol mRNA
p19 p24 p15
polymerase
gag
pol
prt
protease
A n
SPLICING
env mRNA
gp46
p21
env
exon 1
exon 2
A n
SPLICING
tax/rex mRNA
p40 x.IV
tax
rex
exon 1
exon 2
exon 3
A n
p27 x.III

between the *env* gene and the 3' end LTR. This unique sequence is referred to as the pX region and contains four overlapping open reading frames (ORF) named x-I, x-II, x-III and x-IV. Several polypeptides are encoded by this region (75-79), and two of them, Tax and Rex, play important functions in the viral replication cycle. Both proteins are translated from a doubly spliced mRNA transcript. The first exon (non-coding) is identical to exon 1 of the *env* mRNA transcript. Exon 2 encompasses the end of the *pol* gene and the beginning of the *env* gene. Exon 3 contains most of the coding sequences and is located in the pX region. Rex is translated from ORF III while Tax is translated from ORF IV (76, 80, 81). Rex is a 27 kD protein expressed in the nucleoli of infected cells. It is a regulatory protein that acts at a posttranscriptional level and promotes transport of unspliced and singly spliced mRNAs to the cytoplasm, thereby increasing expression of the *gag*, *prt*, *pol* and *env* gene products (78, 82-84).

Tax is a 40 kD nuclear protein (85-88) and a strong transcriptional activator of the viral LTR. Early studies on HTLV-I transcriptional regulation revealed that viral expression was dramatically increased (up to 200 fold) when a LTR-regulated reporter construct was transfected in HTLV-I infected cells. It was suggested that an HTLV-I-encoded protein mediates transcriptional activation of the LTR by a *trans* mechanism (89). Subsequently, the viral pX region was shown to encode a transcriptional *trans*-activator, the Tax protein (90-92). The U3 region of the LTR, upstream of the start site of viral mRNA synthesis, contains sequences that confer responsiveness to Tax *trans*-activation (93-95). There are two types of Tax-responsive elements: TRE-1 and TRE-2 (Fig. 1). The TRE-1 motif is a 21 bp sequence repeated three times within the U3 region (at positions -100, -200 and -250 bp). The CRE (cAMP-responsive element) consensus site is found at the core of the TRE-1 element. The TRE-2 motif

contains four pentanucleotide repeats (CCA/TCC) located between the second and third TRE-1 elements (position -163 to -117). TRE-1 elements are orientation-independent but number- and position-dependent: a minimum of two TRE-1 elements positioned in *cis* will confer Tax responsiveness to a reporter construct. Alternatively, a combination of one TRE-1 and one TRE-2 results in a similar response to Tax *trans*-activation (94-100).

2. The HTLV-I Tax Protein

2.1 *Tax as a transforming protein*

As described above, leukemic cells from ATL patients contain important chromosomal breaks and karyotypic abnormalities. The proviral genome is not spared from these deleterious events: 32% of ATL patients carry deleted viral genomes in their leukemic cells (101). However, the pX region is preferentially retained (101), suggesting a requirement for the Tax (and possibly Rex) protein in maintenance of the transformed phenotype. The presence of *tax/rex* mRNA transcripts in fresh peripheral blood mononuclear cells from ATL patients and HTLV-I carriers further supports a requirement for Tax expression (102).

Oncogenicity of the Tax protein has been thoroughly investigated using both *in vivo* and *in vitro* models. Transgenic mice expressing the Tax protein present quite heterogenous phenotypes ranging from no malignancies to thymic aplasia, neurofibromas, exocrinopathies, arthropathies and sarcomas (103-106). Despite this variety of pathologies, most transgenics share a number of common features. In transgenic animals, cells expressing the Tax protein also

produce elevated amounts of GM-CSF (granulocyte/macrophage-colony stimulating factor) and IL-2 receptors, regardless of the growth phenotype of the cells (107). In additon, involvement of lymphoid cells and development of ATL-like malignancies are never detected in Tax transgenic mice (104, 105, 108).

In vitro transformation by the Tax protein clearly demonstrated the oncogenic potential of the HTLV-I *trans*-activator. Overexpression of Tax immortalized primary T cells. However, these cells were never fully transformed and remained IL-2-dependent (67), which is reminiscent of the early stages of HTLV-I infection. Tax expression immortalized primary fibroblasts (109, 110), and, in cooperation with the *ras* oncogene, transformed primary cells and caused tumorigenicity in nude mice (109). These Tax-transformed cells spontaneously reverted to a normal phenotype when the *tax* gene was lost (111). Therefore Tax initiates a transformation process that results in immortalization of primary cells. More recently, a double transgenic model system was developed. These mice carry HTLV-I LTR-driven c-*myc* and Ig promoter/enhancer-driven *tax* genes. Transgenic animals developed central nervous system tumors and CD4+ T cell lymphomas, similar to those occuring in HTLV-I infected individuals (112). This double transgenic model system provides an important tool for understanding HTLV-I induced pathologies.

2.2 *Tax* trans-*activates cellular genes*

The strong transcriptional activation potential of Tax suggested that Tax *trans*-activation of cellular genes involved in proliferation may be a mechanism by which HTLV-I infection results in transformation (89). Because of their obvious

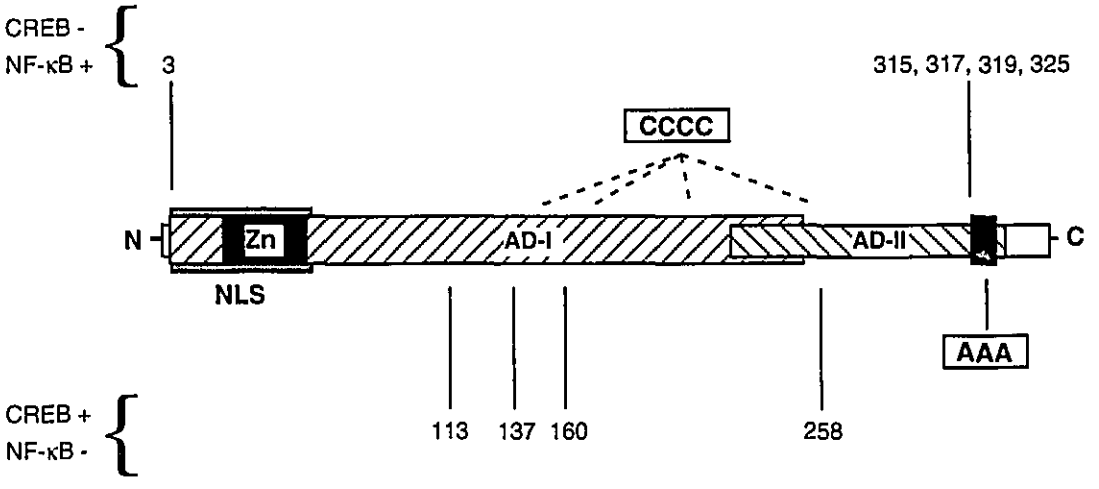
implication in HTLV-I pathogenesis, IL-2 and IL-2Rα genes are likely targets for Tax *trans*-activation. Indeed, both genes are transcriptionally *trans*-activated by the Tax protein and, interestingly, are responsive to Tax *trans*-activation only in CD4+ T lymphocytes, the normal targets of HTLV-I (95, 113-122). Tax strongly *trans*-activated a number of other genes which are also involved in cell activation, proliferation and differentiation. Tax *trans*-activated several cytokines (IL-3, IL-4, tumor necrosis factor or TNFα and β, GM-CSF, transforming growth factor or TGFβ, interferon or IFNγ, nerve growth factor or NGF, proenkephalin, parathyroid hormone-related protein [PTHrP]) (123-132), a cell surface receptor (major histocompatibility complex or MHC class I) (133), a cytoskeleton protein (vimentin) (134), several members of various transcription factors families (fos/jun, NF-κB/Rel, Egr/Krox, c-*myc*) (135-141), viral enhancers (HIV, CMV) (113, 142, 143) and a tyrosine kinase (c-*lyn*) (144). Interestingly, Tax repressed expression of the human β-polymerase gene, the product of which is involved in DNA repair (145). It is not clear if a relationship exists between down regulation of β-polymerase and the increased frequency of chromosomal breaks and abnormalities observed in ATL cells. These observations strongly suggest that the HTLV-I transformation potential resides in the ability of the Tax protein to *trans*-activate expression of cellular genes implicated in cell growth.

The current model of HTLV-I-induced leukemogenesis postulates that Tax *trans*-activation of cellular genes, in particular the IL-2 and IL-2Rα genes, initiates a polyclonal proliferation of CD4+ T lymphocytes. HTLV-I infected cells constitute a pool of proliferating T cells that accumulate mutational events, ultimately resulting in the emergence of a monoclonal, IL-2-independent population of leukemic T cells.

2.3 *Structure-function of the Tax protein*

The transforming properties of Tax correlate with its ability to activate gene expression. However Tax lacks direct DNA binding activity (146). Therefore in order to understand the *trans*-activation potential of Tax at the molecular level, several mutagenesis studies were performed. Tax is not readily dissected into distinct, functional domains (147, 148). However, chimeric fusion proteins, with Tax fused to a DNA binding domain, clearly demonstrated that Tax possesses its own transcriptional activation function (149, 150). Figure 2 schematically illustrates the structure of Tax protein, as deduced from recent mutagenesis studies (151-156). Tax is a 353 amino acids (aa) protein that localizes to the nuclei of HTLV-I infected and Tax expressing cells (85-88). Although a classic nuclear localization signal (NLS), a short stretch of highly basic amino acids, is absent from Tax, the first 50 aa or so (1-48 or 2-59) (151, 152) are required for nuclear localization. This region also contains a cysteine-rich stretch between aa 22 and 53 that is similar to the metal-binding domain of several transcription factors (153). This region can be arranged into two putative zinc-binding fingers (aa 22 to 50 or 28 to 53) (153). Semmes and Jeang reported that Tax binds zinc via the first zinc-binding finger which is required for Tax activities (153). It is not clear whether the zinc-binding domain is required for nuclear localization. An additional interesting observation is that a single amino acid substitution (^{58}Pro ---> Ser) resulted in loss of *ras* cooperative focus formation in rat embryo fibroblasts. The other functions of Tax (immortalization and *trans*-activation) were not affected by this mutation (157). A second cysteine-rich region is found between aa 153 and 261. These residues allow formation of Tax multimers, which are believed to be important for Tax activities (152). Tax is

FIGURE 2. Schematic Organization of the HTLV-I Tax Protein. The structure of the 353 amino acid (aa) Tax protein is illustrated schematically. The N-terminal grey box indicates the nuclear localization signal, NLS. The cysteine-rich zinc binding domain is represented by a black box marked "Zn" (aa 22-53). The white box denoted "CCCC" indicates the second cysteine rich region involved in multimer formation (at aa positions 153, 174, 212 and 261). The C-terminal black box labeled "AAA" denotes the acidic aa stretch (aa 323-332). The transcriptional activation domains (AD-I: aa 2-255, and AD-II: aa 227-337) are represented by hatched boxes. The numbers above and below the diagram indicate the aa position of mutations that abrogate CREB-mediated (above) or NF-κB-mediated (below) *trans*-activation.


CREB -
NF-κB +
3
315, 317, 319, 325
CCCC
N
Zn
AD-I
AD-II
C
NLS
AAA
CREB +
NF-κB -
113
137
160
258

phosphorylated on one or more serine residues (158). Four protein kinase A or C (PKA or PKC) consensus sites, three of them located in the amino-terminal one-third of the protein, are likely targets for phosphorylation (159). The importance of Tax phosphorylation in transformation and/or transcriptional *trans*-activation remains unclear (159). The transcriptional activation domain of Tax is composed of two distinct regions (156): AD-I, aa 2 to 255, contains the NLS, the zinc-binding domain as well as the multimer formation domain. The second region (AD-II, aa 227 to 337) contains an acidic amino acid stretch (aa 323-332) (156). Interestingly, an interaction between Tax and TBP, the TATA binding protein of the TFIID complex, has been reported (160).

At least four different regulatory elements confer responsiveness to Tax *trans*-activation (TRE-1, TRE-2, CArG boxes and NF-κB elements). These elements bind different types of transcription factors (see below). Mutagenesis studies revealed that distinct amino acid residues of the Tax protein are required for *trans*-activation of two different pathways: the CREB (cAMP-Responsive element binding) and NF-κB pathways (Fig. 2 and below) (154, 155). Mutation of a region located between amino acid 315 and 325 abrogates CREB-mediated Tax *trans*-activation without affecting NF-κB-mediated *trans*-activation. Conversely, scattered mutations in Tax (amino acid 113 to 258) result in intact CREB- but deficient NF-κB-mediated *trans*-activation (154, 155). These observations demonstrate that Tax *trans*-activation occurs via distinct regions of the viral protein, and indicate that Tax interacts with cellular transcription factors.

2.4 *Tax physically associates with cellular transcription factors*

Transcriptional activation of the LTR occurs in the absence of *de novo* protein synthesis (131, 162), suggesting an interaction between Tax and preexisting cellular factors. Cellular factors that specifically bind the 21 bp sequence of the TRE-1 element have been purified (TRE-binding or TREBs, TAXRE67, HEFs, HEBs, to name a few) (146, 163-171). The TGACG motif in the center of the TRE-1 element is homologous to the activating transcription factor (ATF) binding sites (172) and cyclic AMP-responsive elements (CREs) (173), and purified TREBs can bind these sites (174). ATF mediates adenovirus early gene expression and E1a-mediated *trans*-activation (175). CREB proteins mediate induction of promoter activity in response to cAMP treatment (176). Members of the ATF/CREB family of transcription factors are characterized by the presence of leucine zipper and basic amino acid domains responsible for protein dimerization and DNA binding, respectively. Analysis of cDNAs encoding the TRE-1 binding proteins TREB7, TREB36 and TAXRE67 revealed that all three are virtually identical to previously characterized ATF/CREB factors (168, 170). These findings demonstrated that *trans*-activation of the viral LTR elements required the binding of CREB/ATF factors to the CRE consensus sites found in TRE-1 elements and suggested an interaction between Tax and CREB/ATF factors.

An initial study demonstrated that Tax interacts with cellular factors that specifically bind the TRE-1 elements (177). Subsequently, these factors were identified as CREB homodimers, CREB/ATF heterodimers and ATF homodimers (178). Using a DNA-affinity precipitation assay, Suzuki *et al.* demonstrated that Tax interacted with CREB and CREM (CRE modulator) (179).

The interaction with Tax resulted in enhanced DNA binding activity of CREB, ATF-1, ATF-2 (178, 180). The exact mechanism by which DNA binding is stimulated remains unclear, but one group reported that it is made possible by the ability of the Tax protein to enhance dimerization of ATF/CREB factors (181). Another group demonstrated that three amino acids immediately adjacent to the conserved DNA binding domain of CREB conferred the ability to interact with Tax and thus enhanced DNA binding (182).

The HTLV-I LTR contains a second type of Tax responsive element, TRE-2 with overlapping binding sites for Sp1, TIF-1, Ets and Myb (183-188). TIF-1, by physically interacting with Tax protein, allows Tax to indirectly bind to TRE-2 elements (100, 184). But most importantly, a Tax-Ets1 interaction at TRE-2 sites permits a cooperative increase in gene expression driven by the HTLV-I LTR (189). Thus, viral gene expression involves a complex interplay between Tax and a number of cellular transcription factors (CREB/ATF, Sp1, Myb, Ets).

Tax *trans*-activation of cellular genes occurs through elements similar to those present in the LTR and also through motifs that are absent from the viral LTR. CArG boxes are found in the promoters of the c-*fos*, *egr-1* and *egr-2* genes (135, 136, 190, 191) and confer responsiveness to Tax *trans*-activation (192). Transcriptional activation of this element requires binding of the serum response factor (SRF) (193-195). It has been shown, both directly and indirectly, that Tax *trans*-activation of CArG boxes occurs through formation of a Tax-SRF complex (192, 196). Lastly, NF-κB elements regulate expression of several cellular genes that are *trans*-activated by Tax. Several reports have demonstrated physical interaction between Tax and members of the NF-κB/Rel family of transcription factors (see below). Thus, Tax clearly has the potential to

associate with a variety of transcription factors that specifically bind Tax-responsive elements, and these associations result in transcriptional *trans*-activation.

3. NF-κB: Pleiotropic Mediator of Gene Regulation

Nuclear Factor κ (kappa) B (B element), NF-κB, was originally described as a DNA binding activity specifically recognizing the decameric sequence 5'-GGGACTTTCC-3' found in the B enhancer of the murine immunoglobulin κ light chain gene (197). Because the activity was observed constitutively only in mature B cells, NF-κB was thought to be a tissue-specific transcription factor (198, 199). Soon however, NF-κB activity was found in a latent inducible form in many other cell types (200-202). NF-κB-like proteins have now been found to be evolutionary conserved among avian, mammalian and insect species. NF-κB-related elements are found in promoters of several genes whose products are implicated in immune and inflammatory functions (203-207). These genes, through NF-κB activation, are triggered by exposure to primary pathogenic stimuli such as bacterial, viral and parasitic infections. NF-κB activity is also induced by agents secondary to the initial challenge such as cytokines, T cell mitogens, viral *trans*-activators as well as detrimental agents (Table 1) (204).

Many biological processes involve NF-κB-regulated genes. B cell differentiation requires κB-mediated immunoglobulin kappa light chain gene expression during the pre-B to B cell transition (197, 200). Cytokine responsiveness and regulation utilize NF-κB at several levels. Cytokines are both inducers of and induced by NF-κB factors (Tables 1 and 2) (203-208). For example, cytokines,

TABLE 1. Inducers of NF-κB Activity[1]

Type	Agent
Primary Pathogenic Stimuli	Poly IC- double stranded RNA
	LPS- bacterial lipid
	Theileria parva- parasite
	Cytomegalovirus- CMV
	Hepatitis virus B- HBV
	Human T cell leukemia virus type I- HTLV-I
	Herpes simplex virus type 1
	Human herpes virus type 6
	Adenovirus
	Sendaivirus
Cytokines	TNF α
	TNF β
	IL-1
T Cell Mitogens	PMA, PDB- phorbol esters
	Lectins
	Calcium inonophores (+PMA)
	Anti-CD28, Anti-CD3- crosslinking agents
Viral *Trans*-Activators	ie1- CMV
	X- HBV
	Tax- HTLV-I
	E1A- Adenovirus
Detrimental Agents	4-Nitroquinolineoxide- DNA damaging agent
	Ultraviolet light- DNA damaging agent
	Cycloheximide- protein synthesis inhibitor
	Anisomycin- protein synthesis inhibitor

1. Adapted from reference (204).

TABLE 2. NF-κB-Regulated Genes[1]

Type	Gene
Cytokines	IFN β
	IL-1
	IL-2
	IL-6
	IL-8
	TNF α
	TNF β
	GM-CSF
	G-CSF
	proenkephalin
Immuno-Receptors	Ig κ light chain
	IL-2 R α chain
	TCR β chain
	MHC class I (H-2k^b)
	MHC class II (Eα^d)
	β_2-microglobulin
	VCAM 1
Transcriptional Regulators	IRF-1
	c-*myc*
	c-*rel*
	*nfkb*1
	*nfkb*2
	mad-3
Viruses	HIV
	CMV
	SV40
	Adenovirus
Others	serum amyloid A precursor
	vimentin

1. Adapted from references (203-208).

such as TNFs and IL-1, are potent inducers of NF-κB activity. Among the genes responding to TNFs- and IL-1-induced NF-κB activation are the genes encoding TNFs and IL-1. Thus these two cytokines are involved in positive autoregulatory loops, through NF-κB activity (208-215). Important immunoreceptors, including IL-2Rα, T cell receptor β chain, MHC molecules, are NF-κB-dependent (122, 216-219). The coupling of both signals (e.g. TNFs, IL-1, IL-2) and responses (e.g. IL-2Rα, T cell receptor or TCRβ, MHCs, Ig κ light chain) by the same transcriptional activator has important implications for cellular growth control and proliferation, as evidenced by the oncogenic potential of NF-κB proteins (see later). NF-κB-like factors also play critical roles in invertebrate development and embryogenesis (220-222), as well as in viral pathogenesis. Viruses, including HIV and CMV, are regulated at the transcriptional level in part by NF-κB elements; thus permitting the utilization of immune stimulatory signals as a mean to increase viral replication (Table 2) (223, 224).

3.1 *NF-κB proteins are targets for Tax* trans*-activation*

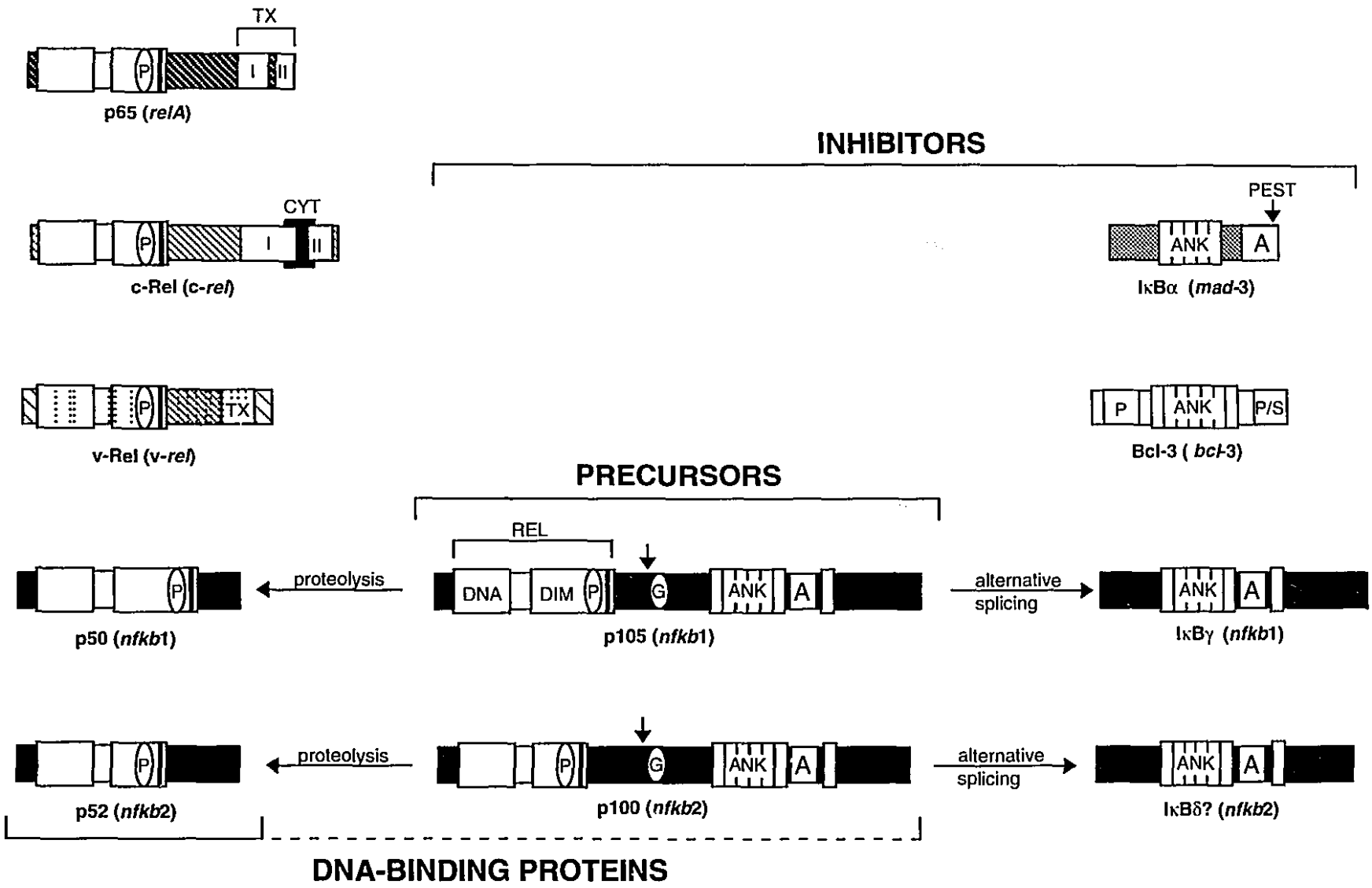
HTLV-I utilizes the NF-κB system to induce cellular gene expression via Tax *trans*-activation. T lymphocyte proliferation requires binding of the growth-promoting lymphokine IL-2 to its specific membrane receptor, the IL-2 receptor (IL-2R). High affinity IL-2R is composed of one α and one β subunit. Resting T cells constitutively express IL-2Rβ which has low affinity for IL-2. Induction of IL-2Rα gene expression by cytokines (e.g. TNFα, IL-1) or mitogens (e.g. phytohemagglutinin, PHA, or phorbol 12-myristate 13-acetate, PMA) results in transient expression of high affinity IL-2R, T-cell proliferation and eventual cell

death (225). The elements present in IL-2 and IL-2Rα gene promoters that permit a response to T-cell mitogenic stimulation are NF-κB motifs (113, 115, 216, 226, 227). Most importantly these elements mediate Tax *trans*-activation of the IL-2 and IL-2Rα genes (115, 122, 216, 226). However Tax stimulation of these genes is sustained, as opposed to the transient induction of gene expression that follows mitogenic stimulation (6, 62-64). Genes such as TNFα and β, GM-CSF, MHC class I, are also *trans*-activated through NF-κB elements (125, 126, 133, 228, 229). Furthermore, soluble extracellular Tax protein has the ability to stimulate proliferation of peripheral blood lymphocytes (230). This function is accompanied by induction of nuclear NF-κB DNA binding activity (231) and, consequently, activation of NF-κB-regulated genes such as IL-2Rα and Ig κ light chain genes, as well as TNFβ (232, 233). Therefore Tax *trans*-activation of NF-κB-regulated genes implicated in cell proliferation likely plays an important role in the oncogenic process initiated by HTLV-I infection. This possibility is further supported by the recent finding that inhibition of NF-κB expression by antisense oligodeoxynucleotides prevents the growth of Tax-transformed fibroblasts as well as an HTLV-I transformed human lymphocytic cells and suggests that NF-κB expression is necessary for the maintenance of the malignant phenotype (234).

3.2 *Structure and function of NF-κB/Rel proteins*

Members of the NF-κB/Rel family of transcription factors bind the consensus site 5'-GGGRANNYYCC-3' (N= any nucleotide, R= G or A residue, Y= T or C residue) (204) as homo- or heterodimers. In uninduced cells, they localize to the cytoplasm and NF-κB induction permits nuclear translocation. Figure 3 is a

FIGURE 3. Schematic Organization of NF-κB/Rel and IκB Proteins. Proteins are grouped according to function: DNA-binding proteins, precursor molecules and inhibitors. The dotted line illustrates the fact that NFKB2 p100 can bind DNA and can therefore be classified as a DNA-binding protein. The name of each protein and gene (in brackets) is indicated below each structure. Tx: transcriptional activation domains, I and II. CYT: cytoplasmic anchorage domain. Vertical dotted lines: mutations in v-Rel. REL: Rel domain. DNA: DNA binding region. DIM: protein dimerization region. P: PKA phosphorylation site. Black vertical line: nuclear localization signal. Arrow: processing site. G: glycine-rich region. ANK: ankyrin repeats. P and P/S: proline and proline/serine-rich regions, respectively. PEST: protein degradation signal.


TX
P
I
II
p65 (relA)
INHIBITORS
CYT
c-Rel (c-rel)
PEST
ANK
A
IκBα (mad-3)
TX
v-Rel (v-rel)
P
ANK
P/S
Bcl-3 (bcl-3)
PRECURSORS
REL
proteolysis
DNA
DIM
G
alternative
splicing
p50 (nfkb1)
p105 (nfkb1)
IκBγ (nfkb1)
p52 (nfkb2)
p100 (nfkb2)
IκBδ? (nfkb2)
DNA-BINDING PROTEINS

schematic representation of NF-κB/Rel and IκB proteins. Structurally, all members of the NF-κB/Rel family of transcription factors share an N-terminal Rel homology domain of approximately 300 amino acids. This Rel domain was originally described in v-*rel*, the transforming oncogene of REV-T (reticuloendotheliosis virus strain T) (235, 236). The Rel domain is responsible for protein dimerization and DNA binding (237-242). It shares no homology with previously characterized structures implicated in DNA binding or protein dimerization (e.g. zinc finger, helix-loop-helix, leucine zipper) (240, 242). Mutagenesis studies demonstrated that the C-terminal half of the Rel domain is sufficient for protein dimerization (243, 244). The N-terminal portion of the Rel domain contains a motif (RXXRXRXXC), conserved in all NF-κB/Rel proteins, that is essential for DNA binding (245, 246). The C-terminal end of the Rel domain also contains a nuclear localization signal (NLS), a stretch of four or five positively charged amino acids that are conserved in all members of the family. This sequence is required for nuclear translocation (247-249). Similar sequences in nuclear proteins were reported to serve as signals for receptor-mediated nuclear uptake (250). Approximately twenty amino acids to the N-terminal side of the NLS is a putative PKA phosphorylation site (Arg-Arg-X-Ser). The PKA site is present in all members of the family, with one exception (251). While the exact role of this motif is unknown, mutagenesis studies suggest that it is important for NF-κB activity (252, 253). Interestingly, a new serine kinase, named NF-κB kinase, has been identified that activates two members of the family (p50 and p65) by direct phoshphorylation *in vitro* (254). Two other members (p105 and c-Rel) have been shown to be tyrosine kinase substrates (255). Finally, in both vertebrates and invertebrates there is evidence that phophorylation of NF-κB/Rel proteins is a response to activating signals (256-258).

NF-κB/Rel proteins may be arbitrarily subgrouped into two categories based on structure and function of the proteins. One subgroup represents proteins synthesized as mature polypeptides. In addition to the N-terminal Rel domain, a sequence responsible for transcriptional activation is found in the C-termini of these proteins. Important members of this subgroup include v-Rel (235, 236, 259), c-Rel (236, 260-262), p65 (237, 238), RelB (251, 263), and the invertebrate homologue of NF-κB, Dorsal (220). The p65 subunit is a strong transcriptional activator (264) and its activation domain maps approximately to the C-terminal 100 amino acids (265-267). The last 30 amino acids are enriched for hydrophobic, serine and acidic residues and adapt an amphipathic α helical structure, typical of acidic activator domains (265). The C-terminal region of c-Rel contains two transcriptional activation domains (268-270) that overlap with a cytoplasmic anchorage domain (260). The C-terminus of v-Rel contains only part of these transcriptional activation domains and also lacks the cytoplasmic anchorage domain (235, 236, 259).

The other subgroup consists of the p105 and p100 proteins (products of the *nfkb*1 and *nfkb*2 genes, respectively), which are precursor molecules containing an N-terminal Rel domain (239, 240, 242, 271-273). The p105 and p100 precursors are proteolytically processed to approximately 50 kD DNA binding proteins (p50 and p52, respectively), essentially corresponding to the Rel domain. This maturation process is slow (274, 275) and occurs through a ubiquitin-proteasome pathway requiring ATP hydrolysis (276, 277). The precise cleavage site has not been identified, but is believed to be close to a glycine rich region that may function as a hinge (Fig. 3) (240, 242).

p50 and p52 possess strong DNA binding activity. However their transcriptional activation potentials are not clearly established. p50 homodimers are active in cell-free systems using recombinant protein (264, 278), but in transfection assays both p50 and p52 repeatedly failed to activate NF-κB-regulated reporter plasmids (266, 271, 272, 279-281). In non-transformed CD4+ T lymphocytes overexpression of p50 homodimers represses IL-2 promoter expression (282). Together these results indicate that p50 and p52 participate mainly in DNA binding while other subunits, such as p65 and c-Rel, contribute to transcriptional activation.

p100 and p105 were originally reported as non-DNA binding proteins (240, 242, 272, 273) localized in the cytoplasm (249, 283). However recent reports demonstrated that p100 can bind DNA (283, 284). Also, the carboxy-terminal half of p105 is a strong transcriptional activator (285). Taken together these results suggest that the precursor proteins p105 and p100 may play more active roles as transcriptional regulators.

3.3 *Structure and function of IκB proteins*

Induction of NF-κB activity does not require *de novo* protein synthesis (200, 286) and can be achieved *in vitro* by treatment of cytosolic extracts with the ionic detergent sodium deoxycholate (287-289). These observations suggest that induction is a posttranslational event involving the release of an inhibitory molecule. The molecule responsible for maintaining NF-κB activity in a latent, inducible cytoplasmic form was biochemically characterized as an inhibitor of NF-κB (IκB) (287). IκB molecules form latent heterotrimeric complexes by

associating with homo- and heterodimers of NF-κB/Rel proteins. This association inhibits the DNA-binding activity of NF-κB/Rel proteins. Different polypeptides with IκB-like activities have been isolated and found to constitute a family of proteins. One of the distinguishing structural features of these proteins is the presence of five to seven copies of a motif known as the ankyrin repeat. The ankyrin repeat is 33 amino acid sequence (Fig. 3) which is also found in ankyrin, a cytoskeleton protein, and in proteins involved in cell-cycle control and tissue differentiation (290). Each individual repeat has greater homology with the corresponding repeat in other ankyrin-containing proteins than with the ones within the same molecule (290). Nonetheless, they contain a conserved core amino acid sequence (TPLH) and enough similarity to predict a secondary structure: It has been proposed that the 33 amino acid ankyrin repeat is composed of an α-helix (residues 4-11), a short turn, and a β-sheet (residues 16-23) that is aligned anti-parallel to the α-helix (291). It is also highly probable that repeats further arrange themselves into higher order structures. The ankyrin repeats of IκBs interact with the Rel domains of NF-κB/Rel proteins (222, 292-294). Mutations within the TPLH sequence of individual ankyrin repeats abolish this interaction (293). Interestingly, a putative PKC site (295) is found in the sixth ankyrin repeat (296), suggesting that phosphorylation might play a role in regulation (see later). This site is adjacent to a PEST motif, which is responsible for rapid protein turnover (297). Another important feature of IκBs is a glutamic and aspartic acid rich sequence generally located before the last ankyrin repeat. This motif is necessary for inhibitory activity of IκB molecules (249, 298, 299). At least two sites within the Rel domain interact with inhibitory molecules: the NLS and a conserved sequence involved in DNA binding (246, 300-303). It is believed that IκBs mask the NLS and prevent nuclear translocation. Sequences in the carboxy-terminal half of some NF-κB/Rel

proteins have also been implicated in interactions with IκBs (303).

Several distinct IκB-like molecules have been described. IκBα, encoded by the *mad*-3 gene, is the prototypical member of the family. IκBα is a 37 kD protein (287, 288, 304) that cytoplasmically retains and inhibits DNA binding activity of p65- and c-Rel-containing hetero- and homodimers (289, 296, 304-310). Curiously, IκBα also enhances DNA binding of p50 and p52 homodimers (311); the biological significance of this however, is unknown. IκBβ is 43 kD in size and has not been cloned yet. Nonetheless, the purified protein, in terms of cytoplasmic retention and DNA binding inhibition, has specificity similar to that of IκBα (304). Cactus is the *Drosophila* homolog of IκBα, and inhibits Dorsal, the invertebrate version of NF-κB/Rel proteins (221, 222). *bcl*-3 was originally identified as the gene present at chromosomal breakpoints in B cell chronic leukemias (312, 313). cDNA analyses demonstrated that the *bcl-3* oncogene product is a member of the IκB family (292). The inhibitory activity of Bcl-3 is selective for homodimers of p50 and p52 (294, 313-319). Unlike other inhibitors, Bcl-3 localizes to the nucleus (315, 320). Surprisingly, Bcl-3 has been shown to transcriptionally activate NF-κB-regulated sites (316, 320, 321). Two reports showed that Bcl-3 acts as a transcriptional activator through its association with p50 or p52 homodimers (320, 321). When fused to heterologous DNA-binding domains, the proline and serine rich sequences that flank the ankyrin repeats of Bcl-3 (Fig. 3) permit transcriptional activation of NF-κB-regulated reporter plasmids (285, 322). It is not clear yet how this protein, through an association with p50 or p52 homodimers, results in either transcriptional activation or cytoplasmic retention.

By virtue of ankyrin repeats present in their carboxy terminal portion, p105 and

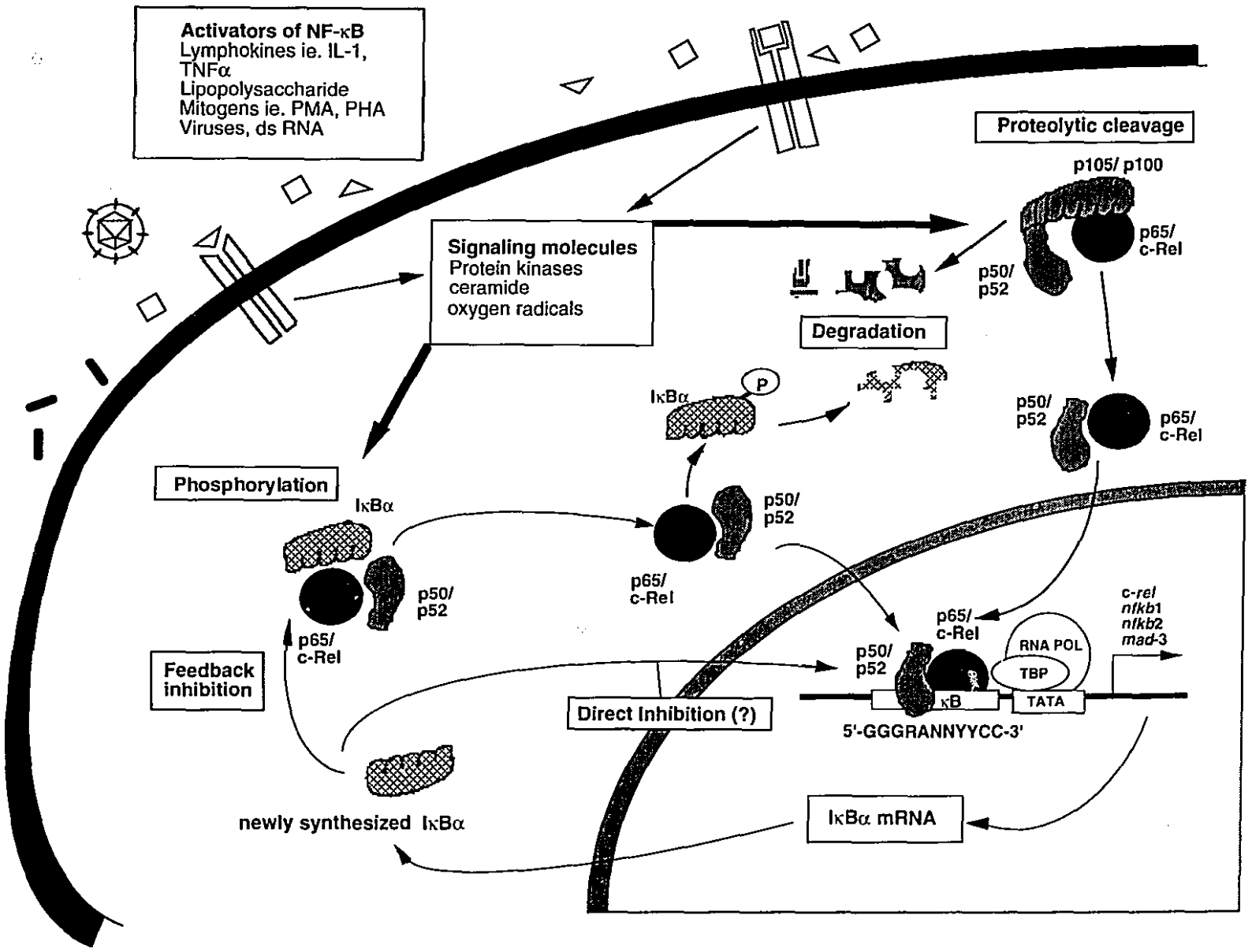
p100, the NFKB1 and 2 precursors, also possess IκB-like activity. Both inhibitory molecules dimerize with and retain p50, p52, p65 and c-Rel in the cytoplasm via Rel domain interactions (274, 275, 283, 318, 323, 324). The ankyrin-containing C-terminus of p105 can fold back and interact with its own N-terminal Rel domain, including the NLS, and thus prevent nuclear translocation (302). Proteolytic removal of the ankyrin repeats is likely to generate active homo- and heterodimers which are susceptible to cytoplasmic retention by other IκB molecules present in the cytoplasm (e.g. α, β, Bcl-3, and γ). Moreover, as discussed earlier, p100 and p105 are capable of transcriptional activation.

Alternative splicing of the primary NFKB1 RNA transcript results in the synthesis of an authentic IκB molecule named IκBγ (70 kD). The NFKB2 gene, by analogy with NFKB1, is predicted to encode an IκB molecule (tentatively named IκBδ, 60 kD) (Fig. 3). IκBγ specifically inhibits p50 homodimers (298, 323) and perhaps c-Rel homodimers and p65-containing dimers (298). This inhibitor is predominantly found in lymphoid cells (298) and capable of activating transcription when fused to a GAL-4 DNA binding domain (285, 322). In addition to their clearly established abilities of cytoplasmically retaining and inhibiting DNA-binding of NF-κB/Rel proteins, IκBs may also possess transcriptional activation capabilities. The acidic amino acids present on the N-terminal side of the last ankyrin repeat may constitute a transcriptional activation domain (Fig. 3). Whether these two apparently opposite functions have any biological significance remains to be determined.

3.4 *Regulation of NF-κB activity*

Given the complexity of the family members and the range of genes regulated by NF-κB, it is not surprising that NF-κB activity is controlled at multiple regulatory levels (Fig. 4). One level of NF-κB/Rel regulation is a posttranslational cytoplasmic retention mechanism. As mentioned earlier, NF-κB binding activity exists in a latent form in virtually all cell types. After cellular stimulation by one of the multiple inducers of NF-κB (Table 1), signal transduction cascades lead to the dissociation of IκB from NF-κB/Rel dimers which translocate to the nucleus. *In vitro* phosphorylation of IκB by several kinases (eg. PKC, PKA, cAMP-dependent kinase, haem-regulated kinase, double-stranded RNA-dependent kinase) abolishes IκB inhibitory activity and activates NF-κB/Rel DNA binding activity (306, 325-332). PKC likely recognizes and phosphorylates the putative PKC site in the C-terminus of IκBα (296). Although it lacks a PKA consensus site, IκBα is a good substrate for PKA-mediated phosphorylation *in vitro* (333), possibly by phosphorylation of a novel PKA consensus sequence. These observations suggest that IκB phosphorylation may be an important regulatory step in NF-κB/Rel activation. This idea is supported by recent studies demonstrating that signals generated by TNFα, IL-1, PMA, and LPS (lipopolysaccharide) treatments result in IκBα phosphorylation, dissociation from NF-κB/Rel dimers, and subsequent degradation (328, 334-338). Rapid proteolysis of IκBα is necessary for activation of NF-κB/Rel (258, 339). It has recently been demonstrated that the rapid degradation of IκBα preceding NF-κB activation occurs through the same ubiquitin-proteasome dependent pathway involved in processing the p105 precursor to the DNA binding form (277). The release of IκB and the concomitant nuclear translocation of NF-κB/Rel dimers permits transcriptional

FIGURE 4. Biochemistry of NF-κB Activation. NF-κB/Rel dimers are found in the cytoplasm as latent complexes coupled to inhibitory molecules (e.g. IκBα, p105 or p100). After cellular stimulation by one of the multiple inducers of NF-κB (Table 1), signal transduction cascades lead to the dissociation of IκBα from NF-κB/Rel dimers which translocate to the nucleus. Dissociation of the inhibitors is preceded by phosphorylation and followed by rapid degradation. Active NF-κB/Rel complexes are also released after processing of p100 or p105. Once in the nucleus, NF-κB/Rel dimers activate target genes including c-*rel*, *nfkb*1, *nfkb*2, and *mad*-3. Newly synthesized IκBα molecules re-assemble with NF-κB/Rel dimers to reform latent cytoplasmic complexes. Alternatively, IκBα may enter the nucleus to directly remove NF-κB/Rel dimers from their target sites.


Activators of NF-κB
Lymphokines ie. IL-1, TNFα
Lipopolysaccharide
Mitogens ie. PMA, PHA
Viruses, ds RNA
Signaling molecules
Protein kinases
ceramide
oxygen radicals
Proteolytic cleavage
p105/ p100
p65/ c-Rel
p50/ p52
Degradation
IκBα
P
Phosphorylation
Feedback inhibition
Direct Inhibition (?)
RNA POL
TBP
κB
TATA
5'-GGGRANNYYCC-3'
c-rel
nfkb1
nfkb2
mad-3
newly synthesized IκBα
IκBα mRNA

activation of NF-κB-regulated genes, which may occur as a result of interactions between NF-κB/Rel factors (p65 and c-Rel) and TBP, the TATA binding component of the TFIID factor in the general transcription machinery (267, 340).

NF-κB/Rel activity is also regulated at the transcriptional level, conferred by the presence of NF-κB sites in the promoters of *nfkb*1, c-*rel*, *mad*-3 and *nfkb*2 genes (332, 341-346). Both posttranslational and transcriptional mechanisms contribute to an NF-κB autoregulatory loop which is important for control of NF-κB activity. Promoter analysis of the *mad*-3 gene revealed that p65-containing dimers, which are highly susceptible to IκBα-mediated inhibition, are the most potent activators of that gene (342). Newly synthesized IκBα re-associates with p65 to restore latent IκBα-p65-containing complexes (334, 335, 337), thus establishing a loop in which both the activator and the inhibitor are mutually regulated. Although the mechanism by which p65-IκBα complexes are re-assembled remains unknown, it has been demonstrated that free IκBα enters the nucleus (285, 301, 347), and that both IκBα and Bcl-3 remove NF-κB/Rel proteins from preformed protein-DNA complexes *in vitro* (304, 348).

Additional levels of regulation are also probable. Various combinatorial associations of NF-κB/Rel subunits, together with distinct DNA binding sites lead to differential NF-κB activity. p50/p65 dimers bind and activate NF-κB sites present in the Ig, HIV, IFN-β, and MHC class I promoters (264, 278, 282, 349, 350), while p52/p65 dimers bind to these sites but only activate the Ig and HIV promoters (271, 273, 280, 281). The target site can also dictate the type of activity of the dimers binding to it. For example, c-Rel homodimers activate the IL-2Rα promoter but repress the κB sites found in the HIV and IL-6 promoters (350-352). Differential mRNA splicing (e.g. p105 versus IκBγ) and precursor

processing (e.g. p105 versus p50) allow expression of proteins with different activities. Therefore, fine control of these two processes is necessary to maintain the proper intracellular balance of activators and repressors. Depending on the type of tissue (eg. B or T cells) or the differentiation stage (eg. pre-B or B cells), cells require different combinations of NF-κB/Rel and IκB proteins to generate constitutive or inducible NF-κB activity. Constitutive NF-κB activity in mature B cells is represented mostly by p50/c-Rel heterodimers whereas in pre-B cells the major inducible form is p50/p65 (353). Finally, interaction with other non NF-κB/IκB molecules can re-model functions and DNA-binding capacities. During virus infection, a cooperation between NF-κB proteins, which bind the PRDII (positive regulatory domain II, NF-κB site) region of the IFNβ promoter, and HMG I(Y) proteins (high mobility group), which bind the minor groove of the NF-κB site, is required for proper virus induction of the IFNβ gene (354). During T cell activation, IL-2 expression is dependent on the binding of a transcription factor supracomplex composed of NF-AT (nuclear factor of activated T cells), a dimer of Rel domain-containing proteins, and AP-1, a dimer of transcription factors that belong to the basic-zipper family (355, 356).

4. Transformation by NF-κB/Rel and IκB Proteins

The discovery that NF-κB/Rel transcription factors shared sequence homology with the v-*rel* oncogene suggested that loss of NF-κB transcriptional regulation contributed to aberrant cell growth. This section will discuss the involvement of NF-κB/IκB regulators (v-*rel* / c-*rel*, *relA* / *relA*Δ, *nfkb2* and *bcl*-3) in cellular transformation.

4.1 *v*-rel / *c*-rel

The reticuloendotheliosis virus strain T (REV-T) is the most virulent of all acutely transforming retroviruses. Young chickens die of multiple lymphomas in liver and spleen within 7 to 10 days after infection (357, 358). REV-T is a replication-defective virus that requires co-infection with REV-A, the replication competent viral counterpart carrying no oncogene (359). The standard *in vitro* assay for REV-T-mediated transformation is colony growth in soft agar after infection of primary cultures of avian cells (359). The transforming oncogene of REV-T is v-*rel*, generated as a result of a recombinatorial event between c-*rel* and REV-A during infection (236, 260, 360). v-*rel* transforms immature and mature B cells, mature T cells, myeloid cells, and, to a lesser extent, primary fibroblasts of avian origin (361-364). v-*rel* is a truncated and mutated version of avian c-*rel*, that recombined within the *env* gene of REV-A and retained small 5' and 3' end *env* amino acids. v-Rel lacks the cytoplasmic anchorage domain, activation domain I, and part of activation domain II of c-Rel. In addition, there are several internal amino acid substitutions and deletions (Fig. 3) (235, 236, 259).

The mechanism of transformation by v-*rel* has not been elucidated. Alterations in the Rel domain of v-*rel* abolish its oncogenic potential (248, 252, 253, 261, 270, 365-368). v-Rel efficiently binds NF-κB elements (240, 242, 369) and non-DNA binding variants fail to transform (369). The integrity of the PKA site is also crucial for v-Rel activity, since mutations of this site abrogate DNA binding and oncogenicity (252, 370). The NLS does not appear to contribute to v-Rel function as there is no correlation between the intracellular localization of v-Rel and its ability to transform cells (247, 248). v-Rel also uses the Rel domain to

heterodimerize with p50 (241-243), as well as with the inhibitory molecules IκBα, p105 and p100 (308, 310, 371-375). Interestingly, the association of IκBα and v-Rel does not inhibit the DNA binding activity of the oncogene (376). Clearly the Rel domain contributes significantly to the transforming ability of v-*rel*. However, overexpression of this domain alone is not transforming (368). Therefore, sequences outside the Rel domain contribute to transformation. In agreement with this, it has been demonstrated that C-terminal sequences in v-Rel are also necessary to promote cellular transformation (367, 368).

The ability of v-Rel to activate NF-κB-regulated genes remains controversial. A number of reports have suggested that v-Rel acts as a dominant repressor of NF-κB-regulated gene expression (241, 266, 270, 352, 369, 370, 377, 378). One model envisions a mechanism whereby transcriptionally inactive v-Rel-containing complexes directly contact NF-κB elements and block transcription. It was also suggested that v-Rel, by associating with other NF-κB/Rel proteins, depletes the cell of functional NF-κB complexes, and indirectly represses transcriptional activity. In contrast, numerous reports proposed that v-*rel* expression actually results in transcriptional activation of NF-κB-regulated genes via direct interactions (261, 368, 370, 378-382). Alternatively v-Rel, by associating with several of the inhibitory molecules (e.g. IκBα, p105 or p100), might allow positively acting factors to stimulate transcription. Transformation by a dominant negative mechanism implies that some NF-κB-controlled tumor suppressor genes fail to be activated, whereas transformation by a positive oncogenic mechanism implies activation of genes promoting cell proliferation. This latter possibility seems more probable since most NF-κB-regulated genes identified so far are involved in cell proliferation. This model is also supported by the observation that overexpression of v-*rel* results in increased expression

of MHC proteins, IL-2 receptor and IκBα (378, 381, 383).

The alterations necessary for conversion of c-*rel* into an oncogene are not unambiguously established. Likewise it is not clear whether overexpression of c-*rel* induces cellular transformation (260, 269, 378). The removal of C-terminal *trans*-activation domains resulted in tumor formation in some cases (260, 269, 378, 384), but had no effects in others (261). Translocations of the c-*rel* gene in certain lymphomas resulted in deletion or amplification of c-Rel C-terminal sequences (385, 386). These *in vivo* observations suggest that c-*rel* overexpression might induce transformation and that C-terminal truncations of c-Rel may be responsible for the acquisition of the tumorigenic potential of v-Rel.

4.2 rel*A*/rel*AΔ*

An alternatively spliced derivative of p65 was identified and named p65Δ (387). This splicing variant lacks amino acid residues 222 to 231, located in the Rel dimerization domain. This deletion prevents p65Δ from dimerizing with other NF-κB/Rel proteins, although a weak interaction with p65 is maintained. As a result of the inability p65Δ to form dimers, DNA binding activity is lost. Overexpressed p65Δ inhibits DNA and transactivation activities by NF-κB, perhaps due to a squelching effect exerted by the intact C-terminal transactivation domain (387). p65Δ was initially reported to induce focus formation, anchorage-independent growth in soft agar and tumor formation in athymic mice. These transformation-specific characteristics were not observed by overexpressing normal p65 or a mutant p65Δ containing a deletion within

the transcriptional activation domain (387). It was proposed that p65Δ transforms cells by sequestering factors whose expression is required to maintain control over cell growth (e.g. tumor suppressor genes). However, the experiments demonstrating the tumor promoting effects of p65Δ could not be reproduced by an independent group (388). Another report demonstrated that antisense inhibition of p65 blocks tumorigenicity and causes tumor regression, thus providing support for an active role of wild type p65 in cellular transformation (389).

4.3 nfkb*2*

The *nfkb2* gene was the first member of the NF-κB/Rel family to be implicated in human cancer. *nfkb2* was originally detected in a B-cell Non-Hodgkins Lymphoma (B-NHL) with a chromosomal translocation, t(10:14)(q24:q32) that juxtaposes the immunoglobulin $C\alpha_1$ locus to a novel gene, *lyt-10* (lymphocyte translocation chromosome 10) (272). Sequencing of the normal *lyt-10* gene revealed a strong homology with the *nfkb*1 gene and showed that it was in fact identical to another recently described NF-κB family member, the *nfkb2* gene (271, 272). As a result of the translocation, the fusion protein lyt-10-$C_{\alpha 1}$ retains the Rel domain but lacks the ankyrin repeats, and, unlike normal lyt-10, binds NF-κB DNA *in vitro* (272), suggesting that removal of the inhibitory ankyrin repeats and substitution with immunoglobulin sequences, produce a constitutively active form of NFKB2. Additional structural alterations of the *nfkb2* gene have been identified in B cell chronic lymphocytic leukemia (B-CLL), cutaneous B cell lymphoma (CBCL), multiple myeloma (MM), and cutaneous T cell lymphomas (CTCL) (390, 391). In all cases, partial or total deletion of the

carboxy terminal ankyrin repeats were observed, suggesting that loss of IκB-like activity in the C-terminus of p100 represents a general mechanism of oncogenic activation of the *nfkb2* gene in multiple B and T cells malignancies.

4.4 bcl-*3*

Several cases of human B-cell chronic lymphocytic leukemia (CLL) contain a t(14:19) translocation which juxtaposes the α constant region exons of the immunoglobulin heavy chain locus (IgH gene, on chromosome 14) adjacent to the *bcl-3* gene on chromosome 19, in a head-to-head manner (392). The translocation does not affect the transcriptional integrity of the *bcl-3* gene. However leukemic cells from CLL patients that carry the translocation express 3 to 4 times more *bcl-3* mRNA than leukemic cells from CLL patients without the translocation (312), indicating that *bcl-3* overexpression is sufficient for tumor development. Several mechanisms of *bcl-3*-induced oncogenesis are possible. Overexpressed Bcl-3 may inhibit DNA binding of p50 or p52 homodimers, thus allowing binding of more transcriptionally active subunits (e.g. p65 and c-Rel) (317). Alternatively, Bcl-3 may associate with these same homodimers and create transcriptionally active complexes that directly stimulate transcription.

In conclusion, members of the NF-κB/IκB family clearly possess oncogenic potential, although the molecular mechanisms of transformation remain to be elucidated.

Specific Research Aims

Molecular, biochemical and epidemiological evidence strongly implicate HTLV-I as an etiologic agent of ATL. HTLV-I infection of T cells initiates a multistep oncogenic process, characterized by an early polyclonal proliferation of T cells by an autocrine mechanism that involves high levels of IL-2 and IL-2Rα gene expression. Over a time course that encompasses decades *in vivo*, a growth factor independent monoclonal proliferation of leukemic T cells emerges. The Tax protein of HTLV-I is a positive transcriptional activator of the viral LTR as well as of cellular genes (e.g. IL-2Rα, IL-2, and GM-CSF). Evidence suggests that the Tax protein mediates transformation by virtue of its ability to interact with cellular proteins involved in transcription control. One of the cellular targets of Tax *trans*-activation is the NF-κB/IκB family of transcriptional regulators. The objective of this research was to characterize the molecular mechanisms by which Tax protein interacts with the NF-κB/IκB transcriptional regulators, and initiates aberrant gene expression, a process that ultimately results in leukemogenic transformation. The specific research aims are the following:

1. Characterize DNA binding and transcriptional activity of NF-κB/Rel proteins in Tax expressing cells.

2. Analyze physical interactions between the Tax protein and NF-κB/Rel and IκB subunits.

3. Characterize phosphorylation and turnover of IκBα in HTLV-I infected and Tax expressing cells.

CHAPTER 2

MATERIALS AND METHODS

1. Tissue Culture and Protein Extractions

1.1 *Cell lines*

Jurkat cells are CD4+/CD8- human immature T helper lymphocytes. Although these cells are immortalized and continuously growing, they resemble normal resting T cells both in morphology and activation requirements. Therefore, throughout the work presented in this thesis, they have been used as the normal control against which Tax expressing and HTLV-I infected T cells were compared. The 19D and 9J cell lines are Jurkat cells stably transformed with a Tax expressing plasmid (119). The NC2.10 cell line was generated by stably transforming Jurkat cells with an anti-sense Tax expressing plasmid (119). The MT-2 cell line is a human T cell line isolated from umbilical cord blood lymphocytes co-cultured with cells from ATL patients. These cells are transformed with and continuous producers of HTLV-I virions (393, 394). The HTLV-I-transformed MT-4 cells are human T cells isolated from a patient with ATL (394-396). C8166-45 cells are human umbilical cord blood lymphocytes co-cultured with cells from ATL patients. The HTLV-I genome contained in these cells is largely deleted, and only the Tax and Rex viral proteins are expressed (397). Cos-7 cells are African Green monkey kidney epithelial cells.

1.2 *Culture conditions*

All cell lines were grown at 37°C in 5.2% CO_2 incubators. T cell lines are suspension cells maintained between $2x10^5$ and 10^6 cells/ml. Cos-7 cells are adherent cells. Jurkat, 19D, 9J and NC2.10 were cultured in 10% calf bovine

serum (CBS) RPMI 1640 (GIBCO), supplemented with 2 mM glutamine and 50 µg/ml gentamycin. NC2.10, 19D and 9J culture media also contained Geneticin (G418, GIBCO) at 50 µg/ml to maintain selective pressure. 19D, 9J and NC2.10 cell lines were obtained from Dr. W.C. Greene (119). MT-2 and MT-4 cells were cultured under biosafety level 2 conditions, in RPMI 1640 supplemented with 15% fetal bovine serum (FBS), 2 mM glutamine and 50 µg/ml gentamycin. Both cell lines were obtained from Dr. Douglas Richman (393-396) through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. Cos-7 cells were cultured in Dulbecco's modification of Eagles medium (D-MEM, high glucose) supplemented with 10% fetal clone serum (FCS), 2 mM glutamine and 50 µg/ml gentamycin. PMA and TNFα inductions were performed at final concentrations of 25 ng/ml and 140 units/ml, respectively. Cycloheximide (CHX) treatments were performed at a final concentration of 50 µg/ml.

1.3 *Preparation of protein extracts*

Whole-cell extracts (WCE): cells were collected by centrifugation (1200 rpm, 7 min), washed once with 1 volume of cold phosphate-buffered saline (PBS), and resuspended (2 ml per 10^8 cells) in WCE buffer (20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 0.5 mM EGTA ([ethylenebis(oxyethylenenitrilo)]-tetraacetic acid), 0.5 mM spermidine, 0.15 mM spermine, 10% glycerol, 10 mM Na_2MoO_4, and 1 mM DTT) containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 0.01 µg/µl each of pepstatin, aprotinin and leupeptin. Cells were lysed by slowly adding 2 M KCl to a final concentration of 0.5 M. Lysates were gently mixed for 30 min at 4°C, and

cellular debris were removed by centrifugation (30 000 rpm, 90 min, 2°C). Supernatants were then diluted to 0.1 M KCl by adding WCE buffer. Final protein concentrations were determined by the Bio-Rad protein assay.

NP-40 lysis: cells were rinsed with PBS twice and lysed by resuspending in 0.5 ml of lysis buffer per $2x10^7$ cells (lysis buffer: 10 mM Tris-Cl, pH 8, 60 mM KCl, 1 mM EDTA ([ethylenedinitrolo]-tetraacetic acid), 1 mM DTT, 0.5% Nonidet P-40 (NP-40), 0.5 mM PMSF, 0.01 µg/µl each of leupeptin, pepstatin and aprotinin). After an incubation on ice for 5 to 10 min, cellular debris was microfuged for 10 min, 4°C. Supernatants were recovered and protein concentrations were determined by the Bio-Rad protein assay.

Freeze-thaw lysis: about 10^7 cells were washed with cold PBS and resuspended in 60 µl of 0.25 M Tris-HCl, pH 7.8. Cells were lysed by three cycles of freeze (15 min on dry ice)-thaw (5 min in 37°C waterbath). Debris was microfuged for 5 min, and supernatants were collected and assayed for protein concentration by the Bio-Rad protein assay.

Nuclear and cytoplasmic extracts: cells were washed once with cold PBS, twice with buffer A (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, 0.01 µg/ml each of pepstatin, leupeptin and aprotinin) (398), and resuspended in buffer A with 0.1% NP-40 (20 µl per 10^7 cells). After a 10 min incubation on ice, nuclei were gently mixed and microfuged 10 min at 4°C. The supernatant (cytoplasmic extract) was removed and diluted with 75 µl of modified buffer D (20 mM Hepes, pH 7.9, 0.05 M KCl, 20% glycerol, 0.5 mM EDTA, 0.5 mM PMSF, 0.01 µg/µl each of leupeptin, pepstatin and aprotinin) (211). The nuclear pellet was resuspended in 15 µl of buffer C (20 mM Hepes,

pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.01 μg/μl each of leupeptin, pepstatin and aprotinin, 0.5 mM DTT) (398), incubated 15 min on ice and finally microfuged 10 min at 4°C. The supernatant was collected (nuclear extract).

2. Gel Migration Assays

2.1 *Oligonucleotides*

Oligonucleotides were synthesized on a Pharmacia Gene Assembler and purified on denaturing polyacrylamide gels. Complementary strands were annealed, and double-stranded oligonucleotides were purified on native polyacrylamide gels. The BUdR (5'-bromo-deoxyuridine)-substituted P2 oligonucleotide was obtained by replacing thymidine residues in the nucleotide sequence of P2 with 5'-bromo-deoxyuridine CED phosphoramidite (Pharmacia) (399). The sequences of the plus strands of the oligonucleotides are as follows: P2, the NF-κB site of the IFN-β gene promoter, 5'-GGGAAATTCC-3'; HIV κB, 5'-GGGACTTTCC-3'; P2-BUdR, 5'-GGGAAABBCCGGGAAA BBCC-3' (B=BUdR). Oligonucleotides were 5' end labeled with [γ-^{32}P]ATP (ICN, 3888 Ci/mmol) and polynucleotide kinase (Pharmacia) for 1 hr at 37°C and subsequently applied to Sephadex G-25 column to remove unincorporated nucleotides.

2.2 *Electrophoretic mobility shift assays*

Gel migration assays were performed by preincubating whole cell protein

extract (see figure legends for exact amounts), in WCE buffer (0.1 M KCl), with 5 µg of poly dI:dC as nonspecific competitor DNA for 10 min on ice. ^{32}P-labeled oligonucleotide probe (0.2 ng, 30 000 cpm) was added to the protein extract and incubated for 30 min at room temperature. To ensure DNA binding specificity, competition assays were performed by incubating a 125 fold excess of unlabeled competitor (25 ng) with the protein for 10 min on ice, prior to the addition of radiolabeled probe. For supershift assays, whole cell extracts were pre-incubated with subunit-specific antiserum (1 µl) in the presence of 5 µg poly dI:dC, 20 min at room temperature, prior to addition of the radiolabeled probe. Supershift specificity was verified by competition with an excess of peptide (1 µg) against which the antiserum was raised. DNA-protein complexes were resolved on native 5% acrylamide gels (60:1 cross-link, in 25 mM Tris-HCl, 195 mM glycine, pH 8.3). After running for about 4 hr at 150 V, gels were blotted to 3MM paper (Whatman), dried under vacuum at 60°C, and autoradiographed (Kodak X-Omat film) at -80°C. Relative DNA binding activity was obtained by laser densitometry of the autoradiographs.

2.3 *UV cross-linking assays*

For ultraviolet light (UV) cross-linking analysis, gel retardation assays were performed using the double-stranded P2-BUdR oligonucleotide probe (399). Protein-DNA complexes were resolved by electrophoresis through a 5% native polyacrylamide gel. The complexes were cross-linked *in situ* by resting the wet gel directly on a Spectroline transilluminator (Model TR-302, 302 nm) and exposing it to UV light for 25 min at 4°C. The region corresponding to the shifted complexes was localized by autoradiography, excised with a scalpel, and

soaked in 30 ml SDS sample buffer (1% SDS, 2% v/v β2-mercaptoethanol, 20% glycerol, 10 μg/ml bromophenol blue, 62 mM Tris-HCl, pH 6.8) for 5 min at 68°C. The SDS sample buffer was aspirated and the gel was rinsed twice in 20 ml stacking buffer (0.1% SDS, 125 mM Tris-HCl, pH 6.8). The gel slice was sandwiched between electrophoresis glass plates and anchored to the top of the plates using 1% agarose solution (prepared in 0.1% SDS, 125 mM Tris-HCl, pH 6.8). An 8% SDS running gel with a 4% SDS stacking gel was cast to within 1 cm of the sandwiched gel slice. The remaining 1% agarose solution was poured around the gel slice to complete the cast. Single-tooth combs were inserted into the agarose to form wells into which molecular weight protein standards (Sigma) were loaded. The gel was run at 45 mA until the tracking dye entered the stacking matrix and then electrophoresis was continued at 100 V for an additional 16 hr. Gels were stained in Coomassie blue to reveal the molecular weight markers, dried and autoradiographed for 1-7 days.

3. Plasmids, Transfections and Transient Expression Assays

3.1 *Reporter plasmids*

The chloramphenicol acetyltransferase (CAT) gene was used as a reporter for transcriptional activity in transient expression assays. The SV1CAT plasmid contains the CAT gene under the control of the GC-rich SV40 promoter but lacks SV40 enhancer activity (400). The SV2CAT construct uses both the promoter and strong enhancer of SV40 to drive CAT gene expression (400). The HIV-CAT construct links the complete HIV-1 LTR (-350 to +80) to the CAT gene (223). The κB(3)-CAT (or HIV enh-CAT) construct was obtained by

inserting three copies of the HIV-1 NF-κB enhancer into the enhancerless *Acc*I-*Sph*I-cleaved SV1CAT vector (401).

3.2 *Expression vectors*

The NF-κB expression plasmids were produced by subcloning different NF-κB genes into a CMV promoter based expression vector (kind gift from A. Cochrane): 1) p105 - a 3205 bp *Eco*R1 fragment of KBF-1 (242) was subcloned into the CMV vector at the *Eco*R1 site; 2) p50 - a 1318 bp *Eco*R1 fragment generated by PCR from the first amino acid (177 bp) to amino acid 440 (1495 bp) of KBF-1 (242) was subcloned into the *Eco*R1 site of CMV; 3) p100 - a 2853 bp *Hind*III- *Eco*R1 fragment from plasmid Bluescript-SK (271) was subcloned into CMV at the *Hind*III- *Eco*R1 sites; 4) p52 - a 1503 bp *Hind*III- *Eco*R1 fragment (271) was also subcloned into CMV at the *Hind*III - *Eco*R1 sites; 5) p65Δ - a 2572 bp *Xba*1-*Xho*1fragment from plasmid Bluescript-SK (387) was subcloned into the BamH1-*Xho*1 site of SVK3; 6) c-*rel* - the 2340 bp *Eco*R1 fragment of *c-rel* cDNA (262) was cloned into the CMV *Eco*R1 site; 7) IκBα - a 1190 bp *Eco*R1 fragment from pGEX-2T (296) was subcloned into the *Eco*R1 site of CMV; 8) the CMIN-p65 vector (237) was used to express the p65 subunit; 9) the p50/p65 fusion - the cDNA encoding *trans*-activation domain of p65 was obtained by PCR amplification with p65 cDNA clone (cloned into plasmid Bluescript-SK) using a specific primer corresponding to positions 1270-1290 (5'-TAGCTCTAGACATGGTATCTGCTCTGGCCC-3') and the KS primer; the *Xba*I-*Xho*I fragment of the PCR product was cloned into the *Xba*I-*Xho*I site of the pSVK3/p105 construct to generate pSVK3/p50-p65. The Tax expression plasmid phtat-1 contains Tax coding sequences under the control of the HTLV-I

LTR (89, 98).

3.3 *Cell transfections*

DEAE-dextran method : $2x10^7$ Jurkat cells were transfected in a volume of 1 ml with the DNA of interest (see figure legends). After 20 min at room temperature, cells were diluted with 10 ml of complete media containing 100 µM chloroquine and incubated for 45 min at 37°C. Cells were pelleted by low-speed centrifugation, resuspended at 10^6 cells/ml and left at 37°C. PMA inductions were carried out 24 hr after transfections (402).

Lipofectamine transfection: COS-7 cells ($7x10^3$) were seeded into a chamber slide (Nunc) two days prior to transfection. Appropriate amounts of DNA (see figure legends) and lipofectamine (GIBCO) were diluted in MEM-NS (without serum), gently mixed together and incubated at room temperature 30 min to allow formation of complexes. Complexes were then diluted with 200 µl of MEM-NS and added to subconfluent cells previously rinsed with MEM-NS. Cells were exposed to DNA complexes for 5 hr and then fed with MEM 20% FBS for 18-24 hr at 37°C. Medium was then replaced with fresh, complete medium and the incubation was continued.

3.4 *CAT assays*

48 hr after transfection, cells were harvested and assayed for accumulated CAT activity by standard procedure (400). The appropriate amount of freeze-thaw

extract (see figure legends) was diluted in 0.25 M Tris-HCl, pH 7.8, to a total volume of 60 µl. The following was then added: 70 µl of 1 M Tris-HCl, pH 7.8, 20 µl of 3.5 mg/ml acetyl coenzyme-A (Pharmacia) and 0.5 µl of D-threo-(dichloroacetyl-1-^{14}C) chloramphenicol (Amersham, 54 mCi/mmol). This mixture was incubated in a 37°C waterbath for the appropriate period of time (see figure legends) and the labeled chloramphenicol was extracted with 1 ml of ethyl acetate which was subsequently evaporated under dry air. Pellets were resuspended in 30 µl of ethyl acetate, spotted on a TLC plate (Whatman), and resolved by ascending thin layer chromatography (TLC) in 95% chloroform- 5% methanol. The plate was dried, enhanced with Amplify (Amersham) and autoradiographed at -80°C. Percent acetylation was determined by dividing the radioactivity in the acetylated forms by the sum of the acetylated and nonacetylated forms. The relative inducibility was obtained by dividing percent acetylation of treated sample by the percent acetylation of the untreated sample. A plasmid carrying the β-galactosidase gene (under the control of the RSV promoter) was co-transfected as a control for transfection efficiencies. CAT activity was normalized to the β-galactosidase activity.

4. RNA Analyses

4.1 *Preparation of RNA*

Total cellular RNA was isolated from approximately 10^8 cells by a modified guanidium isothiocyanate procedure (403). Cells were collected by low-speed centrifugation, washed with PBS, resuspended in 5 ml of solution D (4 M guanidinium isothiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1

M β2-mercaptoethanol), and vortexed briefly to ensure lysis. 0.5 ml of 2 M sodium acetate, pH 4.0, 5 ml phenol:water, 2 ml of chloroform:isoamylacohol (49:1) were then sequentially added and vortexed. After 15 min on ice, phases were separated by a 20 min, 10 000 rpm spin at 4°C (HB-4 rotor, Sorvall). The aqueous phase was collected, mixed to an equal volume of isopropanol and precipitated overnight at -20°C. The RNA was pelleted by a 20 min centrifugation (10 000 rpm, 4°C), resuspended in 0.6 ml of solution D and isopropanol-precipitated again. The RNA was finally resuspended in sterile water and the concentration was determined. For reverse transcriptase-polymerase chain reactions (RT-PCR), RNA was further treated with 1 U of RNase-free DNase (RQ1 DNase; Promega Biotec) for 15 min at 37°C in 40 mM Tris-HCl, pH 7.9, 10 mM NaCl, 6 mM $MgCl_2$. RNA was phenol extracted, ethanol precipitated, and stored at -80°C. Poly A+ RNA was obtained by applying total RNA on Poly-Prep Chromatography Columns (BioRad) containing equilibrated oligo-dT sepharose.

4.2 *Reverse transcriptase-polymerase chain reactions*

Total RNA (2 µg) was reverse transcribed with 200 U of Moloney murine leukemia virus reverse transcriptase (BRL) and 10 pmol of primer B (3' end) in a total volume of 50 µl. PCR amplification (404) was performed in a total volume of 100 µl, using 25 µl of reverse transcriptase product, in PCR buffer containing 100 mM Tris-HCl, pH 8.4, 500 mM KCl, 15 mM $MgCl_2$, 100 µg bovine serum albumin per ml, the four deoxynucleoside triphosphates (Pharmacia) at 2 mM each, 10 pmol of primer A (5' end), 10 pmol primer B (3' end), and 1 U of *Taq* DNA polymerase (Promega). Amplification (28 cycles) was carried out by

denaturing at 95°C for 5 min, annealing at 55°C for 1 min, and extending at 72°C for 1 min, followed by denaturation at 95°C for 1 min. PCR products were analyzed on a 5% denaturing polyacrylamide gel (19:1 cross-link, 7 M urea) and autoradiographed overnight at -80°C. PCR primers were 5' end labeled with [γ-^{32}P]ATP (ICN) and 5 U of T4 polynucleotide kinase (Pharmacia) for 1 hr at 37°C. Markers (plasmid pAT153 cleaved with *Hae*III) were also 5' end labeled with [γ-^{32}P]ATP and polynucleotide kinase. Signals were quantified by laser scanning of the autoradiographs. Table 3 shows the primers used for amplification of the GM-CSF, *nfkb*1 , *tax*, c-*rel*, and GAPDH genes.

4.3 *Northern blots*

Poly A+ RNA (5 µg) was electrophoresed on a 1.2% denaturing formaldehyde agarose gel, transferred to a nylon membrane (Hybond-N, Amersham), cross-linked to the membrane in a Stratalinker UV crosslinker (Stratagene) according to the manufacturer's suggestions and baked for 2 hr at 80°C in a vacuum oven. The *Rsa*I digest fragment of SVK-IκBα vector (296) was labeled by random priming with [α-^{32}P]dCTP (Amersham) using an Oligolabelling Kit (Pharmacia) and hybridized to immobilized poly A+ RNA. Blots were air dried and exposed at -80°C. After exposure, films were scanned using a densitometer. Values were normalized to β-actin expression and plotted as relative mRNA levels.

5. Antisera

Peptide sequences derived from the NF-κB proteins were chosen for highest

TABLE 3. Oligonucleotides Used in mRNA Phenotyping Analyses

Transcripts	Location of primers in nucleotide sequences[1] 5' primer	3' primer	Fragment size (bp)
GM-CSF	668-697 5'-GGC TGC AGA GCC TGC TGC TCT TGG GCA CTG-3'	962-933 5'-CTG GAG GTC AAA CAT TTC TGA GAT GAC TTC-3'	196[2]
*nfkb*1	1365-1388 5'-ACT GGA AGT ACA GGT CCA GGG TAT-3'	1695-1669 5'-TGC ATA GCC TTC TCT AGA AAG AGG TTA-3'	330
tax	5091-5114 5'-GTG GAT CCC GTG GAG ACT CCT CAA-3'	7360-7337 5'-TCC AAA CAC GTA GAC TGG GTA TCC-3'	153[3]
GAPDH	371-388 5'-CCA TGG AGA AGG CTG GGG-3'	565-546 5'-CAA AGT TGT CAT GGA TGA CC-3'	196
c-*rel*	136-159 5'-GAC GCT GGG AGC TGC CTG CGG GAA-3'	477-454 5'-CAA AGG TCT GCG TTC TTG TCC AAA-3'	342

1. The sequence data and nucleotide position numbering were derived from references (71, 242, 405-407).
2. The splice junctions of intron 1 link nucleotides 822 and 921.
3. The splice junction links nucleotides 5183 and 7302.

degree of hydrophilicity and antigenic index; in some cases an additional cysteine residue was added at the C- or N-terminus to facilitate coupling to the carrier protein. HPLC purified synthetic peptides (Core Facility for Protein/DNA Chemistry, Queen's University, Kingston, Ont.) were dissolved in sterile water or 50% dimethylsulfoxide (DMSO) and coupled to preactivated KLH carrier (Pierce) according to manufacturer's instructions. Rabbits received 1 mg of coupled peptide in Freund's incomplete adjuvant (ICN) injected subcutaneously (s.c.) in the dorso-scapular region for the primary immunization. Boosts of 300 μg of peptide also in Freund's incomplete adjuvant were administered s.c. at 3, 6, 9, 15, and 18 weeks after the primary immunization. Rabbits were bled 12 to 14 days following each boost and serum was collected. Antisera were screened by Western blot and immunoprecipitation for specificity and reactivity to NF-κB proteins. Serum from animals receiving the 5th boost was used in these studies.

Antibodies NR1141 and AR27 (anti-NFKB1-N) were raised against a peptide (amino acids 2-15) at the most N-terminal region of NFKB1, and recognize both p105 and p50. Antibodies NR1140 and AR25 were raised against the C-terminus of p105 (amino acids 955-969) and recognize p105 and IκB-γ. Antibodies NR1267 and AR43 (anti-NFKB2-N), were generated against a peptide at the N-terminus of NFKB2 (amino acids 2-17), and recognize both p100 and p52. Antibodies NR1226 and AR28 recognize the C-terminus of p65 and p65Δ (amino acids 537-550). Antibodies NR265 and AR22 recognize the C-terminus of c-Rel (amino acids 573-587). Antibody NR1258 was raised against the C-terminus of IκBα (amino acids 301-317). AR20 recognizes the N-terminus of IκBα (amino acids 2-16). "NR" antibodies were generated by and obtained from Dr. Nancy Rice (unpublished and 275). "AR" antibodies were

generated by Anne Roulston (unpublished and 408). Antiserum to Tax was raised against a C-terminal peptide (amino acids 326-353) and was obtained from Dr. Bryan Cullen (409) through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. A summary is presented in Table 4.

6. Immunoblots

6.1 *Preparation of extracts*

The methods (WCE, NP-40 lysis, or nuclear and cytoplasmic extracts) used to prepare the protein extracts are specified in figure legends. Between 20 and 100 μg were usually used (see figure legends). Samples were prepared for immunoblotting by adding an equal volume of 2xSDS sample buffer, boiling 10 min and loading on sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE).

6.2 *Phosphatase treatment*

In vitro dephosphorylation of IκBα was achieved by incubating 100 μg of extracts with 5 units of calf intestinal alkaline phosphatase (CIP, 1unit/μl, Promega) for 1 hr at 37°C. Final volumes were adjusted to 80 μl with 8 μl of 10x CIP buffer (0.5M Tris-Cl, pH 9.0, 10 mM $MgCl_2$.1 mM $ZnCl_2$, 10 mM spermidine) and water. Additional reactions also contained both CIP and phosphatase inhibitors (Na_3VO_4, 10 mM and NaF, 50 mM, final), to block *in vitro* dephosphorylation of IκBα. 20 μl of 5xSDS sample buffer was added and

TABLE 4. Peptide Specific Antisera Against NF-κB, IκB and Tax Proteins

Protein	Antisera	Peptide Sequence	Peptide Location
p50/p105	NR1141	AEDDPYLGRPEQMFHC	2-15
	AR27		
p105/IκBγ	NR1140	CMPHDYGQEGPLEGKI	955-969
	AR25		
p52/p100	NR1267	GESCYNPGLDGIIEYDD	2-17
	AR43		
p65	NR1226	CADMDFSALLSQISS	537-550
	AR28		
c-Rel	NR265	CNEQLSDSFPYEFFQV	573-587
	AR22		
IκBα	NR1258	CDELPYDDCVFGGQRLTL	301-317
	AR20	FQAAERPQEWAMEGPC	2-16
Tax	Tax	NEKEADENDHEPQISPGGLEPPSEKHFRETEV	321-353

samples were boiled and loaded on 20 cm-long, 12% SDS-PAGE gels.

6.3 *Electrophoresis, transfer, blot and detection*

Gels were run overnight at 20 mA with water circulation. The next day, samples were transfered to nitrocellulose membrane (Schleicher and Schuell) for 4 hr, 400 mA, in 20% methanol-1xTris-Glycine. Mini-gels were run for 1 hr at 100 V and transfered for 1 hr at 100 V. Ponceau Red staining was used to locate protein molecular weight markers. Membranes were blocked 1 to 2 hr in 5% milk-PBS, and incubated overnight with antibody diluted in 5% milk-PBS, with 0.01% Thimerosal to prevent bacterial growth. The next day, the antibody was removed and the membrane was rinsed with PBS 4x15 min, incubated with peroxidase-conjugated anti-rabbit immunoglobulin serum (Amersham) diluted 1:1000 in 5% milk-PBS for 1 hr at room temperature. 25 ml of this secondary antibody solution were used per 96 cm^2 of membrane. The membrane was rinsed with PBS 4x15 min, and finally incubated for 1 min in reagents of the enhanced chemiluminescence detection system (Amersham, mixed 1:1). 0.125 ml of detection reagents were used per cm^2 of membrane. The membrane was exposed to autoradiographic film and the signals were quantified by laser densitometry.

7. Immunoprecipitations

7.1 *Metabolic cell labeling*

Cells were cultured in methionine-free RPMI for 1 hr, then labeled 2 hr at 37°C with ^{35}S-methionine (0.1 mCi/ml, ICN, Trans-label). After labeling, cells were washed and protein extracts were prepared as described above. For each immunoprecipitation reaction (i.e. per lane of gel), between 200 and 500 µg of extract (or between 0.5 and $2x10^7$ cpm) was used.

7.2 *Simple immunoprecipitations*

To the appropriate amount of extract, 50 µl of normal rabbit serum (NRS) per ml of extracts were added. The reaction was completed by adding TNT (20 mM Tris-Cl, pH 7.5, 200 mM NaCl, 1% Triton) to a final volume of 250 µl. Preclearing was done by rotating 1 hr at 4°C. The reaction was transfered on 100 µl of protein A sepharose (previously washed 3 times with 1 ml of TNT) and rotated for 1 hr at 4°C. Protein A sepharose beads were microfuged for 1 min, and the pre-cleared supernatant was transfered into a new tube. The primary antibody was added, usually diluted between 1:500 and 1:1000. Reactions were rotated 1 hr at 4°C, and transfered on 25 µl of washed protein A sepharose beads. Another 1 hr incubation at 4°C was performed. Beads were washed with TNT, 3x1 ml, and finally resuspended in 25 µl of 2xSDS sample buffer. Samples were boiled and loaded on the gel. Gels were run, fixed (30 min in 7.5% acetic acid and 25% methanol), soaked in Amplify (20 to 30 min, Amersham), dried and exposed at -80°C. Peptide competition was used to

confirm the specificity of the antibody by pre-incubating the antibody with 10 µg of cognate peptide for 10 min on ice.

7.3 *Pulse-chase analysis*

Cells were cultured 15 min in methionine-free RPMI supplemented with 2% fetal clone serum, pulse-labeled for 60 min with ^{35}S-methionine (0.5mCi/ml, ICN, Trans-label), and chased with cold methionine for times indicated. The zero time point was harvested immediately after the pulse and aliquots were taken 2, 6, 8 and 12 hr later for preparation of whole cell extracts, as described above, except that TNT buffer (275) was used for the final dilution. Extracts were then subjected to immunoprecipitation as follows: 300 µg of protein were first pre-cleared by an incubation with 50 µl of NRS/ml, 1 hr at 4°C, followed by recovery of non-specific immune reactions on protein A sepharose (4°C, 1 hr). Antibody NR1267 was then added at a final dilution of 1:500 and incubation at 4°C was continued for 1 hr. For competition, both the antibody and the corresponding peptide were added to pre-cleared extracts. Specific immune complexes were recovered on protein A sepharose and processed as described above. Autoradiograms were scanned by laser densitometry to determine the relative intensities of NFKB2.

7.4 *Double and boil immunoprecipitations*

For the double immunoprecipitation, the simple immunoprecipitation procedure was followed up to the boiling in SDS sample buffer. Initial reaction volumes

were adjusted to accomodate the second immunoprecipitation. After boiling and spinning down the beads, the supernatants were collected and divided according to the number of second cycle immunoprecipitations to be performed. TNT was added to 250 µl (1:10 final) and fresh secondary antibody (ies) was added. After an overnight incubation at 4°C, immune complexes were recovered on protein A sepharose beads and processed as described in the simple immunoprecipitation protocol.

For the boil method of immunoprecipitation, TNT was added to the required amount of extracts up to a final volume of 250 µl. Then 50 µl of 5xSDS sample buffer were added, and samples were boiled for 10 min. SDS sample buffer was diluted by adding 900 µl of TNT (1:4 final) and samples were pre-cleared as described and incubated with antibody overnight at 4°C. The following day, samples were finally processed as described in the simple protocol.

8. Immunofluorescence

At 48 hr after transfection, cells were fixed with 50% acetone: 50% methanol at -20°C for 15 min. Cells were blocked for 30 min with 5% FBS/PBS and, after washing with PBS, antiserum was added at 1:100 dilution in 3% bovine serum albumin (BSA)/PBS for 2 hr at room temperature. After washing 3 times with PBS, the cells were incubated with goat anti-rabbit antiserum conjugated with fluorescein at a dilution of 1:200 in 3% BSA/PBS for 1 hr at room temperature. Cells were washed 3 times with PBS and mounted in 50% PBS: 50% glycerol. Fluorescence microscopy was performed with a Leitz Aristoplan microscope. Subcellular localization of Tax or NF-κB fluorescence was quantified as nuclear

or predominantly nuclear (N), distributed equally between the nucleus and cytoplasm (N/C) or cytoplasmic (C).

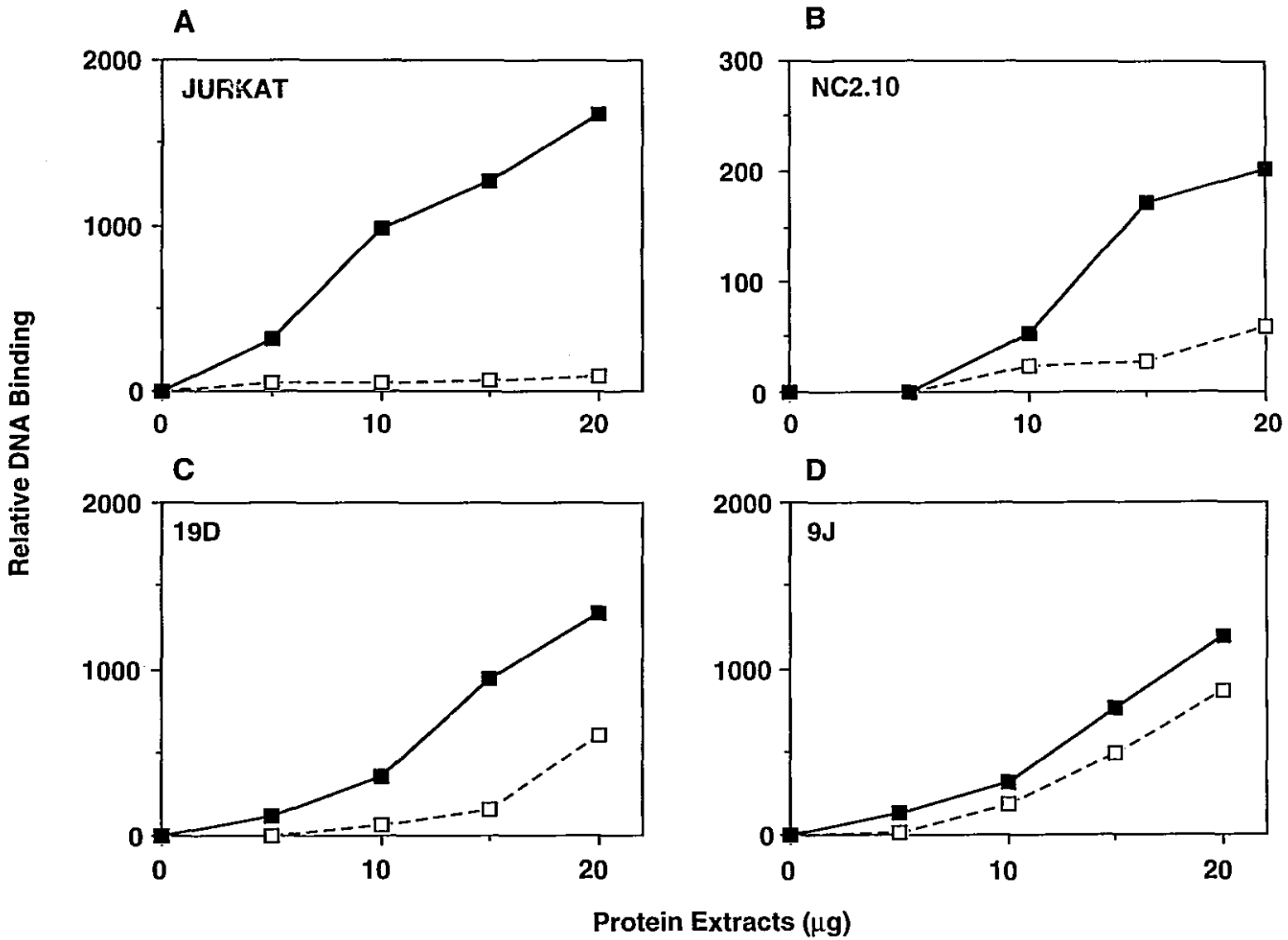
CHAPTER 3

NF-κB ACTIVITY IN T CELLS STABLY EXPRESSING THE TAX PROTEIN OF HTLV-I

1. Constitutive NF-κB Binding Activity in Tax Expressing T Cells

The effect of long-term expression of the *tax* gene product on the DNA binding properties of NF-κB were investigated in T lymphoid Jurkat cells stably expressing the Tax protein (19D and 9J) and in an isogenically matched cell line containing an expression vector with the *tax* gene in the antisense orientation (NC2.10). Uninduced and PMA-induced Tax and anti-Tax cell lines were examined for quantitative or qualitative changes in the DNA binding properties of NF-κB by electrophoretic mobility shift assay (Fig. 5). A radiolabeled oligonucleotide (P2) containing two copies of the NF-κB binding site found in the interferon β PRDII enhanson (401) was used to identify NF-κB-specific DNA binding activity present in increasing amounts of cellular extracts (0-20 μg). In uninduced Jurkat and NC2.10 cell extracts, NF-κB protein-DNA complexes were detected only at higher extract concentrations (15 and 20 μg, Fig. 5A and B). PMA treatment for 6 hr resulted in a 10 to 20 fold increase in detectable NF-κB binding activity in both Jurkat and NC2.10 cells, although NC2.10 extracts contained about 9 fold less NF-κB binding activity than similarly induced Jurkat cells (Fig. 5A and B). In contrast, significant levels of NF-κB binding activity were detected in extracts from unstimulated Tax expressing 19D and 9J cell extracts; PMA treatment further increased the formation of NF-κB complexes about 2 to 3 fold (Fig. 5C and D). This result indicated that Tax expression in 19D and 9J cells was able to induce constitutive level of NF-κB DNA binding activity in the absence of other inducers.

FIGURE 5. Constitutive DNA Binding of NF-κB Proteins in Tax Expressing T Cell Extracts. The radiolabeled P2 oligonucleotide was incubated with 0, 5, 10, 15, and 20 μg of whole cell extracts from uninduced and PMA-induced Jurkat **(A)**, NC2.10 **(B)**, 19D **(C)**, and 9J **(D)** cells. The resulting protein-DNA complexes were resolved on 5% polyacrylamide gels and scanned by laser densitometry. The intensity of the complexes (relative DNA binding) was plotted against the amount of whole cell protein extract. (--□ --): Extracts derived from uninduced cells; (—■ —): extracts derived from PMA-induced cells.


A
JURKAT
2000
1000
0
0
10
20
B
NC2.10
300
200
100
0
0
10
20
C
19D
2000
1000
0
0
10
20
D
9J
2000
1000
0
0
10
20
Relative DNA Binding
Protein Extracts (μg)

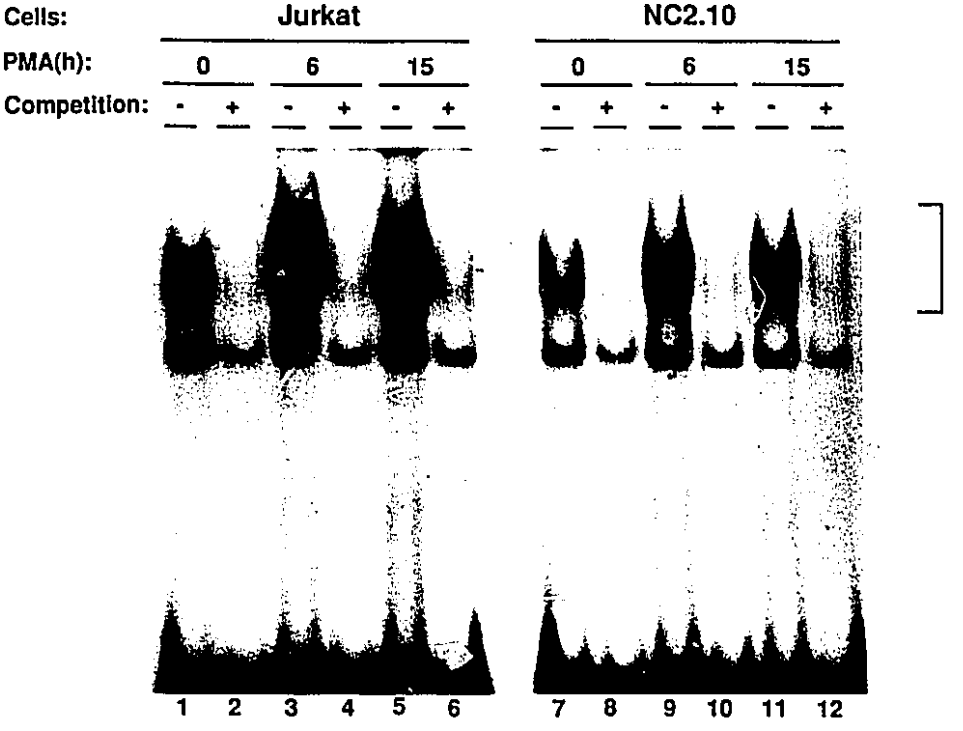
2. Ultraviolet Light Cross-Linking Analysis of NF-κB Binding Proteins

The proteins involved in the formation of the NF-κB complexes were further examined by mobility shift and UV cross-linking analysis using a BUdR-substituted P2 oligonucleotide probe (Figs. 6 and 7). In figure 6A, the electrophoretic mobility shift assay illustrated the inducibility (10 to 20 fold) of the NF-κB binding complexes in Jurkat and NC2.10 cells after 6 and 15 hr of PMA treatment (Fig. 6A, lanes 1, 3, 5, 7, 9, and 11). In contrast, constitutive NF-κB binding activity was clearly present in uninduced extracts from 19D and 9J cells (Fig. 6B, lanes 1 and 7) and was increased only about 2 fold by PMA treatment (Fig. 6B, lanes 3, 5, 9, and 11). With all cell extracts, an excess of unlabeled P2 oligonucleotide was able to compete efficiently for the formation of these complexes (Fig. 6A and B, even-numbered lanes), whereas a mutated NF-κB binding site was unable to block NF-κB binding activity (data not shown), thus demonstrating the specificity of complex formation.

When the NF-κB complexes (indicated by the brackets in Fig. 6A and B) were excised from the gel and analyzed for DNA binding proteins by UV light cross-linking, significant differences in the protein profiles were detected. In uninduced Jurkat and NC2.10 cells, the major cross-linked proteins associated with the BUdR-P2 probe had apparent molecular weights of 65 and 58 kD (Fig. 7, lanes 1 and 4). PMA induction of Jurkat and NC2.10 cells resulted in the binding of induction-specific proteins of 85, 75, and 54 kD (Fig. 7, lanes 2, 3, 5, and 6). In Tax expressing 19D and 9J cells, distinct profiles of NF-κB DNA binding proteins were observed. In uninduced 19D and 9J cells the 85 and 58 kD proteins were constitutively present and the amount of 75 kD protein binding to the P2 the probe was decreased about 4 fold compared to Jurkat cells (Fig. 7,

FIGURE 6. Mobility Shift Analysis of NF-κB Proteins from Tax Expressing T Cell Lines. An electrophoretic mobility shift assay was performed using 10 μg of whole cell extracts and a BUdR-substituted P2 probe. Competition was accomplished using a 125 fold excess of homologous unlabeled P2 DNA (even-numbered lanes). The brackets indicate the protein-DNA complexes analyzed by SDS-PAGE following UV cross-linking of proteins to the BUdR-substituted probe. Assays were performed using extracts derived from **(A)** Jurkat (lanes 1 to 6) and NC2.10 (lanes 7 to 12) cells and **(B)** 19D (lanes 1 to 6) and 9J (lanes 7 to 12) cells. Lanes 1, 2, 7, and 8: extracts derived from untreated cells; lanes 3, 4, 9, and 10: extracts derived from 6 hr PMA-treated cells; lanes 5, 6, 11, and 12: extracts derived from 15 hr PMA-treated cells.

A


Cells:
Jurkat
NC2.10
PMA(h):
0
6
15
0
6
15
Competition:
- + - + - + - + - + - +
1 2 3 4 5 6
7 8 9 10 11 12

B

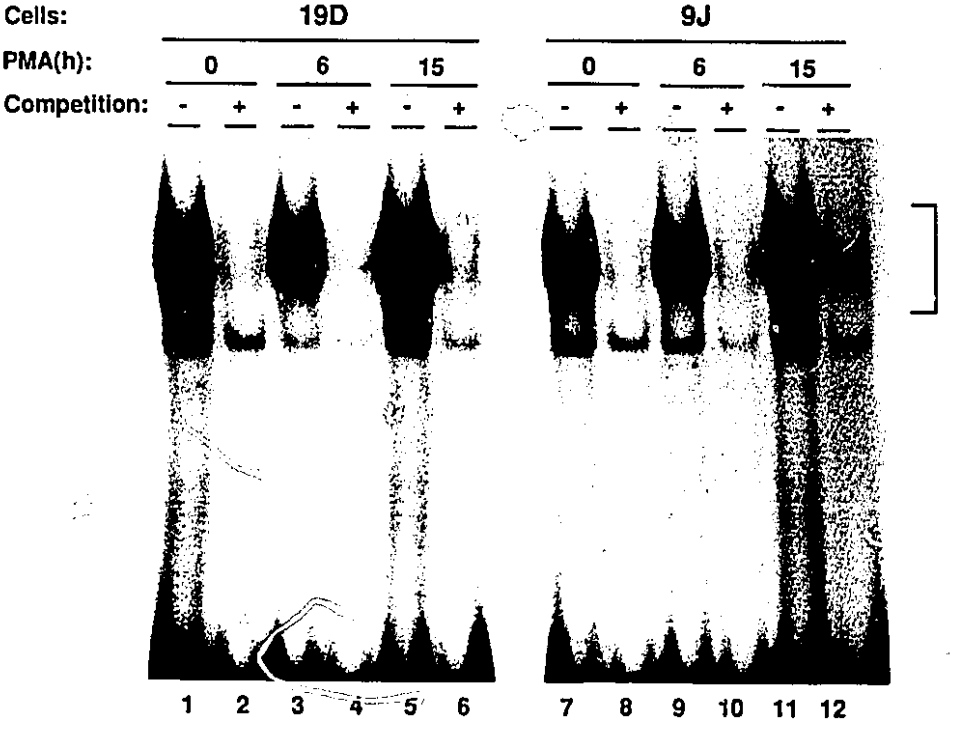
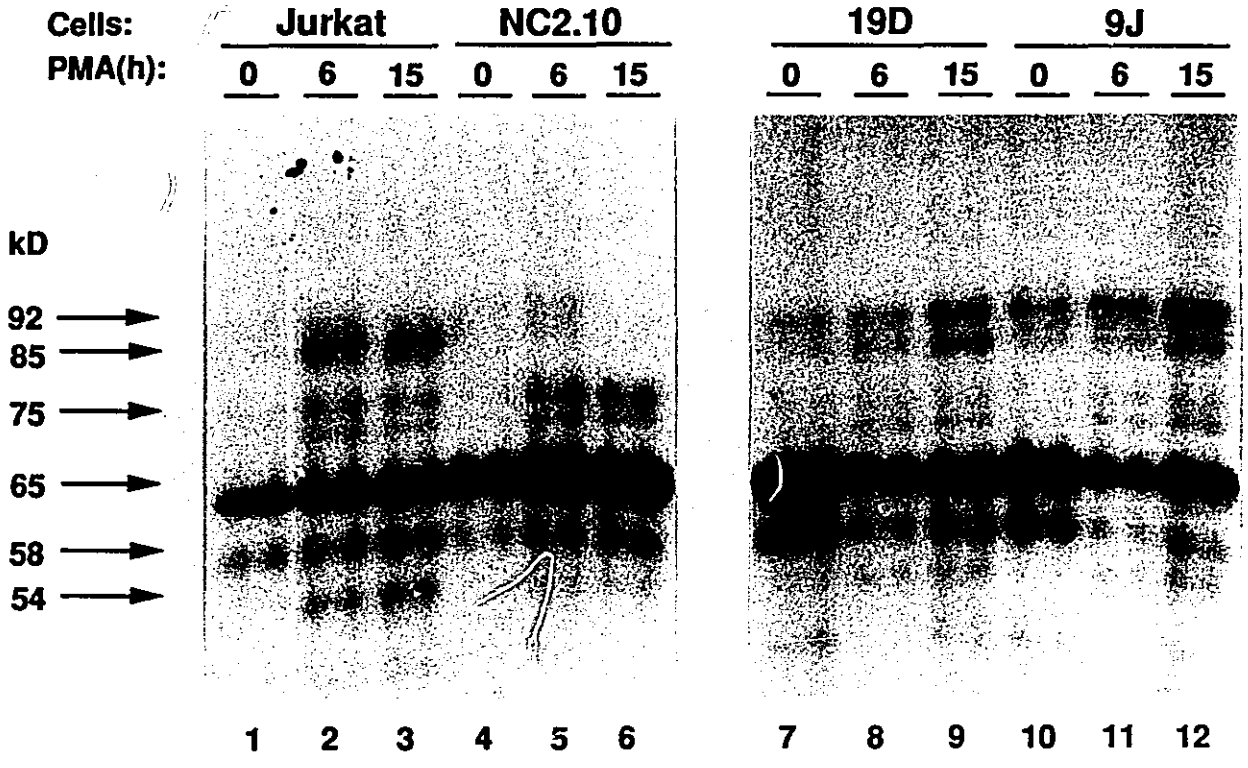

Cells:
19D
9J
PMA(h):
0
6
15
0
6
15
Competition:
- + - + - + - + - + - +
1 2 3 4 5 6
7 8 9 10 11 12

FIGURE 7. Ultraviolet Light Cross-Linking Analysis of NF-κB Protein-DNA Complexes. An electrophoretic mobility shift assay was performed as described in Figure 6 and the wet gel was exposed to UV light. After localizing protein-DNA complexes by autoradiography, gel fragments containing the complexes were resolved on 8% SDS-PAGE. Molecular weights are indicated by arrows. Whole cell extracts from Jurkat (lanes 1 to 3), NC2.10 (lanes 4 to 6), 19D (lanes 7 to 9), and 9J (lanes 10 to 12) cells were used. Lanes 1, 4, 7, and 10: extracts derived from untreated cells; lanes 2, 5, 8, and 11: extracts derived from 6 hr PMA-treated cells; lanes 3, 6, 9, and 12: extracts derived from 15 hr PMA-treated cells.


Cells:
Jurkat
NC2.10
19D
9J
PMA(h):
0
6
15
0
6
15
0
6
15
0
6
15
kD
92
85
75
65
58
54
1
2
3
4
5
6
7
8
9
10
11
12

lanes 7 and 10). Also, a new protein of 92 kD was detected in 19D and 9J cells. PMA induction for 6 and 15 hr increased the amount of the 92 and 85 kD proteins only about 2 fold (Fig. 7, lanes 8, 9, 11, and 12). These results supported observations demonstrating the existence of a family of NF-κB binding proteins (369), and furthermore demonstrated the constitutive expression of distinct NF-κB binding proteins in Tax expressing T cells.

3. Effect of Tax Expression on Endogenous NF-κB-Regulated Gene Expression

To determine the effect of constitutively expressed Tax protein on the transcription of endogenous NF-κB-regulated genes, expression of GM-CSF RNA was analyzed by a reverse transcriptase-PCR amplification procedure (see Table 3 for location of primers). The amounts of amplified PCR product were normalized using the amplification of GAPDH RNA as a constitutively expressed control. Jurkat and NC2.10 cells contained no detectable level of GM-CSF RNA in untreated or PMA-treated cells (Fig. 8A, lanes 1 to 4), although a low level of GM-CSF RNA was observed when cells were treated with both PMA and PHA (data not shown). The Tax expressing cell lines 19D and 9J both contained a constitutive level of GM-CSF RNA (Fig. 8A, lanes 5 and 7), which was further induced about 4 fold by PMA treatment (Fig. 8A, lanes 6 and 8, and 8B). These results demonstrated that constitutive expression of Tax protein in 19D and 9J cell lines was sufficient to *trans*-activate the endogenous NF-κB-regulated GM-CSF gene.

The expression of RNA encoding NFKB1 p105 and p50 was also examined in the same samples. Figure 8A shows that Jurkat and NC2.10 cells contained

FIGURE 8. PCR Amplification of GM-CSF, *nfkb*1, HTLV-I *tax*, and GAPDH RNA. (A) Total cellular RNA was extracted from Jurkat , NC2.10, 19D, and 9J cells, reverse transcribed into cDNA, and amplified by 28 cycles of amplification using ^{32}P-end-labeled primers and *Taq* polymerase. The resulting PCR products were resolved on denaturing polyacrylamide gels, which were dried and autoradiographed. The coamplified band of 140 nucleotides represents an artifact of PCR amplification. The arrows indicate the correctly sized PCR products. Odd-numbered lanes, RNA extracted from untreated cells; even-numbered lanes, RNA extracted from 6 hr PMA-treated cells. M, markers, ^{32}P-labeled fragments of pAT153 cleaved with *Hae*III.

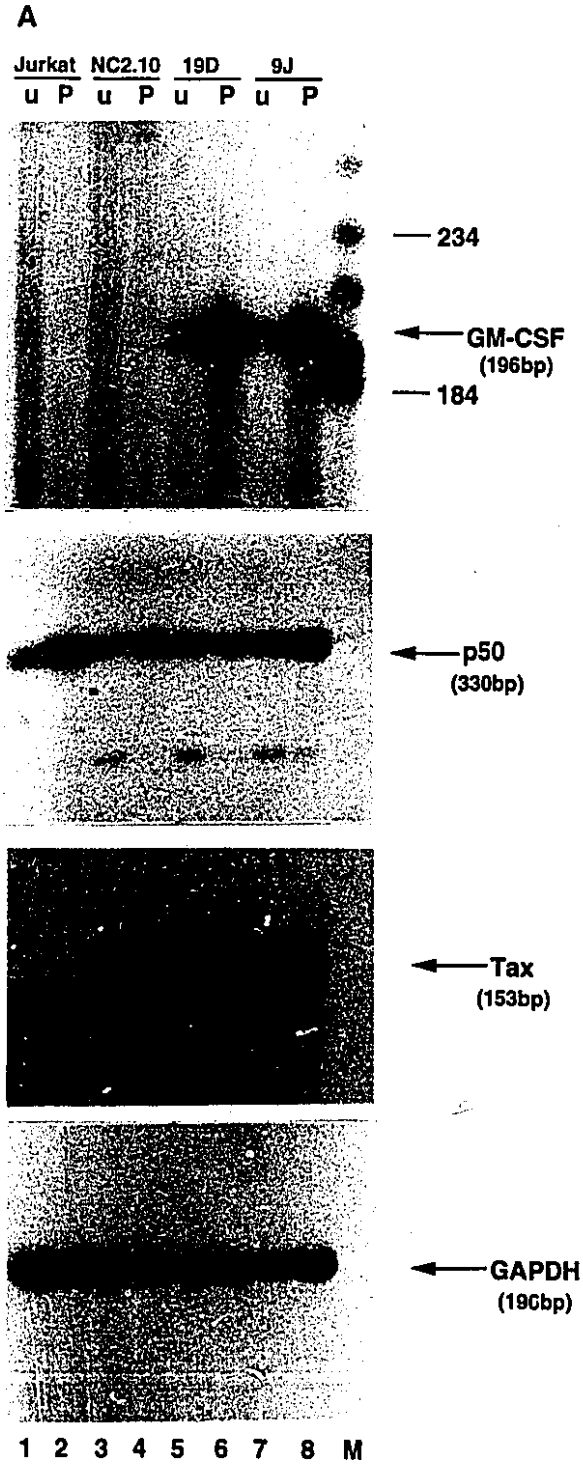
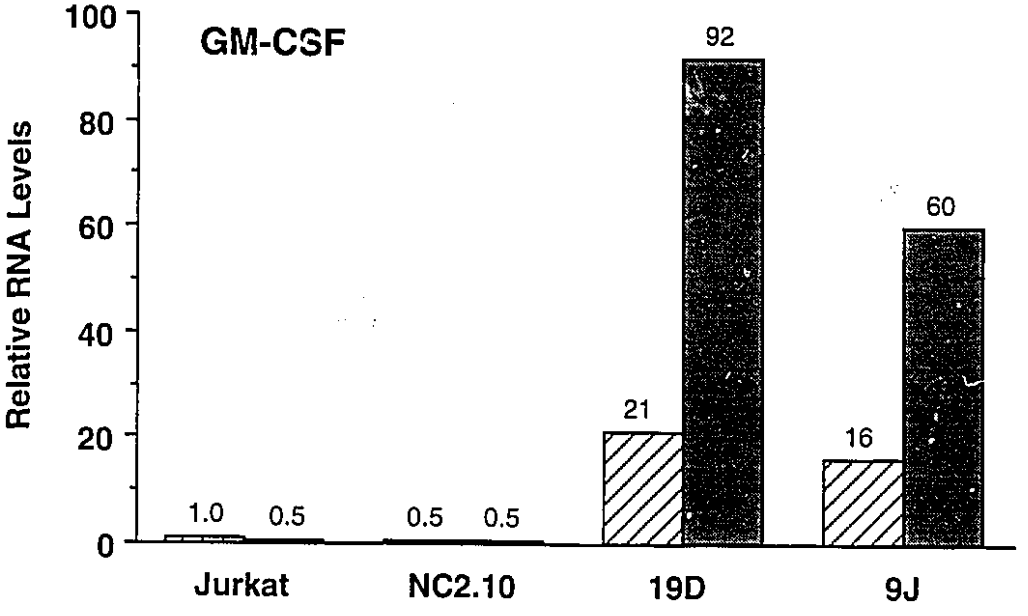
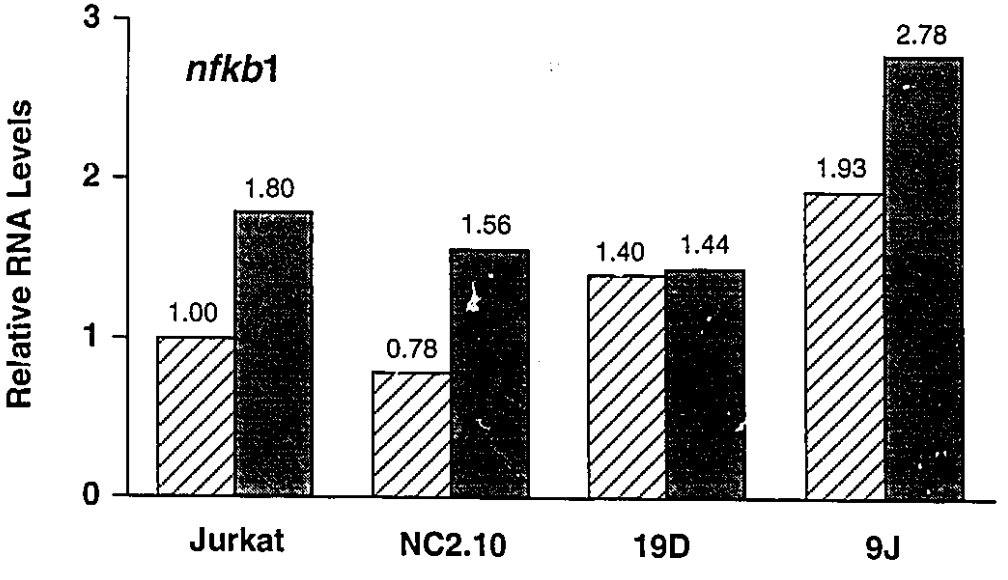

A
Jurkat NC2.10 19D 9J
u P u P u P u P
234
GM-CSF
(196bp)
184
p50
(330bp)
Tax
(153bp)
GAPDH
(190bp)
1 2 3 4 5 6 7 8 M

FIGURE 8... (B) Bands corresponding to amplification of GM-CSF and GAPDH were scanned by laser densitometry to determine their relative intensities. The level of GM-CSF RNA was normalized to the RNA level of constitutively expressed GAPDH. A similar analysis **(C)** was performed for *nfkb*1 RNA. (▨) Derived from uninduced cells; (■) derived from PMA-treated cells.

B


GM-CSF
Relative RNA Levels
100
80
60
40
20
0
1.0
0.5
0.5
0.5
21
92
16
60
Jurkat
NC2.10
19D
9J

C


nfkb1
Relative RNA Levels
3
2
1
0
1.00
1.80
0.78
1.56
1.40
1.44
1.93
2.78
Jurkat
NC2.10
19D
9J

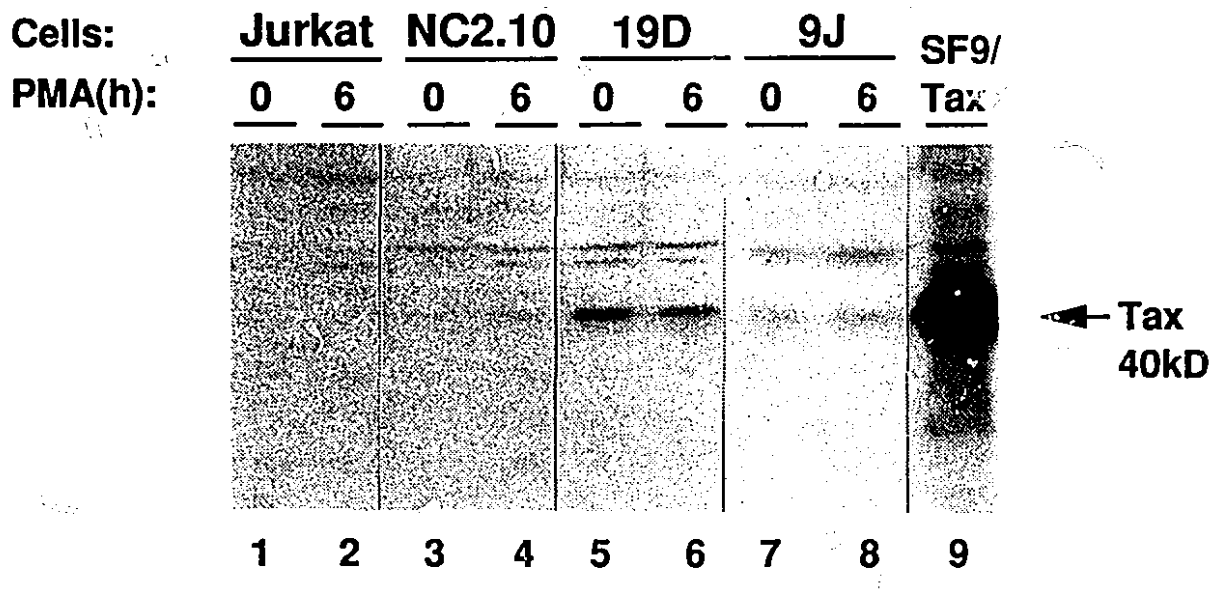
detectable levels of *nfkb*1 RNA; PMA induction of these cells increased RNA levels about 2 fold (Fig.8A, lanes 1 to 4, and 8C). Similarly, in Tax expressing 19D and 9J cells, *nfkb*1 RNA was detectable and only weakly inducible by PMA induction (Fig. 8A, lanes 5 to 8, and 8C), indicating that the constitutive expression of the HTLV-I *trans*-activator had little effect on the production of *nfkb*1 RNA.

Finally, the expression of *tax* mRNA itself was examined to confirm that Tax was produced in 19D and 9J cells (Fig. 8A, lanes 5 to 8) but not in Jurkat cells (Fig. 8A, lanes 1 and 2). Interestingly a low level of *tax* mRNA was detected in NC2.10 cells (Fig. 8A, lanes 3 and 4). Although the *tax* cDNA present in these cells is in the antisense orientation relative to the HTLV-I LTR (119), it is adjacent to the SV40 promoter/enhancer which directs expression of the neomycin gene. Low-level transcription of *tax* mRNA may be due to aberrant transcription initiation from the SV40 sequences. Western blot analysis using an anti-Tax antibody confirmed the constitutive expression of the 40 kD Tax protein in 19D and 9J cells (Fig. 9, lanes 5 to 8) and also detected a low level of Tax protein in NC2.10 (Fig. 9, lanes 3 and 4).

4. Effect of Tax Expression on NF-κB-Regulated Reporter Plasmids

The capacity of constitutively expressed Tax protein to induce NF-κB-regulated reporter plasmids was also measured by means of transient expression assays using NF-κB-regulated CAT reporter plasmids transfected into the non-Tax expressing (Jurkat and NC2.10) and Tax expressing (19D and 9J) cell lines. The SV2CAT, κB(3)-CAT, and HIV-CAT reporter plasmids (Fig. 10A) contain

FIGURE 9. Western Blot Analysis of Tax Protein. Whole cell extracts (200 µg) from Jurkat (lanes 1 and 2), NC2.10 (lanes 3 and 4), 19D (lanes 5 and 6), and 9J (lanes 7 and 8) cells were diluted 1:2 in 2xSDS sample buffer, heated 5-10 min at 68°C, loaded on a 10% SDS-PAGE, and transferred to a nitrocellulose membrane. The nitrocellulose was incubated overnight with the anti-Tax antibody (diluted 1:2000 in 3% BSA/PBS) and finally developed using a chemiluminescene detection system (Amersharn). 0, extracts derived from untreated cells; 6, extracts derived from cells treated with PMA for 6 hr; lane 9, SF9/Tax, positive control for Tax (extracts from SF9 insect cells infected with a Tax-expressing recombinant baculovirus).


Cells:
Jurkat
NC2.10
19D
9J
SF9/
PMA(h):
0
6
0
6
0
6
0
6
Tax
Tax
40kD
1
2
3
4
5
6
7
8
9

NF-κB-dependent regulatory elements that were previously shown to be maximally *trans*-activated by Tax protein in transient expression experiments in Jurkat cells (401). These plasmids were transfected into the four Jurkat cell lines and analyzed for CAT activity following PMA treatment; the averages of three individual transfections are summarized in Figure 10B. In non-Tax expressing Jurkat and NC2.10 cells, the basal level CAT activity varied between 0.7 and 2.3%, depending on the construct; PMA treatment of these cells resulted in a 19 to 35 fold increase in relative CAT activity. Transfection of these plasmids into Tax expressing 19D and 9J cells resulted in 5 to 10 fold higher basal levels of CAT activity (3.6 to 7.3% conversion); these NF-κB-responsive plasmids were still inducible by PMA treatment (Fig. 10B). The relative inducibility in PMA-treated versus untreated cells was decreased 3 to 12 fold due to the higher basal level of CAT activity in the Tax expressing cells. It would appear that constitutive Tax expression in the 19D and 9J cell lines resulted in an increased basal level transcription of transfected NF-κB-dependent promoters; nonetheless, transcription of the transfected genes was further induced by PMA treatment. These results differed significantly from previous experiments which have examined NF-κB *trans*-activation in Tax expressing cell lines (122, 410).

5. Conclusions

The effect of constitutive Tax expression on the interaction of NF-κB with its recognition sequence and on NF-κB-dependent gene expression was examined in T lymphoid Jurkat cell lines (19D and 9J) stably transfected with a Tax expression vector. Tax expressing T cell lines contained a constitutive level

FIGURE 10. *Trans*-Activation of NF-κB-Regulated Reporter Plasmids in Tax Expressing T Cells. (A) Structure of hybrid promoter-CAT constructs. The construction of these constructs was described in Materials and Methods. Arrows denote mRNA start site; open box, the SV40 GC-rich region; black box, the SV40 72-bp repeats; hatched box, the HIV-1 LTR region (-350 to +80), with the enhancer at -105 to -80 shown.

A

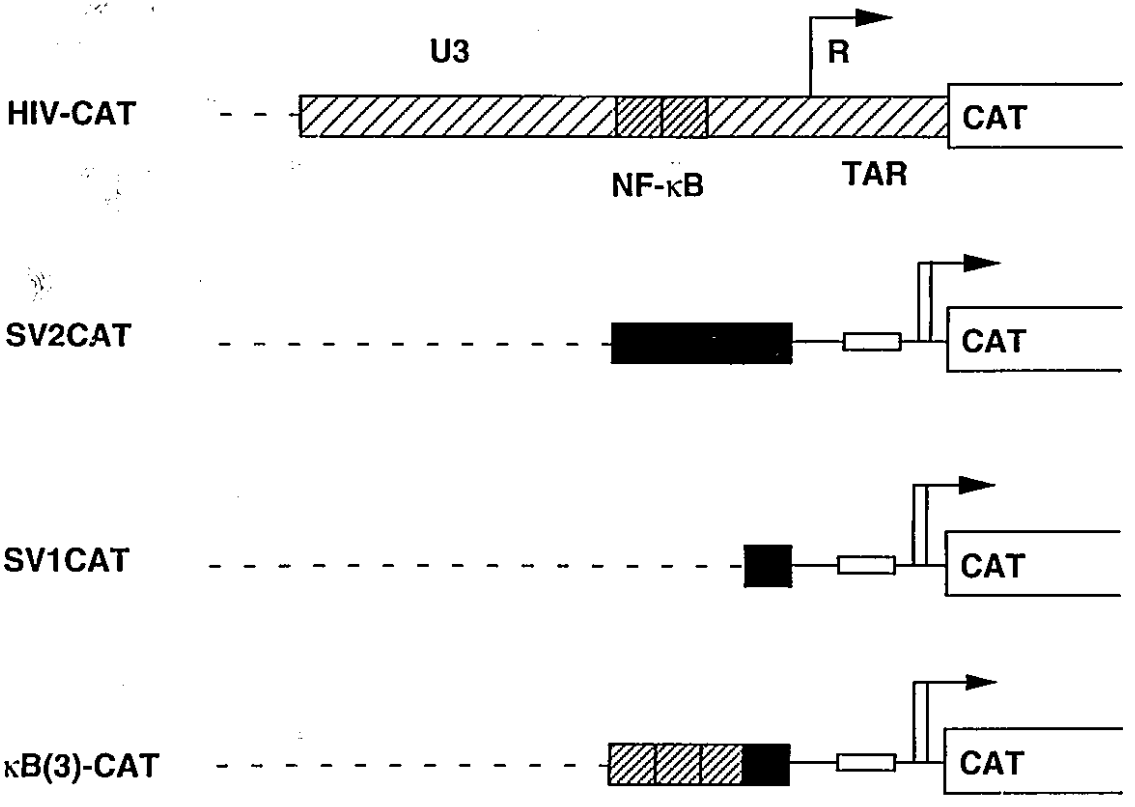
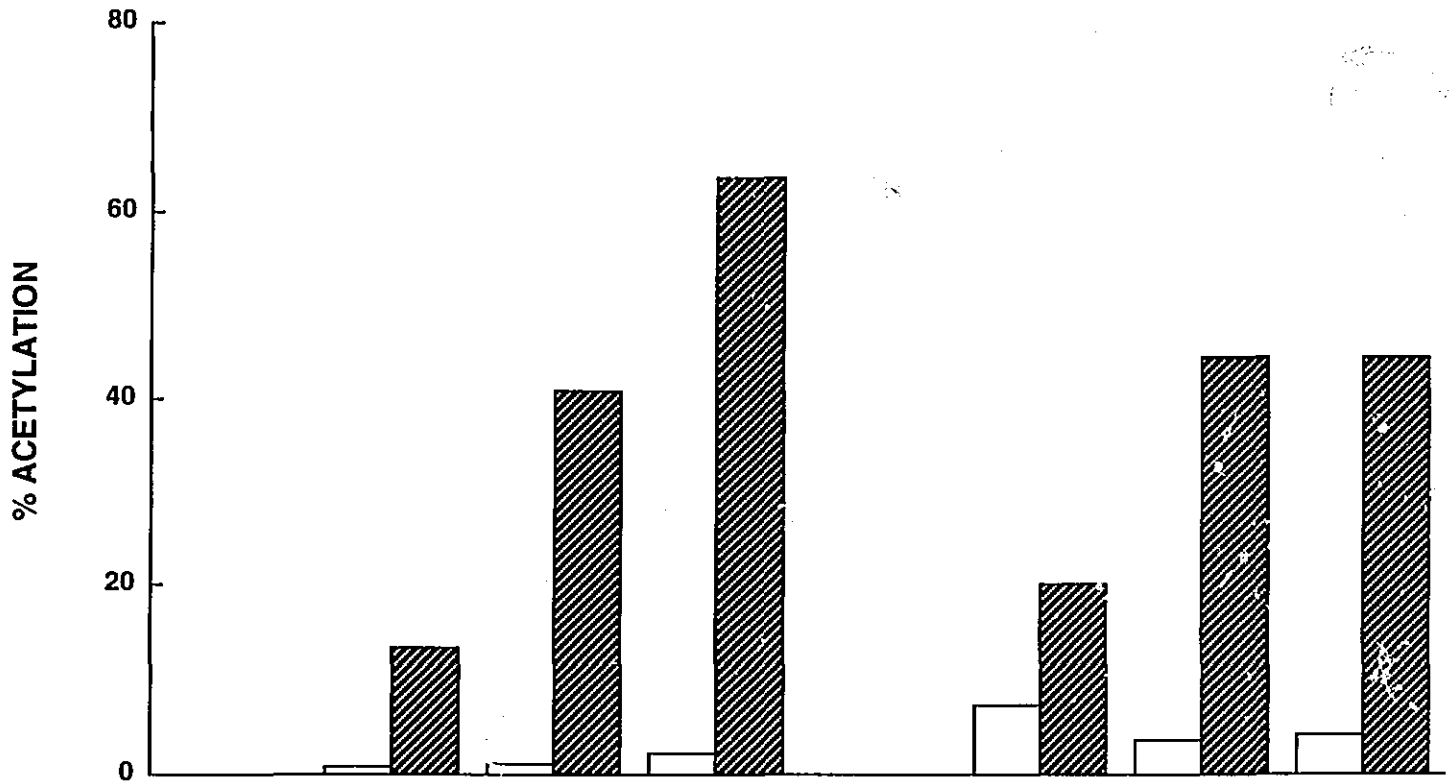


FIGURE 10... (B) Jurkat, NC2.10, 19D, and 9J cells were transfected by the DEAE-Dextran method with each of the constructs described in (A). The transfected cells were induced with PMA (25 ng/ml) at 20 hr after transfection and harvested 24 hr after induction. Freeze-thaw cell lysates were then prepared for CAT assay (20 µg for 45 min). Percentage acetylation is shown as bars and relative inducibility of induced (▨) over uninduced (□) samples is indicated in the table below the bar graph. The values are the average of three experiments; the values from Jurkat and NC2.10 (non-Tax expressing cells) and 19D and 9J (Tax expressing cells) are pooled together.

B



		non Tax expressing cells				Tax expressing cells			
% A	unind	0.1	0.7	1.2	2.3	0.1	7.3	3.6	4.2
	PMA	0.1	13.3	40.9	63.5	0.1	20.1	44.6	44.4
Rel. Induction		1.0	19.0	34.1	27.6	1.0	2.8	12.4	10.6

of NF-κB binding activity, detectable by mobility shift assay and UV cross-linking using a palindromic NF-κB probe homologous to the interferon β PRDII site. In Jurkat and NC2.10 cells induction with PMA resulted in the appearance of new DNA binding proteins of 85, 75, and 54 kD, whereas in Tax expressing cells the 85 kD and 92 kD DNA binding proteins were constitutively induced. Expression of Tax protein in 19D and 9J resulted in transcription of the endogenous NF-κB-dependent granulocyte-macrophage colony stimulating factor gene and increased basal level expression of transfected NF-κB-regulated promoters. Nonetheless transcription of both the endogenous and the transfected genes was inducible by PMA treatment. Tax expression in Jurkat T cells may alter the stoichiometry of NF-κB DNA binding proteins and thus change the expression of NF-κB-regulated promoters.

CHAPTER 4

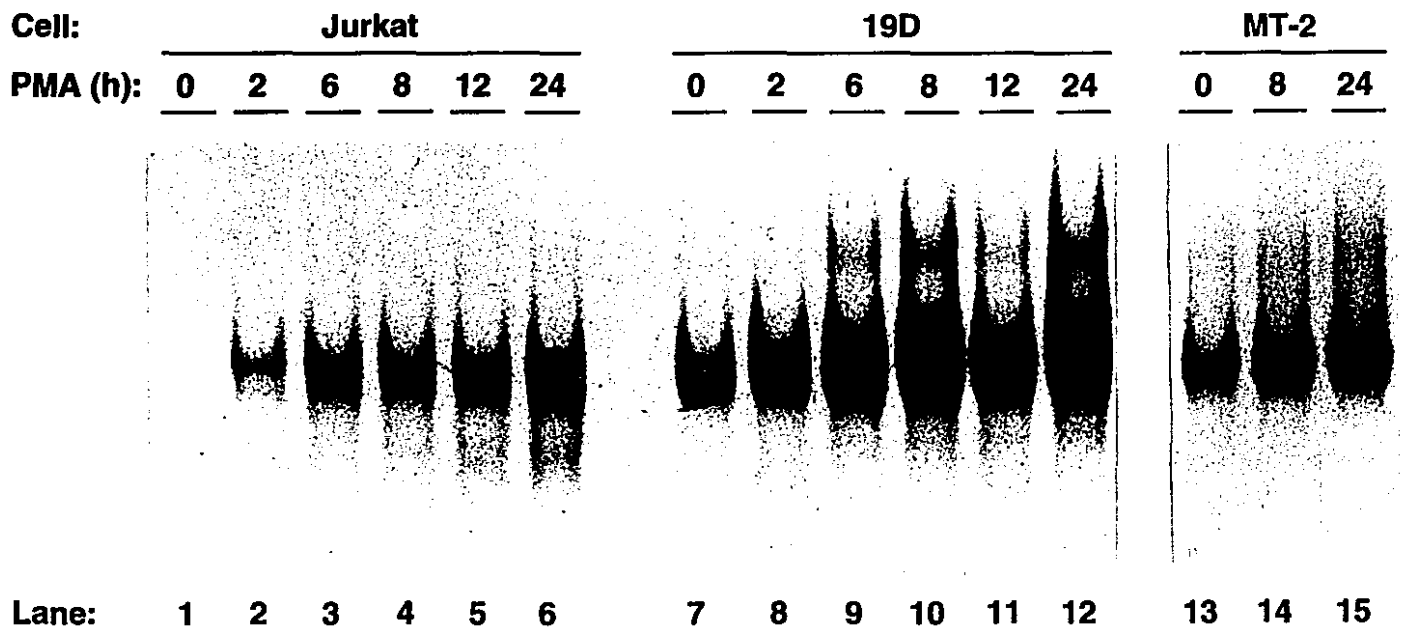
OVERPRODUCTION OF NFKB2 AND c-REL SUBUNITS IN TAX EXPRESSING AND HTLV-I INFECTED T CELLS

1. Constitutive Alterations in NF-κB Binding Complexes in HTLV-I Infected and Tax Expressing T Cells

Whole cell extracts were prepared from non-stimulated Jurkat, 19D and MT-2 cells and from cells induced with PMA for times indicated, and analyzed by electrophoretic mobility shift assay using the NF-κB sequences in the HIV-1 enhancer as a probe. Unstimulated MT-2 (Fig. 11, lane 13) and 19D (Fig. 11, lane 7) cells contained significant levels of constitutive NF-κB DNA binding activity whereas unstimulated Jurkat cells lacked detectable binding activity (Fig. 11, lane 1). In Jurkat cells, the level of NF-κB binding activity increased at least 20 fold after PMA stimulation for 6 hr (Fig. 11, lanes 2 to 6), while in 19D and MT-2 cells, constitutive NF-κB binding activity was further stimulated about 5 fold by PMA induction (Fig. 11, lanes 8 to 12, 14, and 15).

To determine the subunit composition of these DNA-protein complexes, NF-κB subunit-specific antibodies were used in supershift assays. Since unstimulated Jurkat cells contained considerably less detectable NF-κB binding activity (Fig. 6, lane 1, and Fig. 11, lane 1), extracts from 2 hr PMA-stimulated Jurkat cells were compared to unstimulated 19D and MT-2 whole cell extracts. During the early phase of PMA induction of Jurkat cells, NF-κB specific complexes were composed primarily of NFKB1 p50 (Fig. 12A, lane 2) and p65 (Fig. 12A, lane 4); in 2 hr PMA-induced Jurkat cells, interaction with the anti-c-Rel antiserum was very weak (Fig. 12A, lane 6) and interaction with the anti-NFKB2 antiserum was negligible (Fig. 12A, lane 8). However in 24 hr PMA-induced Jurkat extracts, NFKB1 (Fig. 12A, lane 11) and c-Rel (Fig. 12A, lane 15) represented the main NF-κB subunits present in protein-DNA complexes. Specificity of all supershifted complexes was demonstrated by competition with a 200 fold

FIGURE 11. Time Course of NF-κB Binding Activity During T Cell Activation. Human Jurkat T lymphoid cells (lanes 1 to 6), Tax expressing 19D (lanes 7 to 12) and HTLV-I infected MT-2 (lanes 13 to 15) cells were stimulated with PMA (25 ng/ml) for times indicated above each lane. At the indicated times (lanes marked 0 represent non-stimulated cells), cells were harvested for preparation of whole cell extracts, as described in Materials and Methods. Each extract (5 μg) was analyzed by electrophoretic mobility shift assay with the NF-κB sequence (5'-GGGACTTTCC-3') in the HIV-1 enhancer as a probe, in the presence of 5 μg poly dI:dC. Resulting protein-DNA complexes were resolved on a native 5% polyacrylamide gel and visualized by autoradiography.

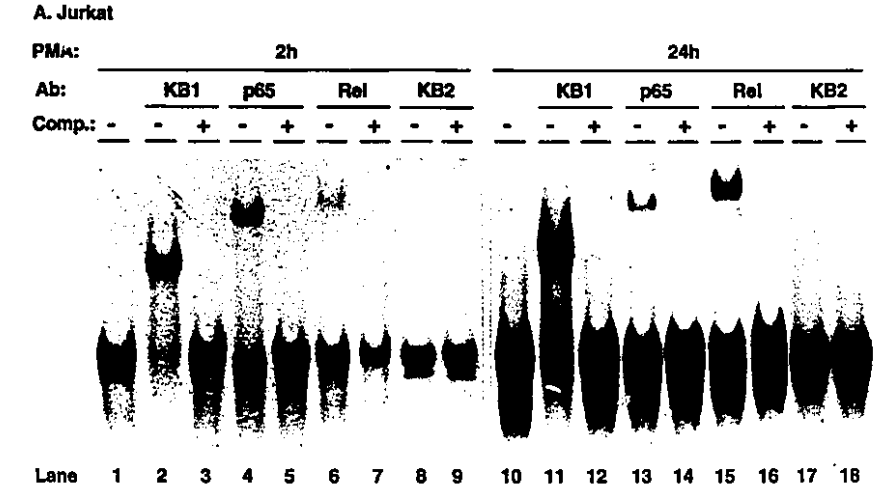

Cell:
Jurkat
19D
MT-2
PMA (h):
0 2 6 8 12 24
0 2 6 8 12 24
0 8 24
Lane:
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

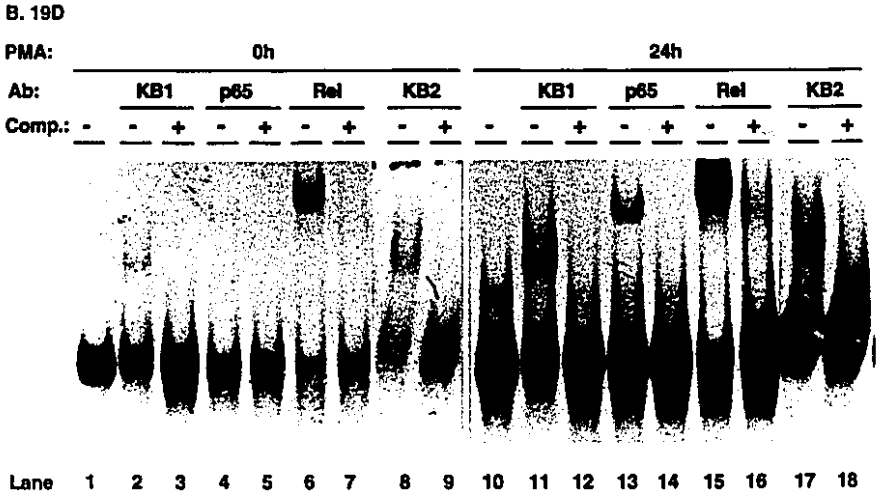
excess of the peptide used to produce the monospecific antibodies (Fig. 12, lanes 3, 5, 7, 9, 12, 14, 16, and 18). Surprisingly, in unstimulated Tax expressing 19D cells, c-Rel (Fig. 12B, lane 6) and NFKB2 (Fig. 12B, lane 8) represented the main subunits present in the NF-κB complexes. In extracts from 24 hr PMA-induced 19D cells, all of the NF-κB subunits (c-Rel, NFKB2, NFKB1 and p65) were detected in the shifted shift assay (Fig. 12B, lanes 11, 13, 15, and 17). Likewise, in unstimulated or 24 hr PMA stimulated MT-2 cells, c-Rel, NFKB2 and NFKB1 were the main proteins present in NF-κB complexes (Fig. 12C, lanes 2, 6, 8, 11, 15, and 17); negligible amounts of p65 binding activity were observed in MT-2 cells (Fig. 12C, lanes 4 and 13). These qualitative results indicated that dynamic alterations in NF-κB binding activity occured in unstimulated and PMA-induced 19D and MT-2 cells; c-Rel and NFKB2 (which was undetectable in activated Jurkat cells) represented major protein constituents of NF-κB complexes in Tax expressing and HTLV-I infected T cells, while the contribution of the p65 subunit to the formation of protein-DNA complexes was reduced.

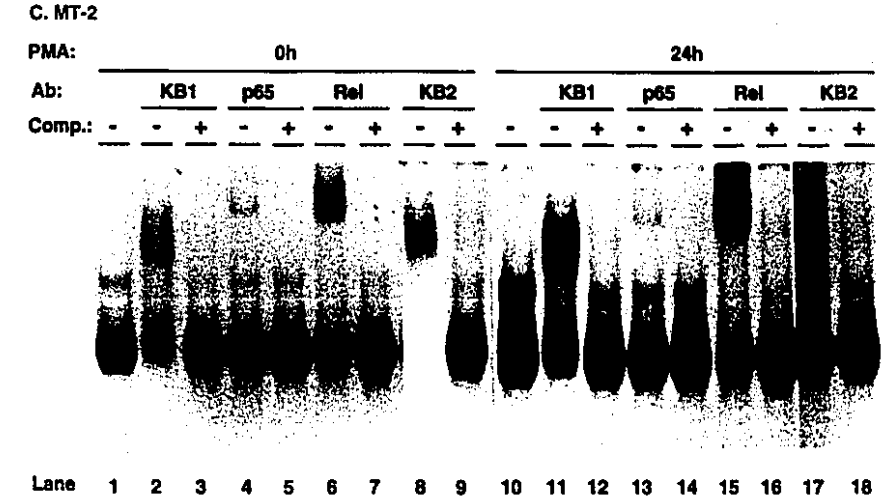
2. Elevated Levels of NFKB2 and c-Rel Proteins in T Cells Expressing Tax

To determine whether the alterations in NF-κB DNA binding complexes observed in 19D and MT-2 cells were a consequence of protein expression modulation, immunoblotting analyses were performed with the same extracts using the subunit-specific antibodies. Expression of NFKB1 proteins (precursor and product) is shown in Figure 13A. p105 was detected in extracts prepared from untreated Jurkat and 19D cells and, to a lesser extent, from untreated MT-2 cells (Fig. 13A, lanes 1, 4, and 7). p50 was detected in untreated Jurkat and

FIGURE 12. Analysis of NF-κB Binding Complexes in Normal, Tax Expressing and HTLV-I Infected T cells. Whole cell extracts were those used in the experiment described in Figure 11: **(A)** 2 hr (lanes 1 to 9) and 24 hr (lanes 10 to 18) PMA-stimulated Jurkat cells; **(B)** unstimulated (lanes 1 to 9) and 24 hr PMA-stimulated (lanes 10 to 18) 19D cells; **(C)** unstimulated (lanes 1 to 9) and 24 hr PMA-stimulated (lanes 10 to 18) MT-2 cells. Each extract (5 μg) was pre-incubated with NF-κB subunit-specific antisera, as indicated above each lane, in the presence of 5 μg poly dI:dC, for 20 min at room temperature, prior to addition of radiolabeled probe (HIV-1 enhancer, as in Figure 11). Specificity of antibody-antigen association was confirmed by competition with excess antibody-specific peptide (lanes marked +).


A. Jurkat
PMA:
2h
24h
Ab:
KB1
p65
Rel
KB2
KB1
p65
Rel
KB2
Comp.: - - + - + - + - + - - + - + - + - +
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18


B. 19D
PMA:
0h
24h
Ab:
KB1
p65
Rel
KB2
KB1
p65
Rel
KB2
Comp.: - - + - + - + - + - - + - + - + - +
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

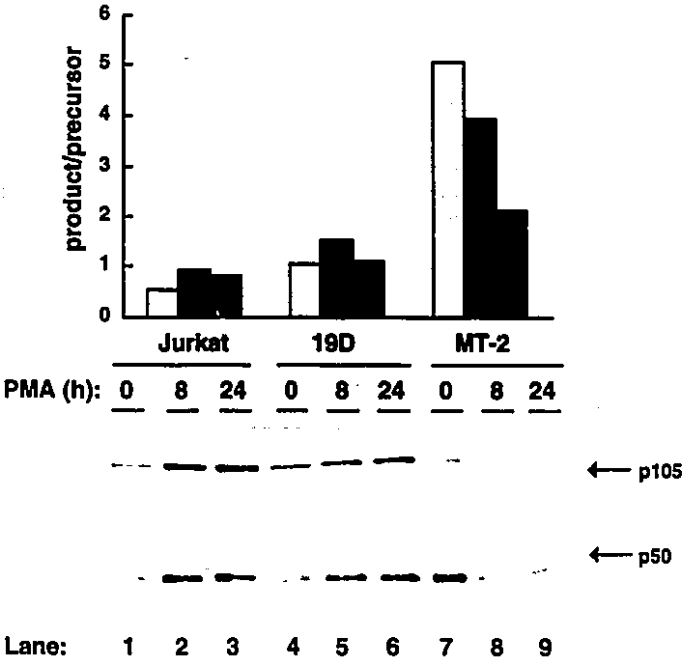

C. MT-2
PMA:
0h
24h
Ab:
KB1
p65
Rel
KB2
KB1
p65
Rel
KB2
Comp.: - - + - + - + - + - - + - + - + - +
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

19D cell extracts (Fig. 13A, lanes 1 and 4), while extracts derived from untreated MT-2 cells contained about 2 fold more p50 (Fig. 13A, lane 7). PMA treatment of Jurkat cells induced production of NFKB1 proteins but had little effects in 19D cells (Fig. 13A, lanes 2, 3, 5, and 6). In contrast, PMA treatment of MT-2 cells reduced expression of both precursor and product forms of NFKB1 (Fig. 13A, lanes 8 and 9). The bar graph in Figure 13A represents the ratio of NFKB1 product to precursor. Jurkat and 19D cells extracts contained similar ratios that varied little upon PMA treatment of the cells (Fig. 13A, lanes 2, 3, 5, and 6). However, this ratio was raised about 5 fold in HTLV-I infected cells, and reduced by about 2 fold following PMA treatment of the cells (Fig. 13A, lanes 8 and 9).

NFKB2 p100 was only weakly detected in extracts from Jurkat cells (Fig. 13B, lane 1) but the level of p100 was 15 to 25 fold higher in unstimulated 19D and MT-2 cells, respectively (Fig. 13B, lanes 4 and 7). NFKB2 p100 protein was induced about 8 fold in Jurkat cells following PMA treatment (Fig. 13B, lanes 2 and 3), whereas the p100 level remained unchanged in 19D and MT-2 cells after PMA induction (Fig. 13B, lanes 5, 6, 8, and 9). Strikingly, the DNA binding NFKB2 p52 product, which was virtually undetectable in Jurkat cells (Fig. 13B, lanes 1 to 3), was present at up to 100 fold higher levels in 19D (Fig. 13B, lanes 4 to 6) and MT-2 (Fig. 13B, lanes 7 to 9) cells. The p52:p100 product:precursor ratios obtained for the three cell lines are shown in the bar graphs of Figure 13B and illustrate the dramatic increase in the amount of NFKB2 p52 in Tax expressing and HTLV-I infected cells. These differences may reflect changes in the stability of the NFKB2 product or an alteration of processing of the NFKB2 precursor in the presence of Tax in 19D and MT-2 cells.

FIGURE 13. Immunoblot Analysis of NF-κB/Rel Protein Levels in Normal, Tax Expressing and HTLV-I Infected T Cells. Whole cell extracts used in experiments described in Figures 11 and 12 were resolved by SDS-PAGE, transferred to nitrocellulose membranes and probed with the indicated antisera, with the use of an enhanced chemiluminescence assay (Amersham). Autoradiograms were scanned and relative amounts of proteins plotted as a function of cell type and time of PMA induction. (□) Derived from uninduced cells; (▨) derived from 8 hr PMA-treated cells; (■) derived from 24 hr PMA-treated cells. **(A)** Total protein extracts (10 μg) were loaded and the anti-NFKB1 (p105 and p50) antiserum was used. **(B)** Total protein extracts (10 μg) were loaded and the anti-NFKB2 (p100 and p52) antiserum was used.

A. NFKB1



B. NFKB2

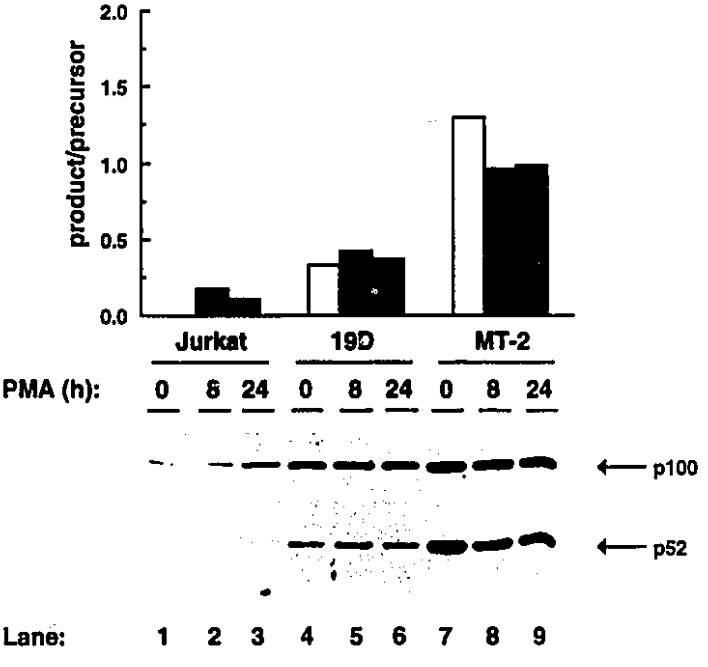
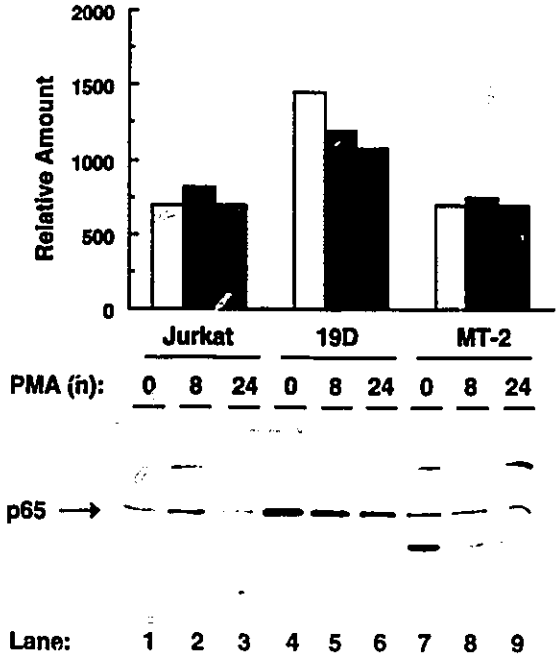


FIGURE 13... (C) Total protein extracts (20 μg) were loaded and the anti-p65 antiserum was used. **(D)** Total protein extracts (3.5 μg) were loaded and the anti-c-Rel antiserum was used.

C. p65


2000
1500
1000
500
0
Relative Amount
Jurkat
19D
MT-2
PMA (h): 0 8 24 0 8 24 0 8 24
p65 →
Lane: 1 2 3 4 5 6 7 8 9

D. Rel

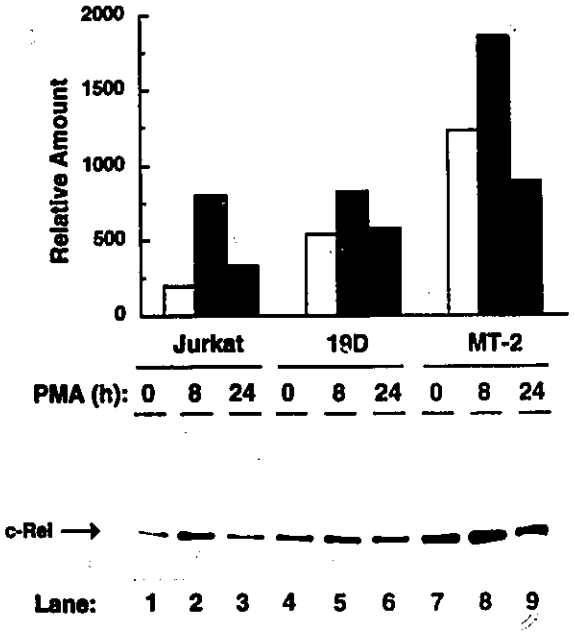

2000
1500
1000
500
0
Relative Amount
Jurkat
19D
MT-2
PMA (h): 0 8 24 0 8 24 0 8 24
c-Rel →
Lane: 1 2 3 4 5 6 7 8 9

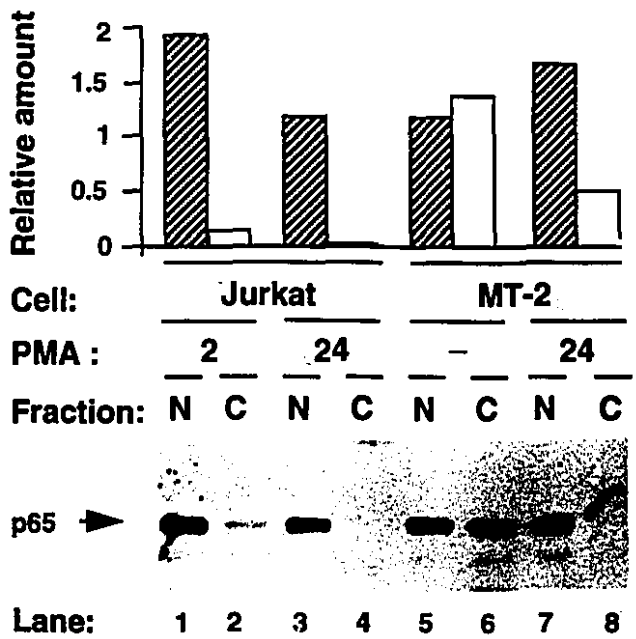
Figure 13C shows analysis of p65 protein expression. Untreated Jurkat and MT-2 cell extracts contained similar amounts of p65 protein (Fig. 13C, lanes 1 and 7), while 19D cells expressed about 2 fold more (Fig. 13C, lane 4). PMA treatment of Jurkat, 19D, and MT-2 had little effect on p65 expression (Fig. 13C, lanes 2, 3, 5, 6, 8, and 9). Levels of c-Rel protein were 3 and 6 fold higher in 19D and MT-2 cells, respectively, compared to Jurkat cells (Fig. 13D, compare lanes 1, 4, and 7). In Jurkat cells, and to a lesser extent in 19D and MT-2 cells, a transient induction of c-Rel protein was observed with PMA stimulation (Fig. 13D, lanes 2, 3, 5, 6, 8, and 9).

Despite similar levels of protein expression (or even higher levels in 19D cells), p65 DNA binding activity was impaired in Tax expressing and HTLV-I infected T cells (Fig. 12B and C). The possibility that p65 activity was in part altered because of a different intracellular localization was verified by preparing cytoplasmic and nuclear fractions of Jurkat and MT-2 cells. In PMA-induced normal T cells, p65 was mostly found in the nuclear fraction (Fig. 14, lanes 1 to 4). In contrast, in either uninduced or PMA-treated MT-2 cells, p65 was equally distributed between the cytoplasm and the nucleus (Fig. 14, lanes 5 to 8). These results showed that p65 intracellular distribution was altered in HTLV-I infected cells, and the observed reduced DNA binding activity of p65 might result from this relocalization.

3. Processing of NFKB2 in HTLV-I Infected T Cells

To assess the rate of processing of the NFKB2 p100 protein *in vivo*, MT-2 cells (which produce the highest levels of Tax and NFKB2) were metabolically labeled with ^{35}S-methionine for 60 min, and chased with cold methionine over

FIGURE 14. Subcellular Distribution of p65 Protein in Normal and HTLV-I Infected T Cells. Nuclear and cytoplasmic extracts were prepared from untreated MT-2 (lanes 5 and 6), 2 hr PMA-treated Jurkat (lanes 1 and 2), and 24 hr PMA-treated Jurkat (lanes 3 and 4) and MT-2 (lanes 7 and 8) cells as described in Materials and Methods. Extracts (20 μg) were separated on SDS-PAGE, transferred to nitrocellulose membranes, probed with an anti-p65 antiserum, and visualized using a chemiluminescence detection system. Autoradiograms were scanned and relative amounts of proteins plotted. (▨) Nuclear extracts; (□) cytoplasmic extracts.

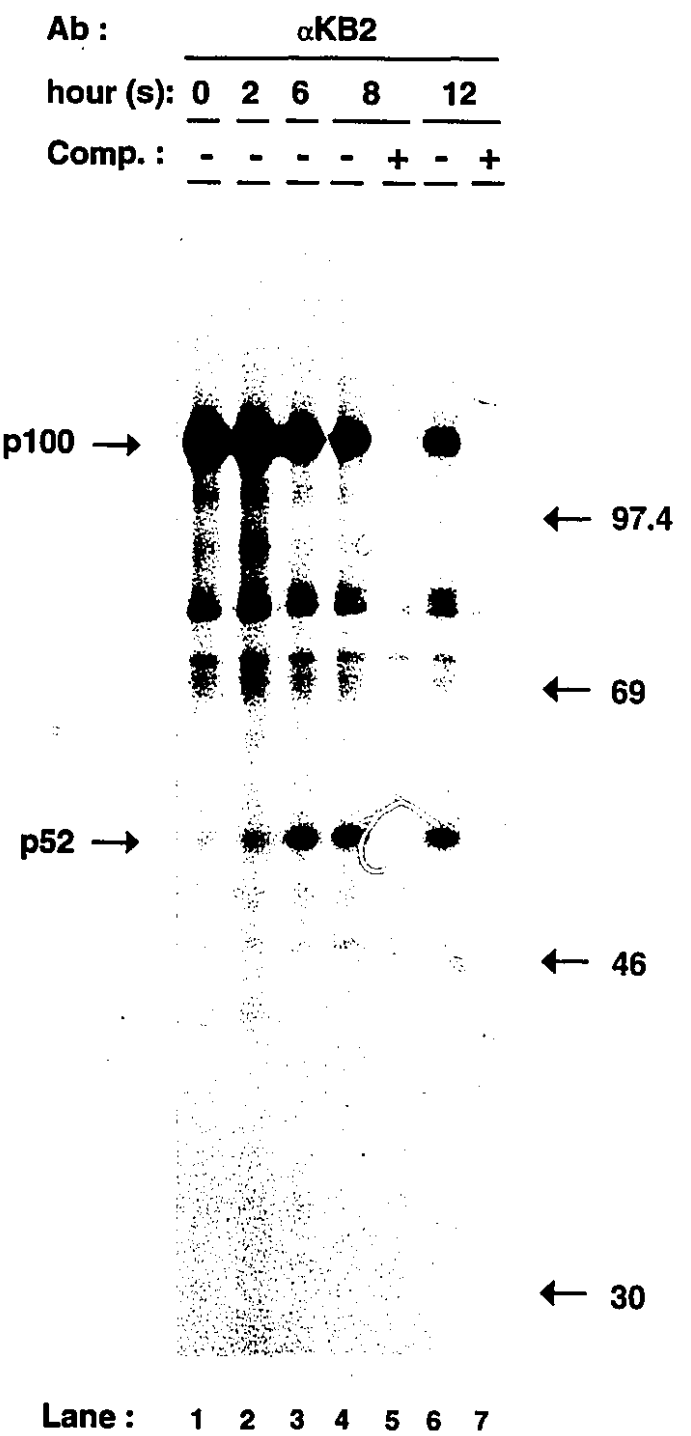

Relative amount
2
1.5
1
0.5
0
Cell:
Jurkat
MT-2
PMA :
2
24
–
24
Fraction:
N C N C N C N C
p65
Lane:
1 2 3 4 5 6 7 8

a period of 12 hr. Whole cell extracts, prepared at different time intervals, were subjected to immunoprecipitation using an anti-NFKB2 antiserum that specifically recognized the N-termini of both p100 and p52. After the 60 min labeling period, greater than 90% of the ^{35}S-methionine label was associated with the NFKB2 p100 precursor (Fig. 15, lane 1). Following a 2 hr chase, 10% of the label was associated with NFKB2 p52 (Fig. 15, lane 2) and by 12 hr the remaining radioactivity was distributed 3:2 between p100 and p52 (Fig. 15, lanes 2 to 4, and 6); as noted previously, the processing of the NFKB2 precursor occurred slowly with time (274). The amount of radioactivity associated with the precursor decreased linearly with only 35% of the total remaining at the end of the 12 hr chase period. Specificity of the interactions with NFKB2 p100 and p52 was demonstrated by competition with an excess of cognate peptide (Fig. 15, lanes 5 and 7). The protein migrating above the 69 kD marker appeared to be c-Rel, which has been shown to physically interact with NFKB2 as well as with NFKB1 *in vivo* (274, 275). These results demonstrated the presence of a precursor:product relationship between p100 and p52 in HTLV-I infected cells; however the rate of conversion from the p100 precursor to the p52 product occured slowly in MT-2 cells with a $T_{1/2}$ greater than 12 hr. Similar rates of processing were obtained in PMA-induced Jurkat cells (data not shown). Therefore it appeared that processing of p100 to p52 was inducible in T cells either by Tax expression or PMA induction.

4. Dose-dependent Effect of Tax on NF-κB Activity and Protein Expression

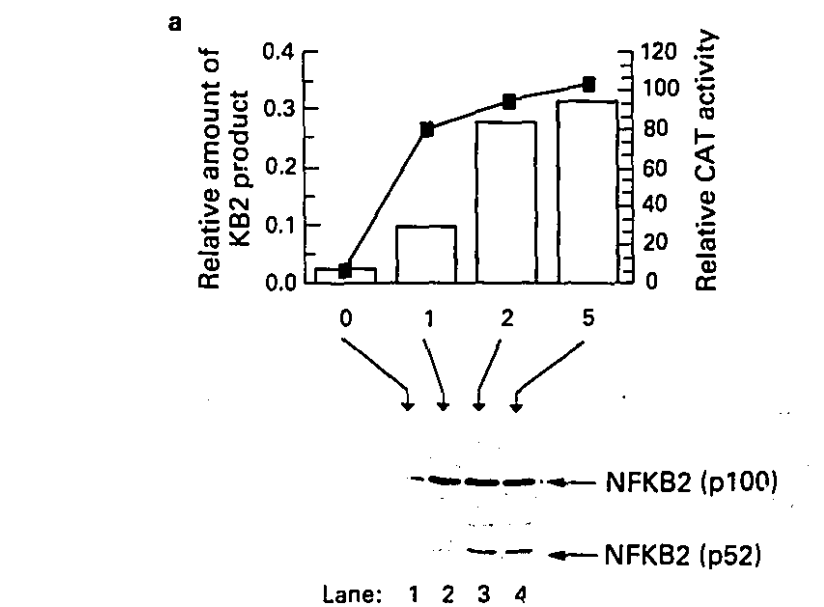
To examine whether the amount of NFKB2 p52 product derived from p100 increased in a dose-dependent manner with the amount of Tax, transient

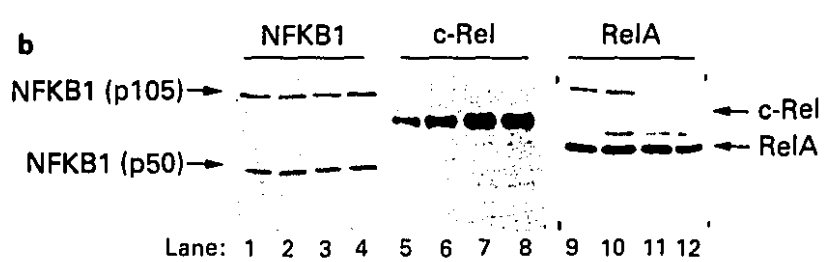
FIGURE 15. Pulse-Chase Analysis of NFKB2 Proteins in HTLV-I Infected T Cells. MT-2 cells were starved and pulsed 1 hr with ^{35}S-methionine. A chase was then initiated by transferring the cells to complete RPMI. Samples were taken, starting with a zero time point, followed by chases of 2, 6, 8 and 12 hr. Whole cell extracts were prepared from cells harvested at each time point. After pre-clearing using normal rabbit serum (NRS), the extracts were subjected to immunoprecipitation using an NFKB2 N-terminus specific antibody. Competition was performed by incubating the pre-cleared extracts with the antibody and the corresponding antigenic peptide (lanes 5 and 7). Immune complexes were recovered on protein A Sepharose and resolved on SDS-PAGE. Arrows on the right indicate migration of the molecular weight markers. The precursor and product are pointed by arrows on the left.


Ab : αKB2
hour (s): 0 2 6 8 12
Comp. : - - - - + - +
p100
p52
97.4
69
46
30
Lane : 1 2 3 4 5 6 7

expression assays were carried out in Jurkat cells. As demonstrated in Figure 13B, Jurkat cells contain very low levels of endogenous NFKB2 p52. Therefore any increase in the processing of NFKB2 p100 occurring as a consequence of Tax expression should be easily monitored. Jurkat cells were transfected with an HIV-enhancer-CAT reporter construct in the absence or presence of increasing concentrations of a Tax expression plasmid. Cell extracts were analyzed for *trans*-activation of NF-κB-regulated reporter gene activity by CAT assays, and for NFKB2 protein levels by Western blotting, using the anti-NFKB2 antiserum. The low basal level of NF-κB-regulated CAT reporter gene activity observed when the construct was transfected alone was increased 12 to 15 fold with increasing concentrations of co-transfected Tax plasmid (Fig. 16A, solid line). In the same extracts, the NFKB2 p52:100 ratio was 0.03 in cells transfected with the CAT reporter construct alone (Fig. 16A, lane 1 and open bar); strikingly, the NFKB2 product:precursor ratio increased in a dose-dependent manner with co-transfection of the Tax expression plasmid from 0.1 to 0.3 (Fig. 16A, lanes 2 to 4), thus reflecting a 3 to 10 fold increase in the amount of NFKB2 p52 protein in extracts from Tax-transfected cells. In addition, NFKB2 p100 protein levels increased about 5 fold in Tax-transfected cells (Fig. 16A, lanes 1 to 4), suggesting that increased synthesis of NFKB2 was also associated with Tax expression. A similar dose-dependent increase in the p105 and p50 forms of NFKB1 was not observed (Fig. 16B, lanes 1 to 4), whereas the amount of c-Rel protein was also increased 3 to 6 fold with Tax co-expression (Fig. 16B, lanes 5 to 8). Finally, the high level of p65 protein did not fluctuate with increasing amounts of Tax (Fig. 16B, lanes 9 to 12). These results implied a direct correlation between Tax expression, increased c-Rel and NFKB2 synthesis and *trans*-activation of NF-κB-regulated reporter gene activity.

FIGURE 16. Dose-Dependent Effect of Tax on NF-κB-Regulated Gene Expression and Expression of NF-κB Subunits. Jurkat cells were transfected with the HIV-1 enhancer CAT construct alone (2 μg) (lane 1), or together with 1 μg (lane 2), 2 μg (lane 3) or 5 μg (lane 4) of the Tax expression plasmid. A plasmid carrying the β-galactosidase gene was co-transfected as a control for transfection efficiency. The total amount of transfected DNA was normalized by addition of non-specific pUC DNA. Cells were harvested 48 hr post-transfection, analyzed for NF-κB activity by CAT assay and for NFKB2 protein production by Western blotting. **(A)** Transcriptional activity (solid line) refers to percent acetylation normalized to β-galactosidase activity and represents an average of at least two independent experiments. Open bars represent relative amounts of NFKB2 (p52) protein, determined by densitometric scanning of the Western blot. **(B)** NFKB1, c-Rel, and p65 (RelA) protein production was analyzed by immunoblotting using anti-NFKB1 (lanes 1 to 4), anti-c-Rel (lanes 5 to 8), and anti-p65 (lanes 9 to 12) antisera.


a
Relative amount of KB2 product
0.4
0.3
0.2
0.1
0.0
120
100
80
60
40
20
0
Relative CAT activity
0
1
2
5
NFKB2 (p100)
NFKB2 (p52)
Lane: 1 2 3 4


b
NFKB1
c-Rel
RelA
NFKB1 (p105)
NFKB1 (p50)
c-Rel
RelA
Lane: 1 2 3 4 5 6 7 8 9 10 11 12

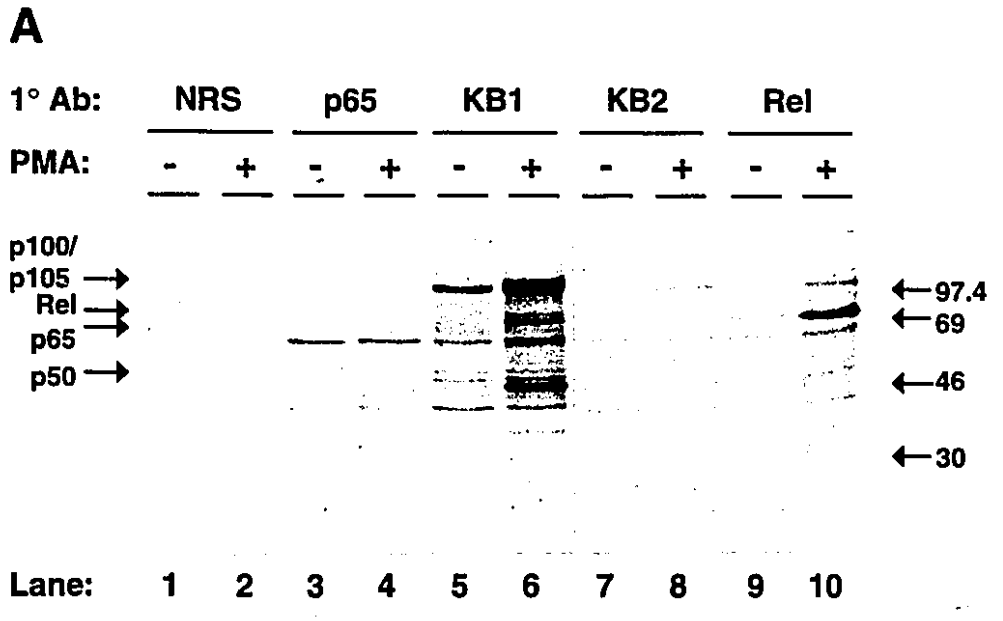
5. Effect of Tax Expression on NF-κB/Rel Protein-Protein Interactions

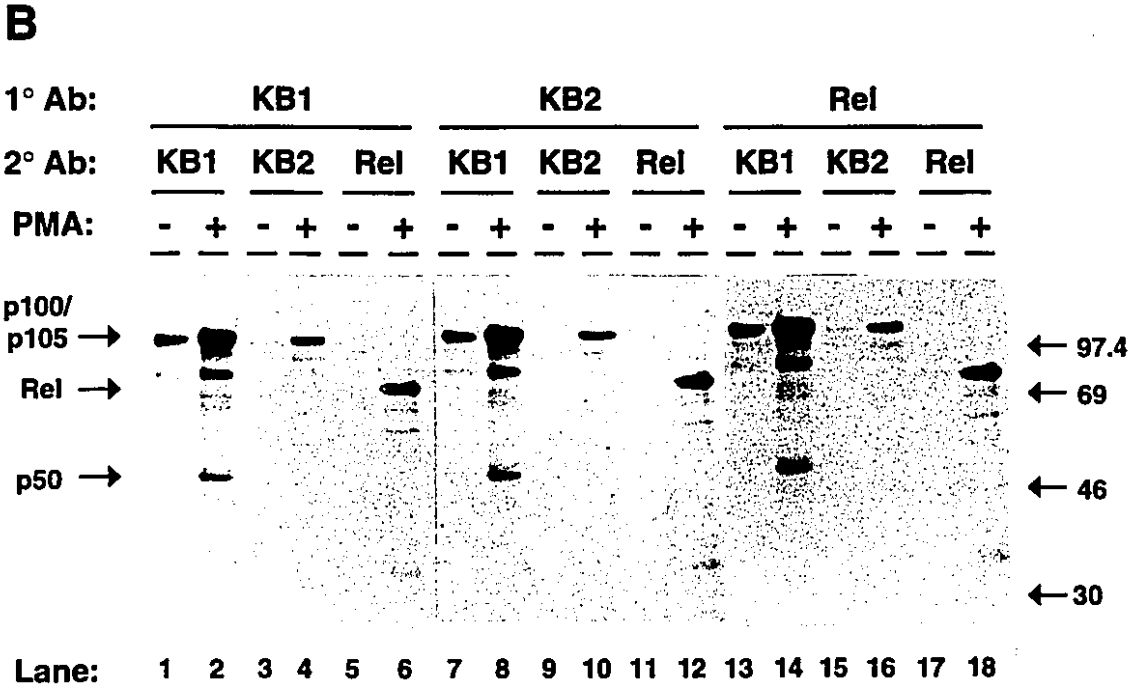
5.1 *Immunoprecipitation analysis of NF-κB/Rel proteins in normal T cells*

Formation of NF-κB/Rel heterodimers was next analyzed in Jurkat T cells by immunoprecipitation. Uninduced and PMA-induced Jurkat cells were metabolically labeled with ^{35}S-methionine, and lysates were prepared and subjected to immunoprecipitation (Fig. 17). As a control, lysates from uninduced and PMA-induced cells were immunoprecipitated with NRS to reveal non-specific immune complexes (Fig. 17A, lanes 1 and 2). A major band of 65 kD was immunoprecipitated using the anti-p65 antiserum (Fig. 17A, lanes 3 and 4). A minor band at 40 kD was also co-immunoprecipitated from PMA-treated cells (Fig. 17A, lane 4). Several polypeptides were immunoprecipitated with the anti-NFKB1 antiserum (Fig. 17A, lanes 5 and 6). The 100 and 50 kD bands correspond to the NFKB1 precursor and product, respectively. In addition, proteins co-migrating with c-Rel and p65, as well as a 40 kD protein co-immunoprecipitated (Fig. 17A, lanes 5 and 6). An antiserum specific to the N-terminus of NFKB2 immunoprecipitated a 100 kD protein, p100, but not the 52 kD protein (Fig. 17A, lanes 7 and 8), supporting previous results that demonstrated low levels of p52 expression in Jurkat cells (Fig. 13). Finally, the anti-c-Rel antiserum immunoprecipitated c-Rel as well as proteins migrating at 100, 50 and 40 kD.

Double immunoprecipitations were performed to establish the identity of the co-immunoprecipitating proteins (Fig. 17B). Immune complexes recovered from the first immunoprecipitation were divided into aliquots, boiled in SDS sample

FIGURE 17. Co-Immunoprecipitation Analysis of NF-κB/Rel Subunits in Normal T Cells. (A) NP-40 lysis protein extracts were prepared from uninduced (odd numbered lanes) and PMA-induced (even numbered lanes) Jurkat cells after a 2 hr metabolic labeling with ^{35}S-methionine. Immunoprecipitations were performed as described in Figure 15. The antisera used were: normal rabbit serum (NRS) (lanes 1 and 2), anti-p65 (lanes 3 and 4), anti-NFKB1 (lanes 5 and 6), anti-NFKB2 (lanes 7 and 8), and anti-c-Rel (lanes 9 and 10). **(B)** Extracts from untreated (odd-numbered lanes) and 8 hr PMA-induced (even-numbered lanes) Jurkat cells were subjected to a first cycle of immunoprecipitation using anti-NFKB1 (lanes 1 to 6), anti-NFKB2 (lanes 7 to 12) and anti-c-Rel (lanes 13 to 18) antisera. Immune complexes immobilized on protein A sepharose were eluted from the beads by boiling for 10 min in TNT, 1% SDS and 0.5% β2-mercaptoethanol. The supernatant was collected, divided, diluted 1:10 and re-precipitated overnight with the same (lanes 1, 2, 9, 10, 17, and 18) or different (lanes 3 to 8, 11 to 16) antisera, as indicated above the lane. Immune complexes were processed as described in Figure 15. Arrows on the right indicate migration of molecular weight markers. Immunoprecipitated proteins are identified by arrows on the left.


A
1° Ab:
NRS
p65
KB1
KB2
Rel
PMA:
- + - + - + - + - +
p100/
p105
Rel
p65
p50
97.4
69
46
30
Lane:
1 2 3 4 5 6 7 8 9 10


B
1° Ab:
KB1
KB2
Rel
2° Ab:
KB1 KB2 Rel KB1 KB2 Rel KB1 KB2 Rel
PMA:
- + - + - + - + - + - + - + - + - +
p100/
p105
Rel
p50
97.4
69
46
30
Lane:
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

buffer to remove any protein noncovalently associated with the immunoreactive species, and subjected to a second cycle of immunoprecipitation with the same or a different antibody. Uninduced and 2 hr PMA-induced Jurkat cell lysates were subjected to a first cycle of immunoprecipitation using anti-NFKB1 (Fig. 17B, lanes 1 to 6), anti-NFKB2 (Fig. 17B, lanes 7 to 12), and anti-c-Rel (Fig. 17B, lanes 13 to 18) antisera. A second cycle of immunoprecipitation with the same antibodies confirmed the expression of p105 in uninduced cells (Fig. 17B, lane 1), and also p50, p100, and c-Rel in PMA-treated cells (Fig. 17B, lanes 2, 10, and 18). The use of anti-NFKB2 or anti-c-Rel antisera in the second cycle, immunoprecipitated bands corresponding to p100 and c-Rel, respectively (Fig. 17B, lanes 4 and 6), suggesting the presence of NFKB1-NFKB2, as well as NFKB1-c-Rel, heterodimers. This was confirmed by reciprocal double immunoprecipitations in which the anti-NFKB2 antiserum immunoprecipitated NFKB1 (Fig. 17B, lanes 7 and 8), and the anti-c-Rel antiserum immunoprecipitated NFKB1 (Fig. 17B, lanes 13 and 14). Double immunoprecipitations using either anti-NFKB2 or anti-c-Rel antisera in the first or second cycle also demonstrated the presence of NFKB2-c-Rel complexes (Fig. 17B, lanes 12 and 16).

5.2 *Immunoprecipitation analysis of NF-κB/Rel proteins in HTLV-I infected T cells*

Lysates from uninduced and PMA-induced MT-2 cells were subjected to a similar analysis (Fig. 18). Surprisingly, the anti-p65 antiserum failed to immunoprecipitate any detectable p65 (Fig. 18A, lanes 3 and 4), although immunoblots confirmed p65 protein expression in these cells (Figs. 13C and

FIGURE 18. Co-Immunoprecipitation Analysis of NF-κB/Rel Subunits in HTLV-I Infected T Cells. (A) NP-40 lysis protein extracts were prepared from uninduced (odd numbered lanes) and PMA-induced (even numbered lanes) MT-2 cells after a 2 hr metabolic labeling with ^{35}S-methionine. Immunoprecipitations were performed as described in Figure 15. The antisera used were: normal rabbit serum (NRS) (lanes 1 and 2), anti-p65 (lanes 3 and 4), anti-NFKB1 (lanes 5 and 6), anti-Tax (lanes 7 and 8), anti-NFKB2 (lanes 9 and 10), and anti-c-Rel (lanes 11 and 12). **(B)** Extracts from 8 hr PMA-induced MT-2 cells were subjected to a first cycle of immunoprecipitation using NRS (lane 1), anti-NFKB2 (lanes 2 to 4), anti-Tax (lanes 5 to 8) and anti-c-Rel (lanes 9 to 11) antisera. Immune complexes immobilized on protein A sepharose were eluted from the beads by boiling for 10 min in TNT, 1% SDS and 0.5% β2-mercaptoethanol. The supernatant was collected, divided (except NRS), diluted 1:10 and re-precipitated overnight with the same (lanes 1, 2, 5, and 9) or different (lanes 3, 4, 6 to 8, 10, and 11) antisera, as indicated above the lane. Immune complexes were processed as described in Figure 15. Arrows on the right indicate migration of molecular weight markers. Immunoprecipitated proteins are identified by arrows on the left.

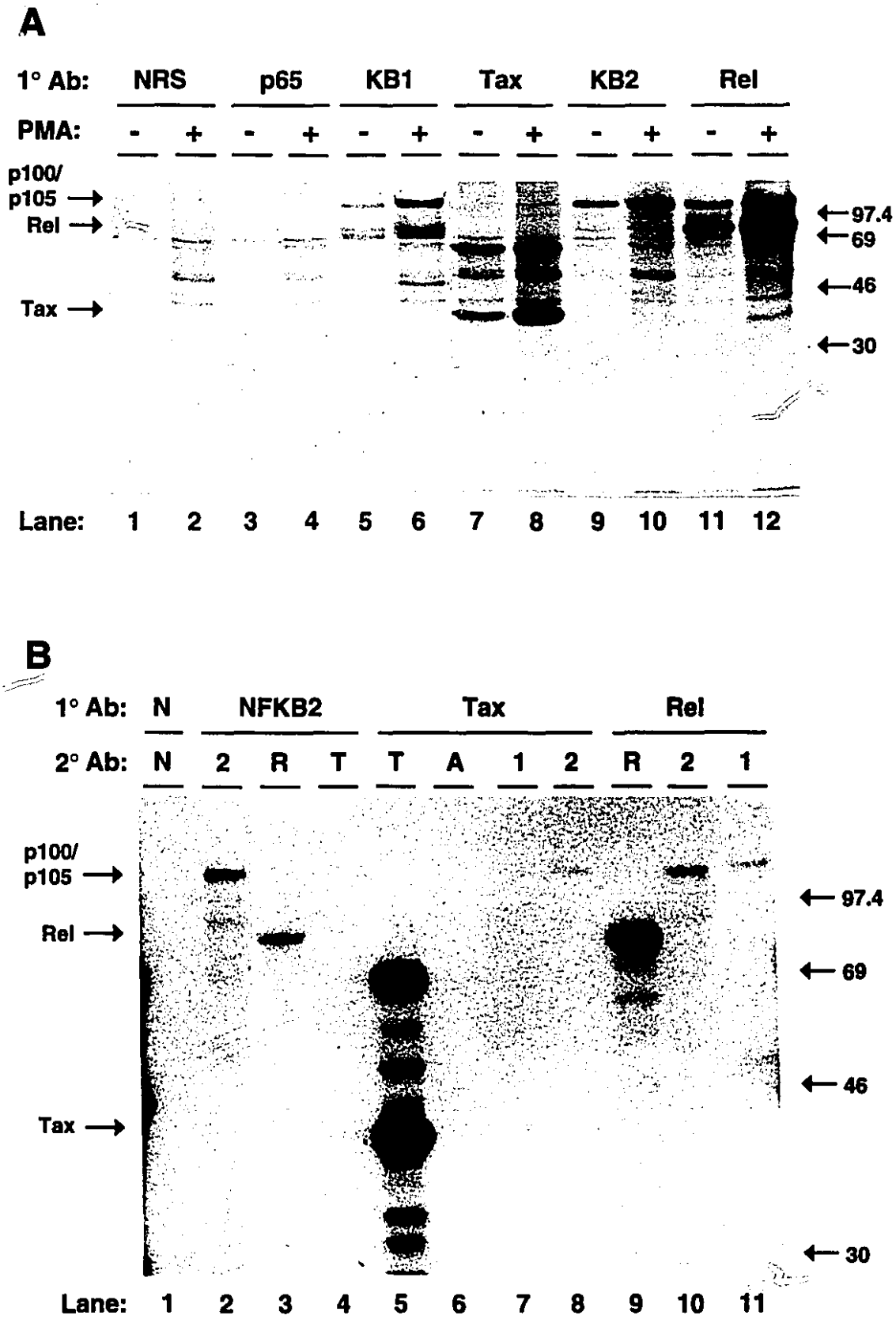
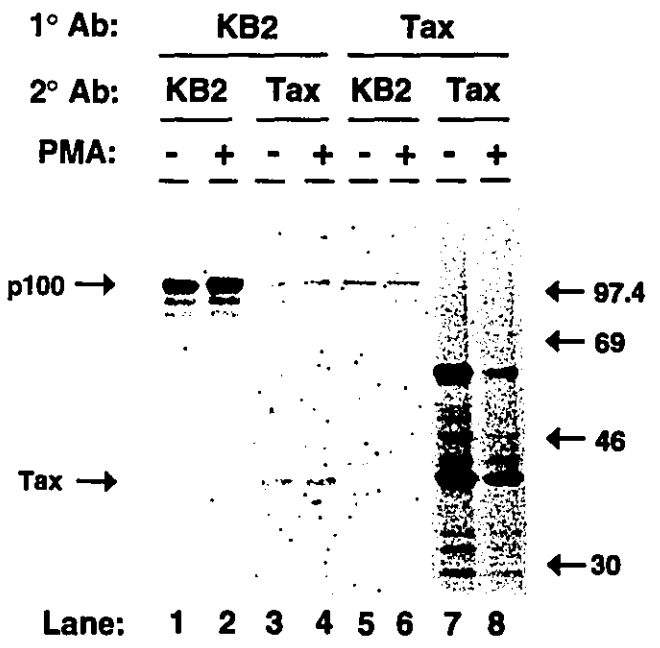

A
1° Ab:
NRS
p65
KB1
Tax
KB2
Rel
PMA:
- + - + - + - + - + - +
p100/
p105
Rel
Tax
97.4
69
46
30
Lane: 1 2 3 4 5 6 7 8 9 10 11 12
B
1° Ab:
N
NFKB2
Tax
Rel
2° Ab:
N 2 R T T A 1 2 R 2 1
p100/
p105
Rel
Tax
97.4
69
46
30
Lane: 1 2 3 4 5 6 7 8 9 10 11

FIGURE 18... (C) Double immunoprecipitations were performed as described in Materials and Methods. Anti-NFKB2 (lanes 1 to 4) and anti-Tax (lanes 5 to 8) were used in the first cycle of immunoprecipitation. These same antibodies were used in a second cycle of immunoprecipitation (anti-NFKB2 for lanes 1, 2, 5, and 6 and anti-Tax for lanes 3, 4, 7, and 8). Molecular weights are indicated on the right and the immunoprecipitated proteins on the left.

C


1° Ab:
KB2
Tax
2° Ab:
KB2
Tax
KB2
Tax
PMA:
- + - + - + - +
p100
Tax
97.4
69
46
30
Lane:
1 2 3 4 5 6 7 8

14). The anti-NFKB1 antiserum immunoprecipitated p105, p50, as well as other minor polypeptides (Fig. 18A, lanes 5 and 6). Simple immunoprecipitations using anti-c-Rel and anti-NFKB2 antisera confirmed that c-Rel and NFKB2 were both present at high levels in MT-2 cells, and suggested the presence of NFKB2-c-Rel complexes in these cells (Fig. 18A, lanes 8 to 12). Two major bands precipitated with the anti-Tax antiserum: the 40 kD Tax protein and a protein of approximately 65 kD (Fig. 18A, lanes 7 and 8). Two other minor proteins of approximately 100 and 50 kD were also precipitated with the anti-Tax antibody, and these proteins co-migrated with NFKB1 p105 or NFKB2 p100, and with p52 proteins (Fig. 18A, compare lanes 8 and 10). PMA treatment was also able to increase the amount of both NFKB2 p100 and p52 (Fig. 18A, compare lanes 9 and 10). The anti-NFKB2 antiserum also weakly precipitated a protein that co-migrated with c-Rel (Fig. 18A, compare lanes 10 and 11). Using the anti-Rel antiserum, major proteins of approximately 100 and 80 kD were immunoprecipitated, as well as several weaker proteins (Fig. 18A, lanes 11 and 12).

To examine directly the possibility of NFKB2-c-Rel-Tax complexes in HTLV-I infected MT-2 cells, double immunoprecipitations were carried out using 8 hr PMA stimulated MT-2 cell extracts. When the anti-NFKB2 antiserum was used in the primary immunoprecipitation (Fig. 18B, lanes 2 to 4), the same antibody re-precipitated the 100 kD NFKB2 precursor (Fig. 18B, lane 2). The anti-c-Rel antiserum as the second antibody immunoprecipitated c-Rel (Fig. 18B, lane 3), demonstrating that c-Rel was co-precipitated as part of the complex with NFKB2. Surprisingly, a faint band co-migrating with Tax was precipitated with the anti-Tax antibody (Fig. 18B, lane 4). p65 was not detected in the double co-precipitation analysis (data not shown).

When anti-Tax antiserum was used in the primary immunoprecipitation (Fig. 18B, lanes 5 to 8), the same antibody immunoprecipitated two proteins in the second cycle: a 40 kD protein Tax protein and a 65 kD protein which migrated at the same position as p65 (Fig. 18B, lane 5). When the second precipitation was performed with anti-p65 (Fig. 18B, lane 6), the 65 kD protein was not immunoprecipitated, indicating that it was not the NF-κB p65 subunit. Also the 65 kD protein was directly recognized by the anti-Tax antiserum in Western blots (data not shown), indicating direct specificity for the anti-Tax antiserum. A protein migrating at the same position was previously observed in co-immunoprecipitations with Tax, and shown to be an Tax-env fusion protein present in MT-2 cells (411, 412). Strikingly, when the anti-NFKB2 antiserum was used in the secondary immunoprecipitation, a protein of ~100 kD was detected, indicating that the NFKB2 precursor was part of the immune complex initially precipitated with the anti-Tax antiserum (Fig. 18B, lane 8). Surprisingly, no proteins were immunoprecipitated when the anti-NFKB1 antiserum was used as the second antibody (Fig. 18B, lane 7), although NFKB1 was previously shown to associate with Tax in HTLV-I infected cells (413).

When the anti-c-Rel antiserum was used in the primary immunoprecipitation (Fig. 18B, lanes 9 to 11), the same antibody re-precipitated c-Rel (Fig. 18B, lane 9). The precursor forms of both NFKB2 (p100) and NFKB1 (p105) co-immunoprecipitated with c-Rel (Fig. 18B, lanes 10 and 11) but again p65 was not immunoprecipitated (data not shown). These results confirmed previous studies demonstrating an association between c-Rel and NFKB1 or NFKB2 (274, 275) and suggested an association between Tax protein and NFKB2 p100.

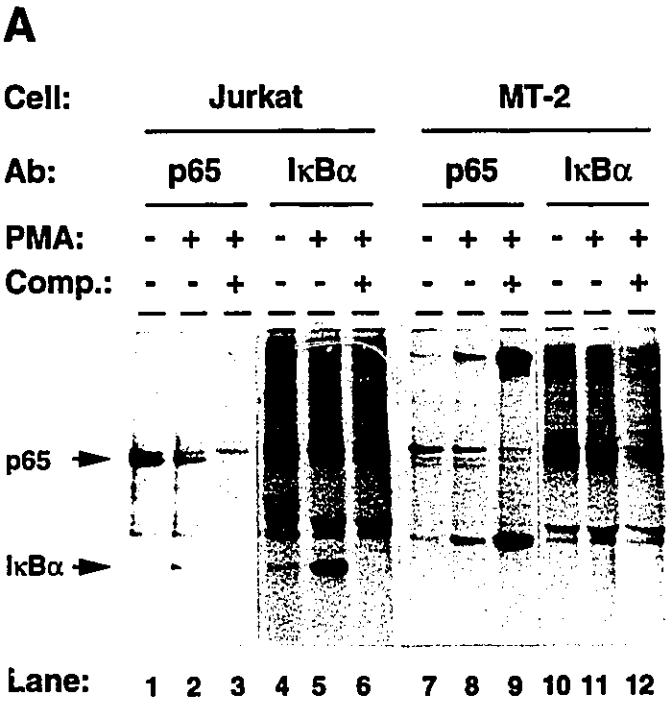
5.3 *Tax physically associates with NFKB2 p100 in HTLV-I infected T cells*

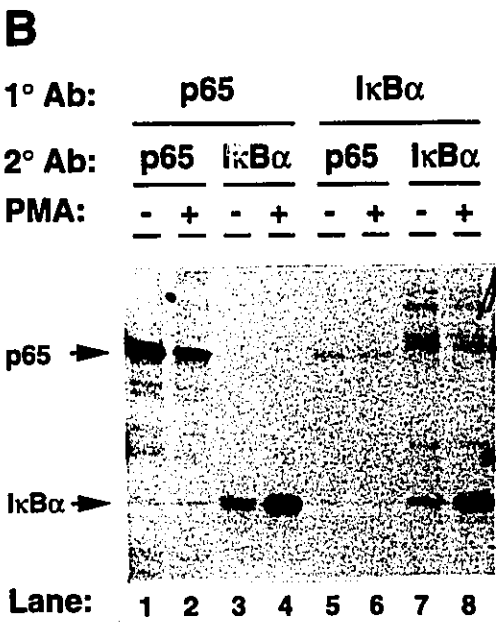
Double immunoprecipitations with the anti-NFKB2 and anti-Tax antisera were used to demonstrate an association between Tax protein and NFKB2 in uninduced and PMA induced MT-2 cells (Fig. 18C). When the anti-NFKB2 antiserum was used in the first immunoprecipitation, secondary immunoprecipitation with the homologous antiserum readily detected NFKB2 p100 (Fig. 18C, lanes 1 and 2); conversely, the anti-Tax antiserum weakly recognized the 40 kD form Tax protein present in MT-2 cells (Fig. 18C, lanes 3 and 4). When the anti-Tax antiserum was used in the first immunoprecipitation, secondary immunoprecipitation using the same antiserum readily detected both the 40 and 65 kD forms of Tax in MT-2 cells (Fig. 18C, lanes 7 and 8); secondary immunoprecipitation with the anti-NFKB2 anti-serum also weakly detected NFKB2 p100 (Fig. 18C, lanes 5 and 6). Using similar procedures it was not possible to convincingly demonstrate an association between Tax protein and other NF-κB specific subunits (data not shown); nonetheless, these results demonstrated for the first time an interaction between Tax protein and the NFKB2 p100 precursor in HTLV-I infected cells.

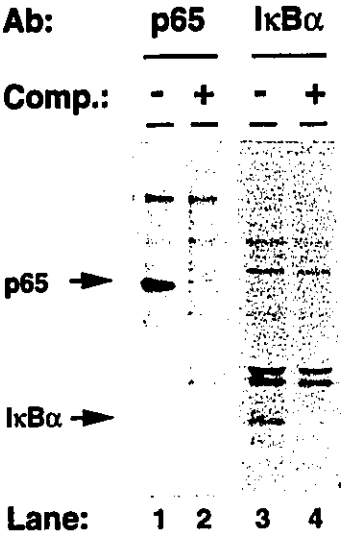
5.4 *Analysis of p65 and IκBα subunits*

Figure 17A, lane 4 showed that p65 was immunoprecipitated from Jurkat cell extracts together with a 40 kD protein. However, p65 was not immunoprecipitated from MT-2 cell extracts (Fig. 18A, lanes 3 and 4). These

FIGURE 19. Analysis of p65 and IκBα in Normal and HTLV-I Infected T Cells. **(A)** NP-40 lysis protein extracts were prepared from uninduced (lanes 1, 4, 7, and 10) and PMA-induced (lanes 2, 3, 5, 6, 8, 9, 11, and 12) Jurkat (lanes 1 to 6) and MT-2 (lanes 7 to 12) cells after a 2 hr metabolic labeling with ^{35}S-methionine. Immunoprecipitations were performed as described in Figure 15. The antisera used were: anti-p65 (lanes 1 to 3, and 7 to 9), and anti-IκBα (lanes 4 to 6, and 10 to 12). Competition with excess peptide was performed to ensure specificity (lanes 3, 6, 9, and 12). **(B)** The extracts above were subjected to a first cycle of immunoprecipitation using anti-p65 (lanes 1 to 4) and anti-IκBα (lanes 5 to 8) antisera. Immune complexes were processed as described in Figure 18 and re-precipitated overnight with the same (lanes 1, 2, 5, and 6) or different (lanes 3, 4, 7, and 8) antisera, as indicated above the lane. Immunoprecipitations were completed as described in Figure 15. Arrows on the right indicate migration of molecular weight markers. Immunoprecipitated proteins are identified by arrows on the left. **(C)** The same extracts were diluted with an equal volume of SDS sample buffer, boiled for 10 min and pre-cleared with NRS as described in Materials and Methods. Immunoprecipitations were then performed using anti-p65 (lanes 1 and 2) and anti-IκBα (lanes 3 and 4) antisera. Immune complexes were processed as described in Figure 18. Competition with excess peptide was performed to ensure specificity (lanes 2 and 4).


A
Cell:
Jurkat
MT-2
Ab:
p65
IκBα
p65
IκBα
PMA:
- + + - + + - + + - + +
Comp.:
- - + - - + - - + - - +
p65
IκBα
Lane:
1 2 3 4 5 6 7 8 9 10 11 12


B
1° Ab:
p65
IκBα
2° Ab:
p65
IκBα
p65
IκBα
PMA:
- + - + - + - +
p65
IκBα
Lane:
1 2 3 4 5 6 7 8


C
Ab:
p65
IκBα
Comp.:
- + - +
p65
IκBα
Lane:
1 2 3 4

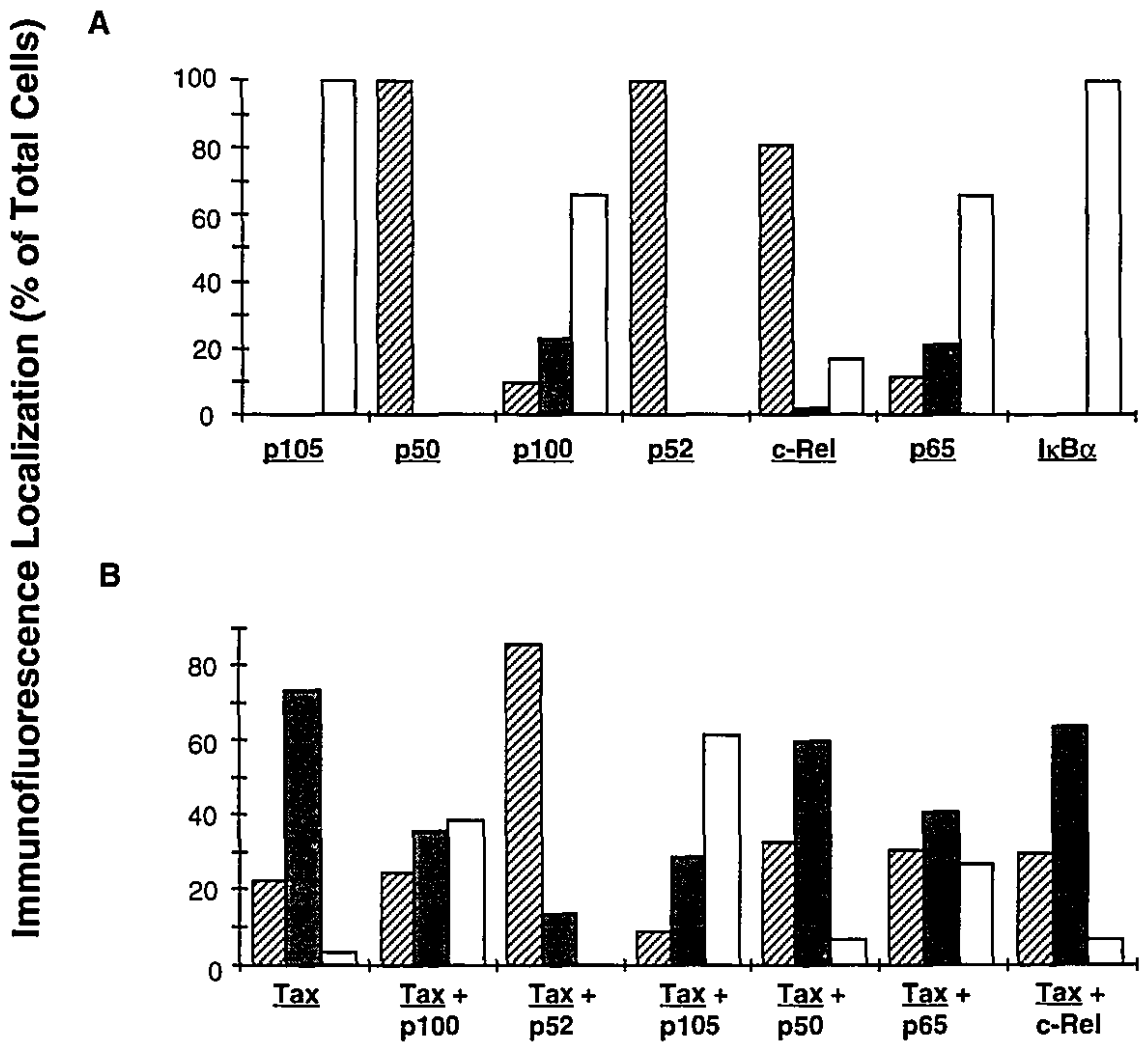
observations prompted further investigation of p65 and IκBα in normal Jurkat and HTLV-I infected T cells. Immunoprecipitation of p65 from uninduced and induced Jurkat cells also co-immunoprecipitated a 40 kD protein (Fig.19A, lanes 1 and 2). Precipitation of both p65 and the 40 kD protein was specific, as demonstrated by competition with excess peptide (Fig. 19A, lane 3). Immunoprecipitation of Jurkat cell extracts with the anti-IκBα antiserum revealed a 40 kD protein that co-migrated exactly with the p65 co-immunoprecipitated protein (Fig.19A, lanes 4 and 5). A double immunoprecipitation procedure was applied to uninduced and PMA-induced Jurkat cell extracts using anti-p65 and anti-IκBα antisera (Fig. 19B). Lanes 3 and 4 showed that IκBα was present in immune complexes formed by the anti-p65 antiserum. Similarly, lanes 5 and 6 showed that p65 protein was present in immune complexes formed by the anti-IκBα antiserum. These results demonstrated the formation of p65-IκBα complexes in uninduced and PMA-induced normal T cells. In contrast, anti-p65 and anti-IκBα antisera did not specifically immunoprecipitate their target proteins from uninduced or PMA-induced MT-2 cell extracts, and neither antibodies co-immunoprecipitated IκBα and p65 proteins, respectively (Fig. 19A, lanes 7 to 12). To ensure that both antibodies were capable of recognizing their target proteins, lysates were first boiled in SDS sample buffer, and then immunoprecipitated with anti-p65 or anti-IκBα antisera. As shown in Fig. 19C, both anti-p65 and anti-IκBα antisera specifically immunoprecipitated p65 (Fig.19C, lanes 1 and 2) and IκBα (Fig. 19C, lanes 3 and 4) proteins when lysates were denatured prior to the incubation with the antibodies. These results demonstrated that although both p65 and IκBα proteins were present in HTLV-I infected cells, they could not be detected as protein-protein complexes.

6. NF-κB-Tax Co-Transfection Studies

6.1 *Localization of NF-κB/Rel subunits*

An additional approach was used to assess the Tax-NF-κB protein interactions. cDNA sequences corresponding to distinct NF-κB/Rel subunits (p50/p105, p52/p100, p65, p65Δ, c-Rel and IκBα) were cloned into a CMV driven eukaryotic expression vector and Cos-7 cells were transfected with these plasmids. At 48 hr after transfection, cells were fixed and stained for NF-κB specific proteins. The immunofluorescence was quantified into three categories on the basis of nuclear or cytoplasmic staining properties, and expressed as a percentage of the total number of positive cells: 1) nuclear staining, cells with exclusive or predominant nuclear staining (at least 2 fold higher nuclear versus cytoplasmic staining); 2) nuclear and cytoplasmic staining, cells with approximately equal nuclear and cytoplasmic staining; 3) cytoplasmic staining, cells with exclusive or predominant cytoplasmic staining (at least 2 fold higher cytoplasmic versus nuclear staining). The results of these transfection-immunofluorescence studies are summarized in Figure 20. As shown in Figure 20A, transfection with plasmids expressing NFKB1 p105 and IκBα resulted in exclusively cytoplasmic fluorescence when stained with anti-NFKB1 and anti-IκBα antisera, respectively. Conversely, transfected NFKB1 p50, NFKB2 p52, and c-Rel subunits localized mainly to the nucleus (Fig. 20A). Interestingly, a fraction of NFKB2 p100 was distributed to the nucleus. About 65% of the cells displayed exclusive cytoplasmic staining, consistent with the role of p100 as an ankyrin-repeat containing inhibitory protein; however, about 10% of the cells displayed nuclear fluorescence and about 25% of the cells had both

FIGURE 20. Tax-NF-κB Co-Transfection-Immunofluorescence Studies. Cos-7 cells were transfected with the indicated Tax and NF-κB expression plasmids by lipofection (Lipofectamine, GIBCO), as described in Material and Methods. At 48 hr post-transfection, cells were fixed and analyzed for expression of NF-κB or Tax by indirect immunofluorescence using specific antisera (underlined). The immunofluorescence was quantified into three categories on the basis of nuclear or cytoplasmic staining properties and expressed as a percentage of the total number of positive cells: 1) nuclear staining (▨); 2) nuclear and cytoplasmic staining (▩); 3) cytoplasmic staining (□). **(A)** Localization of NF-κB/Rel subunits by indirect immunofluorescence. **(B)** Subcellular relocalization of Tax by NF-κB/Rel subunits.


Immunofluorescence Localization (% of Total Cells)
A
100
80
60
40
20
0
p105
p50
p100
p52
c-Rel
p65
IκBα
B
80
60
40
20
0
Tax
Tax + p100
Tax + p52
Tax + p105
Tax + p50
Tax + p65
Tax + c-Rel

cytoplasmic and nuclear fluorescence (Fig. 20A). This result indicated that both nuclear and cytoplasmic distributions of p100 exist, consistent with recent biochemical data demonstrating that the nuclear protein H2TF1 is in fact NFKB2 p100 (283, 284). The strong transcriptional activator protein p65 also displayed a similar profile of predominantly cytoplasmic fluorescence with a portion of nuclear staining (Fig. 20A). In general, the subcellular localization of NF-κB/Rel proteins observed in these co-transfection-immunofluorescence assays was consistent with extensive biochemical data on the nuclear-cytoplasmic partitioning of these proteins (reviewed in 333 and 414).

6.2 *Subcellular relocalization of Tax by NF-κB/Rel subunits*

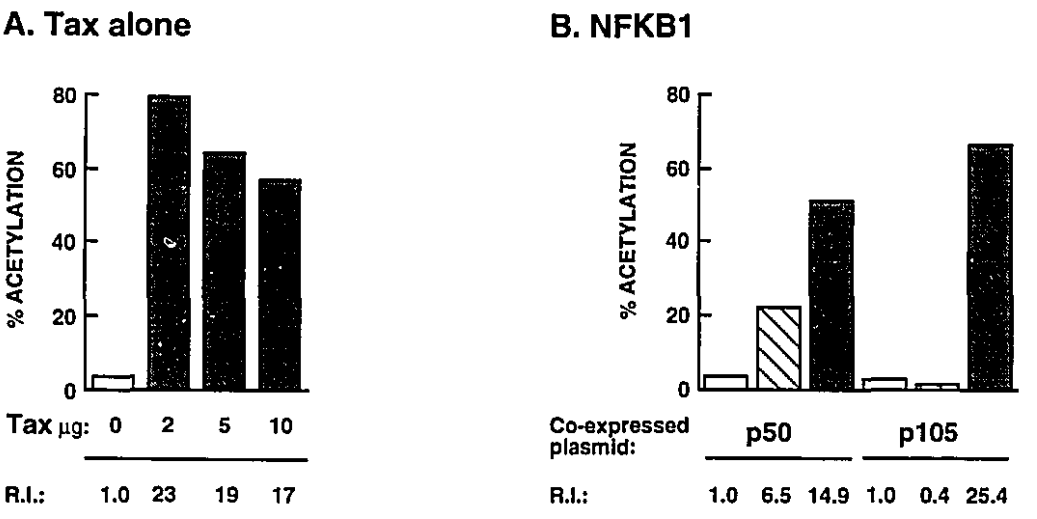
HTLV-I Tax protein was described as a predominantly nuclear viral protein with some cytoplasmic distribution (85, 86). Likewise, in these studies, the profile of Tax staining was either predominantly nuclear (>20%) or a mixture of nuclear and cytoplasmic (>70%) localization (Fig. 20B). Based on the results presented in Figure 18 demonstrating a physical association between Tax and specific NF-κB subunits, the effects of individual NF-κB proteins on the location of Tax were examined. An indirect immunofluorescence assay was performed on cells co-transfected with plasmids expressing individual NF-κB subunits together with the Tax expressing vector, and analyzed at 48 hr for Tax using a rabbit polyclonal anti-Tax antibody. Co-expression of either NFKB1 p50 or c-Rel only slightly altered the profile of Tax staining by increasing from 20% to 30% the fraction of cells with nuclear fluorescence (Fig. 20B). Expression of p65 also increased the number of cells displaying cytoplasmic Tax from less than 5% to about 25%, thus implying a weak interaction between Tax and p65 as

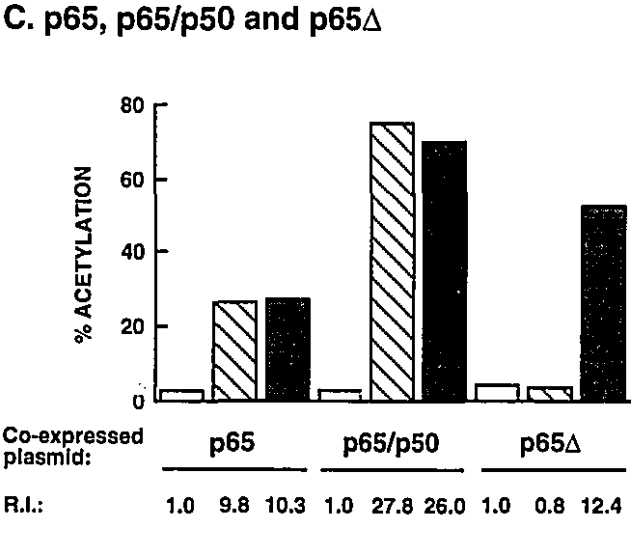
previously suggested (Fig. 20B) (415, 416). Dramatic alterations in Tax distribution occurred with the co-expression of NFKB2 p100 and p52, and with NFKB1 p105. Upon transfection of Tax and p100, about 40% of the transfected cells displayed cytoplasmic localization of Tax (Fig. 20B). Strikingly, co-expression of p52 resulted in an exclusively nuclear localization of Tax in more than 80% of the cells examined (Fig. 20B). This distinctive pattern of cytoplasmic or nuclear localization of Tax confirmed the Tax-p100 interaction presented in Figure 18, and further supported an interaction between Tax and the Rel homology domain of p52. Co-expression of Tax and p105 also resulted in a striking redistribution of Tax to the cytoplasm with more than 60% of the cells exhibiting preferential Tax localization to the cytoplasm (Fig. 20B).

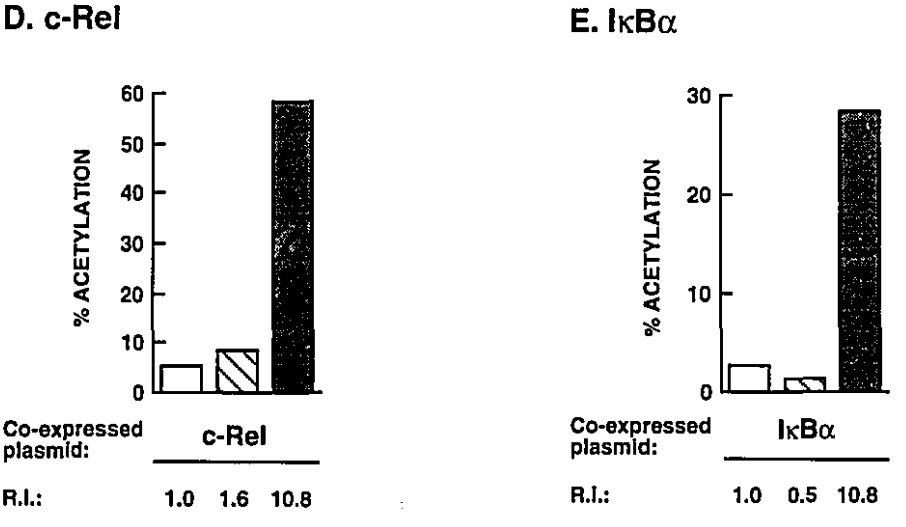
6.3 *NF-κB-mediated transcriptional activity*

Tax *trans*-activation of NF-κB-mediated transcriptional activity was next examined by transient expression assays. CMV-based plasmids expressing the individual NF-κB proteins were co-transfected into Jurkat cells along with an NF-κB regulated CAT gene (HIV-1 enhancer) in the presence or absence of a Tax-expression vector; the results of these experiments are summarized in Figure 21. p50 alone *trans*-activated the reporter gene about 6 fold; co-expression of Tax and p50 further increased the activity about 2 fold (Fig. 21B) to a level similar to that of Tax alone (Fig. 21A). The precursor p105 alone did not stimulate transcription, consistent with its role as an inhibitory molecule, but a 25 fold increase in reporter gene activity was observed with Tax co-expression (Fig. 21B). p65 expression alone *trans*-activated reporter gene activity about 10 fold and Tax did not further increase gene activity (Fig. 21C).

FIGURE 21. Tax-NF-κB Co-Transfection Studies. Jurkat cells ($2x10^7$) were transfected by the DEAE dextran method with an HIV enhancer-CAT reporter construct and co-transfected with NF-κB subunit expression plasmids in the absence or presence of a Tax expression vector as follows: **(A)** reporter alone; **(B)** NFKB1; **(C)** p65, p65/p50 and p65Δ; **(D)** c-Rel; **(E)** IκBα. Lysates for CAT assays were prepared from cells harvested 48 hr post-transfection. % acetylation: percent of acetylated ^{14}C-chloramphenicol. RI: relative induction: relative to transcriptional activity in the absence of NF-κB or Tax. Results are averages of different experiments. □ : reporter gene transfected alone; ▨ : co-transfection of reporter gene and NF-κB expression vectors; ▩ : co-transfection of reporter gene, NF-κB expression vectors and Tax expression vector.


A. Tax alone
% ACETYLATION
80
60
40
20
0
Tax μg: 0 2 5 10
R.I.: 1.0 23 19 17
B. NFKB1
% ACETYLATION
80
60
40
20
0
Co-expressed plasmid:
p50
p105
R.I.: 1.0 6.5 14.9 1.0 0.4 25.4

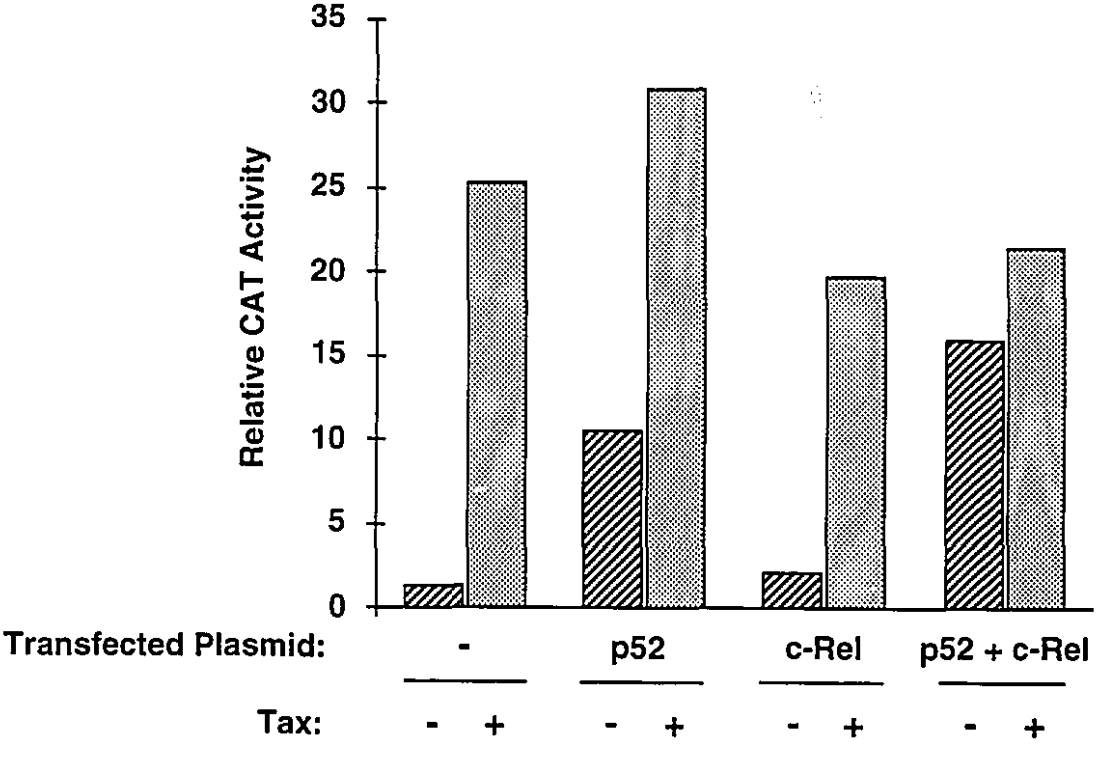

C. p65, p65/p50 and p65Δ
% ACETYLATION
80
60
40
20
0
Co-expressed plasmid:
p65
p65/p50
p65Δ
R.I.: 1.0 9.8 10.3 1.0 27.8 26.0 1.0 0.8 12.4


D. c-Rel
% ACETYLATION
60
50
40
30
20
10
0
Co-expressed plasmid:
c-Rel
R.I.: 1.0 1.6 10.8
E. IκBα
% ACETYLATION
30
20
10
0
Co-expressed plasmid:
IκBα
R.I.: 1.0 0.5 10.8

The most potent activator of NF-κB regulated gene expression was the combination of p65 and p50, the prototypical NF-κB, which stimulated activity more than 25 fold (Fig. 21C). Again, Tax did not further augment reporter gene activity. Unlike p65, expression of p65Δ, a naturally occuring splicing variant of p65 (387), did not activate but rather inhibited basal level transcription about 3 fold (Fig. 21C). Also, the combination of p65 and p65Δ inhibited NF-κB-regulated gene expression (data not shown). Despite the expression of p65Δ, Tax expression was able to overcome the inhibitory effects of p65Δ (Fig. 21C). c-Rel protein had little effect on the HIV-CAT construct whereas the combination c-Rel and Tax *trans*-activated about 10 fold (Fig. 21D). In a similar manner, IκBα-mediated inhibition of CAT activity was relieved by overexpression of Tax (Fig. 21E). Taken together, these results showed that Tax protein does not further increase reporter gene activity when co-transfected with strong NF-κB *trans*-activator proteins such as p65 and p65-p50. However, when co-transfected with subunits capable of inhibiting NF-κB mediated gene activity (p105, p65Δ, IκBα), Tax protein functioned as a *trans*-dominant activator of NF-κB activity.

Since NFKB2 and c-Rel proteins are major constituents of Tax expressing cells, the capacity of NFKB2 and c-Rel to *trans*-activate an NF-κB-regulated promoter was evaluated in Jurkat cells. The HIV-1-enhancer-CAT reporter construct alone or in combination with an NFKB2 p52 or c-Rel expression plasmid, in the presence or absence of a Tax expressing plasmid was introduced into Jurkat cells; as shown in Figure 22, transfection of p52 alone resulted in a 5 fold increase of reporter activity; co-expression of p52 and Tax increased the relative level of CAT activity 15 fold, to the level observed with Tax alone (Fig. 22). A c-Rel expression plasmid did not *trans*-activate the HIV-1 enhancer

FIGURE 22. Effect of c-Rel and NFKB2 (p52) on NF-κB-Dependent Gene Activity. Jurkat cells were transfected by DEAE-Dextran method with an NF-κB-dependent CAT reporter plasmid (HIV enhancer-CAT) alone or in combination with NFKB2 p52, c-Rel, or both expression plasmids (▨). The same samples were also co-transfected with a Tax expression plasmid (▩). Extracts were prepared from cells harvested 2 days after transfection and analyzed for CAT activity. The results represent an average from three independent experiments.


Relative CAT Activity
35
30
25
20
15
10
5
0
Transfected Plasmid:
-
p52
c-Rel
p52 + c-Rel
Tax:
- + - + - + - +

construct significantly, whereas the combination of c-Rel and Tax stimulated reporter gene activity about 10 fold (Fig. 22). However, when p52 and c-Rel were co-transfected into Jurkat cells, an 8 fold enhancement of reporter gene activity was observed; this level of *trans*-activation was increased moderately to about 11 fold with Tax co-expression (Fig. 22). Together, these transient expression assays suggested that the majority of the *trans*-activation potential of HTLV-I Tax protein may be contributed by the overexpression of c-Rel and NFKB2 in Tax expressing and HTLV-I infected T cells.

7. Conclusions

Results presented in this chapter demonstrated that NFKB2 and c-Rel are overexpressed in HTLV-I infected and Tax expressing cells and, together account for the majority of the constitutive NF-κB binding activity in these cells before and after PMA stimulation. However, participation of the p65 subunit in the formation of protein-DNA complexes was reduced. Also, a Tax-dependent correlation was established between expression of NFKB2 p100 and processing to the NFKB2 p52 DNA binding form, induction of c-Rel, and *trans*-activation of NF-κB-mediated gene expression. Co-immunoprecipitations were used to examine protein-protein interactions in non-Tax and Tax expressing cells. NFKB1-c-Rel, NFKB2-c-Rel, NFKB1-NFKB2, and p65-IκBα complexes were detected in Jurkat T cells. In HTLV-I infected MT-2 cells the predominant NF-κB heterodimers were composed of NFKB2-c-Rel and, to a lesser extent, NFKB1-c-Rel. Most importantly, NFKB2 p100 was found to physically associate with Tax in HTLV-I infected cells. Co-transfection immunofluorescence studies also revealed that Tax subcellular localization was affected by co-expression of

p100, p52 and p105, suggesting interactions between the Rel homology domain of these proteins and Tax. These results suggest that constitutive NFKB2 synthesis and processing allow continuous nuclear expression of an otherwise cytoplasmic protein and, in conjunction with overexpression of c-Rel, alter the NF-κB signaling pathway, thus contributing to leukemic transformation of T cells by HTLV-I.

CHAPTER 5

CONSTITUTIVE PHOSPHORYLATION AND TURNOVER OF IκBα IN HTLV-I INFECTED AND TAX EXPRESSING T CELLS

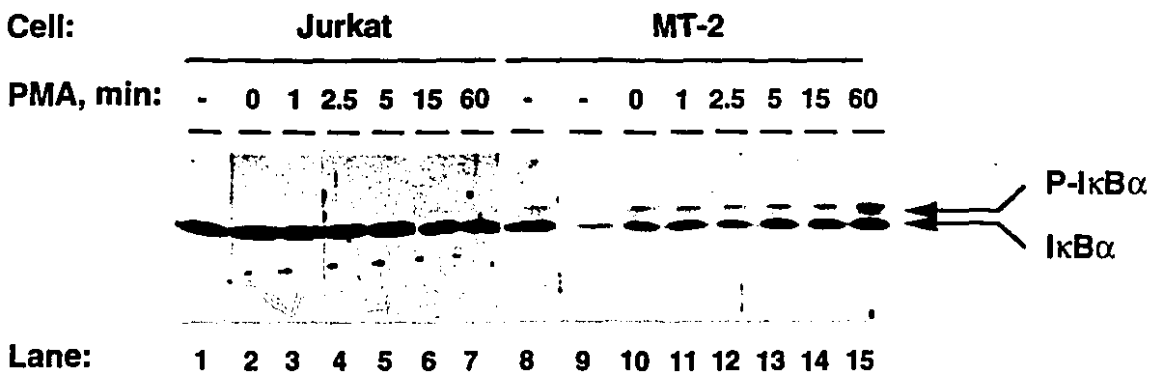
1. Constitutive IκBα Phosphorylation

Since phosphorylation and degradation of IκBα have been implicated as critical steps leading to NF-κB activation following induction (334, 335, 337, 417), the possibility that the constitutive NF-κB binding activity observed in HTLV-I infected and Tax expressing cells correlated with the modification of IκBα phosphorylation state and increased IκBα turnover was examined. Extracts from untreated or PMA-treated Jurkat and MT-2 cells were examined for the presence of phosphorylated forms of IκBα by SDS-polyacrylamide gel electrophoresis and immunoblotting using an IκBα-specific antisera, as described in Materials and Methods. Phosphorylated IκBα was not detected in Jurkat cells following PMA treatment for times ranging from 0 to 60 min (Fig. 23A, lanes 1 to 7, a dark exposure is shown). On the other hand, a band migrating just above IκBα was detected immediately after PMA addition to MT-2 cells (Fig. 23A, lane 10). Most importantly, darker exposure of the immunoblot revealed the slower migrating band in untreated MT-2 cells, indicating a constitutive level of IκBα phosphorylation in MT-2 cells (Fig. 23A, lane 8). PMA treatment of MT-2 cells increased IκBα levels and also increased the amount of the putative phosphorylated form (Fig. 23A, lanes 10 to 15).

To ensure that the slower migrating form represented phosphorylated IκBα, MT-2 cell extracts (0 and 30 min) were treated with calf alkaline intestinal phosphatase (CIP) to dephosphorylate IκBα *in vitro* (Fig. 23B). Dephosphorylation was achieved by incubating extracts (100 μg) with 5 units of CIP for 1 hr at 37°C. Another reaction contained both CIP and phosphatase inhibitors (10 mM sodium vanadate and 50 mM sodium fluoride), to block *in vitro* dephosphorylation of IκBα, as previously described (335). The

FIGURE 23. Phosphorylated IκBα in HTLV-I Infected and Tax Expressing T Cells. (A) Detection of phosphorylated forms of IκBα. Whole cell extracts were prepared from untreated Jurkat (lane 1) and MT-2 (lanes 8 and 9), and from cells treated with PMA for 0 (lanes 2 and 10), 1 (lanes 3 and 11), 2.5 (lanes 4 and 12), 5 (lanes 5 and 13), 15 (lanes 6 and 14) and 60 (lanes 7 and 15) min. Denaturing electrophoresis was performed on 12% SDS-polyacrylamide, 20 cm long gels. After transfer to nitrocellulose, IκBα signals were detected by immunoblotting with an anti-IκBα antibody, and chemiluminescence (ECL, Amersham). The phosphorylated form of IκBα is indicated as P-IκBα. Lane 8 represents a darker exposure of lane 9. **(B)** *In vitro* dephosphorylation of P-IκBα. Extracts from MT-2 cells treated for 0 (lanes 1 to 3) and 30 (lanes 4 to 6) min with PMA were incubated at 37°C for 1 hr in the absence (lanes 1 and 4) or presence (lanes 2, 3, 5 and 6) of 5 units of calf alkaline intestinal phosphatase (CIP). Inhibitors of phosphatases (10 mM sodium vanadate and 50 mM sodium fluoride) were also included in reactions loaded on lanes 3 and 6. After incubation, SDS sample buffer was added and reactions were immediately loaded on 12% SDS-PAGE. IκBα signals were detected as described previously.

A


Cell:
Jurkat
MT-2
PMA, min:
- 0 1 2.5 5 15 60 - - 0 1 2.5 5 15 60
P-IκBα
IκBα
Lane:
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

B

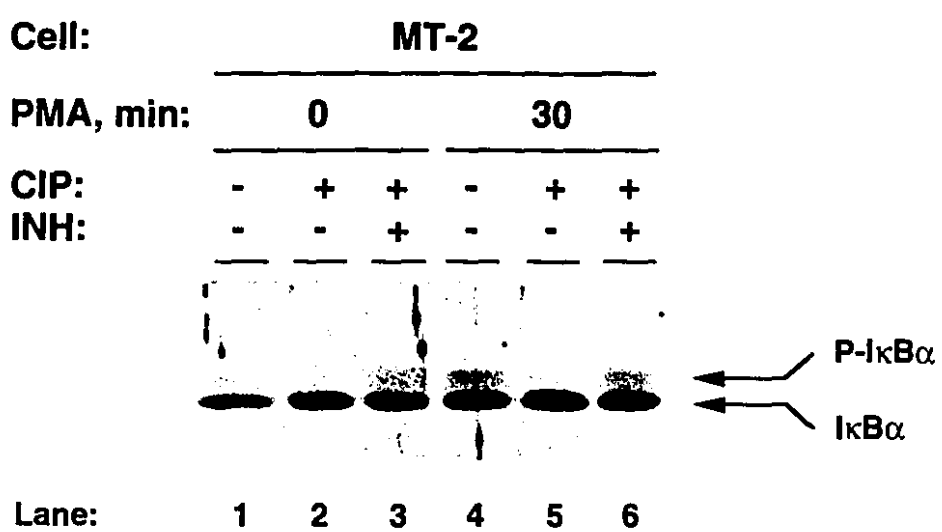
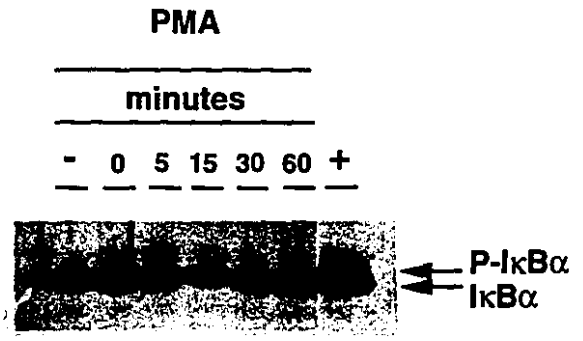
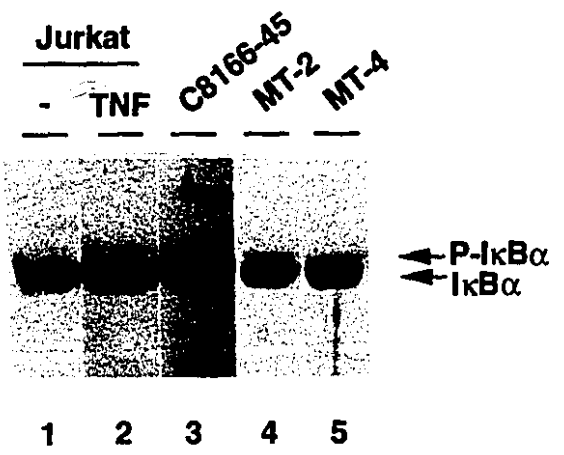

Cell:
MT-2
PMA, min:
0
30
CIP:
- + + - + +
INH:
- - + - - +
P-IκBα
IκBα
Lane:
1 2 3 4 5 6

FIGURE 23... (C) Phosphorylated IκBα in Tax expressing 19D cells. Whole cell extracts were prepared from untreated 19D (lane 1) and from cells treated with PMA for 0, 5, 15, 30, and 60 min (lanes 2 to 6). Samples were examined for phosphorylated IκBα as described above. Lane 7 is a positive control loaded with MT-2 cell extracts. **(D)** Analysis of phosphorylated IκBα in untreated and TNFα-treated Jurkat, and in untreated C8166-45, MT-2, and MT-4 cells. Extracts were prepared and analyzed as described above. The amount of extract loaded in each well was normalized to obtain similar signal intensities: lane 1: untreated Jurkat, 100 µg; lane 2: 5 min TNFα-treated Jurkat, 100 µg; lane 3: untreated C8166-45, 200 µg; lane 4: untreated MT-2, 100 µg; lane 5: untreated MT-4, 50 µg.

C


PMA
minutes
- 0 5 15 30 60 +
P-IκBα
IκBα
Lane: 1 2 3 4 5 6 7

D


Jurkat
- TNF
C8166-45
MT-2
MT-4
P-IκBα
IκBα
Lanes: 1 2 3 4 5

disappearance of the upper band with CIP treatment (Fig. 23B, lanes 2 and 5) and its reappearance in the presence of CIP plus inhibitors (Fig. 23B, lanes 3 and 6), demonstrated that the slower migrating band is phosphorylated IκBα. Appropriately exposed autoradiograms were scanned by laser densitometry to determine the ratio of phosphorylated to unphosphorylated IκBα. The ratio of IκBα:P-IκBα varied from about 5:1 in unstimulated MT-2 cells (Fig. 23B, lanes 10 and 11) to 2:1 in MT-2 cells treated with PMA for 60 min (Fig. 23B, lane 15). These results indicated that phosphorylated IκBα was present constitutively in HTLV-I infected cells.

In order to extend the observation that Tax expression correlated with the presence of constitutively phosphorylated IκBα, similar analyses were performed using Tax expressing 19D Jurkat cells (Fig. 23C) and HTLV-I infected cell lines C8166-45 and MT-4 (Fig. 23D). Unphosphorylated IκBα was detectable in 19D cells and immediately after addition of PMA, induced expression of phosphorylated IκBα was also detectable (Fig. 23C, lanes 1 and 2); this result was in contrast to Jurkat cells in which phosphorylated IκBα was not detected (Fig. 23A). PMA treatment further increased expression of phosphorylated IκBα in 19D cells without affecting levels of the non-phosphorylated form (Fig. 23C, lanes 3 to 6). Laser densitometry established that the IκBα:P-IκBα ratio was 10:1 after induction with PMA (Fig. 23C).

C8166-45 cells, derived from human cord blood lymphocytes co-cultured with ATL-derived cells, carry a deleted HTLV-I genome and express Tax and Rex proteins only (397), whereas MT-4 cells, although transformed by HTLV-I, do not express Tax (NP, data not shown). Phosphorylated IκBα was easily detected in C-8166-45 cells by immunoblot analysis (Fig. 23D, lane 3) as well

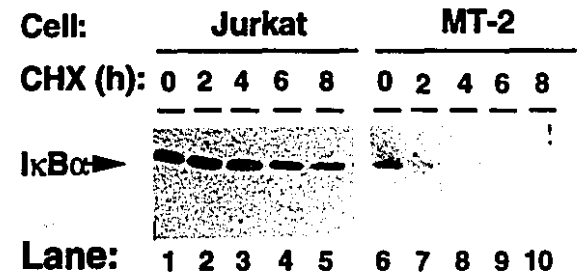
as in MT-2 cells (Fig. 23D, lane 4). Control reactions with CIP and phosphatase inhibitors demonstrated the phosphorylated nature of the upper band (LP, data not shown). Interestingly, MT-4 cells did not express any detectable phosphorylated IκBα (Fig. 23D, lane 5). Taken together, these results demonstrated that expression of Tax proteins in T cells was correlated with the presence of phosphorylated IκBα.

Figure 23D also illustrated that treatment of Jurkat cells with TNFα (140 units/ml) for 5 min resulted in the appearance of a slower migrating phosphorylated IκBα species (Fig. 23D, lane 2). This phosphorylated IκBα form appeared to be quite unstable, since it was not detected after 15 min of TNFα treatment as described previously (332) or after PMA treatment of Jurkat cells (Fig. 23A).

2. Increased IκBα Turnover

The possibility that constitutive phosphorylation of IκBα in MT-2 and C8166-45 cells was accompanied by increased IκBα turnover was next examined. IκBα protein expression and turnover were analyzed by immunoblotting in Jurkat and MT-2 cells treated with the protein synthesis inhibitor cycloheximide for times ranging from 0 to 8 hours (Fig. 24). Jurkat cell extracts contained about 7 fold more IκBα protein than MT-2 cells (Fig. 24, lanes 1 and 6). Also, C8166-45 cells contained about 2 to 3 fold less IκBα protein than MT-2 cells (NP, data not shown). Furthermore, the turnover of IκBα was more rapid in MT-2 cells (Fig. 24, lanes 7 to 10) than in Jurkat cells (Fig. 24, lanes 2 to 5).

FIGURE 24. IκBα Turnover in Normal and HTLV-I Infected T cells. Jurkat cells (lanes 1 to 5) and MT-2 cells (lanes 6 to 10) were treated with cycloheximide (50 μg/ml) for times ranging from 0 to 8 hr, as indicated above the lanes. Whole cell extracts (20 μg) were analyzed for IκBα by immunoblotting using an IκBα-specific antibody (AR20) and visualized by a chemiluminescence detection system (Amersham). Bands corresponding to the IκBα signal were quantified by laser densitometry and IκBα half life was determined; the graph represents the average of three separate experiments. The level of IκBα in Jurkat (□) and MT-2 (■) cells at a given time, divided by the IκBα level at 0 time, is plotted.


Cell:
Jurkat
MT-2
CHX (h): 0 2 4 6 8 0 2 4 6 8
IκBα
Lane: 1 2 3 4 5 6 7 8 9 10

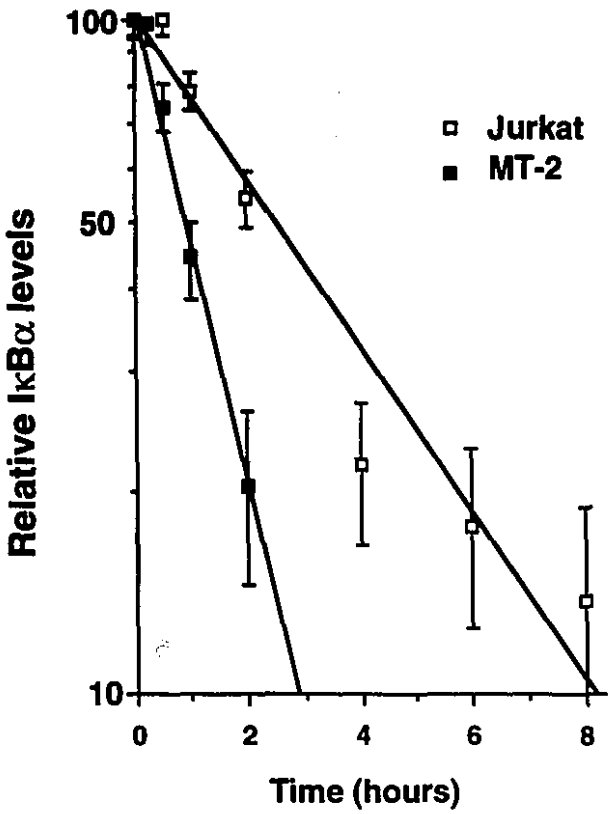

100
50
10
Relative IκBα levels
Jurkat
MT-2
0
2
4
6
8
Time (hours)

TABLE 5. IκBα Turnover in Normal, HTLV-I Infected and Tax Expressing T Cells

Cells	IκBα Turnover (hr)[1]
Jurkat	2.6 ± 0.1
Jurkat-PMA[2]	1.0 ± 0.2
MT-2	0.85 ± 0.04
C8166-45	0.72 ± 0.27

[1] The indicated cells were treated with cycloheximide (50 µg/ml) for 0 to 8 hours. Whole cell extracts were prepared and analyzed for IκBα by immunoblotting as described in figure 24. IκBα signals were quantified by laser densitometry and plotted against time of treatment. Turnover rates were averaged from 2 to 3 separate experiments.

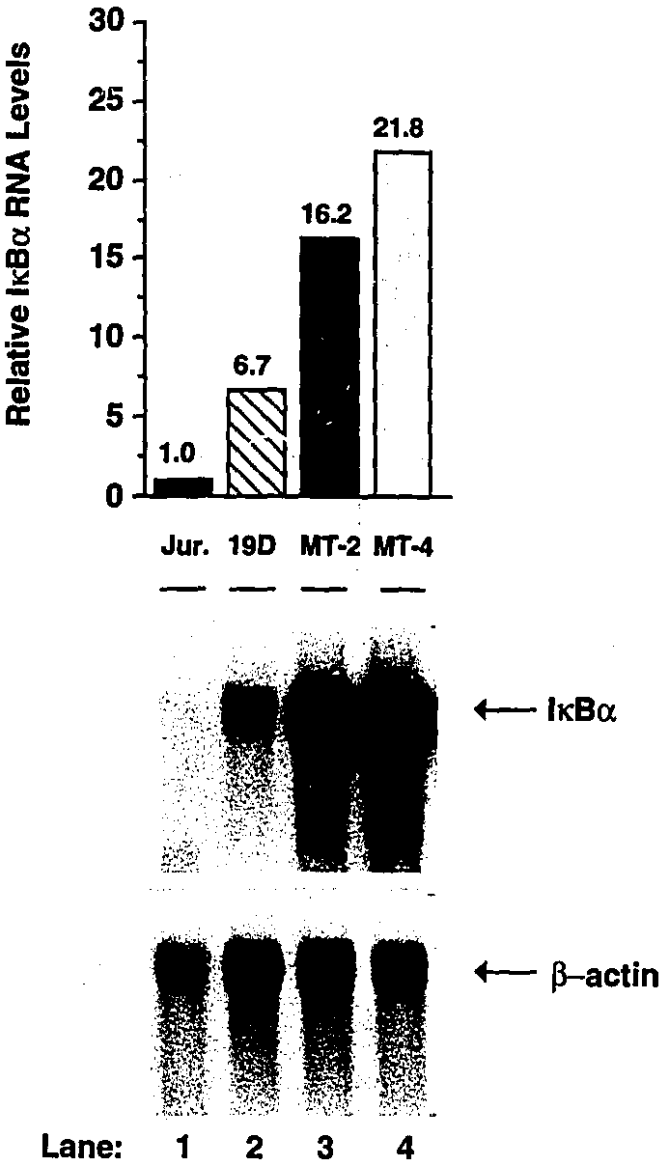
[2] Cells were co-treated with PMA (25 ng/ml) through out cycloheximide treatment.

The graph shown in Figure 24 represents a quantitative analysis of the decay of IκBα in Jurkat and MT-2 cells derived from three independent experiments. These results are summarized in Table 5. The half life of IκBα in unstimulated Jurkat cells was calculated as 2.6±0.1 hr (Table 5). In PMA-treated Jurkat, MT-2, and C8166-45 cells, the half life of IκBα was reduced 3 to 4 fold to 1.0±0.2h, 0.85±0.1h, and 0.72±0.3h, $p<0.01$, respectively (Table 5). These results demonstrated that IκBα turnover was increased 3 fold in PMA-treated, HTLV-I infected, and Tax expressing T cells, and supported the model that phosphorylation may target IκBα for rapid degradation (334, 335, 337, 417).

3. Overexpression of IκBα mRNA

One consequence of constitutive NF-κ B binding activity (119, 138, 418-420) is increased expression of NF-κB-regulated genes, including transcriptional induction of the *mad*-3 (IκBα) gene itself (334, 335, 337, 342, 417). Therefore, to examine whether rapid turnover of IκBα and increased NF-κB binding activity correlated with increased transcription, *mad*-3 (IκBα) expression was analyzed by Northern blotting using poly A+ mRNA (5 μg) from Jurkat, Tax expressing 19D, and HTLV-I infected (MT-2 and MT-4) T cells (Fig. 25). An *Rsa*-1 fragment of IκBα, labeled by random priming with [α-^{32}P]dCTP, served as IκBα specific probe. RNA signals were scanned by laser densitometry, normalized to β-actin expression and plotted as relative mRNA levels. *mad*-3 (IκBα) gene activity was strikingly higher in Tax expressing and HTLV-I infected cells compared to Jurkat cells. IκBα mRNA levels were 7 to 20 fold higher in 19D, MT-2 and MT-4 cells (Fig. 25, lanes 2 to 4) than in normal Jurkat cells (Fig. 25, lane 1). In similar experiments, the mRNA levels of *nfkb*1, *RelA*, and c-*rel* genes were also

FIGURE 25. Northern Blot Analysis of *mad*-3 (IκBα) Gene Expression. Poly A+ RNA (5 μg) was prepared from Jurkat (lane 1), 19D (lane 2), MT-2 (lane 3) and MT-4 (lane 4) cells as described in Materials and Methods. After resolution by formaldehyde agarose gel electrophoresis, RNA was transferred to nylon membrane and immobilized by UV light exposure. The filter was hybridized with a ^{32}P-labeled Rsa-1 fragment of *mad*-3 (IκBα) and with a β-actin probe as an internal control. The signals were quantified by laser densitometry; IκBα levels were expressed relative to β-actin mRNA.


Relative IκBα RNA Levels
30
25
20
15
10
5
0
1.0
6.7
16.2
21.8
Jur.
19D
MT-2
MT-4
IκBα
β-actin
Lane:
1
2
3
4

transcriptionally stimulated about 2 to 4 fold in MT-2 and MT-4 cells compared to Jurkat cells (Fig. 8, and data not shown). Thus, decreased IκBα protein level detected in MT-2 cells was not due to impaired *mad*-3 gene activity; rather IκBα mRNA expression was upregulated by the constitutive NF-κB activity observed in Tax expressing cells.

4. Conclusions

Results presented in this chapter demonstrated that constitutive phosphorylation and increased turnover of the regulatory IκBα protein in HTLV-I infected MT-2 and C8166-45 cells and in Tax expressing 19D cells contributed to constitutive NF-κB binding activity which was previously shown to consist of c-Rel, NFKB2 p52 and NFKB1 p50. IκBα mRNA expression was also increased 7 to 20 fold in these cells, although the steady state level of IκBα protein was reduced in HTLV-I infected and Tax expressing T cells. These results indicated that the viral Tax protein, by indirectly mediating phosphorylation of IκB, may target IκBα for rapid degradation, thus leading to constitutive NF-κB activity.

CHAPTER 6

GENERAL DISCUSSION

1. Constitutive Tax Expression Induces NF-κB Activity

Initially, Tax *trans*-activation of NF-κB activity was characterized in Jurkat cell lines stably expressing the Tax protein and in HTLV-I infected T cells. Constitutive levels of NF-κB DNA binding activity were observed by mobility shift assays in the 19D, 9J, and MT-2 cell lines. In these cells, NF-κB activity was PMA inducible, but to a lesser extent than in normal T cells. Constitutive NF-κB binding activity in the 19D and 9J cell lines was accompanied by elevated transcription of the endogenous NF-κB-regulated gene GM-CSF and a higher basal level expression of transfected NF-κB-dependent promoters. Nevertheless, both the endogenous and transfected promoters remained inducible by PMA treatment. These results demonstrated that expression of Tax induced constitutive NF-κB DNA binding activity and transcription of NF-κB-regulated promoters.

The results demonstrating higher basal level expression of transfected NF-κB-dependent promoters differed from experiments demonstrating full *trans*-activation of NF-κB-regulated promoters in Tax expressing cell lines (122) and from observations showing that high-level Tax expression completely suppressed NF-κB-dependent gene transcription and inducibility (410). In the latter studies, gene activation regulated by cAMP-dependent signal transduction pathways was not affected by constitutive Tax expression, indicating the specificity of Tax-mediated suppression for the NF-κB pathway (410). Possibly the 19D and 9J cell lines represent one of a number of distinct cellular phenotypes with regard to the long-term capacity of Tax protein to *trans*-activate NF-κB-dependent gene activity.

With continued passage in culture for 2-3 months, a decrease in the level of Tax protein expression was observed in 19D and 9J cells and was accompanied by a loss in constitutive NF-κB binding activity (unpublished observation). Somewhat surprisingly, transcription of the GM-CSF gene remained elevated in these cells, suggesting that once the GM-CSF gene was activated continued expression of Tax was not required. Similarly, the MT-4 cell line, an HTLV-I transformed line, contained little NF-κB DNA binding activity (J. Lanoix, unpublished observation), and no detectable Tax protein (N. P., unpublished observation). Nonetheless, the NF-κB-regulated *mad*-3 gene was overexpressed in these cells. Previous studies in adult T cell leukemia cells have shown that Tax expression may or may not be maintained in leukemic cells (23, 102). Therefore it appears that interactions of Tax with the NF-κB/Rel transcription factors at early stages of HTLV-I infection alter cellular gene expression and may be sufficient to initiate a multistep leukemogenic process.

2. Tax Expression Upregulates c-Rel and NFKB2 Subunits

Further investigations were performed to determine which of the several NF-κB/Rel subunits were present in Tax-induced constitutive NF-κB activity. Distinct patterns of NF-κB binding proteins were observed in PMA-induced or Tax expressing cells by UV cross-linking analysis. In Jurkat cells, PMA induction resulted in the appearance of DNA binding proteins of 85, 75, and 54 kD, whereas in Tax expressing cells two polypeptides, 92 and 85 kD, were constitutively present, while the amount of the 75 kD protein was reduced relative to Jurkat and NC2.10 cells. The 85 kD protein was also inducible in normal Jurkat cells by PMA and, based on its kinetics of appearance, DNA

binding specificity, and molecular weight, appeared to be identical to c-Rel (369, 421). The 92 kD protein was constitutively expressed in Tax expressing cells but not in normal T cells. Based on the recent finding that NFKB2 p100 possesses DNA binding activity (283, 284) and that NFKB2 was overexpressed in Tax expressing and HTLV-I infected T cells (see below), the 92 kD protein might be identical to p100.

From the qualitative mobility shift analyses utilizing subunit specific antisera, it was apparent that c-Rel, NFKB2 and NFKB1 were the major NF-κB subunits present in complexes from 19D and MT-2 cells. In effect, the profile of NF-κB proteins in unstimulated Tax expressing cells was similar to the profile in Jurkat cells activated with PMA for 24 hr (421). A dynamic shift in the relative abundance of NF-κB subunits was observed following PMA stimulation of 19D and MT-2 cells, including the detection of high levels of c-Rel binding activity which has also been observed in Tax-transfected cells (422). Also, a distinct aspect of the binding profiles in Tax expressing cells was the appearance of the NFKB2 binding activity which was not detected in normal Jurkat cells. Consistent with these results, immunoblot analyses revealed that the amounts of c-Rel and NFKB2 were upregulated in Tax expressing and HTLV-I infected cells. These results suggested that Tax-induced constitutive NF-κB activity was a consequence of upregulated c-Rel and NFKB2 expression. Moreover these observations supported recent findings demonstrating that Tax-mediated *trans*-activation of c-*rel* expression was regulated at the transcriptional level (138, 352, 419), probably through the NF-κB site present in the c-*rel* promoter (341).

Tax-NF-κB co-transfection experiments were performed to correlate these *in vivo* observations with *in vitro* results. Tax was able to further *trans*-activate NF-

κB-dependent reporter gene activity resulting from p50, c-Rel, and p52 expression. Tax expression also overcame the inhibitory effects of IκBα, p105, and p65Δ. But most importantly, when c-Rel and p52 were co-transfected together, reporter gene activity was stimulated about 10 fold and Tax protein co-expression did not significantly augment gene activity. This result was interpreted as an indication that one of the consequences of Tax expression in T cells was to increase the level of the c-Rel-p52 heterodimer, which in turn *trans*-activated NF-κB-regulated gene expression. Moreover a direct correlation between increasing concentrations of Tax protein, increased production of NFKB2 proteins and *trans*-activation of NF-κB-regulated gene expression was observed in these transfected cells. The level of c-Rel protein also increased in response to increasing concentrations of Tax, confirming the above observations that synthesis of c-Rel was increased in the presence of Tax.

The upregulation of NFKB2 was examined by pulse-chase and immunoprecipitation analysis in HTLV-I infected cells and a precursor-product relationship was demonstrated between the non-DNA binding form of NFKB2 (p100) and the proteolytically processed, DNA binding NFKB2 form (p52). The increase in the amount of both p100 and p52 appeared to be caused by an increase in synthesis of the NFKB2 precursor. In unstimulated Jurkat cells, p100 was present in low amounts, whereas following PMA treatment of Jurkat cells for 8 hr, there was a 5 fold increase in the ^{35}S-labeled p100 protein and a similar precursor:product relationship was observed (data not shown). It should be noted that the amount of NFKB2 proteins was about 5 fold higher in unstimulated MT-2 cells than in PMA-stimulated Jurkat cells. Mercurio *et al.* also reported that the NFKB2 product (p52) was not detectable in unstimulated Jurkat cells, although the precursor was present; these studies demonstrated

that processing of the precursor was stimulus-responsive and occurred after mitogen activation of the cells (274). In both PMA-stimulated Jurkat cells and MT-2 cells, the $T_{1/2}$ for the generation of the p52 product from the p100 form of NFKB2 varied between 10 to 16 hr (J. Lacoste, data not shown), indicating that processing of the precursor was a slow event occurring over several hours. In essence, Tax protein appeared to be a functional equivalent of PMA stimulation with regard to the capacity of T cells to process the NFKB2 precursor.

3. Tax Alters the NF-κB/Rel Subunit Stoichiometry

A characteristic feature of the NF-κB/IκB system of transcriptional regulators is the formation of multimeric complexes which can strongly bind DNA, activate transcription, or inhibit NF-κB activity by cytoplasmic retention. These interactions must remain under stringent control in order to maintain proper NF-κB activity (i.e. transiently inducible versus constitutive). Both the NFKB1 and NFKB2 precursors have IκB-like activity, provided by the ankyrin repeat containing C-terminal domains (249, 275). These precursors serve as reservoirs for the cytoplasmic retention of NF-κB subunits and are capable of forming stable complexes with p65, c-Rel, p50, p52 and with each other (249, 255, 274, 275). In Jurkat cells, c-Rel formed stable complexes with both NFKB2 and NFKB1 and NFKB1-NFKB2 complexes were detected as well. Upon cell stimulation, NFKB1 and NFKB2 were processed to yield mature p50-c-Rel and p52-c-Rel complexes (274).

As discussed in Chapter 1, changes in the balance of NF-κB subunits available in a particular cell may alter the pattern of gene expression and consequently

contribute to oncogenic transformation. Therefore, the consequences of Tax expression and c-Rel and NFKB2 upregulation on NF-κB subunit stoichiometry were examined. A complex involving NFKB2 and c-Rel was immunoprecipitated by antisera directed against either the N-terminal portion of NFKB2 or the C-terminal region of c-Rel in MT-2 cells. The identification of this complex was consistent with the increased expression of both of these NF-κB subunits in HTLV-I infected and Tax expressing T cells. Co-immunoprecipitation analysis also demonstrated the formation of a minor c-Rel-NFKB1 complex in HTLV-I infected cells; similar NFKB1-c-Rel complexes were also observed previously (419). Most importantly, co-immunoprecipitation experiments revealed that at least a portion of the intracellular Tax protein was complexed with p100 in HTLV-I infected cells. This observation raises several intriguing questions concerning the biological consequences of this interaction. For example it is not clear whether the association of Tax protein with p100 facilitates proteolytic processing in T cells or possibly stabilizes the p52 DNA binding form. Similarly, the association of Tax protein with NFKB2 suggested that the protein may be sequestered in the cytoplasm as part of a NFKB2 complex and subsequently translocated to the nucleus together with the c-Rel-p52 complex. The physical interaction between Tax and a member of the NF-κB family was reported previously; NFKB1 p105 was shown to co-immunoprecipitate with Tax from HTLV-I infected and Tax transfected cells, suggesting that Tax formed a complex with the p105 precursor (413). These complexes were present in the cytoplasm as well as in the nuclei of these cells (413). Such Tax-NFKB1 complexes could not be detected in the co-immunoprecipitation studies presented here. One possibility to explain this discrepancy is that Tax may interact with different NF-κB proteins with distinct affinities, possibly via the Rel homology domain. Tax-Rel domain interactions

would provide Tax with the potential to modulate the activity of several NF-κB/Rel family members and to intervene at multiple steps in the NF-κB/Rel signaling pathway.

The observation that Tax physically interacted with p100 suggested that Tax may mediate its transforming potential at least in part via association with an NF-κB protein previously implicated in B cell leukemogenesis (271, 272). In the B cell lymphoma-associated chromosomal translocation t(10;14)(q24;q32), the amino-terminal DNA binding domain of p100 is juxtaposed to the immunoglobulin C_α locus potentially generating a NFKB2-Ig$C_{\alpha 1}$ fusion protein. NFKB2-$C_{\alpha 1}$ protein, transcribed and translated *in vitro* from lymphoma-derived RNA, retained the capacity to interact with NF-κB binding sites *in vitro*. These results indicated that the t(10;14) chromosomal translocation produced two biological consequences: 1) a constitutively activated, DNA binding NFKB2 molecule via loss of the ankyrin repeat-containing C-terminus; and 2) transcriptional deregulation of NFKB2 caused by translocation into the transcriptionally active immunoglobulin locus (272). By analogy, HTLV-I, utilizing the Tax protein, may initiate T cell transformation by interfacing with the NF-κB/Rel signaling pathway at the level of the NFKB2 protein. Tax-NFKB2 interactions appears to mimic the regulatory alterations observed in the t(10;14) translocation: 1) constitutive synthesis of NFKB2, combined with overexpression of c-Rel, results in high levels of NF-κB DNA binding activity which in turn may disrupt the transcriptional autoregulation of NF-κB/Rel genes (335, 337, 341); and 2) continuous proteolysis of NFKB2 to remove the C-terminal, IκB regulatory domain allows continuous nuclear expression of the DNA binding form of NFKB2, in a manner reminiscent of the NFKB2-$C_{\alpha 1}$ fusion protein.

Another important complex present in normal T cells is p65-IκBα. p65 is the most potent transcriptional activator of the NF-κB/Rel family and, together with IκBα, constitutes an autoregulatory loop important for NF-κB regulation. In contrast to Jurkat T cells, immunoprecipitations using anti-p65 or anti-IκBα antisera failed to detect these two proteins in HTLV-I infected T cells. Curiously however, both proteins were detected as denatured proteins in immunoblot analyses. Although p65 protein levels were similar or elevated in Tax expressing or HTLV-I infected cells compared to Jurkat cells, p65 DNA binding activity was inhibited in these cells. Similar reductions of p65 DNA binding activity have been observed during the pre-B to mature B cell transition (353), and in the early to late stages of Tax expression in T cells (423). Several possibilities may account for decreased p65 binding activity: 1) p65 may be targeted for rapid degradation because of its association with phosphorylated IκBα ; 2) despite its rapid turnover, IκBα may be able to sequester p65 in the cytoplasm; 3) competition for target sites by other NF-κB proteins such as c-Rel and NFKB2 may limit p65 activity, as suggested by a recent report demonstrating that p52 overexpression down-regulated p65-dependent transcription (281); or 4) p65 could be sequestered in NFKB2-overexpressing cells by the p100 precursor rather than by IκBα. The observation that p65 subcellular distribution was altered from an exclusively nuclear localization in PMA-treated normal T cells, to an equal cytoplasmic-nuclear distribution in HTLV-I infected cells supported the latter possibility. Moreover it has recently been demonstrated that p100 cytoplasmically retained p65 and inhibited p65-mediated transcription (332, 424).

4. Tax Induces Constitutive Phosphorylation and Turnover of IκBα

To explain the mechanism by which Tax expressing and HTLV-I infected T cells maintain constitutive NF-κB DNA binding activity, the regulatory activity of IκBα was examined. These investigations demonstrated that constitutive phosphorylation and increased turnover of IκBα protein occurred in HTLV-I infected and Tax expressing T cells. In general, an inverse correlation between Tax protein expression and steady state levels of IκBα was observed in these studies; i.e. higher levels of Tax in HTLV-I infected cells (MT-2 and C8166-45) resulted in lower levels of IκBα, higher levels of phosphorylated IκBα, and faster IκBα turnover. IκBα mRNA transcript levels were also increased 7 to 20 fold in Tax expressing cells, probably as a consequence of constitutive NF-κB binding activity and subsequent induction of the *mad*-3 gene. Similar results were recently reported (423, 425). Kanno *et al.* analyzed a permanently transfected Jurkat T cell line in which expression of Tax was inducible by the addition of heavy metals to the culture media. Upon induction of Tax expression, *mad*-3 gene expression was increased dramatically (423). Although IκBα protein levels remained unaffected by Tax expression, turnover was significantly increased compared to IκBα turnover in cells expressing a mutant Tax protein (423). Sun *et al.* also concluded that Tax expression induced constitutive phosphorylation and rapid turnover of IκBα (425). However the $T_{1/2}$ of IκBα in C8166-45 cells (<5 min) (425) differed significantly from the one shown in Table 5 (43 min). This discrepancy may be explained by the fact that Sun *et al.* measured turnover of the phosphorylated form of IκBα, while the turnover of the whole IκBα pool (both unphosphorylated and phosphorylated forms) was measured in the experiments presented in Table 5. Together these results support a model in which a HTLV-I Tax mediated phosphorylation of

IκBα targets IκBα for degradation, possibly through the ubiquitin-proteasome pathway (258, 296). Disruption of the NF-κB/IκB autoregulatory pathway results in constitutive NF-κB DNA binding activity that may promote aberrant NF-κB-dependent gene expression in T cells.

Despite increased IκBα mRNA expression in HTLV-I T cells, IκBα protein levels were on average 7 fold lower than in Jurkat cells; this apparent discrepancy was partially resolved by the rapid turnover of IκBα in these cells. In unstimulated Jurkat cells, IκBα half life was 2.6 hr (156 min) while in MT-2 and C8166-45 cells, IκBα half life was reduced to 0.85 hr (51 min) and 0.72 (43 min), respectively. However, the activity of the protease(s) involved in IκBα degradation must also be increased at least 10 fold in HTLV-I infected cells to account for the rapid and continuous turnover of IκBα. A similar conclusion was also reached in a recent study of NF-κB activity and IκBα turnover during B-cell differentiation (420).

Interestingly, pre-B to B cell differentiation involves changes in NF-κB activity that are very analogous to those described in normal T cells versus HTLV-I infected T cells. NF-κB activity is highly inducible in pre-B cells and in normal T cells, and is composed mainly of p65-p50 heterodimers (353). In both mature B cells and Tax expressing T cells, NF-κB activity is constitutive and composed primarily of c-Rel, and *mad*-3 and c-*rel* mRNAs are overexpressed. Importantly, IκBα turns over rapidly with a half life between 40 and 50 min in mature B cells and Tax expressing T cells, whereas in pre-B and normal T cells, IκBα half life is between 150 and 160 min. Moreover, in mature B cells, increased IκBα turnover is due to increased activity of a serine protease, since TPCK (tosylsulfonyl-phenylalanyl chloromethyl ketone), an inhibitor of serine

proteases, stabilizes IκBα and inhibits NF-κB binding activity (353, 420). The kinetic analysis of NF-κB activation in the inducible Tax expressing Jurkat cell line also confirmed our results demonstrating a shift in NF-κB subunits. Initial NF-κB DNA binding activity was composed of p65-p50 heterodimers (423) and with prolonged expression of Tax, a transition occurred toward NF-κB complexes composed of p52 and c-Rel (423). As mentioned earlier, IκBα also turned over more rapidly in these cells (423). These results suggested that constitutive NF-κB activity contained in Tax expressing and HTLV-I infected cells resulted from increased IκBα turnover and p52-c-Rel binding activity.

The recent observation that HTLV-I Tax interacts with the ankyrin repeat domain of p105 and IκBγ (426) suggests that Tax-IκB interactions may influence the phosphorylation state of IκB. However, it should be noted that direct Tax-IκBα interactions have not been demonstrated (see below). An alternative model is that HTLV-I Tax may physically associate with and activate a host kinase that phosphorylates IκBα; phosphorylation may target IκBα for degradation by serine protease(s). One candidate kinase is the 68 kD dsRNA dependent kinase (PKR) (327), which binds to Tax *in vitro* (NP, data not shown). Tax would thus disrupt IκB-NF-κB interactions and release free NF-κB/Rel and Tax complexes.

5. Tax-NF-κB/Rel Interactions Affect Intracellular Localization of Tax

Co-transfection-immunofluorescence studies have demonstrated that NF-κB/Rel subunits display distinct patterns of nuclear or cytoplasmic compartmentalization. Importantly, these studies also showed that Tax

intracellular localization was affected by co-transfection of p100, p52, p105, and to a lesser extent, p65, implying physical interactions between Tax and these proteins. The Tax-p100 complex was clearly identified by double immunoprecipitations, but no Tax-p52 complex was observed. In the case of the p52 subunit, which lacks a distinct *trans*-activation domain (271, 280), the Tax *trans*-activation function may alter the regulatory potential of the DNA binding subunit. The physical interaction between Tax-p105 has been controversial since only one group has been able to co-immunoprecipitate Tax and p105 complexes from HTLV-I infected and Tax transfected cells (413). However, the co-transfection-immunofluorescence studies presented here clearly provided immunological evidence that Tax is sequestered in the cytoplasm by p105. Finally, these experiments also demonstrated the ability of p65 to moderately retain Tax in the cytoplasm. Therefore Tax appears to interact with different NF-κB/Rel proteins with distinct affinities and consequences, possibly through the Rel homology domain. Support for differential response of NF-κB/Rel proteins to Tax association comes from the observation that Tax-p105 interactions enhanced p65-p50 nuclear localization, while Tax-p100 interactions inhibited p65-dependent transcription through p100-mediated cytoplasmic retention (332, 424, 427).

6. Multiple Mechanisms of Tax-Mediated Transcriptional *Trans*-Activation

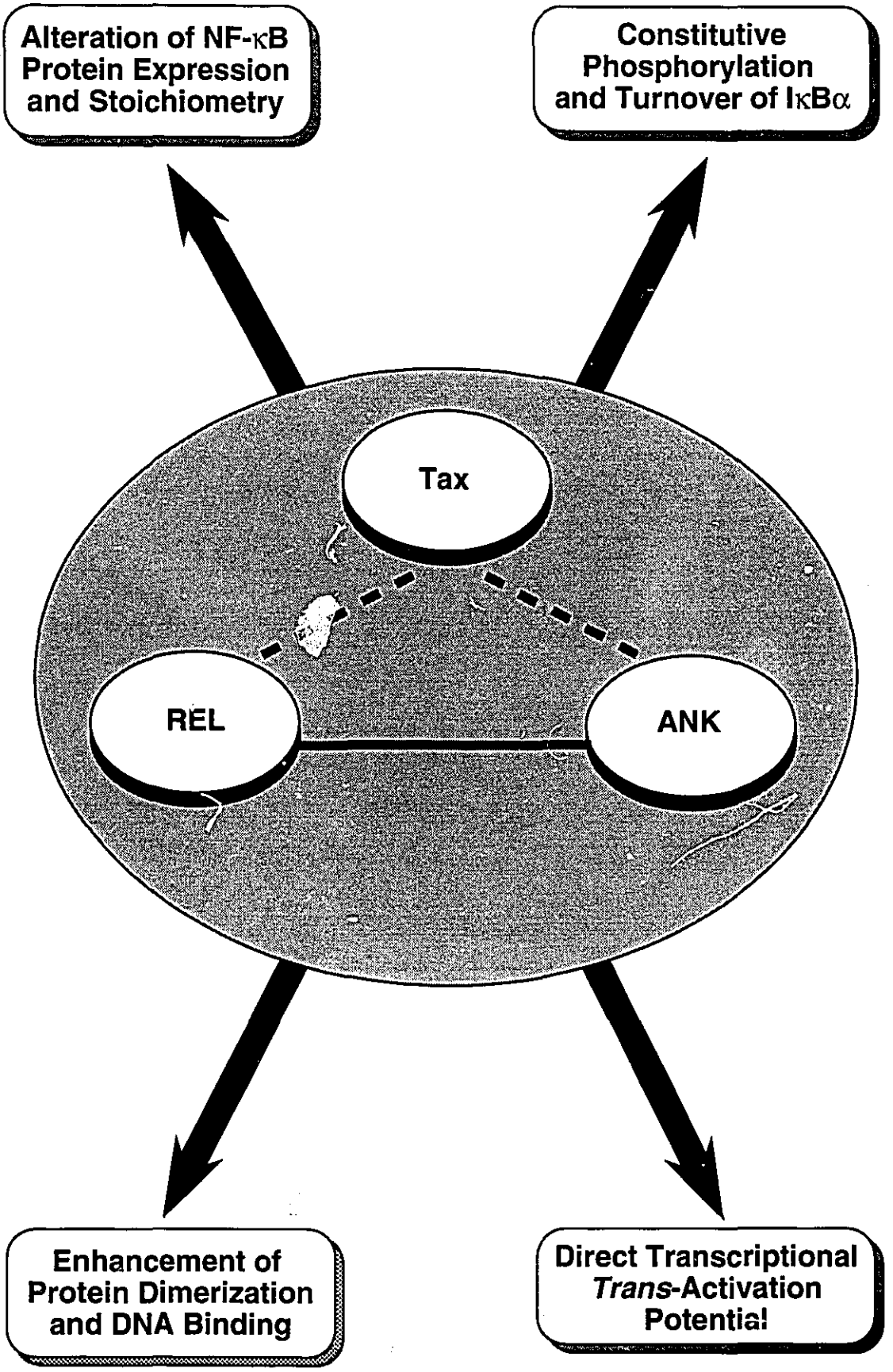
Tax-mediated transformation results from the ability of Tax to *trans*-activate cellular genes involved in cell growth control. Transcriptional *trans*-activation is a complex phenomenon triggered by the physical interaction of the Tax protein with numerous cellular transcription factors such as the NF-κB/Rel proteins.

Although the molecular basis for these interactions is not completely understood, it appears that, based on the results presented here and other published reports (160, 179, 181, 192, 428), multiple mechanisms of Tax-mediated transcriptional *trans*-activation exist (Fig. 26).

The predominant eifect of Tax expression on the NF-κB/IκB family of transcriptional regulators was the strong induction of c-Rel and NFKB2 protein expression and the resulting DNA binding and transcriptional activities. Therefore overexpression of these two subunits and the resulting alteration of the NF-κB subunits stoichiometry might be one mechanism by which Tax induces aberrant gene expression.

Results presented here suggested that Tax interacted with the Rel domain of NF-κB/Rel proteins and promoted constitutive phosphorylation and rapid degradaton of the ankyrin repeat-containing protein IκBα. It is possible that Tax-Rel domain-Ankyrin repeats interactions bring in close vicinity a kinase that would promote phosphorylation and degradation of ankyrin repeat containing proteins through the ubiquitin-proteasome pathway. Depending on the type of inhibitory molecules initially present in the complex (IκBα, p105 or p100), this would result in degradation of IκB or processing of precursors into a DNA binding proteins. The net result in both cases is the formation of free NF-κB/Rel dimers ready to translocate into the nucleus. Such a mechanism would transform p100-Tax complexes into p52-Tax complexes. This model is supported by the observation that both p105 processing to p50 and IκBα degradation use the same ubiquitin-dependent degradation mechanism, and that phosphorylation precedes degradation of both inhibitory molecules (258, 277).

FIGURE 26. Multiple Mechanisms of Tax-Mediated Transcriptional *Trans*-Activation. See text for a description of the figure.


Alteration of NF-κB Protein Expression and Stoichiometry
Constitutive Phosphorylation and Turnover of IκBα
Tax
REL
ANK
Enhancement of Protein Dimerization and DNA Binding
Direct Transcriptional *Trans*-Activation Potential

It is not clear whether or not Tax remains associated with the dimers translocating to the nucleus. However, other functions attributed to Tax suggest that it does. Tax has been shown to enhance the DNA binding activity of the ATF/CREB cellular proteins that bind to the HTLV-I 21-bp repeats. This activity was mediated through the DNA binding and dimerization domains of these proteins and did not require formation of stable Tax-containing complexes (180). Wagner and Green further demonstrated that Tax increased the *in vitro* DNA binding activity by stimulating dimerization of ATF/CREB proteins, and this, in turn, facilitated the DNA binding reaction (181). Enhancement of dimerization and DNA binding may allow dimers containing at least one strong transcriptional activation domain (e.g. p65 or c-Rel) to efficiently turn on transcription, perhaps without further involvement of Tax.

The capacity of Tax protein to increase the binding of cellular transcription factors to DNA via an increase in dimerization represents one mechanism by which Tax can mediate its effects on gene expression. However, Tax, by possessing *trans*-activation domains, could play a more direct role in stimulating transcription. Tax may associate with subunits such as p52 or p50, which are strong DNA binding proteins that lack a *trans*-activation function. The resulting complexes would combine both DNA binding and *trans*-activating functions necessary to induce transcription.

The picture emerging from the results presented in this thesis is that expression of the Tax protein in HTLV-I infected cells induces IκBα phosphorylation, rapid IκBα turnover, Tax-p100 complexes and constitutive NF-κB activity composed of c-Rel and NFKB2. Together these Tax-NF-κB interactions culminate in

transcriptional activation of NF-κB-regulated genes. In particular, transcriptional activation of the IL-2 and IL-2Rα genes initiates a polyclonal proliferation of CD4+ T lymphocytes. These rapidly growing cells presumably undergo multiple mutational events which, over a time period that involves decades *in vivo*, favor the appearance of a leukemic population of T cells.

Contributions to Original Knowledge

The Tax protein of HTLV-I, a positive transcriptional activator of HTLV-I gene expression, is a viral oncogene that also increases transcription of cellular genes including IL-2, IL-2Rα, and GM-CSF. One of the cellular targets of the *trans*-activating effects of Tax is the NF-κB/IκB family of transcriptional regulators. The objective of this thesis was to characterize the molecular mechanisms by which Tax interacts with the NF-κB/IκB family of proteins to initiate aberrant gene expression. Results presented in this thesis contributed to original knowledge in the following manner:

1. Tax expressing T cell lines contained a constitutive level of NF-κB binding activity, a detectable level of transcription from the endogenous NF-κB-dependent GM-CSF gene and increased basal level expression of transfected NF-κB-regulated promoters.

2. NFKB2 and c-Rel subunits were overexpressed in HTLV-I infected and Tax expressing T cells and, together accounted for the majority of the constitutive NF-κB binding activity observed in these cells.

3. A Tax-dependent correlation existed between expression of NFKB2 p100 and processing to p52, induction of c-Rel, and *trans*-activation of NF-κB-mediated gene expression.

4. A novel protein-protein complex composed of NFKB2 p100 and Tax was detected in HTLV-I infected T cells. Additional support for direct Tax-NF-κB physical interactions was provided by the ability of p100, p105 and p52 to

modify the intracellular localization of Tax.

5. In HTLV-I infected and Tax expressing T cells, Tax expression promoted constitutive phosphorylation of the inhibitor IκBα. Although the *mad*-3 gene was overexpressed, protein levels were 7 to 10 fold lower than in normal T cells, suggesting an increased IκBα turnover. Indeed, IκBα turnover rates were 3 fold more rapid in cells that constitutively expressed the Tax protein.

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