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Mutants of IκBα interfere with NF-κB regulated gene expression and HIV-1 replication

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Doctor of Philosophy.

To my wife Monica
for your love and understanding

To my mother and my brother Hyungju
for all your support

Abstract

Human immunodeficiency virus type 1 (HIV-1) utilizes the NF-κB/Rel pathway to mediate, in part, its transcriptional regulation through NF-κB binding sites present in the HIV-1 LTR. In unstimulated cells, DNA binding NF-κB subunits are retained in the cytoplasm by inhibitory IκB proteins. Upon stimulation by cytokines, viruses or dsRNA, IκBα is rapidly phosphorylated and degraded, resulting in the release of NF-κB and the subsequent increase in NF-κB-regulated gene expression.

The effect of transdominant mutants of IκBα (TD-IκBα) on the synergistic activation of the HIV-1 LTR by TNFα and the HIV-1 transactivator Tat, was examined in Jurkat T cells. Co-expression of WT IκBα and TD-IκBα inhibited Tat-TNF activation of HIV-1 LTR in a dose dependent manner. TD-IκBα also effectively inhibited HIV-1 multiplication in a single cycle infection model in COS-1 cells. To examine the effect of inducible expression of TD-IκBα on *de novo* HIV-1 multiplication, we generated Jurkat T cells inducibly expressing TD-IκBα by doxycycline (Dox). Dox induction of TD-IκBα dramatically reduced both NF-κB DNA binding activity and LTR directed gene activity. The time course of *de novo* HIV-1 infection was altered by Dox induction of TD-IκBα in Jurkat cells , resulting in a dramatic inhibition of HIV-1 multiplication.

Interestingly, induced expression of TD-IκBα also progressively decreased the expression of endogenous IκBα to undetectable levels by 24h after induction, indicating that TD-IκBα was able to repress endogenous NF-κB dependent gene expression. We also demonstrated that production of TD-IκBα reduced endogenous IκBα gene transcription, due to the continued cytoplasmic sequestration of RelA(p65) by TD-IκBα. *In vivo* genomic footprinting revealed stimuli-responsive protein-DNA binding not only to the -63 to -53 κB1 site but also to the adjacent -44 to -36 Sp1 site of the IκBα promoter. *In vivo* protection of both sites was inhibited by Dox-inducible TD-IκBα expression. Prolonged NF-κB binding and a temporal switch in the composition of NF-κB complexes bound to the -63 to -53 κB1 site of the IκBα promoter were also observed; with time after induction, decreased levels of transcriptionally active p65-p50 and increased c-rel-p50 heterodimers were detected at the κB1 site. Mutation of either the κB1 site or the Sp1 site abolished transcription factor binding to the respective sites, as well as the inducibility of the IκBα promoter in transient transfection studies. Based on these results, specific activation of TD-IκBα can block NF-κB-regulated gene expression and HIV-1 infection.

Resumé

La régulation du niveau transcriptionnel du Virus de l'Immunodéficience Humaine de type 1 (HIV-1) se fait en partie, par la voie de signalisation NF-κB/Rel due à la présence de sites de fixation à NF-κB au niveau du LTR (Long Terminal Repeat) de HIV-1. En absence de stimulation, les sous-unités de NF-κB capables de lier l'ADN sont séquestrées au niveau du cytoplasme par les proteines inhibitrices IκB. Suite à une stimulation induite soit par des cytokines, soit par une infection virale ou par la présence d'ARN double brin, il y a phosphorylation de la sous-unité α de IκB et dégradation de la protéine IκB. Ainsi, ce processus conduit à une libération de NF-κB et à une augmentation de l'expression des genes régulés par ce facteur.

La caractérisation de mutants transdominants de IκBα (TD-IκBα) a été effectuée dans des cellules Jurkat (lymphocyte T) en évaluant leurs effets sur le degré d'activation du LTR de HIV-1 suite à l'action simultanée du TNF-α (Tumor Necrosis Factor) et de la protéine transactivatrice Tat du HIV. Nos études démontrent que la co-expression de la protéine IκBα de type sauvage avec la protéine mutée transdominante, TD-IκBα, inhibe l'effet synergique de Tat-TNFα sur l'activation du LTR de HIV. Ce degré d'inhibition est proportionnel au niveau d'expression de la protéine mutée. De plus, le mutant TD-IκBα est capable d'inhiber la multiplication virale lors d'un premier cycle d'infection dans les cellules COS-1. Afin d'observer l'effet de l'expression de TD-IκBα sur la mutilplication *de novo* de HIV, nous avons choisi d'établir des lignées de cellules Jurkat pouvant exprimer TD-IκBα de façon régulée. Ainsi, le contrôle de l'expression génique dans ces cellules est modulé par la presence ou l'absence de doxycycline (Dox) dans le milieu de culture. En présence de Dox, l'expression induite de TD-IκBα diminue la capacité de NF-κB à lier l'ADN de même que niveau d'activation de gènes régulés par le LTR. Un suivi de l'infection dans le temps de ces cellules démontre une inhibition de la multiplication du HIV suite à l'expression de TD-IκBα de manière induite. 24 heures suivant l'expression de TD-IκBα, nous observons une diminution du niveau d'expression de la protéine endogène IκBα. TD-IκBα serait donc capable de réprimer l'expression de gènes régulés par NF-κB. La production de protéine TD-IκBα provoque également une réduction du niveau transcriptionnel du gène IκBα, due à une sequestration au niveau du cytoplasme de la protéine Rel A (p65) par TD-IκBα. Une empreinte génomique effectuée *in vivo*, révèle la présence d'éléments régulateurs de la transcription non seulement au site κB1 en position –63 à –53 mais également à un site adjacent au site Sp1 (-44 à –36) du promoteur IκBα. Des essais de protection *in vivo* démontrent que ces sites ne sont plus protégés suite à une induction de l'expression du mutant transdominant. L'attachement de NF-κB à son inhibiteur est prolongé et on observe

un changement dans la composition des complexes NF-κB capables de lier le site κB1 du promoteur IκBα. En effet, on note une réduction au site κB1 de la forme transcriptionnellement active (p65-p50) et une augmentation de la forme hétérodimérique c-rel-p50. Des mutations affectant soit le site κB1 ou Sp1 empêchent l'attachement du facteur de transcription à ces sites de même que l'activation transcriptionnelle du promoteur IκBα. L'ensemble de ces résultats démontre que l'activation d'un répresseur transdominant de IκBα peut bloquer non seulement l'expression des gènes régulés par NF-κB mais également une infection virale à HIV.

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Preface

In accordance with the guidelines for thesis preparation, I have chosen to present the results of my research in classical form. A General Introduction is presented in Chapter I, followed by detailed Materials and Methods in Chapter II. The results are described in Chapters III, IV and V and appear in the following articles:

1. Beauparlant P*, **Kwon H***, Clarke M, Lin R, Wainberg M, Sonenberg N, Hiscott J. 1996 Transdominant mutants of IκBα block the Tat-TNF synergistic activation of HIV-1 gene expression and virus multiplication. *J. Virol.* **70**: 5777-5785.
(*both authors contributed equally to this paper)

2. Hiscott J, Beauparlant P, Crepieux P, DeLuca C, **Kwon H,** Lin R, Petropoulos L. 1997 Cellular and viral protein interactions regulating IκBα activity during human retrovirus infection. *J. Leukocyte Biol.* **62**: 82-91.

3. **Kwon H**, Pelletier N, DeLuca C, Genin P, Cisternas S, Lin R, Wainberg M, Hiscott J. 1998 Inducible expression of IκBα repressor mutants interferes with NF-κB activity and HIV-1 replication in Jurkat T cells. *J. Biol. Chem.* **273**: 7431-7440.

4. Algarte M*, **Kwon H***, Genin P*, Hiscott J. 1999 Identification by In Vivo genomic footprinting of a transcriptional switch containing NF-κB and Sp1 that regulates the IκBα promoter. *Mol. Cell. Biol.* **19**:6140-6153.
(*These authers contributed equally to this paper)

5. DeLuca C, **Kwon H**, Lin R, Wainberg M, Hiscott J. 1999 NF-κB activation and HIV-1 induced apoptosis. *Cytokine and Growth Factor Reviews* **10**:235-253.

A General Discussion and summary of the results appear in Chapter VI. References cited in this thesis are found Chapter VII.

The candidate was responsible for all the research described in this thesis and would like to acknowledge the contribution of several experiments carried out by colleagues. Results obtained by Dr. Pierre Beauparlant and Michelle Clarke are shown in Figure 8, 9, 11 and 12. Dr. Rongtuan Lin generated inducible expression vectors used in Chapter IV. Nadine Pelletier is responsible for the results presented in Figure 18. In vivo genomic footprinting presented in Figure 22 to 25 were performed by Drs. Pierre Genin and Michelle Algarte. The candidate also wishes to acknowledge Sonia Cisternas and Dana Zmeureanu for technical assistance.

The candidate was also involved in studies with other reseachers in the laboratory which resulted in the following publications:

6. Crepieux P, **Kwon H**, Leclerc N, Spencer W, Richard S, Lin R, Hiscott J. 1997 IκBα physically interacts with a cytoskeleton-associated protein through its signal response domain. *Mol. Cell. Biol.* **17**: 7375-7385.

7. DeLuca C, **Kwon H**, Pelletier N, Wainberg M, Hiscott J. 1998 NF-κB protects HIV-1-infected myeloid cells from apoptosis. *Virology* **244**: 27-38.

8. Spencer W, **Kwon H**, Crepieux P, Lin R, Hiscott J. 1999 Taxol selectively blocks microtubule dependent NF-κB activation by phorbal ester via inhibition of IκBα phosphorylation and degradation. *Oncogene* **18**:495-506.

9. Genin P, Mamane Y, **Kwon H**, Hiscott J. 1999 Differential regulation of CC chemokine gene expression in human immunodeficiency virus-infected myeloid cells. *Virology* **261**:205-215.

10. Mamane Y, Heylbroeck C, Genin P, Algarte M, Servant M, LePage C, DeLuca C, **Kwon H**, Lin R, Hiscott J. 1999 Interferon Regulatory Factors: The Next Generation. *Gene* **237**:1-14.

11. Ishii T, **Kwon H**, Hiscott J, Mosialos G, Koromilas A. Enhanced IκBα phosphorylation and NF-κB activation in NIH-3T3 cells expressing mutants of the IFN-inducible protein kinase PKR (Submitted to JBC).

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Abbreviations

AIDS	Acquired immunodeficiency syndrome
ARM	Arginine rich RNA binding motif
CAK	cyclin-dependent kinase-activating kinase
CBP	CREB-binding protein
CD4	Cluster determination antigen 4
CDK	Cyclin-dependent kinase
CKII	Casein kinase II
CMV	Cytomegalovirus
CREB	cAMP response element binding protein
CTD	C-terminal domain (Pol II)
ddC	2',3'-Dideoxycytidine , Zalcitabine
ddI	2',3'-Dideoxyinosine, Didanosine
d4T	2',3'-Didehydro-3'-deoxythymidine, Stavudine
DNA	Deoxyribonucleic acid
Dox	Doxycycline
dsRNA	Double stranded RNA
EBV	Epstein-Barr virus
FADD	Fas associated death domain protein
G-CSF	Granulocyte colony stimulating factor

GM-CSF	Granulocyte/macrophage colony stimulating factor
HIV-1	Human immunodeficiency virus-1
HLH	helix-loop-helix
HTLV-1	Human T cell leukemia virus-1
IAP	Inhibitor of apoptosis protein
ICAM-1	Intercellular adhesion molecule-1
IFN-β	β-interferon
IκB	Inhibitory kappa B protein
IKK	IκB kinase
IL	Interleukin
IL-1RAcP	IL-1 receptor accessory protein
IL-1RI	cell surface type-I receptor
IRAK	IL-1 receptor associated kinase
IRF	Interferon regulatory factor
LBP	Leader binding protein
LEF-1	lymphoid enhancer binding factor-1
LMB	leptomycin B
LPS	Lipopolysaccharide
LTR	Long terminal repeat
LT-β	Lymphotoxin β
LZ	leucine zipper
MAP3K	Mitogen-activated protein kinase kinase kinase

MEKK1	Mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1
MHC	Major histocompatibility complex
mRNA	Messenger RNA
NES	Nuclear export sequence
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor kappa B transcription factor
NIK	NF-κB Inducing kinase
NLS	Nuclear localization sequence
NRE	Negative regulatory element
PBMC	Peripheral blood mononuclear cells
p/CAF	p300/CBP-associated factor
PDGF	Platelet-derived growth factor
PEST	Proline, glutamic acid, serine, threonine rich domain
PHA	phytohemagglutinin
PICOT	PKC-interacting cousin of thiredoxin
PI3K	phosphatidylinositol 3-kinase
PKA	cAMP-dependent protein kinase A
PKC	protein kinase C
PKR	dsRNA dependent protein kinase
PMA	phorbol myristate acetate
PP2A	protein phosphatase 2A
P-TEFb	positive transcriptional elongation factor b

Rev	
RHD	Rel homology domain
RIP	receptor interacting proteins
RNA	Ribonucleic acid
RNAPII	RNA polymerase II
RRE	Rev response element
SV40	Simian virus 40
TA1	transactivation domain 1
TAB1	TAK1 binding protein 1
TAHs	Tat-associated histone acetyltransferases
TAKs	Tat-associated kinases and
TAK1	TGF-β Activated Kinase 1
TAR	Tat responsive element
Tat	trans-activator of transcription
TBP	TATA-binding protein
TFIIH	transcription factor II H
TGF-β	Transforming growth factor-β
TNFα	Tumor necrosis factor-α
TNFR	TNF receptor
TRADD	TNFR associated death domain
TRAF	TNFR associated factor
UBP	Upstream binding protein
VCAM-1	Vascular cell adhesion molecule-1

WT	wild type

CHAPTER I

GENERAL INTRODUCTION

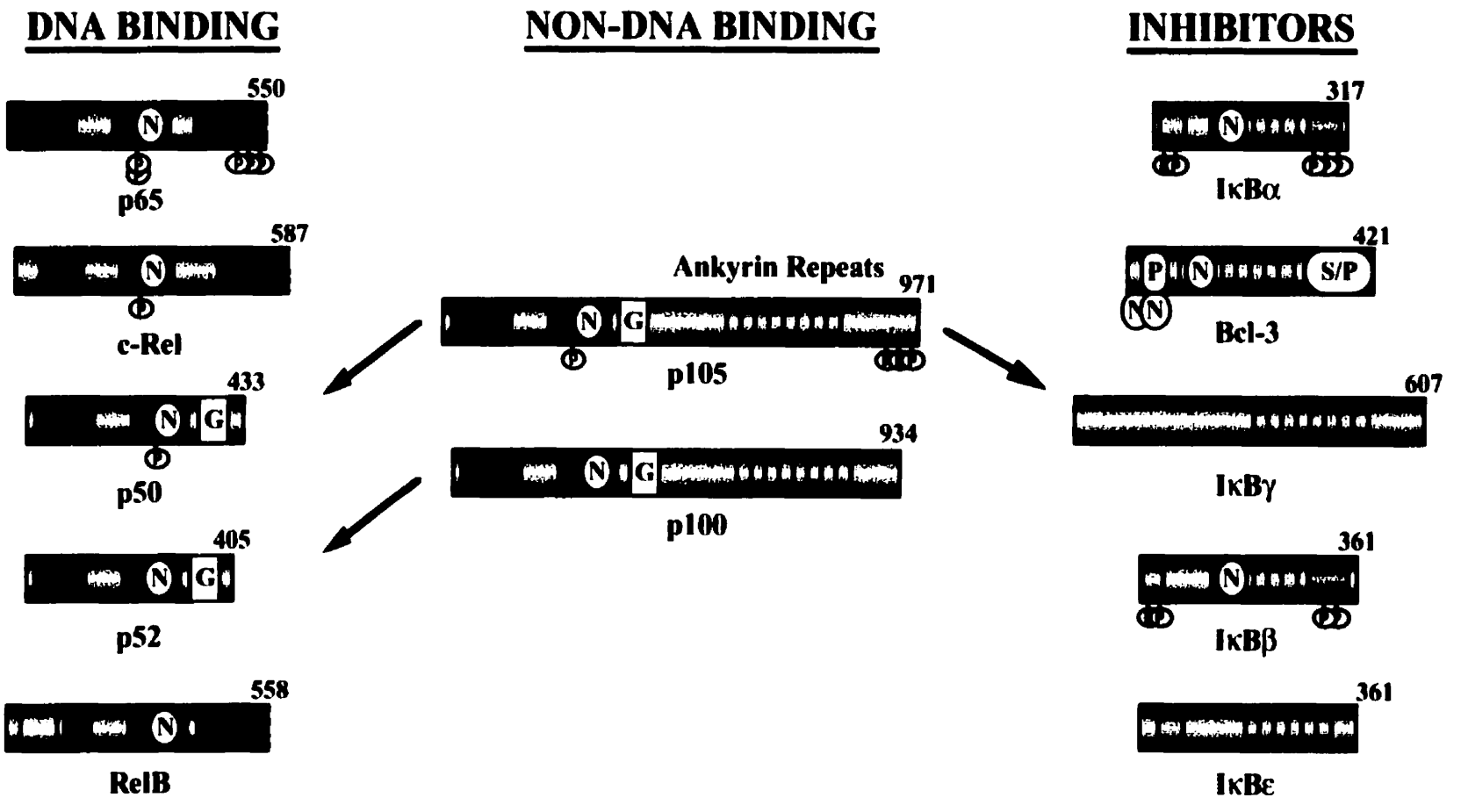
1.0 The NF-κB family of transcription factors and the IκB family of inhibitory proteins

The NF-κB/Rel family of transcription factors plays a pivotal role in immunomodulation through the activation of cellular genes that encode cytokines, cell surface receptors, and acute phase proteins as well as viral genes such as the human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR) (16, 20, 365, 429). In unstimulated cells, NF-κB/Rel proteins are sequestered in the cytoplasm by the IκB inhibitory proteins. Upon stimulation with cytokines, viruses or dsRNA (double stranded RNA), IκBα is rapidly phosphorylated and degraded, resulting in the release of NF-κB and a subsequent increase in NF-κB-regulated gene expression. The IκBα gene contains NF-κB binding sites in its promoter, so NF-κB is able to regulate the transcription of its own inhibitor (55, 74, 185, 228, 407). This autoregulatory control of IκBα expression by NF-κB is in part responsible for the transient nature of NF-κB activation. The first section will discuss each member of the NF-κB/Rel and IκB families and some recent findings in this field of research.

1.1 The NF-κB family of transcription factors

The NF-κB/Rel (here on referred to as NF-κB) family can be subdivided into two subgroups based on structure and function (Figure 1): the DNA binding and non-DNA binding proteins. The DNA binding proteins include NF-κB1 (p50) (52, 140, 208), NF-κB2 (p52) (50, 302, 381), RelA (p65) (309, 368), c-Rel (56) and RelB (369, 370). The non-DNA binding proteins include NF-κB precursor proteins NF-κB1 (p105) and NF-κB2 (p100) which are proteolytically cleaved to generate the DNA binding proteins p50 and p52, respectively (50, 52, 140, 208, 302, 381). Each member of the family shares an N-terminal 300 amino acid domain known as the Rel homology domain (RHD), which is involved in the formation of a DNA binding dimer complex that associates with a

Figure 1. The NF-κB family of transcription factors and IκB family of inhibitory proteins. NF-κB family members contain well-conserved N-terminal rel homology domains (RHD): , which are composed of the DNA binding domain: , dimerization domain, and nuclear localization sequence (NLS): Ⓝ. p50 and p52 are produced by a unique cotranslational process involoving 26S proteasome or by proteolysis of the precursor proteins, p105 and p100, respectively. Both p105 and p100 contain a glycine rich region (GRR): [G], which is important for proteolysis of these precursors, and the C-teminal ankyrin repeats: which are present in IκB proteins. There is also a well-conserved protein kinase A (PKA) site: Ⓟ in the RHD of p65, p50, c-rel, -105. p65, c-rel and rel B contain transactivation domain: in their C-termini. Rel B also contains a leucine zipper ( ) in the N-terminus. IκB proteins contain ankyrin repeats: , through which, they interact with NF-κB proteins. NLSs are also present in IκBα, IκBβ, and Bcl-3. N-terminal signal response and C-terminal PEST domains are designated by and , respectively. N- and C-terminal proline- and serine-rich domains in bcl-3 are shown as P and S/P. IKK complex and CKII phosphorylation sites are shown by Ⓟ and Ⓟ, respectively, as well as other sites (Ⓟ) phosphorylated by unidentified kinases.


DNA BINDING
NON-DNA BINDING
INHIBITORS
550
p65
587
c-Rel
433
p50
405
p52
558
RelB
Ankyrin Repeats
971
p105
934
p100
317
IκBα
421
Bcl-3
607
IκBγ
361
IκBβ
361
IκBε

decameric consensus sequence 5'-GGGPuNNPyPyCC-3' in the promoters of target genes. The RHD is also responsible for nuclear localization and dimerization between different NF-κB members. Nuclear import of NF-κB proteins is accomplished by virtue of a nuclear localization sequence (NLS) that is located at the C-terminal region of the RHD and is characterized by a short stretch of basic amino acids which resembles the classical NLS (RKKKRKV), as typified by the NLS of simian virus 40 (SV40) large T protein (29, 128, 143, 467). Although a direct involvement of the importin-α-importin-β (importin-α-β) receptor in the nuclear import of rel proteins has not been demonstrated, the presence of a classical NLS within NF-κB proteins strongly suggests that nuclear import may be mediated by this pathway. Among NF-κB family members, p65, c-rel and relB contain C-terminal transactivation domains. The dimer composition of different NF-κB subunits and the sequence context of NF-κB sites in different promoters contributes to the differential specificity of gene activation (66, 125, 223, 229, 251, 331).

1.1.1 *RelA (p65)*

The most abundant complex of NF-κB in cells is the p50/p65 heterodimer, in which the p65 subunit contains the transcriptional activation domain. Along with phosphorylation of IκB proteins, p65 is also phosphorylated during the activation of NF-κB by inducers such as tumor necrosis factor-α (TNFα), lipopolysaccharide (LPS) and hydrogen peroxide (101, 298, 475). An inactive form of the cAMP-dependent protein kinase A catalytic subunit of PKA, PKAc, has been shown to be associated with the NF-κB/IκB complex (475). During induction of NF-κB by LPS, the associated PKAc becomes active and the concomitant phosphorylation of the p65 subunit on serine 276 in the RHD occurs. This phosphorylation is required for efficient transcriptional activation by NF-κB and occurs after the degradation of IκB proteins. This phosphorylation of p65 by PKA is required for the association of NF-κB with CBP (CREB-binding protein) and p300, which plays an essential role in NF-κB transcriptional activity (138, 330, 476). NF-κB-dependent

transcription has been shown to be inhibited by adenovirus E1a protein, which blocks the ability of CBP to associate with the histone acetylase p/CAF (p300/CBP-associated factor), which suggests that histone acetylation plays an important role in transcription through NF-κB (323, 462).

Recently, p65 has been shown to be phosphorylated in vitro by the immunoprecipitated IκB kinase (IKK) complex (composed of IKKα/β/γ, see below) and by a recombinant wild type IKKα or a constitutively active mutant of IKKβ with a specificity constant similar to that of IκBα (281, 282). Two other phosphorylation sites in p65 have been identified: TNFα-induced phosphorylation of p65 at serine 536 has been shown to be mediated by IKKs prior to the nuclear translocation, although the functional importance of this phosphorylation site has not been investigated (373). An additional TNFα-induced phosphorylation site was mapped at serine 529 within the C-terminal transactivation domain 1 (TA1) of p65 (435). This phosphorylation was shown to increase NF-κB transcriptional activity without affecting its nuclear translocation or DNA binding affinity. The kinase responsible for phosphorylation at serine 529 and the mechanism by which this phosphorylation potentiates transcriptional activity is currently unknown. The transactivational potential of p65 was also shown to be mediated by p38 and mitogen-activated protein kinases (MAPKs) (427). In addition, phosphorylation and transcriptional activity of a defined region within the TA2 domain were shown to be stimulated upon phorbol myristate acetate (PMA) (383). On the other hand, activation of NF-κB by Interleukin (IL)-1α has been shown to be accompanied by casein kinase II (CKII)-mediated phosphorylation of p65 in which two potential CKII phosphorylation sites located at serine 276 and 539 (41). Finally, phosphatidylcholine-specific phospholipase C, protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3K) have also been shown to be implicated in the phosphorylation and activation of p65 upon IL-1β or TNFα treatment (36, 403). Therefore, these studies strongly suggest that a second

signaling pathway, which is activated in parallel to the cascade leading to IκBα phosphorylation and degradation by NF-κB inducers mentioned above, is required for p65-mediated transcriptional competency .

1.1.2 *c-rel*

p50/p65 complexes have been the most extensively studied NF-κB dimers, therefore much less is known about the regulation and function of the transactivating subunit c-rel. As c-rel is the cellular homolog of the viral oncogene v-rel, it is not surprising that there is a correlation between the amplification/overexpression of the c-rel gene and oncogenesis (142). c-rel is present in both nuclear and cytoplasmic NF-κB complexes (21, 289). While p65 is found in the nucleus during early phase of NF-κB activation, the induced expression of nuclear c-rel is largely delayed for several hours, which may be due to the fact that c-rel gene expression is also regulated by NF-κB (151, 154, 289). Like p65, c-rel also contains a C-terminal transactivation domain which was shown to have a weaker transactivation potential and which lacks homology with the corresponding domain in p65 (57, 184, 196, 350). Therefore, in promoters such as the HIV-LTR and the IL-2Rα promoters, c-rel acts as a specific inhibitor of nuclear p65 function through interference with p65 binding to the κB enhancer (105). Both proteins have been shown to interact with TATA-binding protein (TBP) although, unlike p65, c-rel has not yet been shown to directly interact with p300/CBP (60, 138, 206, 330, 384). In addition, the cytoplasmic and nuclear regulation of c-rel has recently been shown to be controlled by the discrete functional domains of IκBα (263).

1.1.3 *Rel B*

Rel B is a strong transcriptional activator that exhibits many of the classical features of the NF-κB family (49, 50, 103, 370). However, unlike other NF-κB members, RelB

cannot form homodimers and only heterodimerizes efficiently with p50 and p52 (104). Rel B heterodimers have a much lower affinity for IκBα than other NF-κB complexes, which makes them less susceptible to inhibition by IκBα. As a result, RelB heterodimers are predominantly localized to the nucleus, where they represent constitutive NF-κB activity (104, 236). In lymphoid tissues, high levels of RelB heterodimers are expressed, which suggests that they play an important role in the constitutive expression of κB-regulated genes in lymphoid tissues (65, 236, 237, 334, 439). Unlike p65 and c-rel, Rel B lacks a PKA phosphorylation site in the RHD which has been shown to be important for the transactivational potential of p65 (475, 476) and is also quite divergent in sequence in that region of the Rel domain. Recently, RelB was shown to be an important transcriptional suppressor in fibroblasts by limiting the expression of proinflammatory mediators such as IL-1α, IL-1β and TNFα upon LPS stimulation and by modulating the stability of IκBα protein (457). It is also possible that RelB can inhibit p65/RelA or c-rel activities by driving the expression of IκBα since it can strongly induce the expression of IκBα and is inefficiently inhibited by IκBα (104, 114). Finally, RelB has been shown to be an important player for κB-dependent gene demethylation in B cells, which represents the first demonstration of a trans-acting factor involved in cell-type-specific demethylation (211, 212, 457).

1.1.4 *p105/50 and p100/52*

Unique among the NF-κB family members, the NFKB1 and NFKB2 genes encode two proteins each: p105/p50 and p100/p52 (50, 140, 208, 302, 381). The N-terminal portion of p105 and p100 correspond to the p50 and p52 subunits of NF-κB, respectively, which appear to be produced by proteolytic processing. The C-terminal portions of p105 and p100 contain ankyrin repeats, which allow them to function as an IκB (15, 20, 273, 349). Proteolytic processing of p105 to p50 appears to be a constitutive event because p105 and

p50 are produced in nearly stoichiometric amounts in most cells (248, 249, 299). Ubiquitination and the 26S proteasome have been shown to be involved in the generation of p50, which requires a glycine-rich region in the C-terminal region of p105 (112, 249, 320). Some research publications have indicated that like IκBα, induced processing of the precursor p105 is followed by phosphorylation of the proteins upon stimulation with TNFα, dsRNA or phorbol ester, therefore, signal-induced enhanced processing to p50 has been proposed (265, 278, 280, 298, 303). However, recent information suggests that p105 can be completely degraded without giving rise to the processing product p50 when the MAP3K, Cot/Tpl-2, is overexpressed (32). In addition, only modest changes were observed in the p50/p105 ratios upon stimulation. Likewise, in LPS-stimulated monocytes, p105 was shown to undergo degradation without enhanced generation of p50 by processing (157). Therefore, a classical product-precursor relationship between p105 and p50 is not probable. Instead, processing of p105 and p100 has recently been shown to occur co-translationally by a proteasome-mediated process which also does not follow a classical product-precursor relationship (169, 248). In addition, like p65 and IκBα, p50 can also be phosphorylated upon induction such as PMA/Phytohemagglutinin (PHA) induction and this phosphorylated form of p50 binds to κB site with a much greater affinity (239).

1.2 IκB family of inhibitory proteins

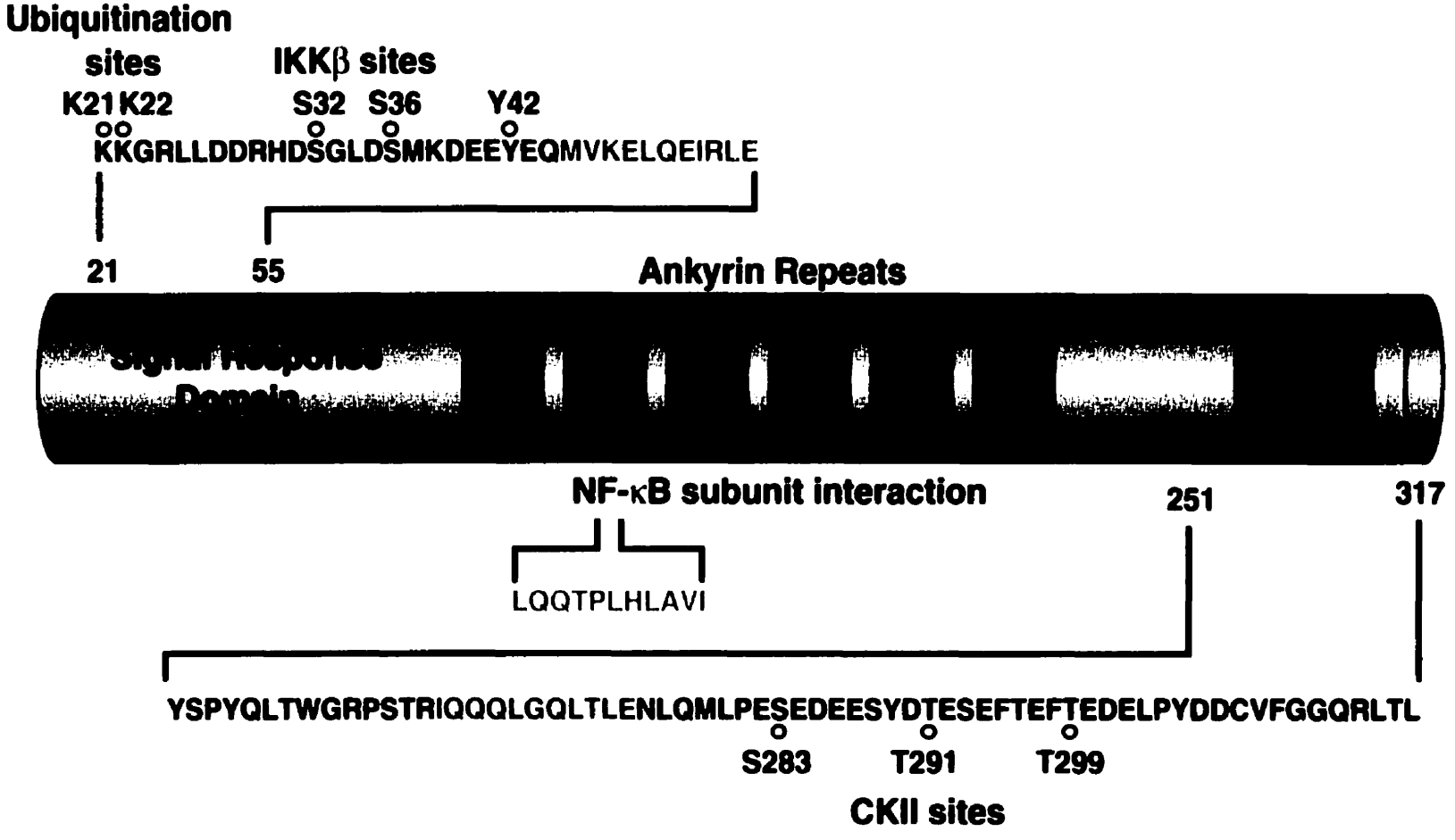
NF-κB activity is regulated in part at the level of subcellular localization. In unstimulated cells, the NF-κB complex is retained in the cytoplasm through the interaction with the inhibitory IκB proteins. The members of the IκB family (Figure 1) include IκBα (162), IκBβ (415), IκBγ (137), IκBε (448) and Bcl-3 (164), as well as the precursor proteins p105 (256) and p100 (280). These proteins contain multiple ankyrin repeats that bind to NF-κB and mask the NLS within the RHD, thus preventing the translocation of the DNA-

binding proteins (27, 29). Each IκB family member has a different specificity for different NF-κB proteins: p100 and p105 can bind efficiently to all mammalian NF-κB factors (163, 164, 183, 256, 280, 299, 349, 379); IκBα, IκBβ and IκBε strongly prefer dimers containing p65 or c-rel (162, 255, 415, 421, 448); Bcl-3 has a strong preference towards p50 or p52 homodimers (51, 308, 456).

1.2.1 *IκBα*

The structure and degradation mechanism of IκBα has been the most extensively studied among the IκB family members. IκBα, which was the first IκB protein to be identified, is encoded by the human cDNA clone MAD3, which is highly homologous to cDNA clones isolated from chicken, rat and pig, called pp40, RL-IF1, and ECI-6, respectively (14, 89, 90, 162, 414). The IκBα proteins can be divided into three domains: the N-terminal signal response domain, the ankyrin repeat domain and the PEST (proline, glutamine, serine, threonine) domain (Figure 2). The C-terminal 35 amino acid PEST region is composed of negatively charged residues which contain multiple CKII phosphorylation sites. While this portion of the protein is not required for inducible degradation or interaction with NF-κB, phosphorylation in this domain regulates the constitutive degradation of the protein (7, 22, 250, 276, 386, 406, 424). Mutations of Ser-283, Thr-291 and Thr-299 of the CKII phosphorylation residues to alanine increased the intrinsic stability of IκBα without affecting inducer-mediated degradation (250). In contrast, C-terminal truncation of IκBα has been shown to prevent inducer-mediated degradation and the PEST regions have been involved in protein breakdown catalyzed by the proteasome (26, 53, 54, 320, 351, 449). Therefore, the function of the IκBα C-terminus still remains unclear. Furthermore, the PEST domain of IκBα has also been implicated in the inhibition of p65/RelA and c-rel DNA binding but not for cytoplasmic retention of c-rel or p65/relA homodimers (111, 163, 372).

Figure 2. Regulatory elements in human IκBα. IκBα can be divided into three domains; N-terminal signal response domain which is composed of Lys 21/22 for ubiquitination and Ser 32/36 for phosphorylation by IKK complex as well as Tyr 42 phosphorylation site; the six ankyrin repeats indicated by red boxes , which mediate the interaction with, and sequestration of, NF-κB family members; and PEST domain (rich in proline, glutamic acid, serine and threonine residues; indicated by green box) which contains three CKII phosphorylation sites, which are Ser 283, Thr 291 and Thr 299 and important in regulating constitutive IκBα protein turnover. Two nuclear export sequences (NESs, in purple letters) are found in the N- and C-terminal regions and permits nuclear IκBα to remove DNA bound NF-κB and translocate it to the cytoplasm. A nuclear localization signal (NLS, in green letters) is located in the second domain of the ankyrin repeats.


Ubiquitination sites
K21 K22
IKKβ sites
S32 S36
Y42
KKGRLLDDRHDSGLDSMKDEEYEQMVKELQEIRLE
21
55
Ankyrin Repeats
Signal Response Domain
NF-κB subunit interaction
LQQTPLHLAVI
251
317
YSPYQLTWGRPSTRIQQQLGQLTLENLQMLPESEDEESYDTESEFTEFTEDELPYDDCVFGGQRLTL
S283
T291
T299
CKII sites

Ankyrin repeats, the common structural feature of all IκB proteins, are multiple, closely adjacent and homologous 33 amino acid sequences (46, 307). The ankyrin repeats are not only restricted to IκB proteins but are also present within a family of proteins that regulate interactions between a variety of membrane structures and the cytoskeleton (264). The repeats bind specifically to the dimerization domain of NF-κB proteins (164, 183, 256, 456). Structure-function analysis reveals that the entire repeat domain is required for interaction with NF-κB, and association with NF-κB results in the dissociation of preformed NF-κB-DNA complexes as well as the hindrance of nuclear translocation of NF-κB (45, 164, 167, 256, 467). Recently, the structure of IκB-NF-κB complexes was revealed by crystallography (13, 180, 186). Binding of IκBα to the dimerized RHDs of NF-κB (p65/p50) through ankyrin repeat domains may inhibit NF-κB from binding to DNA due to the rotation of the N-terminal Immunoglobulin (Ig)-like domain of the p65 RHD. As expected, this interaction also masks the nuclear localization sequence (NLS) of p65 by inducing the unstructured NLS into an α-helical conformation, thus preventing translocation of p65 to the nucleus.

Most attention was focused on the N-terminal signal-response domain, since phosphorylation at Ser-32 and Ser-36 caused inducer-mediated degradation of IκBα. Therefore, the main and first task for researchers was to identify the kinase responsible for the phosphorylation at Ser-32 and Ser-36. Long term efforts by many researchers recently resulted in the identification of the IκB kinase (IKK) complex (discussed later in the thesis). Phosphorylation at serines 32 and 36 marks IκBα for ubiquitination at lysines 21 and 22; which leads to subsequent degradation by the proteasome (4, 19, 53, 54, 72, 99, 353, 354, 380, 416, 449). When the N-terminal IκBα sequences containing these serine and lysine residues were fused to a heterologous protein, they served as an inducible destruction box (455). Even in the absence of stimuli, the IκBα protein is continuously degraded. This continuous basal turnover may be controlled by a different

mechanism, since neither the IKK phosphorylation sites nor the ubiquitination attachment are required, but this process is also mediated by the proteasome (218).

Newly synthesized IκBα accumulates in the cytoplasm but has also been shown to be able to enter the nucleus and dissociate NF-κB from its DNA binding sites and export NF-κB back to the cytoplasm, thus terminating NF-κB-dependent transcription in the nucleus (8, 9, 466). Recently, the NLS and two NESs (Nuclear export sequences) of IκBα were identified, which strongly supported the idea that IκBα is able to shuttle between the nucleus and the cytoplasm. These two leucine-rich NES elements in IκBα are homologous to the NES found in shuttle proteins such as the HIV-1 Rev protein and protein kinase A inhibitor. N- and C-terminal NES are located from amino acid 45 to 55 and from 265 to 277, respectively (9, 116, 124, 193, 442). The IκBα-derived NES can functionally substitute for the NES in HIV Rev (9, 124). The nuclear protein CRM1 (also known as exportin 1) has also been recently identified as the NES receptor (119, 127, 315, 405). In addition, nuclear IκBα appears to be resistant to signal-induced phosphorylation and degradation. This results in nuclear accumulation of transcriptionally inactive IκBα/NF-κB complexes when cells are treated with leptomycin B (LMB), an inhibitor of NES-mediated nuclear export (352). However, contradictory results were obtained by Hope and co-workers which indicated that nuclear NF-κB/IκBα complexes are also sensitive to signal-induced degradation of IκBα through a proteasome-mediated mechanism (193). Moreover, proteasomes were also found in the nucleus, suggesting that degradation can occur directly in the nucleus (347).

Nuclear localization of IκBα has recently been shown to be mediated by a novel nuclear import sequence within the second ankyrin repeat of IκBα. This sequence does not resemble the classical NLS, which is characterized by a short stretch of basic amino acids (371). Although the second ankyrin repeat of IκBα contains the predominant nuclear

import sequence, other ankyrin repeats may also contribute to the nuclear localization of the full length IκBα protein (371). Recently, IκBα has been shown to be imported into the nucleus by a piggy-back mechanism (371) that involves additional protein(s) which contain a basic NLS and are able to interact with the ankyrin repeats of IκBα. This process requires GTP hydrolysis by Ran (a small GTPase) as well as the basic NLS receptors importin α and β (419). However, an unidentified NLS sequence in the first ankyrin repeat has been shown to be sufficient for temperature-dependent nuclear import of IκBα (193). Interestingly, when IκBα is overexpressed as an NF-κB-bound form, it localizes exclusively in the cytoplasm, which suggests that NF-κB interaction with IκBα in the cytoplasm could mask a region of IκBα involved in its nuclear import (467). Therefore, a mutual masking of the sequences responsible for the nuclear import of both NF-κB and IκBα causes cytoplasmic retention of both proteins (419). In contrast, Johnson *et al.* recently proposed a shuttling model in which individual proteins or a complex of NF-κB and IκBα can shuttle between the nucleus and the cytoplasm (193). Thus, further analysis is required to resolve the mechanism of the nuclear import and export of NF-κB and IκB proteins.

1.2.2 *IκBβ*

IκBβ protein was first identified by Zabel and Baeuerle (466) and, subsequently, human cDNAs for IκBβ were isolated (232). Unlike IκBα, basal phosphorylation of IκBβ is required for interaction with NF-κB (255). IκBβ has been shown to associate with different NF-κB members depending on its phosphorylation status. Recombinant, unphosphorylated IκBβ could bind to p65 but not to c-rel, however, IκBβ phosphorylated at the C-terminal PEST domain could efficiently bind to c-rel (77). Human T-cell leukemia virus type 1 (HTLV-1) Tax-induced degradation of IκBβ leads to the release of c-rel-containing complexes, which emphasizes the physiological importance of c-rel-IκBβ

complexes (145). In different studies, recombinant IκBβ did not exhibit a preference between p65 and c-rel (415). Furthermore, the major sites of constitutive phosphorylation of IκBβ have been mapped to Ser 313 and Ser 315. These residues have been shown to be phosphorylated by CKII, which also phosphorylates the C-terminal region of IκBα (77). Carboxy-terminal phosphorylation by CKII of IκBβ has also been shown to increase its affinity to NF-κB (417). Similar to IκBα, IκBβ is also proteolytically degraded after stimulation with LPS, IL-1, TNFα, or HTLV-1 Tax and contains the conserved N-terminal serines and lysines, suggesting that these molecules are controlled by similar mechanisms (99, 277, 415). Inducible phosphorylation residues were identified as Ser 19 and Ser 23 (99, 277), which were shown to be phosphorylated by a recently identified IKK complex (100, 346). Therefore, mutations of serine 19 and 23 to alanines in IκBβ creates a dominant-negative form that is no longer degraded upon induction (99, 156, 277). Inducible degradation of IκBβ was also blocked by the proteasome inhibitor MG132, which suggests that ubiquitination is required (99, 277). Furthermore, T cells from transgenic mice expressing a dominant negative mutant of IκBβ showed a complete inhibition of persistent NF-κB activation (see below) upon PMA/PHA stimulation (11).

In contrast to IκBα, IκBβ is not resynthesized immediately after stimulation and is degraded with delayed kinetics, which allows for persistent activation of NF-κB (415). Newly synthesized IκBβ first accumulates as an underphosphorylated species which interacts with p65 without masking the NLS, thus maintaining NF-κB activity by protection of p65 from IκBα in both the nucleus and the cytoplasm (408). A nuclear localization sequence (NLS) has not been identified in IκBβ, but has been suggested to be within the second ankyrin repeat (371) . Previously, it was proposed that, due to the failure of masking NF-κB's NLS by newly synthesized underphosphorylated IκBβ, the NF-κB/IκBβ complex is able to enter the nucleus by a piggy-back mechanism and activate

transcription (408, 417). Recently, nuclear IκBβ has been shown to maintain persistent NF-κB activation in HIV-1-infected myeloid cells (95). In addition, two different forms of human IκBβ, termed IκBβ1 (43kDa) and IκBβ2 (41kDa) were identified. The former is the most similar to IκBα in responsiveness to inducing agents while the latter, which contains a truncated PEST domain in the C-terminus, is either degraded incompletely or inert in response to certain inducers (170). This emphasizes the importance of the C-terminal PEST domain on efficient proteolysis of IκBβ (159, 441).

1.2.3 *IκBγ*

IκBγ is the C-terminal half of p105 containing multiple ankyrin repeats which was believed to be produced by an alternative transcription start site in the intron of NFKB1 gene, thus p105, p50 and IκBγ are produced from the same gene (183, 256, 287). Unlike p105 which is ubiquitously expressed, IκBγ mRNA is specifically present in murine lymphoid cell lines (183). It associates with p65/p50, p50/p50 and c-rel, and inhibits c-rel nuclear translocation and c-rel-mediated transactivation. It was suggested that the activity of IκBγ is also regulated by phosphorylation since it contains a consensus sequence for phosphorylation by CKII and others (183). Moreover, the amount of IκBγ mRNA was decreased during B-cell differentiation suggesting a cell-type specific role of IκBγ (256).

1.2.4 *IκBε*

IκBε, the most recently identified IκB family member, was isolated by two hybrid screens using p52, p50 and p65 as baits (245, 402, 448). Human IκBε, a 45 kDa protein, exists as multiple phosphorylated isoforms in resting cells and contains conserved serine residues (at 18 and 22) which closely resemble the signal response domain of IκBα but has no

PEST-like sequences in the C-terminus (448). IκBε binds predominantly to p65 and c-rel, weakly to p50 and p52, and is able to displace NF-κB from its DNA binding sites as well as inhibit NF-κB-mediated transcription (245, 402, 448). Like IκBα and IκBβ, upon induction with PMA/ionomycin and LPS, IκBε is proteolytically degraded with slower kinetics compared to IκBα. The two conserved serine residues are required for the induced degradation of IκBε (448). Interestingly, IκBε expression is also upregulated by similar stimulants that activated IκBα expression, suggesting that IκBε gene is also NF-κB regulated (245, 448). Furthermore, the C-terminal region of murine IκBε is required for efficient inhibition of NF-κB DNA binding (402).

1.2.5 *bcl-3*

Bcl-3, which was identified as a putative oncoprotein , is a unique IκB member, since it is most abundant in the nucleus and is not degraded upon activation of NF-κB-stimulating pathways (51, 120, 206, 308, 311). Bcl-3 was shown to bind p50 or p52 homodimers and induce p50 homodimers from cytosolic p105/p50 complexes without enhancing the processing of p105 (436). Recently, cellular Bcl-3 was shown to bind p50 but not p52 homodimers (166). Lower expression of p52 and less efficient processing of p100 to p52 may explain this observation, along with the fact that Bcl-3-p50 complexes are more abundant in some cell types (40, 61, 436). As p50 and p52 do not contain transactivation domains, it has been proposed that Bcl-3 may antagonize p50-mediated inhibition by dissociating p50 or p52 homodimers from DNA (121, 182, 205, 456). Conversely, ectopic expression of Bcl-3 enhanced the DNA-binding activity of p50 homodimers in the murine thymus (61). Supporting the latter, Bcl-3 has been demonstrated to form ternary complexes with p50 or p52 homodimers bound to DNA and act as a transcription activator which requires N- and C-terminal proline- and serine-rich domains (51, 126, 171, 321). The activation potential of Bcl-3-p50 complexes can be further stimulated by

interaction of Bcl-3 with the histone acetylase Tip60 (91). The presence of two basic NLS in the N-terminal domain of bcl-3 and the inability of bcl-3 to mask NF-κB NLS results in the nuclear import of the Bcl-3/p50 homodimer complex, mediated either by p50 NLS or bcl-3 NLS (473).

1.3 Homozygous disruption of NF-κB and IκB proteins

The generation of single and double knockouts and transgenic mice has yielded invaluable information about the physiological functions of various NF-κB and IκB proteins. Recently, Gerondakis *et al.* reviewed these genetic approaches in order to understand the function of these proteins (136). The phenotypes of single knockout mice are briefly discussed here.

1.3.1 Mice lacking NF-κB proteins

One of the interesting phenotypes of mice lacking p65 was a massive liver degeneration due to apoptosis implicating p65 in the inhibition of apoptosis (31). This was shown to be due to an increased sensitivity to TNFα-induced apoptosis (28, 423, 433). In addition, TNFα/p65 double deficient mice were found to be viable and have normal liver (106). These data further suggest that p65 protects the cells from TNFα-induced apoptosis. Although homozygous *nfkb1*-deficient mice develop normally, these mice are defective in B-cell activation (153, 392). Knockout mice lacking p52/p100 also develop normally but exhibit multiple defects in the immune system, which is consistent with the knowledge that p52-containing dimers, p52-c-rel and p52-RelB appear to play a particularly important role in the terminal stages of B-cell differentiation (62, 122, 150, 257). Based on studies with homozygous *c-rel*-deficient mice, c-rel plays an essential role in lymphocyte proliferation, immune and inflammatory responses, and T-cell

development (48, 216). A dramatic reduction in constitutive κB-binding activity and specific defects in lymphoid tissues were observed in mice lacking relB (59, 440).

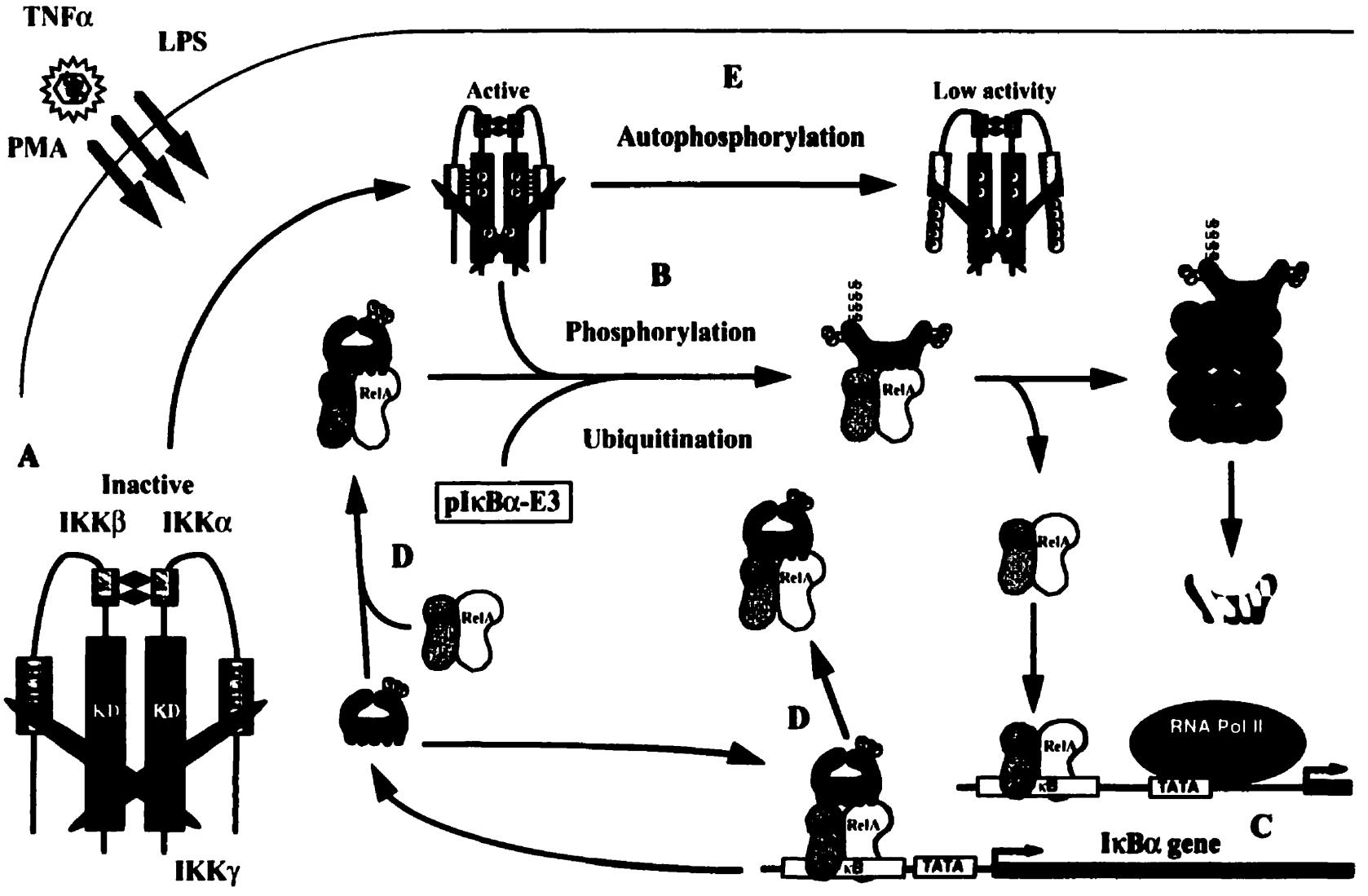
1.3.2 Mice lacking IκB proteins

Knockout mice lacking IκBα develop normally but die post-natally due to severe widespread inflammatory dermitis and granulocytosis (30, 214). Fibroblasts from these mice exhibited a prolonged NF-κB activation upon stimulation, suggesting that IκBα is required for post-induction repression of NF-κB. At present, no information on *IkBg*$^{-/-}$ and *IkBb*$^{-/-}$ mice is available. However, one would expect a similar phenotype in *IkBb*$^{-/-}$ mice as seen in IκBα knockout mice due the functional similarity between the two proteins. Homozygous *bcl-3*-deficient mice also develop normally, but show defects in antigen-specific B- and T-cell responses (123, 387). Surprisingly, unlike *IkBa*$^{-/-}$ and *bcl-3*$^{-/-}$, mice lacking IκBε are indistinguishable from wild type animals, although IκBε has been shown to have common features with IκBα (245, 279, 402, 448)

2.0 Mechanism of NF-κB activation

In response to a variety of stimuli, including TNFα, IL-1β, LPS or viral infection (319), the most studied IκB protein, IκBα is phosphorylated at serine 32 and 36 residues by the recently identified 500-900 kDa IκB kinase (IKK) complex. Phosphorylation targets IκBα for ubiquitination, which is ATP dependent and involves the covalent attachment of multiple ubiquitin molecules to IκBα lysine residues 21 and 22 by the recently identified IκBα-ubiquitin ligase-E3 (pIκBα-E3, (463)), targeting IκBα for degradation by the 26S proteasome. This results in the release of NF-κB proteins (72) which can now translocate to the nucleus to activate gene transcription (Figure 3). The function of the IKK complex and the upstream kinase that activate the IKK complex will be discussed in this section.

Figure 3. The biochemistry of NF-κB activation. NF-κB is sequestered in the cytoplasm by inhibitory IκB proteins. Stimulation by a diverse collection of agents and pathogens including TNFα, PMA, LPS and viruses lead to the activation of signaling cascades that are believed to culminate with activation of the IKK complex. The IKK complex is preferentially composed of IKKα, IKKβ and IKKγ (A). Cell stimulation enhances the phophorylation of all three IKK subunits which activates IKK complex (92). Activated IKK in turn phosphorlyates IκBα, which marks it for ubiquitination by recently identified pIκBα-E3 and subsequent proteosome mediated degradation (B). NF-κB is released and translocates to the nucleus where it transactivates κB responsive genes (C). Target genes are selectively regulated by the distinct transcriptional activation potential of different subunit combinations. The IκBα gene is regulated by NF-κB leading to its resynthesis (C). *De novo* synthesized IκBα can associate with free NF-κB sequestering it in the cytoplasm and can enter the nucleus removing DNA bound NF-κB, thereby establishing an autoregulatory mechanism by which NF-κB activation is limited (D). In addition, activated IKKs become autophosphorylated at the C-terminal serine cluster, which decrease IKK activity (E).


TNFα
LPS
PMA
A
Inactive
IKKβ
IKKα
KD
KD
IKKγ
Active
E
Autophosphorylation
Low activity
B
Phosphorylation
Ubiquitination
RelA
pIκBα-E3
D
D
RelA
RNA Pol II
YATA
IκBα gene
C

2.1 NF-κB Activation by the IκB Kinase (IKK) Complex

Except for induction with UV radiation and anoxia, NF-κB activation requires the phosphorylation of IκBα at the two N-terminal serines and its subsequent degradation (33, 181, 242). Early IκBα degradation induced by UV irradiation requires intact N- (positions 1-36) and C-terminal (positions 277-287) sequences of IκBα, while later IκBα degradation requires the phosphorylation of the N-terminal serines (32 and 36) that is caused by the autocrine and paracrine action of IL-1α (33, 242). However, IκBα degradation by a different type of photonic radiation, γ radiation, required the phosphorylation of two serine residues (242). On the other hand, phosphorylation at tyrosine 42 of IκBα induced by reoxygenation of hypoxic cells, led to NF-κB activation in the absence of IκBα degradation. This represents a proteolysis-independent mechanism of NF-κB activation which implicates the tyrosine kinase $p56^{lck}$ (181). Recently, tyrosine 42 phosphorylated IκBα was shown to be sequestered by the p85 subunit of PI3 kinase via its SH2 domain from NF-κB (35).

Based on mutational analysis of IκBα, it was suggested that the physiological IκB kinase should be serine specific, as substitution of the serine residues with threonines prevented inducible phosphorylation of IκBα (99). Two high-molecular-weight (500-900 kDa) kinase complexes that appeared to specifically phosphorylate Ser-32 and -36 of IκBα were recently identified (73, 100, 230, 282, 346, 451, 471). The difference between these two kinase complexes was that the activity of one complex was regulated via ubiquitination by an unknown mechanism while the other was not. The latter kinase complex has been shown to contain at least two catalytic subunits IKKα and IKKβ and the regulatory subunit IKKγ /NF-κB essential modulator (NEMO)/IKK-associated protein 1 (IKKAP1) (100, 281, 282, 346, 362, 451, 459, 471). IKKγ is devoid of a catalytic domain but contains three large α-helical motifs and a LZ, and has been shown to be

absolutely essential for NF-κB activation by HTLV-1 Tax, LPS, PMA and IL-1 (281, 362, 459). IKKα was previously known as CHUK [conserved helix-loop-helix (HLH) ubiquitous kinase], whose function was unknown (85). By searching the expressed sequence tag (EST) database, a protein kinase exhibiting an overall homology to IKKα of 52% was identified and named IKKβ (282, 346, 451, 471). Both IKKα and IKKβ are ubiquitously expressed. The structures of IKKα and IKKβ are similar to each other, however they are unlike other known Ser/Thr kinases. Each protein is composed of an N-terminal kinase domain followed by a leucine zipper (LZ) region and a C-terminal helix-loop-helix (HLH) domain. They can form homo- or heterodimers, which is mediated through binding between the LZ domains (282, 451, 470, 471). However, the predominant form of IKK complex (Figure 3A) is an IKKα/IKKβ heterodimer associated with a dimer of IKKγ (282, 362) which is mediated by an interaction between IKKβ and IKKγ (281, 459). Furthermore, the HLH region is not required for dimerization between IKKα and IKKβ but is indispensable for efficient kinase activity through interaction with the kinase domain or possibly with upstream activators (92, 471). The LZ domain present in the C-terminal region of IKKg was also suggested to be involved in the interaction with the upstream kinases and/or activators (362). Some differences in the activities between IKKα and IKKβ were identified by overexpressing wild type (WT) or dominant negative (kinase inactive) forms of each subunit: 1) when transfected alone, the activity of IKKα is relatively low compared to IKKβ, which is constitutively active and requires costimulation with TNFα to become fully activated (282, 451, 471), 2) A dominant negative form of IKKβ efficiently inhibited NF-κB activation upon TNFα or IL-1 while inhibition of NF-κB activation by a dominant negative form of IKKα was not consistent (100, 282, 346, 451, 471), 3) Both IKKα and IKKβ phosphorylate Ser-32 and -36 of IκBα *in vitro* , with IKKβ exhibiting much greater activity than IKKα (231, 470). Conversely, IKKβ could phosphorylate both Ser-19 and -23 of IκBβ equally, while IKKα phosphorylated poorly and preferentially Ser-23 alone (100, 282, 346, 451). Once

activated, IKKβ became autophosphorylated at a carboxy terminal serine cluster, which decreased IKK activity and prevented prolonged activation of the inflammatory response which can be an additional regulatory step to inhibit prolonged NF-κB activation (Figure 3E) (92). Furthermore, IKK activity was inhibited by the protein phosphatase 2A (PP2A) catalytic subunit, which was also found in the IKK complex (100). The *in vivo* function of PP2A on IKK activity is currently unknown. Recently, two different functions of IKKα in the IKK complex have been suggested: Inhibition of IKKβ activity by IKKα in the absence of inducers and the induction of IKKβ by IKKα in the presence of inducers (310). In the latter case, IKKα plays a role in relaying upstream signals to IKKβ in a directional manner, therefore IKKα is a pre-requisite for induction of IKKβ activity, which is contradictory to the previous suggestion (92).

2.2 Upstream kinases activating IKK

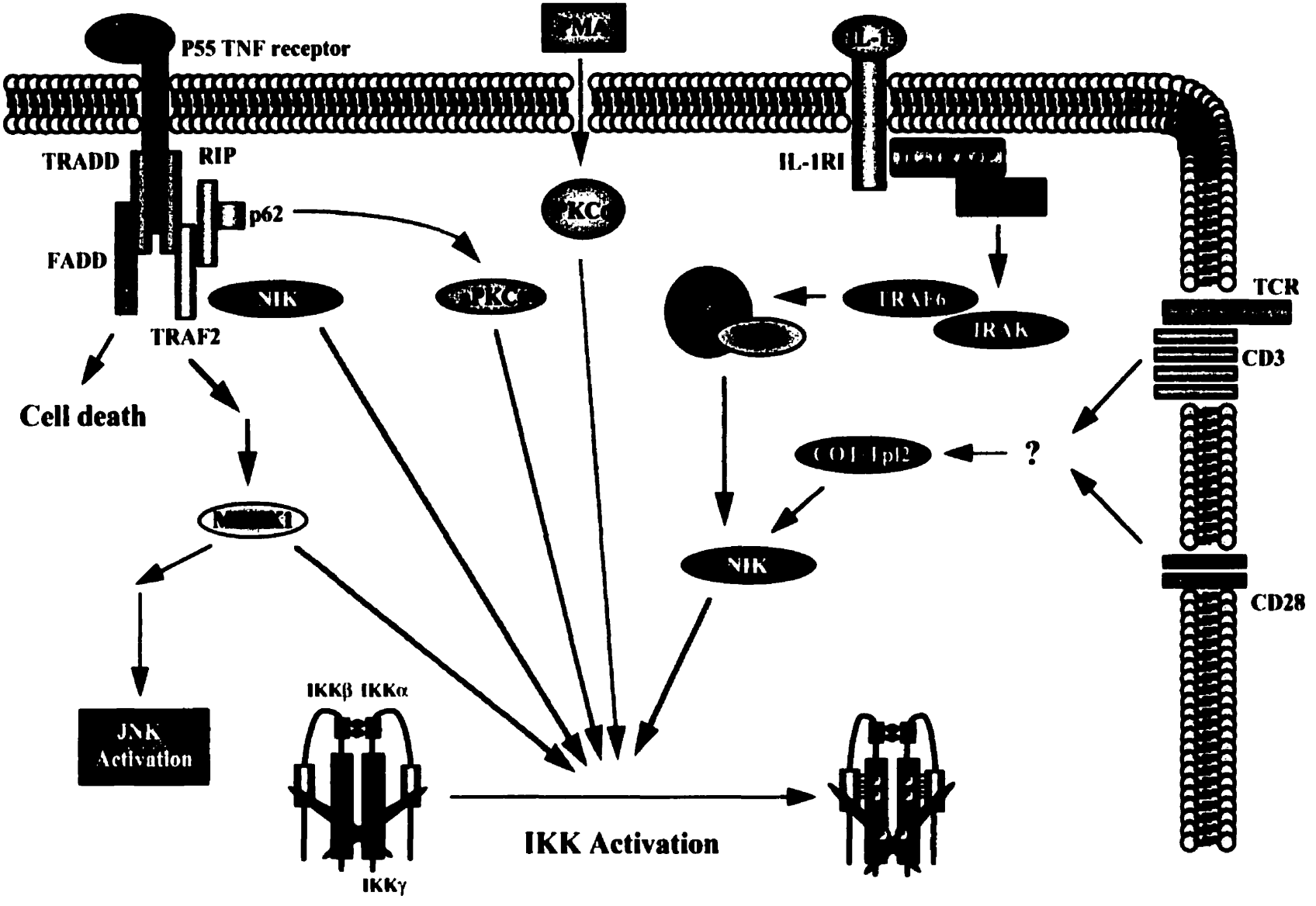
After the identification of the IKK complex, attention focused on the upstream kinases in many different signal transduction pathways that all converge to the IKK complex. Among the upstream kinases, four are MAP3Ks. This is not surprising because MAP kinase consensus sequences were identified in both IKKα and IKKβ. Throughout the course of studies on these upstream kinases, many contradictory results have emerged. This section summarizes the recently identified upstream kinases of IKK complex.

2.2.1 *NF-κB Inducing kinase (NIK)*

NIK (MAP3K, serine/threonine kinase) was initially identified as a TNFR associated factor-2 (TRAF-2) interacting protein by two-hybrid screening of a human B-cell cDNA library (268). Subsequently, IKKα was also isolated through a yeast two hybrid screening as a protein that interacts with NIK (346). NIK was shown to be a part of IKK

complex isolated from IL-1-stimulated cells (83). Overexpression of NIK enhanced the ability of the IKKs to phosphorylate IκB and activate NF-κB (100, 282, 346, 451, 471) while dominant negative mutants of NIK suppressed NF-κB activation by TNFα, IL-1, TNFR associated death domain (TRADD), receptor interacting proteins (RIP), TRAF-2, TRAF-5 and TRAF-6, which suggests that the convergence of these signaling pathways leading to NF-κB activation might be at the level of this kinase (Figure 4) (268, 404). NIK physically associates with and activates both IKKα and IKKβ, and the carboxy-terminal domain of NIK, which is also responsible for interaction with TRAF-2, mediates its interaction with IKKα (253, 268, 346, 451). Selective deletion of each of the three structural domains did not affect the ability of NIK to bind to IKKβ, suggesting that this association occurs via multiple interactions between the two proteins through different domains of IKKβ (451). However, IKKα has been shown to be a better substrate than IKKβ for phosphorylation by NIK *in vitro* and the Ser-176 in the kinase activation loop of IKKα was identified as the phosphorylation target by NIK (254). Thr-559 within the activation loop of NIK kinase domain plays a critical role in regulating the activation and autophosphorylation of this kinase (253). Nevertheless, involvement of NIK in NF-κB activation has become controversial based on three publications: binding of NIK to TRAF2 and its catalytic activity was not stimulated by TNFα (23). NIK is implicated in lymphotoxin (LT) signaling rather than in TNFα signaling (397). NF-κB release was intact upon TNFα treatment of cells expressing a catalytically inactive mutant of NIK (469). However, inhibition of TNFα-induced NF-κB dependent gene expression was observed, suggesting that NIK may affect transactivation potential of NF-κB but not IKK activation and release of NF-κB from inhibitory complexes (469). Further analysis is required to elucidate the involvement of NIK in NF-κB signaling under more physiological conditions, since many studies have been performed by overexpression, which almost certainly does not accurately reflect physiological signaling events. Knockout mice lacking NIK as well as transgenic mice expressing catalytically inactive

Figure 4. Signal transduction pathways of different activators of NF-κB and upstream kinases of IKK complex. Binding of TNFα to its receptor causes recruitement of TRADD to the receptor. TRADD interacts with TRAF2 which is connected to IKK through NIK. RIP is interacting with TRADD, TRAF2 and p62 which transduces TNF signal to IKK through aPKC. Signal transduction pathway leading to IKK activation by TNFα is outlined in red arrows. IL-1 induced IKK activation (outlined in blue arrows) requires the recruitement of IL-RAcP, MyD88 and IRAK to IL-1RI upon the engagement of IL-1. IRAK then dissociates from the receptor complex and interacts with TRAF6 that transduces the IL-1 signal to NIK and then to the IKK complex through TAK1/TAB1. CD3/CD28 costimulatory signaling pathways converge on NIK, leading to activation of the IKK complex (in black arrows). PMA induces IKK activation through PKCα (in green arrows).


P55 TNF receptor
TRADD
RIP
p62
FADD
TRAF2
NIK
PKC
IL-1RI
TRAF6
IRAK
TCR
CD3
CD28
Cell death
?
NIK
IKKβ IKKα
IKKγ
JNK Activation
IKK Activation

mutants of NIK would provide more reliable information on the role of NIK in NF-κB activation.

2.2.2 *Mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1 (MEKK1)*

NF-κB activation occurs under conditions that also stimulate the MAPK pathway and, vice versa, the MAP kinase pathway can be activated by other known inducers of NF-κB such as the small GTPases Cdc42 and Rac1, which have been shown to activate NF-κB (172, 230, 268, 283, 332, 345). MEKK1, which is a mammalian serine/threonine kinase in the MAP3K group (226) was copurified during the purification of the IKK complex, and both IKKα and IKKβ contained a canonical MAP kinase kinase (MAP2K) activation loop (Ser-X-X-X-Ser) within their catalytic domains (282). Mutational analysis revealed that these serine residues (Ser-177 and Ser-181) in IKKβ were essential for kinase activity, suggesting activation of IKKβ by MEKK1 (Figure 4) (282). Recombinant MEKK1 has been shown to stimulate IKKβ-mediated phosphorylation of IκBα, and overexpression of MEKK1 induced IKKβ activity and NF-κB reporter activity in the presence or absence of TNFα or IL-1 (231, 295, 301, 465). Many publications showed that MEKK1 is a much weaker activator of the NF-κB transcriptional reporter compared to NIK (231, 297, 404). Some data suggest that there is the existence of a differential specificity of MEKK1 and NIK to IKK complex subunits: IKKβ is the substrate of MEKK1, whereas IKKα is the substrate of NIK (254, 295). HTLV-1 Tax-induced NF-κB activation has been shown to mediated through the action of MEKK1, which then directly activates IKKβ but not IKKα (465). In addition, recent studies show that IKK activation by Tax occurs through IKKγ, which acts as a bridge between two molecules (78, 158, 192) and also through NIK (420). Involvement of MEKK1 also became controversial since MEKK-1 did not play a role in NF-κB activation in some studies, inconsistent with

those described above (100, 259, 297, 404). Other MAP3Ks, including MEKK2 and MEKK3, have been shown to activate IKKs *in vivo*, but MAP3Ks such as MEKK4, ASK1 and MLK3 failed to activate NF-κB (108, 474), suggesting a specificity of NF-κB activation by only a subset of MAP3Ks.

2.2.3 *TGF-β Activated Kinase 1(TAK1)*

TAK1, a recently identified MAP3K, together with its activator protein, TAK1 binding protein 1 (TAB1) (396), has been shown to be a kinase that links TRAF6 to the NIK-IKK cascade in IL-1 signaling (306). Therefore, the signaling pathway of NF-κB activation by IL-1 may be as follows (Figure 4). IL-1 binds to the cell surface type-I receptor (IL-1RI), which forms a complex with the accessory protein IL-1RAcP (IL-1 receptor accessory protein) and the adapter protein MyD88 as well as a Ser/Thr kinase named IRAK (IL-1 receptor associated kinase) (147, 292, 444). IRAK then dissociates from the receptor complex and interacts with TRAF6, which transduces the IL-1 signal to NIK and then to the IKK complex through TAK1 (63, 64, 306, 443). Although TAK1 was first shown to be activated by TGF-β and bone morphological protein (458), no functional relationship between TGF-β signaling and the NF-κB activation has been documented. In other words, TGF-β treatment could not induce the nuclear translocation of NF-κB (375). Therefore, TAK1 may be involved in the NF-κB activation induced by stimuli other than TGF-β, such as IL-1 and TNFα (see below). TAK1 has also been shown to interact with both IKKα and IKKβ and induce IκBα degradation (374). Furthermore, TAK1/TAB1 complex also plays a role in TNFα-induced NF-κB activation (374). In contrast to the above study which suggests that TAK1-induced IKK activation is mediated through NIK, TAK-1 mediated NF-κB activation has been shown to be independent of NIK and the further study revealed that dominant negative (DN) mutants of NIK partially inhibited TAK1-induced NF-κB activation (374). Therefore, understanding the precise relationship

between TAK1 and NIK in TNFα-induced NF-κB activation requires further investigation.

2.2.4 *Cot/Tpl-2*

The proto-oncogene Cot/Tpl-2, another MAP3K related serine-threonine kinase which stimulates both the ERK and JNK pathways (288, 324), has been shown to physically assemble and phosphorylate NIK *in vivo* (252). It was proposed to be an upstream kinase of NIK upon TCR/CD3 (Figure 4) and CD28 costimulation, but not TNFα in NF-κB activation (Figure 4). In contrast, another study by Belich *et al.*, showed that Cot/Tpl-2 was involved in TNFα-induced NF-κB activation through increased complete degradation of p105 (32). Both studies used almost identical systems, except for two different kinase-inactive mutants of NIK to analyze their effects on TNFα-induced NF-κB activation. The latter observed an inhibitory effect of the Cot/Tpl-2 mutants on TNFα-induced NF-κB activation. Therefore, at present, it is not clear whether Cot/Tpl-2 is involved in TNFα signaling. Belich *et al.* also demonstrated a direct interaction between p105 and Cot/Tpl-2, however, no direct phosphorylation by Cot/Tpl-2 could be demonstrated which suggests that it may regulate p105 phosphorylation through an unidentified downstream kinase, reminiscent of the regulation of IκBα phosphorylation by NIK (32). To date, no evidence exists for the ability of IKKα or IKKβ to phosphorylate other IκB isoforms such as IκBε and IκBγ, which also contain N-terminal serine residues crucial for its regulation (448). However, Heissmeyer *et al.* recently demonstrated that NF-κB p105 (434) but not p100 precursors are equivalent targets of the IKK complex like IκBα and IκBβ, and phosphorylation occurs at the C-terminal serines (921, 923 and 932) of p105 (166). It would be interesting to see whether Cot/Tpl-2 interacts with IKKα because both kinases interacted with a similar region at the C-terminus of p105 as well as whether IKK complex can phosphorylate IκBγ, which contains exactly identical C-terminal region of p105 including these IKK phosphorylation sites (32, 166, 183).

2.2.5 *PKR ,PKC and Akt*

Before the identification of the IKK complex, many kinases such as PKCζ, PKA, Raf-1, PKR and pp90-rsk were candidates for IκB kinases (98, 115, 139, 221, 385). However, these kinases did not fulfill the requirement for IκB kinase, which should phosphorylate IκBα in vivo at both of the N-terminal serine residues (Ser-32 and -36). For example, the 90 kDa ribosomal S6 kinase (pp90-rsk), downstream of the Raf-MAPK/extracellular signal-regulated kinase pathway, is involved in phorbol ester-induced NF-κB activation by phosphorylating Ser 32 but not Ser 36. Interestingly, recent studies revealed the involvement of some of these kinases in IKK activation. As opposed to a direct kinase, PKR has been shown to be implicated in dsRNA-induced NF-κB activation which is mediated by NIK and IKK activation (76, 469). Also, overexpression of PKCζ activated IKKβ but not IKKα activity and dominant-negative mutants of PKCζ inhibited IKKβ activation in TNFα, but not in PMA-stimulated cells (224). This suggests the involvement of the atypical protein kinase C (aPKCs) in TNFα-mediated NF-κB activation through the regulation of IKKβ activity. A more precise mechanism showing the link between the TNFα receptor and aPKC was revealed recently by Sanz *et al* (378). In this study, they proposed that the interaction of p62 (a novel aPKC-interacting protein) with RIP, which interacts with TRADD, serves to link aPKCs to NF-κB activation by TNFα (Figure 4). Previous studies also demonstrated the existence of a trimolecular complex composed of TRADD, RIP and TRAF2 (177, 178). TRAF2 has been shown to interact with NIK in NF-κB activation pathway (268). Therefore, RIP is at the center of NF-κB activation through two independent and redundant pathways, connecting TRAF2 to NIK and p62 to aPKCs. This may explain why TNFα could induce NF-κB in TRAF-2-deficient or TRAF-2 dominant transgenic mice (234, 464), while TNFα-induced NF-κB activation was severely defective in RIP-deficient mice (203). PMA-mediated NF-κB

activation was also shown to involve PKCα, which binds to IKKs in vitro and in vivo, and PMA-induced IKKβ activation was dependent on the activity of PKCα (Figure 4) (224). Interestingly, PKCθ-mediated NF-κB activation has been shown to occur exclusively via IKKβ homodimers (310), which together with other studies shown above strongly suggest that IKKα and IKKβ are selective for different upstream kinases, which may be due to different structural determinants for this specificity in both IKKs and upstream kinases. Furthermore, a novel PKCθ-interacting protein named PICOT (PKC-interacting cousin of thioredoxin) has recently been shown to inhibit CD3/CD28 induced NF-κB activation, therefore it would be interesting to see whether PICOT can inhibit PKCθ-mediated NF-κB activation (450).

The serine-threonine kinase Akt (also known as protein kinase B, PKB) which is a downstream target of PI3K, has also been shown to be involved in TNFα-, platelet-derived growth factor (PDGF)- and PMA-induced NF-κB activation (197, 207, 318, 356). In TNFα-treated and serum-starved IKKα-transfected cells, Akt was shown to mediate IKKα phosphorylation at threonine 23 (318). IKKβ does not contain a putative Akt phosphorylation site, which is another example of differential regulation of IKKα and IKKβ by upstream kinases (318). Although no direct interaction was observed between Akt and IKKs in TNFα-stimulated cells, in PDGF-stimulated cells, NF-κB activation was shown to be mediated by Akt which transiently interacted with IKKα and IKKβ (356). Since PDGF has been shown to activate NF-κB and inhibit c-Myc-induced apoptosis by activating the Ras/PI3K/Akt (202, 204, 314), it was suggested that activation of NF-κB by PDGF contributes to inhibition of c-Myc-induced apoptosis by induction of NF-κB regulated anti-apoptotic genes (see below) (75, 152, 356, 432, 433, 478). Interestingly, Akt-mediated NF-κB DNA binding activity and NF-κB specific reporter activity was inhibited by DN mutants of NIK in Akt-transfected cells in the absence or presence of PMA treatment (197), suggesting the involvement of NIK in Akt-mediated NF-κB

activation. Recently, in contrast to the above reports, indicating Akt-mediated NF-κB activation occurs through IKK dependent degradation of IκBα, Madrid *et al.* observed that activated PI3K or Akt stimulated NF-κB-dependent transcription; however, this acts through stimulating TA1 domain of the p65 subunit rather than inducing NF-κB nuclear translocation via IκBα degradation (266). Furthermore, mutation of Serine 529, previously shown to be important for the transcriptional activity of p65, inhibited the ability of Akt to activate p65 transcription (266, 435). Therefore, further investigation is required to analyze Akt-mediated NF-κB activation.

2.3 Mice lacking IKKα and IKKβ

Initially, IKKα and IKKβ were thought to be interchangeable IκB kinases in the complex. Based on a number of experiments, IKKβ was later shown to be a stronger kinase with greater activity for phosphorylation of IκB proteins than IKKα. However, it is now clear that these two kinases play different roles in the IKK complex after observations of knockout mice lacking IKKα or IKKβ. Homozygous *ikkβ*-deficient mice died at about 14 days of gestation due to massive hepatic cell apoptosis, a phenotype remarkably similar to that seen in mice deficient in the RelA(p65) subunit of NF-κB (31, 244, 246, 410). This may be due to the loss of the anti-apoptotic effects of NF-κB as shown by decreased NF-κB activation upon TNFα or IL-1 in fibroblasts from these mice (244, 410). Although IKKα has IκB kinase activity and is involved in phosphorylation of the IκB proteins , this observation clearly provided that IKKα homodimers found to be still in the complex cannot perform IκB kinase activity (244, 410). In contrast, mice lacking IKKα showed functional NF-κB activation upon TNFα treatments but exhibit profound epidermal and morphogenic abnormalities and die *in utero* or soon after birth (179, 243, 409). These findings are clearly contradictory to earlier studies showing that catalytically inactive mutants of IKKα or addition of anti-sense IKKα RNA inhibits NF-κB activation

in response to many inducers. Moreover, a similar phenotype of skin abnormality was observed in mice lacking IκBα compared to *ikkα*-/- mice (214). Further studies are required to understand whether this phenotype is due to uncontrolled NF-κB activation by IKKβ homodimers in the absence of IKKα, which was shown to be a negative regulator of IKKβ in uninduced condition (241, 310, 470) and to search for the real function of IKKα, which may be related to morphogenic and epidermal development.

3.0 NF-κB REGULATED GENES

Numerous genes regulated by NF-κB are listed in Table 1 (319). About one third of known NF-κB-regulated genes are involved in the host immune response, which include cytokines/chemokines, immunoreceptors, cell adhesion molecules and genes encoding for proteins that are involved in antigen presentation. In addition to its important role in the immune response, NF-κB also activates genes that are involved in the stress response, apoptosis and cell growth. Therefore, NF-κB is a central mediator of the human stress response (319). A number of anti-apoptotic genes are positively regulated by NF-κB, including cellular Inhibitor of apoptosis protein 1 (c-IAP1), c-IAP2, TRAF1, TRAF2 and the Bcl-2 homologue A1/Bfl-1 (75, 259, 432, 434, 454). Therefore, when NF-κB activity is inhibited, cells become sensitive to TNFα or DNA damaging drug-induced apoptosis (28, 222, 423, 433). Conversely, NF-κB is also involved in promoting apoptosis (149, 247). It is possible that this discrepancy of the role of NF-κB in promoting or inhibiting apoptosis is dependent on cell type and which apoptosis-inducing agent is used. Interestingly, NF-κB has also been implicated in the activation of Fas, FasL and TRAIL gene expression, which are TNFα superfamily members involved in apoptosis. Expression of these genes is upregulated when cells are treated with inducers that activate NF-κB (68, 191, 200). Therefore, the mechanisms of how many of these anti-apoptotic gene products protect cells from apoptosis still remain unclear. In addition, NF-κB

Table 1. Genes regulated by the NF-κB family of transcription factors.

Table 1. Genes regulated by the NF-κB family of transcription factors.

Class	Target Genes
Cytokines/chemokines	Interleukin 1α,β (IL-1α,β) Interleukin 2, 6, 8, 9, 11, 12, 15 (IL-2, 6, 8, 9, 11, 12, 15) γ-interferon (IFN-γ) β-interferon (IFN-β) Tumor necrosis factor α (TNF-α) Lymphotoxin α (LT-α)/Tumor necrosis factor β (TNF-β) Lymphotoxin β (LT-β) Cytokine-induced neutrophil chemoattractant (CINC) CXC chemokine ligand for CXCR3 (CXCL11) Macrophage chemotactic protein-1 (MCP-1) Macrophage inflammatory protein-1α,β (MIP-1α,β) Macrophage inflammatory protein-2 (MIP-2) Regulated upon Activation Normal T lymphocyte Expressed and Secreted (RANTES)
Immunoreceptors	Immunoglobulin κ light chain Immunoglobulin ε heavy chain IL-2 receptor α-chain CC chemokine receptor-5 (CCR-5) TNF receptor, p75/p80 T cell receptor β chain T cell receptor α chain (human) Major histocompatability class II ($E\alpha^d$) β2-microglobulin
Cell adhesion molecules	Endothelial leukocyte adhesion molecule-1 (ELAM-1) Vascular cell adhesion molecule-1 (VCAM-1) Intercellular adhesion molecule-1 (ICAM1) P-selectin Mucosal addressin cell adhesion molecule -1 (MadCAM-1) Tenascin-C
Acute phase proteins	Angiotensinogen Serum amyloid A precursor Complement factor B Complement factor C4 Urokinase-type plasminogen activator Pentraxin PTX3 Lipopolysaccharide binding protein C-reactive protein Tissue-factor 1
Antigen presentation	Proteasome subunit LMP2 Peptide transporter TAP1

Class	Target Genes
Stress response	Angiotensin II Cyclooxygenase (COX)-2 Ferritin H chain 5-Lypoxygenase 12-Lypoxygenase Inducible nitric oxide (NO) synthase NAPDH quinone oxidoreductase Mn superoxide dismutase (SOD) Phospholipase A2
Apoptotic regulators	Bcl-2 Pro-survival Bcl-2 homologue Bfl1/A1, Bcl-xL and Nrl3 c-IAP1 and 2 TRAF-1 and -2 Fas ligand and receptor (CD95) Fas like interleukin-1β converting enzyme (FLICE) TNF receptor 1 and 2
Growth factors	Granulocyte/macrophage colony stimulating factor (GM-CSF) Granulocyte colony-stimulating factor (G-CSF) Macrophage colony-stimulating factor (M-CSF) Platelet-derived growth factor (PDGF) B chain Proenkephalin Thrombospondin Vascular endothelial growth factor (VEGF) C Insulin-like growth factor binding protein-1,2 (IGFBP-1,2)
Transcription factors	c-Rel NFκB1 (p105) and NFκB2 (p100) IκBα c-myc Interferon regulatory factor 1 and 2 (IRF-1 and IRF-2) A20 p53
Cell cyle	p53 cyclin D1 and D3
Viruses	Human immunodefiency virus (HIV-1) Cytomegalovirus (CMV) Simian virus 40 (SV40) Adenovirus (E3 region) Avian Leukosis virus Bovine Leukimia virus Epstein-Barr virus (EBV) Simian immunodeficiency virus (SIV) Measles virus

induces expression of many transcription factors such as c-myc, A20, interferon regulatory factor-1 (IRF-1), IRF-2, *jun*B and p53 which would result in amplification of many other genes that are under the control for these transcription factors, as well as its own family members and inhibitors (see below).

3.1 Regulation of NF-κB/IκB gene expression: autoregulation of NF-κB activation

Since the IκBα gene contains NF-κB binding sites in its promoter, NF-κB is able to autoregulate the transcription of its own inhibitor (55, 74, 90, 162, 185, 228, 407). Autoregulatory control of IκBα expression by NF-κB is in part responsible for the transient nature of NF-κB activation of gene expression as mentioned earlier. The IκBα gene has also been shown to be regulated by RelA(p65) both at the mRNA and protein levels: RelA(p65)-IκBα protein interactions increased the half-life of the inhibitory protein and IκBα mRNA was induced by RelA(p65) as a consequence of increased IκBα gene transcription (44, 389). Stimulation of Jurkat T cells by TNFα or PMA induced degradation of the IκBα protein concomitant with NF-κB release and activation (238, 286). Activation is followed by *de novo* IκBα synthesis in an NF-κB-dependent manner, and cycloheximide treatment prior to induction resulted in the inhibition of IκBα resynthesis, as well as prolonged NF-κB DNA binding (407). Furthermore, NF-κB also upregulates expression of other NF-κB family members such as c-rel, NFKB1 and NFKB2, which all contain NF-κB binding sites in their promoters (80, 81, 151, 154, 260, 412). Many studies suggest that p50 and p52 homodimers which do not contain transactivation domains, act as the transcriptional repressors by competing for NF-κB binding sites in the promoter, while p105 and p100 sequester NF-κB in the cytoplasm (51, 69, 105, 223, 335, 381, 382). This further controls the transient nature of NF-κB activation.

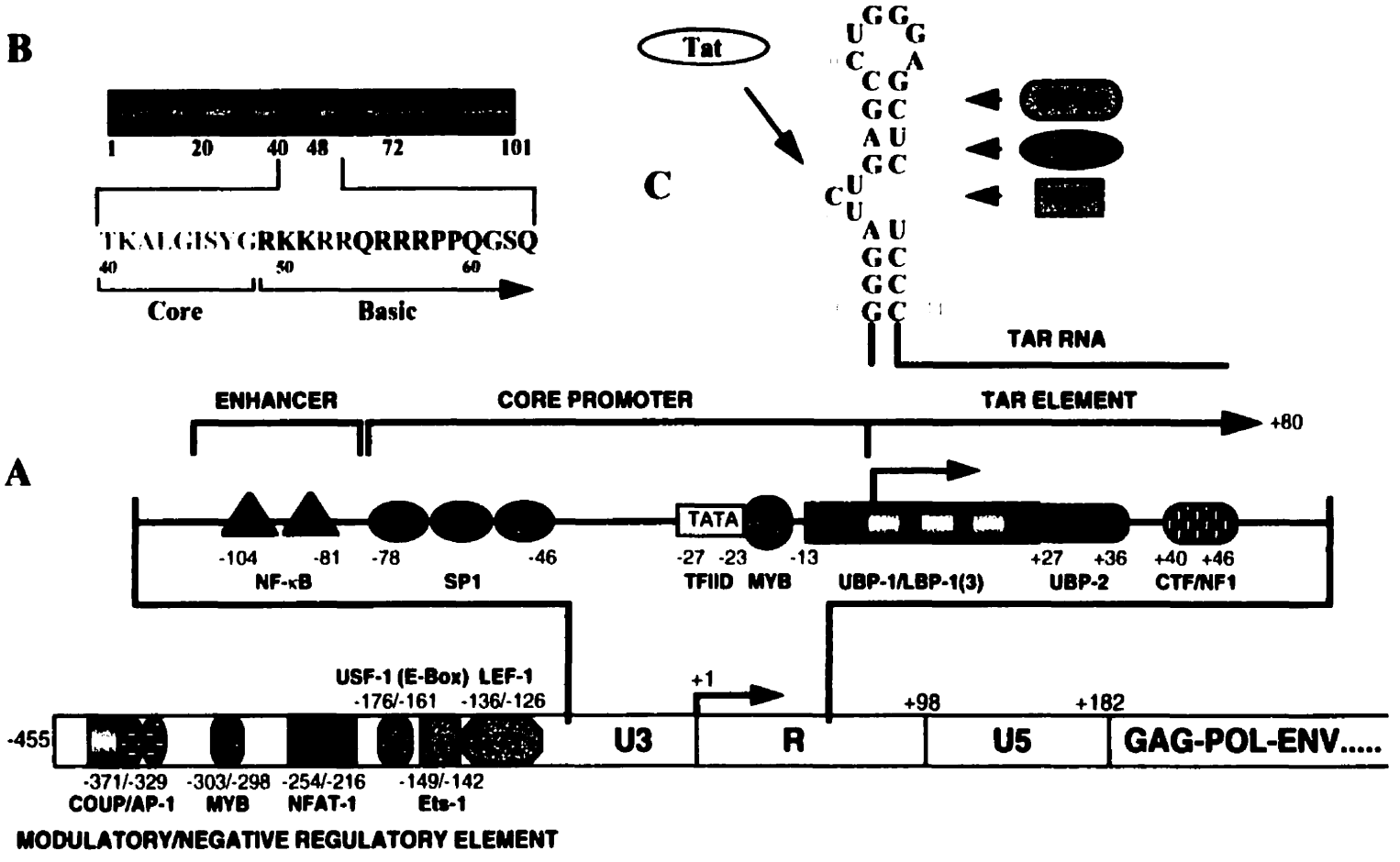
3.2 Promoter elements regulating HIV-1 gene expression

Viral gene expression of Cytomegalovirus (CMV), Simian virus 40 (SV-40) and HIV-1 (Table 1) is induced by NF-κB and vice versa, viral infection can lead to activation of NF-κB (148, 198, 293, 363-365, 376). Therefore, chronic HIV-1 infection may be caused in part by constitutive NF-κB activation due to constitutive activation of the IKK complex, leading to increased degradation of IκBα (94, 96, 173, 275, 363-365). Many viral proteins also participate in their gene expression, for example, the HIV-1 Tat (trans-activator of transcription) protein has been shown to be involved in induced expression of the HIV-1 LTR. Thus, a complex interaction between host and viral proteins is involved in viral gene expression. This section will discuss host transcription factors and viral proteins involved in HIV-1 gene expression.

3.2.1 *host transcription factors*

The HIV-1 promoter, located in the U3 region of the viral long terminal repeat (LTR), is composed of positive and negative transcriptional regulatory elements (Figure 5A) that are required for the expression of the integrated proviral genome (135, 195, 365). The intracellular efficiency of HIV-1 gene expression and replication is due in part to the ability of HIV-1 to utilize host signaling pathways to mediate its own transcriptional regulation. The promoter-proximal enhancer region of the HIV-1 LTR contains two NF-κB binding sites (-109 to -79) (148, 210, 293, 359) whose functional significance in inducible HIV-1 gene expression was demonstrated by mutational analysis in transient transfection assays using HIV LTR or HIV enhancer reporter constructs in the presence of inducers such as TNFα and IL-1 (16, 107, 125, 219, 251, 342, 365, 429). Although NF-κB proteins play a central role in HIV-1 long terminal repeat (LTR) driven transcription, these κB sites are not absolutely required for viral growth (70, 290)

Figure 5. HIV-1 Tat protein and regulatory elements in HIV-1 LTR. The viral LTR is composed of three regions designated U3, R and U5. U3 region includes the enhancer (two NF-κB binding sites), the core promoter containing three Sp-1 binding sites and TATA box and the upstream modulatory domain which contains binding sites for a number of cellular factor binding sites. Transcription starts at the U3/R border and R region encodes the transactivation response element, TAR. HIV-1 Tat protein binds to the TAR element in the nascent RNA and enhances viral transcription. It is composed of five domains. Domain III and IV are important for its transactivation potential and RNA binding activity, respectively. Domain II is rich in cysteines which are important for Tat function.


B
1
20
40
48
72
101
TKALGISYGRKKRRQRRRPPQGSQ
40
50
60
Core
Basic
Tat
C
TAR RNA
A
ENHANCER
CORE PROMOTER
TAR ELEMENT
+80
-104
-81
-78
-46
NF-κB
SP1
TATA
-27
-23
-13
TFIID
MYB
UBP-1/LBP-1(3)
+27
+36
+40
+46
UBP-2
CTF/NF1
USF-1 (E-Box)
-176/-161
LEF-1
-136/-126
+1
+98
+182
-455
U3
R
U5
GAG-POL-ENV.....
-371/-329
COUP/AP-1
-303/-298
MYB
-254/-216
NFAT-1
-149/-142
Ets-1
MODULATORY/NEGATIVE REGULATORY ELEMENT

Recently, HIV-1E, a naturally occurring subtype of HIV-1 that contains a single NF-κB binding site in the enhancer region, has been shown to display reduced TNFα-responsiveness, which further demonstrates the importance of NF-κB in HIV-1 gene expression (290). Sp-1, which binds to three sites adjacent to the NF-κB sites in the core promoter region of the HIV-1 LTR, has been shown to be important for basal as well as HIV-1 Tat induced transcription of the HIV-1 LTR (38, 161). In addition, NF-κB activity was further enhanced by synergistic transactivation with the transcription factor Sp-1 (326, 329, 401). Sequences upstream of the enhancer region have been defined as the negative regulatory element (NRE) because elimination of this region increases basal and induced HIV-1 LTR activity in transfected cells or *in vitro* (261, 262, 313, 358, 399). In contrast, a region between -130 and -201 was shown to be important for viral replication in peripheral blood lymphocytes (PBLs) and some T cell lines (210). This region contains binding sites for USF-1 (E-box), which is a bHLHZip protein and Ets proteins (EBS), as well as for the DNA bending transcription factor LEF-1 (lymphoid enhancer binding factor-1). Both Ets-1 and LEF-1, expressed highly in T cells, were shown to be able to relieve nucleosomal repression of the HIV-1 LTR in conjugation with Sp-1 suggesting an important role of these transcription factors in the distal region of HIV-1 LTR (71, 235, 395, 418, 437). Moreover, cooperative binding and synergistic transactivation of the HIV-1 LTR between USF-1 and Ets-1 has recently been shown to occur at the distal enhancer region of the HIV-1 LTR (400). NFAT binding sites were also shown to be involved in HIV-1 gene expression upon T-cell activation (399). The functional importance of other upstream transcription factor binding sites for COUP, AP-1 and Myb and binding sites downstream of transcription start site for UBP-2 and CTF/NF1 is not currently known, except for upstream binding protein/leader binding protein (UBP-1/LBP-1) which was shown to inhibit HIV-1 gene expression by interfering with TFIID binding to the TATA element (201).

3.2.2 *HIV-1 Tat*

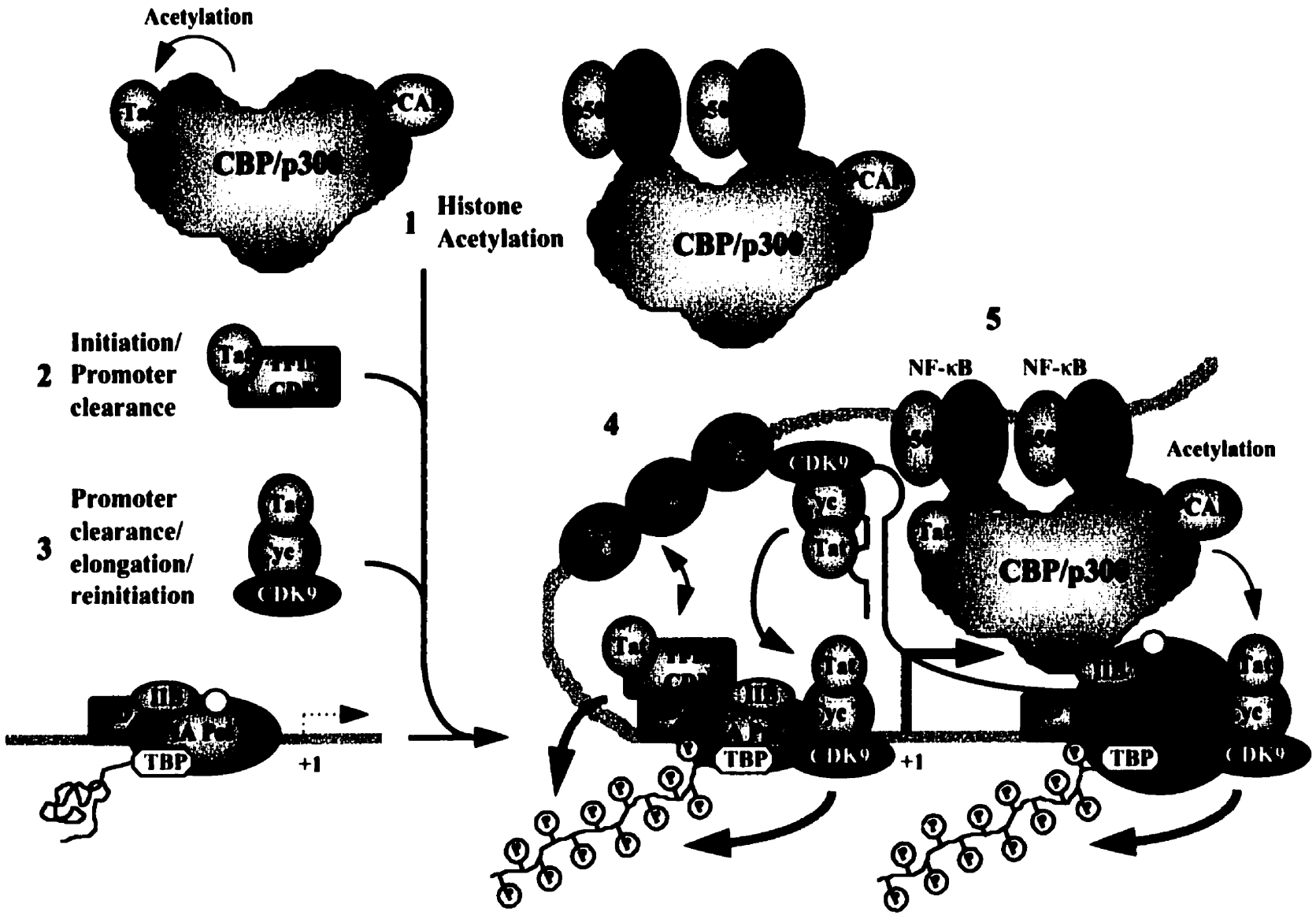
A small nuclear protein, Tat is a 101 amino acids arginine-rich RNA-binding protein that is absolutely required for productive HIV-1 replication (190). HIV-1 Tat protein enhances LTR-derived gene expression and is required for high-level expression of all viral genes (87). Pleiotropic effects of Tat on cellular gene expression have also been well documented; modulation of cytokine secretion, inhibition of antigen-induced lymphocyte proliferation, downregulation of MHC (Major histocompatibility complex) class I surface expression and decrease in manganese-dependent superoxide dismutase activity (58, 176, 316, 340, 341, 422, 430, 446, 447). Furthermore, Tat has been shown to be involved in promoting or inhibiting apoptosis and acts as a growth factor for Kaposi's sarcoma cells (109, 215, 240, 267, 296, 304, 305, 445, 472). Among these pleiotropic effects of Tat, transcriptional activity of Tat on the HIV-1 LTR will be discussed in this section. Tat can be separated into five functional domains (Figure 5B). Cysteine-rich domain II is important for Tat function as well as domain III and IV, which are essential for its transactivational capacity and RNA binding activity (102, 134, 344, 391). However, domain I has a minimal effect and domain V is dispensable for its transcriptional function. Unlike other transcription activators, Tat acts by binding to RNA through its RNA binding domain, composed of basic amino acids, not to DNA, but to a 59-base stem-loop RNA structure called the transactivation response (TAR) sequence (Figure 5C), which is located at the 5' ends of all nascent HIV-1 transcripts causing a substantial increase in transcript levels (39, 93, 130, 195, 366, 367). The core secondary stem-loop structure including the bulge region (Figure 5, nucleotides +18 to +44) is critical for Tat-TAR interactions, Tat-mediated transactivation as well as HIV replication (39, 93, 213, 367, 390). The bulge region in TAR (+23 to +25) serves as the primary binding site for Tat, and the loop sequences (+30 to +35) also contribute to this interaction (366, 367, 390). Certain cellular factors such as TRP-185 and p68 also bind to TAR RNA and facilitate interactions with promoter elements such as TATA, Sp1 and

the HIV-1 enhancer, stimulating transcription initiation and stabilizing elongation complexes (37, 38, 199, 270, 393).

The optimal activity of Tat to induce transcriptional initiation and elongation, requires its association with two classes of cellular proteins, Tat-associated kinases (TAKs) and Tat-associated histone acetyltransferases (TAHs) (189, 194, 195, 227). TAKs, which include RNA polymerase II (RNAPII) C-terminal domain (CTD) kinases, a basal transcription factor II H (TFIIH) and a positive transcriptional elongation factor b (P-TEFb), induce processive transcription of RNAPII from the HIV-1 LTR promoter by phosphorylating the CTD of RNAPII (79, 86, 129, 131, 312, 322, 438, 461, 477). Kinases responsible for CTD phosphorylation are CDK7 found in the cyclin-dependent kinase-activating kinase (CAK) complex of TFIIH and CDK9/PITALRE in P-TEFb complex. CDK7 has been shown to be responsible for phosphorylating the CTD of the largest subunit of RNAPII in the preinitiation complex during its transition from initiation to promoter clearance while CDK9 phosphorylates RNAPII during transcriptional elongation as well as initiation/reinitiation (86, 131, 144, 269, 274, 300, 460, 477). CDK9 is recruited to the TAR element by indirect interaction with Tat, and this association is mediated by cyclin T (Figure 6), therefore forming a ternary complex (327, 438). Association between Tat and TFIIH has also been demonstrated (86, 131, 322). Therefore, two CTD kinases which are important for initiation, promoter clearance and elongation gain access to the HIV-1 LTR through Tat interaction. Tat has also been shown to interact with TAHs, which contain p300/CBP and P/CAF capable of acetylating histones and therefore causing the activation of chromatinized HIV-1 (34, 175, 271, 272). Thus, Tat gains access to the integrated HIV-1 LTR (394, 426, 428). Furthermore, acetylation of Tat by p300/CBP and PCAF has recently been demonstrated to be important for Tat binding to the TAKs and dissociation of Tat from TAR RNA that occurs during early transcription

elongation (209). Figure 6 describes the current model for Tat-mediated HIV-1 LTR activation.

Figure 6. Model for transcriptional activation of the HIV-1 LTR by HIV-1 Tat and cellular transcription factors. Tat which is expressed from integrated HIV-1 genome possibly by cellular activation and/or taken up by the cells associates with CBP/p300 (1) therefore causing the activation of chromatinized HIV-1, which can also occur by cellular activation, for example, TNFα stimulation. Now, Tat/CDK7/TFIIH (2) and Tat/CDK9/CycT (3) complexes gain access to the HIV-1 LTR, and initiate transcription by phosphorylating CTD of RNAPII. Tat/CDK9/CycT complex binds to the TAR element in nascent viral transcrips. (4) Basal transcription factors such as Sp-1 may further activate HIV-1 transcription by bringing CBP/p300 which acetylates Tat and regulates its activity, as well as through interaction with TFIID complex (141, 285, 339). (5) NF-κB upon cellular activation even further increases HIV-1 transcription by bringing CBP/p300 to the HIV-1 LTR. CBP/p300 also interacts with TFIIB (188). By interacting with many cellular factors, Tat can increase HIV-1 transcription, along with transcription factor which are activated by cellular events.


Acetylation
CBP/p300
1
Histone
Acetylation
2
Initiation/
Promoter
clearance
3
Promoter
clearance/
elongation/
reinitiation
4
5
NF-κB
NF-κB
Acetylation
CDK9
TBP
+1

Specific Research Aims

The primary objective of the research presented in this thesis was to elucidate the effects of transdominant mutants of IκBα (TD-IκBα) on NF-κB regulated genes and HIV-1 replication in Jurkat T cells. To accomplish this, the work has been divided into three specific research aims:

1) To examine the effect of TD-IκBα on the synergistic activation of HIV-1 LTR by HIV-1 Tat protein and TNFα .

2) To study the effect of inducible TD-IκBα expression on *de novo* HIV-1 replication in Jurkat T cells.

3) To analyze the IκBα promoter by *in vivo* genomic footprinting in Jurkat T cells inducibly expressing TD-IκBα.

This work presents the effects of TD-IκBα on HIV-1 replication. The experiments performed focus on T cells which are the main targets for HIV-1 infection as well as cells of the monocytic lineage. NF-κB activity was carefully examined at the same time due to the fact that the HIV-1 LTR is largely regulated by NF-κB. This work further investigates the effect of TD-IκBα on stimulus induced transcriptional activation of the IκBα gene promoter. Based on these experiments, we demonstrate that TD-IκBα effectively blocks HIV-1 replication in T cells, furthermore inducer-mediated regulation of IκBα gene expression is dependent on NF-κB and Sp-1 binding sites in the proximal region of IκBα promoter.

CHAPTER II

MATERIALS AND METHODS

Plasmids.

1) Expression vectors. Plasmids SVK3-IκBα and SVK3-IκBα (3C) encoding wild type IκBα and IκBα(3C) respectively were described elsewhere (174, 250). IκBα (3C) is a full length human IκBα in which serine 283, threonine 291 and threonine 299 were substituted for an alanine residue. Substitutions S32A/S36A in mutant IκBα (2N) and substitutions S32A;S36A;S283A; T291A;T299A in mutant IκBα (2N + 3C) were generated by overlap PCR mutagenesis using Pfu DNA polymerase. The resulting mutated IκBα cDNAs were inserted in the expressing vector SVK3 (Pharmacia) and the plasmid pREP-9 CMVt (26). Mutations were confirmed by sequencing. The plasmids expressing wild type and R52Q,R53Q mutant of Tat, HIV LTR ΔB and ΔB-ACU, as well as the plasmid containing the HIV-1 proviral DNA (pSVC21 BH10) were previously described (82, 93, 366). CMVt-rtTA contains the Moloney murine leukemia virus-based pBABE vector backbone, which contains a puromycin (puro) resistance gene under the control of the CMV promoter. Construction of the plasmid consisted of the consecutive insertion of three components into the polylinker site: the doxycycline-responsive promoter CMVt from the CMVtBL vector (a kind gift from A. Cochrane), the rtTA gene from the pUHD172-1neo plasmid (398), and the polyA fragment from the pSVK3 vector. neo CMVt BL was constructed in two steps. First, an intermediary plasmid (neo BL) was generated by ligation of a 3 kb XhoI/EcoRI fragment from the pMV7 vector (contains the neomycin (neo) resistance gene) to a 3.8 kb XhoI/EcoRI fragment from the CMV BL vector (contains the poly A site and the ampicillin (Amp) resistance gene). Second, a 450 bp XhoI (blunt)/NotI fragment of CMVt BL (contains the CMVt promoter) was cloned into the EcoRI (blunt)/NotI sites of neo BL. CMVt-IκBα 2N was constructed by cloning an EcoRV (blunt)/BamHI IκBα 2N mutant cDNA fragment downstream of CMVt at the EcoRI (blunt, filled with Klenow enzyme)/BamHI site of neo CMVt BL. SVK3-IκBα 2NΔ4 was constructed by replacing a XhoI fragment of SVK3-IκBα 2N with a XhoI fragment from SVK3-IκBα Δ4. CMVt-IκBα 2NΔ4 was constructed by cloning an

EcoRI/BamHI IκBα 2NΔ4 mutant cDNA fragment from SVK3-IκBα 2ND4 downstream of CMVt at the EcoRI/BamHI site of neo CMVt BL.

2) Reporter plasmids. HIV LTR CAT plasmids ptzIIICAT, -109/-79, IIIΔ23 and IIIΔA were a kind gift from Dr. Eric Cohen (for a schematic map, see Fig 8). 0.4SK-pGL3 Luc was obtained by subcloning the 0.4 kb fragment of IκBα promoter (a kind gift of Dr. A. Israël) into *Kpn*I/*Sac*I-digested pGL3. The plasmids carrying respectively point mutations into the κB1 or Sp1 or in both sites were obtained by the subcloning of polymerase chain reaction-amplified fragments into *Kpn*I/*Sac*I-digested pGL3. Briefly, these constructs were obtained in two steps by a procedure previously described (225). The first round of amplification used 0.4SK-pGL3 Luc as template, 5'-CACGCGTAAGA GCTCCACCG-3' (*Sac*I primer) as 3'-primer and 5'-GGAAATTCaaCGAGCCTGAC-3' (small nucleotides indicate point mutations) as 5'-primer for κB1 site mutagenesis or 5'-CCTGACCaaGCCCCAGAGAA-3' as 5'-primer for Sp1 site mutagenesis. The amplified fragments were used as the 3'-primer in a second polymerase chain reaction using 0.4SK-pGL3 Luc as template and 5'-CTATCGATAGGTACCGGGCC-3' (*Kpn*I primer) as 5'-primer. In each case, the final products were purified, digested by *Kpn*I and *Sac*I, and inserted between these sites in the pGL3 polylinker. The construct carrying both κB1 and Sp1 mutations was similarly obtained using Sp1-mutated IκBα promoter as template. The promoter constructs carrying internal deletions or insertions were obtained by the ligation of two separately amplified fragments, one digested by *Sac*I, the other by *Kpn*I, to *Kpn*I/*Sac*I-digested pGL3. Thus, Δ8-IκBα plasmid was constructed by ligation of two fragments amplified with 5'-CCCCGCCCCAGAGAAATC-3'/*Sac*I primers and *Kpn*I/5'-GGGGAATTTCCAAGCCAGT-3' primers, in the presence of 0.4SK-pGL3 Luc as template. The +5 and +9-IκBα plasmids were similarly constructed using 5'-<u>TGCAG</u>CTGACCCCGCCCCAGAGAAA-3'/*Sac*I primers (inserted nucleotides are underlined) and *Kpn*I/5'-GCTCGGGGAATTTCCAAGCCA-3' primers and with 5'-<u>GCA</u>

GCATCGCTGACCCCGC CCCAGAGAAA-3'/*Sac*I primers and *Kpn*I/5'-GCTCG GGG AATTTCCAAGCCA-3' primers, respectively. The correct sequences of the constructs presented were confirmed by DNA sequence analysis.

Cell culture and generation of IκBα expressing cell lines. Cell lines inducibly expressing wild type IκBα or IκBα (3C), tTA-IκBα (wt) and tTA-IκBα (3C) respectively, were described elsewhere (26). tTA-3T3 cells were transfected with pREP9-CMVt-IκBα (2N) or (2N + 3C) plasmids. Cells which inducibly expressed IκBα (2N) or (2N + 3C) were selected and maintained in DMEM media containing 10% calf serum, 300 μg/ml Hygromycin B and 400 μg/ml G418 (Gibco BRL). Jurkat cells were transfected with CMVt-rtTA plasmid by the DEAE-dextran method. The precipitated CMVt-rtTA plasmid (15 μg) was resuspended in TS solution (8 mg/ml NaCl, 0.38 mg/ml KCl, 0.1 mg/ml $Na_2HPO_4 \cdot 7H_2O$, 3.0 mg/ml Tris, 0.1 mg/ml $MgCl_2$, 0.1 mg/ml $CaCl_2$, pH 7.4) and subsequently DEAE-dextran (Pharmacia) was added. For transfection, $1x10^7$ cells in exponential phase were washed once in TS, resuspended with the DNA solution and incubated at room temperature for 20 min and then incubated at 37°C for 30 min in 10 ml with RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 10 μg/ml gentamicin (Schering Canada, Pointe Claire, Quebec) and 0.1 mM chloroquine (Sigma Chem. Co.) after which they were centrifuged and resuspended in fresh medium. Cells were selected beginning at 24h after transfection in RPMI 1640 medium containing 10% FBS, 2 mM glutamine, 10 μg/ml gentamicin and 2.5 μg/ml puromycin (Sigma). Resistant cells carrying the CMVt-rtTA plasmid (rtTA-Jurkat cells) were then transfected with CMVt-Neo, CMVt-2N and CMVt-2NΔ4 plasmids by DEAE-dextran. Cells were selected beginning at 24h after transfection for approximately 4 weeks in RPMI containing 10% FBS, 2 mM glutamine, 10 μg/ml gentamicin, 2.5 μg/ml puromycin and 400 μg/ml G418 (Life Technologies, Inc.). Initially, pools of transformants corresponding to rtTA-, rtTA-neo-, rtTA-IκBα-2N-, rtTA-IκBα-2NΔ4

expressing Jurkat cells were analyzed for inducible IκBα expression; subsequently, 6-10 individual clones from each transformant pool were selected for further analysis. To analyze growth kinetics, rtTA-neo-, rtTA-IκBα-2N-, rtTA-IκBα-2NΔ4-expressing Jurkat cells were cultured in the presence of 1 μg/ml Dox for various times at an initial cell density of $1x10^5$cells/ml and then counted every other day. All cell lines grew well in the above medium with doubling times of 50 ± 4h. Values obtained are the average of two experiments.

Analysis of HIV-1 LTR transcription in reporter gene experiments. Jurkat cells or rtTA-TD-IκBα Jurkat cells were transiently transfected by DEAE -dextran method as above with precipitated DNAs (0.5 to 8 μg), representing either HIV-LTR or HIV-enhancer containing CAT reporter plasmids with or without pSVexTat plasmids (wt Tat or R52Q,R53Q Tat mutant) (93). At 32 h after transfection, Jurkat cells were induced with 5 ng/ml TNFα (Boeringer Mannheim). At 16 h after induction, cells were harvested and lysed. rtTA-Neo or rtTA-2NΔ4 were incubated with or without Dox in the presence of 10 ng/ml TNFα (Boeringer Mannheim) or PMA (100ng/ml, ICN)/PHA (1 μg/ml, ICN) at 24 h after transfection and subsequently harvested at 24 h after treatment. Extracts (10 to 400 μg) were assayed for CAT activity during 10 min - 8 h, depending on the experiment. The percent acetylation was determined by ascending thin layer chromatography as previously described (132) and quantified using the BioRad Gelscan Phosphoimager and the Molecular Analyst (BioRad) software program.

Analysis of apoptosis. To identify apoptotic cells, cells were treated with TNFα after 24h culture in the absence or presence of 1 μg/ml Dox, and stained using the TUNEL assay (Boehringer Mannheim) and the Hoescht dye 33258. The mixture was then viewed under UV illumination using a Leica fluorescent microscope. To calculate percent apoptosis, a minimum of 200-400 cells were counted. Apoptosis was also analyzed by

DNA fragmentation assay. A total of $3X10^6$ cells were collected, resuspended in 0.25 ml TBE containing 0.25 % NP-40 and 0.1 mg/ml RNAse A and incubated for 30 min at 37°C. Extracts were then treated with 1 mg/ml proteinase K for 30 min at 37°C. DNA preparations (30 µl) were loaded on 1.8 % agarose gel; DNA fragmentation was visualized under UV light.

Western blot analysis. Cells were washed with phosphate-buffered saline (PBS) and lysed in Western Lysis Buffer (WLB) containing 10 mM Tris-Cl pH 8.0, 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.5 % Nonidet P-40 (NP-40), 0.5 mM phenylmethysulfonyl fluoride (PMSF), 10 µg/ml leupeptin, 10 µg/ml pepstatin, and 10 µg/ml aprotinin. After incubation on ice for 10 min, protein extracts were collected by centrifugation at 14000 rpm for 15 min at 4°C. Equivalent amounts of whole cell extract (20 µg) were subject to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in a 10% or 15 % polyacrylamide gel. After electrophoresis, the proteins were transferred to Hybond transfer membrane (Amersham Pharmacia Biotech) in a buffer containing 30 mM Tris, 200 mM glycine and 20% methanol for 1 h. The membrane was blocked by incubation in PBS containing 5 % dried milk for 1h and then incubated overnight with anti-IκBα MAD10B (187), -p24 (ID Laboratories), -actin (Sigma) monoclonal or anti-p65 (328) polyclonal antibodies in 5 % milk/PBS, at dilutions of 1:500 or 1:1000. These incubations were done at 4°C overnight. After four 10min washes with PBS, membranes were incubated with a peroxidase-conjugated secondary goat anti-rabbit or anti-mouse antibody (Amersham Pharmacia Biotech) at a dilution of 1:1000. The reaction was then visualized with the enhanced chemiluminescence detection system (ECL) as recommended by the manufacturer (Amersham Pharmacia Biotech or NEN™ Life Science, Boston, MA).

Immunoprecipitation. rtTA-Neo and rtTA-2NΔ4 Jurkat cells were induced TNFα (10 ng/ml) and PMA (50 ng/ml) for various times in the presence or absence of Dox and lysed in TNN buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.5 % NP-40, 2 mM PMSF, 5 μg/ml Leupeptin, 5 μg/ml Pepstatin, 0.5 mM Spermidine, 0.15 mM Spermine and 5 μg/ml Aprotinin). Cell lysates (500 μg) were precleared with preimmune sera. Precleared lysates were incubated with 10 μl of p65 antibody (328) or IκBα antibody and 30 μl of protein A-Sepharose beads (Pharmacia Biotech, Upsalla, Sweden) for 1 h at 4°C. Beads were washed five times with TNN buffer and the immunoprecipitates were eluted by boiling the beads 5 min in SDS loading dye. Eluted proteins were electrophoresed on 10 % SDS-polyacrylamide gel electrophoresis and detected by using anti-goat p65 antibody (SantaCruz Biotechnology, Santa Cruz, CA) and MAD10B IκBα antibody.

Nuclear extracts. Cells were washed in Buffer A [10 mM HEPES, pH 7.9; 1.5 mM $MgCl_2$; 10 mM KCl; 0.5 mM dithiothreitol (DTT); and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)] and were resuspended in Buffer A containing 0.1 % NP-40. Cells were then chilled on ice for 10 min before centrifugation at 14000 rpm. Pellets were then resuspended in Buffer B (20 mM HEPES, pH 7.9; 25 % glycerol; 0.42 M NaCl; 1.5 mM $MgCl_2$; 0.2 mM EDTA; 0.5 mM DTT; 0.5 mM PMSF; 5 μg/ml leupeptin; 5 μg/ml pepstatin; 0.5 mM spermidine; 0.15 mM spermine; and 5 μg/ml aprotinin). Samples were incubated on ice for 15 min before being centrifuged at 14000 rpm. Nuclear extract supernatants were diluted with Buffer C (20 mM HEPES, pH 7.9; 20% glycerol; 0.2 mM EDTA; 50 mM KCl; 0.5 mM DTT; and 0.5 mM PMSF).

Electrophoretic mobility Shift Assay (EMSA) Nuclear extracts were incubated for 20 min at room temperature with ^{32}P-labeled probes corresponding to the PRDII region of the IFN-β promoter and κB1, Sp1, κB1/Sp1 sites from IκBα promoter either in NF-κB DNA binding buffer (20 mM HEPES, pH 7.9, 5 % glycerol, 0.1 M KCl, 0.2 mM EDTA,

pH 8.0, 0.2 mM EGTA, pH 8.0, 1 mM dithiothreitol) or in NF-κB/Sp1 DNA binding buffer (20 mM HEPES, pH 7.9, 100 mM KCl, 20 % Glycerol, 0.1 mM EDTA, 0.25 mM $ZnSO_4$, 0.05 % NP-40, 1mM dithiothreitol), together with 5 or 0.5 μg of poly(dI-dC) (Amersham Pharmacia Biotech), respectively. Oligonucleotides used are as follow: PRDII: 5'-GGGAAATTCCGGGAAATTCC-3'; κB1: 5'-GATCTTGGAAATTCCCCGA-3'; Sp1: 5'-TCGAGACCCCGCCCCAG-3'; consensus Sp1: 5'-ATTCGATCGGGGCGG GGCGAG C-3'; mutated Sp1: 5'-ATTCGATCGGTTCGGGGCGAGC-3'; κB1/Sp1: 5'-T CGATTGGAAATTCCCCGGCCTGACCCCGCCCCAG-3'; mutκB1/Sp1: 5'-TCGATTG TCAATTCCCCGAGCCTGACCCCGCCCCAG-3'; κB1/mutSp1: 5'-TCGATTGGAAAT TCCCCGAGCCTGACCAAGCCCCAG-3'; +5 κB1/Sp1: 5'-TCGATTGGAAATTCCCC GAGCTGCAGCTGACCCCGCCCCAG-3'. Underlining delineates the Sp1 site, and bold face letters indicate mutations in either κB1 or Sp1. Recombinant proteins (GST-NF-κB fusion proteins (251, 333) and Sp1(Promega Inc.) were also incubated with the probes in a different DNA binding buffer (10 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM DTT, 0.1 mg/ml BSA, 50 μM $MgCl_2$, 1 mM ATP, 5 μg/ml poly dI-dC). The resulting protein-DNA complexes were resolved by 5-6 % Tris-glycine or Tris-borate-EDTA gels and exposed to X-ray film. To demonstrate the specificity of protein-DNA complex formation, 125-fold molar excess of unlabeled oligonucleotide was added to the nuclear extract before adding labeled probe. Supershift analysis was performed by incubating anti-p65, anti-p50, anti-c-Rel and anti-Sp1 antibodies (Santa Cruz Biotechnology Inc) with nuclear extracts for 20 min at room temperature.

RT-PCR analysis of HIV RNA. Total RNA was isolated from rtTA-Neo and rtTA-2NΔ4 Jurkat cells infected with HIV in the presence or absence of Dox using RNeasy Mini Kit (QIAGEN) and treated with 1U RNase free DNase (RQ1 DNase; Promega Biotec, Madison, WI) for 30 min at 37°C, phenol:chloroform:iso-amylalcohol extracted, ethanol precipitated. RT was performed on 2 μg of RNA and 0.2 pmol random hexamers

using 200 U of MMLV reverse transcriptase (BRL Burlington, Ont.) in buffer containing 50 mM Tris HCl, pH 8.3, 75 mM KCl, 10 mM dithiothreitol, 3 mM $MgCl_2$, 500 nM dNTP, 0.1 mg/ml BSA, 272.5 U/ml RNase Inhibitor (Pharmacia Biotech). PCR assays were performed using 7 µl of RT product, in PCR buffer containing 100 mM Tris HCl, pH 8.4, 500 mM KCl, 15 mM $MgCl_2$, 200 mM dNTP, 15 pmol γ-^{32}P labeled primers, and 1.25 U Taq DNA polymerase (Pharmacia Biotech). Nucleotide sequences of primers used are as follows : M667, 5'-GGCTAACTAGGGAACCCACTG-3'; M668, 5'-CAGGT CCCTGTTCGGGCGCC-3'; LA45, 5'-GCCTTAGGCATCTCCTATGGC-3'; LA41, 5'-T GTCGGGTCCCCTCGTTGCTGG-3'; M669, 5'-GTGTGCCCGTCTGTTGTGTGACTC TGGTAAC-3';LA23, 5'- GCCTATTCTGCTATGTCGACACCC-3'. The PCR reaction mixture was subjected to 24-30 cycles of denaturation for 1 min at 95°C, annealing for 2 min at 61°C and polymerization for 2 min at 72°C. PCR products were then ethanol precipitated and analyzed on a 6 % denaturing polyacrylamide gel. Primers for glyceraldehyde phosphate dehydrogenase (GAPDH) were used as previously described (88) to normalize all reactions.

RNAse Protection Assay. A 221 bp XbaI-PstI fragment was obtained by PCR amplification with IκBα cDNA clone (cloned into pSVK3) using specific primers containing restriction enzyme sites corresponding to positions 824-839 (5'ATCATCTAGAAACAGAGTTACCTACC3') and 1030-1045 (5'ATCACTGCAGTA ACGTCAGACGCTGG3'); the XbaI-PstI fragment of the PCR product was cloned into the XbaI-PstI site of the pDP18-T7/T3 transcription vector (Ambion, Austin, TX) to generate pDP18CU-/CTERM. ^{32}P-labeled antisense RNA probe was transcribed by using *In Vitro* Transcription Kit (Pharmingen, San Diego, CA) and RNase protection carried out using RPA Kit (Parmingen). A β-actin antisense probe (pTRI-β-actin; Ambion, Inc) was synthesized by the same protocol and used in the same reaction with IκBα probe. 5-10 µg of total RNA extracted using RNeasy Mini Kit (QIAGEN, Valencia,

CA) from unstimulated or stimulated rtTA-Neo or rtTA-2NΔ4 Jurkat cells was used. The resulting protected RNAs were resolved by 5 % denaturing gel and exposed to X-ray film.

Analysis of HIV-1 protein and RNA synthesis in a single cycle infection model. Cos-1 cells were transfected with 10 μg of HIV-1 proviral DNA (pSVC21 BH10) and 1, 5 or 10 μg of IκBα expressing plasmid : either SVK3-IκBα -IκBα (2N), -IκBα (3C), or -IκBα (2N + 3C). In all experiments, the total amount of DNA transfected was completed to 20 μg with unrelated DNA (pUC8). DNA was introduced in cells by lipofection (Lipofectamine^{TM}) according to manufacturer's instructions (Promega inc.). Three days after transfection, the media and the cells were collected. The relative amount of virion protein p24 present in the media was determined by ELISA (357). Proteins were extracted from a portion of the collected cells by resuspending them in WLB buffer. Proteins were analyzed by immunoblotting as described above using IκBα monoclonal antibody, human sera from a HIV-1 seropositive individual or actin monoclonal antibody (ICN). Total RNA was extracted from the remaining cells using an RNeasy kit (Qiagen) according to manufacturer's instructions. RNA was subjected to Northern blot analysis using ^{32}P labeled, random-primed HIV-1 proviral DNA or β–actin cDNA probes (see below).

Northern blot analysis. Total RNA (10-20 μg) was electrophoresed in a 1.2 % denaturing formaldehyde agarose gel and transferred to a nylon membrane (Hybond-N; Amersham) with a Vacu-Gene blotting system (Pharmacia). RNA was cross-linked to the membrane in a UV Stratalinker 2400 (Stratagene) and prehybridized overnight in Prehybridization solution: 25 mM KPO_4 pH 7.4, 5X SSC (750 mM NaCl, 75 mM sodium citrate pH 7.0), 50% deionized formamide, 50 μg/ml salmon sperm DNA (Boehringer Mannheim) and 5X Denhardt's solution containing 0.5 % (w/v) Ficoll 400 (Sigma), 0.5

% (w/v) polyvinylpyrrolidine, and 0.5 % (w/v) BSA at 42°C in a hybridization oven. The HIV-1 proviral cDNA probes were the 2 and 2.2 kbp Hind-III fragments derived from pSVC21 BH10. The β-actin cDNA used as probes was the 1 kbp Pst-1 fragments derived from pβ–actin plasmid. Probes were labeled to approximately 1 x 10^9 cpm/μg with the Oligolabeling kit (Pharmacia) and [α–^{32}P]CTP. Hybridization occurred overnight at 42-50°C in Prehybridization solution supplemented with 10 % (w/v) dextran sulfate (Pharmacia) and approximately 50 million cpm of labeled probe. The blots were washed, wrapped in plastic and exposed to X-OMAT film (Kodak) at -80°C.

Analysis of HIV-1 multiplication. rtTA-neo, rtTA-IκBα-2N, rtTA-IκBα-2NΔ4 expressing Jurkat cells after preincubation with or without Dox for 24 h were infected with HIV-IIIB, derived from the HXB2D molecular clone of HIV-1 (117) in serum-free medium for 2 h at a MOI of 0.01 pfu/ml and then grown in complete medium for 36 days. Cell supernatants (precleared by centrifugation at 3000 rpm for 30 min at 4°C) were collected every four days and analyzed for virus reverse transcriptase (RT) activity as previously described (233). The relative amount of virion protein p24 present in the media was determined by ELISA (357). Proteins were extracted from a portion of the collected cells by resuspending them in WLB. Proteins were analyzed by immunoblotting as described above using IκBα MAD10B monoclonal antibody, p24 specific antibody (ID Labs) or actin monoclonal antibody (ICN).

Reverse transcriptase analysis. Cell culture supernatants were clarified by centrifugation at 3000 RPM for 30 min. A 50 μl aliquot of the supernatant was incubated in 50 mM Tris-HCl pH 7.9, 5 mM $MgCl_2$, 150 mM KCl, 0.05% Triton X-100, 0.3 mM glutathione (reduced) 0.5 mM [Ethylenebis(oxyethylenenitrilo)]-tetraacetic acid (EGTA). 50 μg/ml poly rAdT (Pharmacia), and 10 mCi tritiated thymidine triphosphate (^{3}H-TTP; ICN Biomedicals) for 22 h at 30°C. Reverse transcription was stopped by adding 2 ml of

0.01M sodium pyrophosphate in 1 N HCl, 2 ml of ice cold 10% trichloroacetic acid (TCA) and placing the tubes on ice. The resulting precipitates were then collected on Whatman GF/C filters and washed several times with cold 5 % TCA and once with 70 % ethanol. Filters were air dried, then counted by liquid scintillation fluorography.

***In vivo* genomic footprinting.** Jurkat cells (10^8) were harvested and resuspended in 1 ml of RPMI 10 % FBS containing 20 mM HEPES (pH 7.3). The methylation reaction was performed in presence of 10 µl of concentrated dimethyl sulfate (Aldrich Chemical Company, Milwaukee, USA) for 1 min. The reaction was then quenched by 2 washes in cold PBS containing 2 % β-mercaptoethanol. Genomic DNA extraction was performed as previously described (3). Briefly, cells were lysed in 2 ml of Tris buffer pH 7.5 containing 10 mM NaCl, 10 mM EDTA and supplemented with 100 µl of Proteinase K (20 mg/ml), 100 µl SDS (20 %), 100 µl NP40 (10 %) and incubated at 50°C overnight. Proteins were precipitated by adding 1.2 ml of NaCl 5M and centrifuged 40 minutes at 8000 rpm. Cleared supernatant was ethanol precipitated to obtain genomic DNA; the pellet was resuspended in 200 µl H_20 with 20 µl of piperidine (Aldrich Chemical Company) and incubated 30 minutes at 90°C to provide cleavage of methylated G (or A) residues. The DNA control (Naked DNA) was first extracted from cells, then submitted to DMS treatment and subsequently to piperidine cleavage to allow methylation and cleavage to all G residues of the sequence. For each sample, 2 µg of DNA were submitted to LM-PCR using Vent DNA Polymerase (New England Biolabs, Mississauga, USA) as described (133, 291). PCR amplification step was 2 minutes for the first cycle and was progressively increased to 10 minutes in the last cycle. A total of 18 cycles were performed for DNA amplification. The third primer was radiolabeled by end-labeling using T4 polynucleotide kinase (Pharmacia Biotech) and [γ^{32}P]ATP (ICN). Two more PCR cycles were performed to radiolabeled elongated DNA. The final labeled PCR product was analyzed on a 5 % Hydrolink™ Long Ranger sequencing gel (Baker,

Phillipsburg, USA) in 0.6X TBE at 65 W, and exposed from 12 to 36 hours with a BioMax sensitive film (Kodak, New-York, USA). For the LM-PCR, several set of oligonucleotides were used (see Fig 22C):

For the non-coding strand:

primer 1: 5'- CTCATCGCAGGGAGTTTCT -3', T_m 55°C

primer 2: 5'- CCCAGCTCAGGGTTTAGGCTTCTTT -3', T_m 63°C

primer 3: 5'- GGGTTTAGGCTTCTTTTTCCCCCTAGCAG -3', T_m 66°C

For the coding strand:

primer 1B: 5'- ACTGCTGTGGGCTCTGCA -3', T_m 63°C

primer 2B: 5'- TAAACGCTGGCTGGGGATTTCTCTG -3', T_m 63°C

primer 3B : 5'- TGGGGATTTCTCTGGGGCGGGGTCAGGCT -3'. T_m 71°C

Luciferase Assay. Jurkat T cells were transiently transfected by the DEAE-dextran method with 1 μg of 0.4SK/pGL3 (wild type IκBα promoter) luciferase reporter plasmids or mutant 0.4SK/pGL3 (mutκB1, mutSp1 and mutκB1/Sp1) along with pRL-TK (for transfection normalization, Promega Inc.). At 30h after transfection, cells were induced with TNFα or PMA/PHA. At 16h after induction, cells were harvested, lysed by 1X Passive Lysis Buffer and then luciferase activity was analyzed by Dual-Luciferase™ Reporter Assay System (Promega Inc.) as specified by the manufacturer. The background obtained from mock transfected cells was subtracted from each experimental value. The experiments were performed in triplicate in 24-well plates, and the average fold induction was calculated.

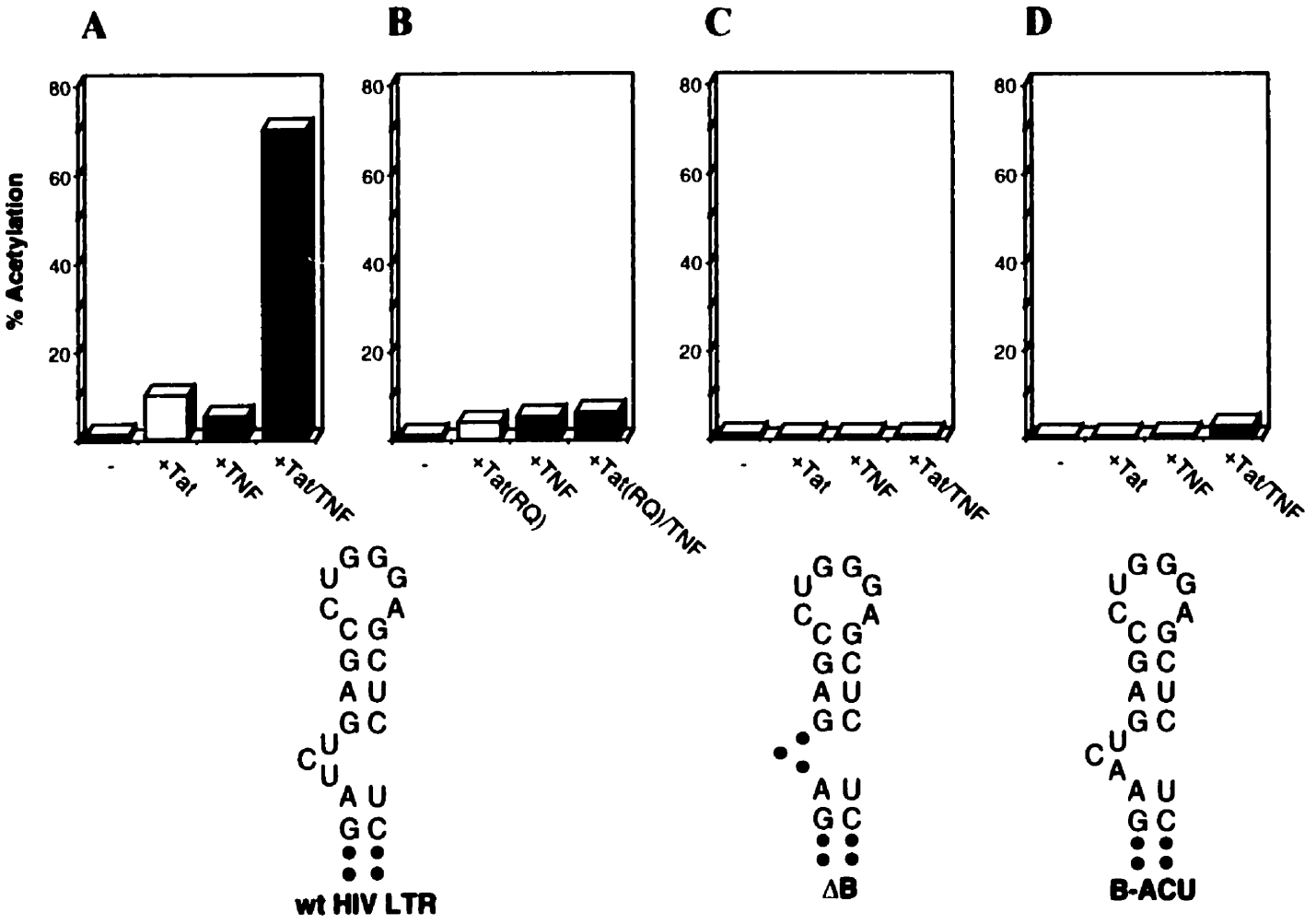
CHAPTER III

TRANSDOMINANT MUTANTS OF IκBα BLOCK TAT-TNFα SYNERGISTIC ACTIVATION OF HIV-1 GENE EXPRESSION AND VIRUS MULTIPLICATION

Experiments using HIV-1 LTR reporter constructs in lymphoid cells showed that mutation of NF-κB motifs reduced gene expression in the presence and absence of the HIV-1 Tat protein (1, 12, 37, 38, 118). This prompted us to analyze the effect of transdominant mutant IκBα (TD-IκBα) on the Tat-TNFα synergistic activation of the HIV-1 LTR and HIV-1 multiplication in a single cycle infection model.

Tat-TNFα Synergism requires Tat binding to the TAR element. To determine the specific conditions required for Tat-TNFα synergistic activation of HIV-1 LTR driven reporter constructs in Jurkat T cells, titration of wt Tat expression plasmid and/or recombinant TNFα was initially performed (data not shown). In subsequent experiments, Tat plasmid was used at a concentration of 2 μg, while TNFα was used at a concentration of 5 ng/ml. Tat stimulated the HIV-1 LTR CAT reporter plasmid approximately 10-fold while TNFα treatment alone induced reporter gene expression about 5-fold (Figure 7A). Addition of both activators produced a true synergistic stimulation of the HIV-1 LTR, resulting in a 50-70 fold induction of gene expression (Figure 7A). Previous studies demonstrated that efficient transactivation of HIV-1 gene expression by Tat required physical interaction between Tat and the TAR element (93). The R52Q,R53Q point mutations of the HIV-1 Tat protein abrogated Tat binding to the TAR element and gene transactivation. To test whether the Tat-TNFα synergism defined in the above model system required Tat-TAR association, the R52Q,R53Q mutant of Tat (designated TatRQ) was used together with TNFα to stimulate expression of the HIV-1 LTR driven CAT reporter (Figure 7B). The combination of TNFα treatment and TatRQ co-expression was only weakly effective (5-fold induction) in mediating activation of the HIV-1 LTR (Figure 7B), compared to wt Tat plus TNFα (Figure 7A). Similarly, mutations within the TAR element (ΔB and ΔB-ACU) that altered the Tat protein binding site (93, 366) also were not activated by Tat-TNFα treatment (Figure 7C and 7D). These results thus reflect

Figure 7. Tat-TNFα activation of the HIV LTR requires Tat binding to the TAR element. Jurkat T cells were co-transfected with 5μg of ptzIIICAT (A and B) and 2μg of pSVexTat (A) or Tat (R52Q,R53Q) (B) expression plasmids; cells were also transfected with 2μg of LTR-ΔB (C) or LTR-ΔB-ACU (D) and 2μg of pSVexTat. At 32h after transfection, cells were incubated for an additional 16h in the presence or absence of TNFα. CAT activities were assayed using whole cell extracts (50μg for 30min). Cells transfected only with ptzIIICAT in the absence of activators were used as negative control. The results are the average of three experiments.


A
B
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D
% Acetylation
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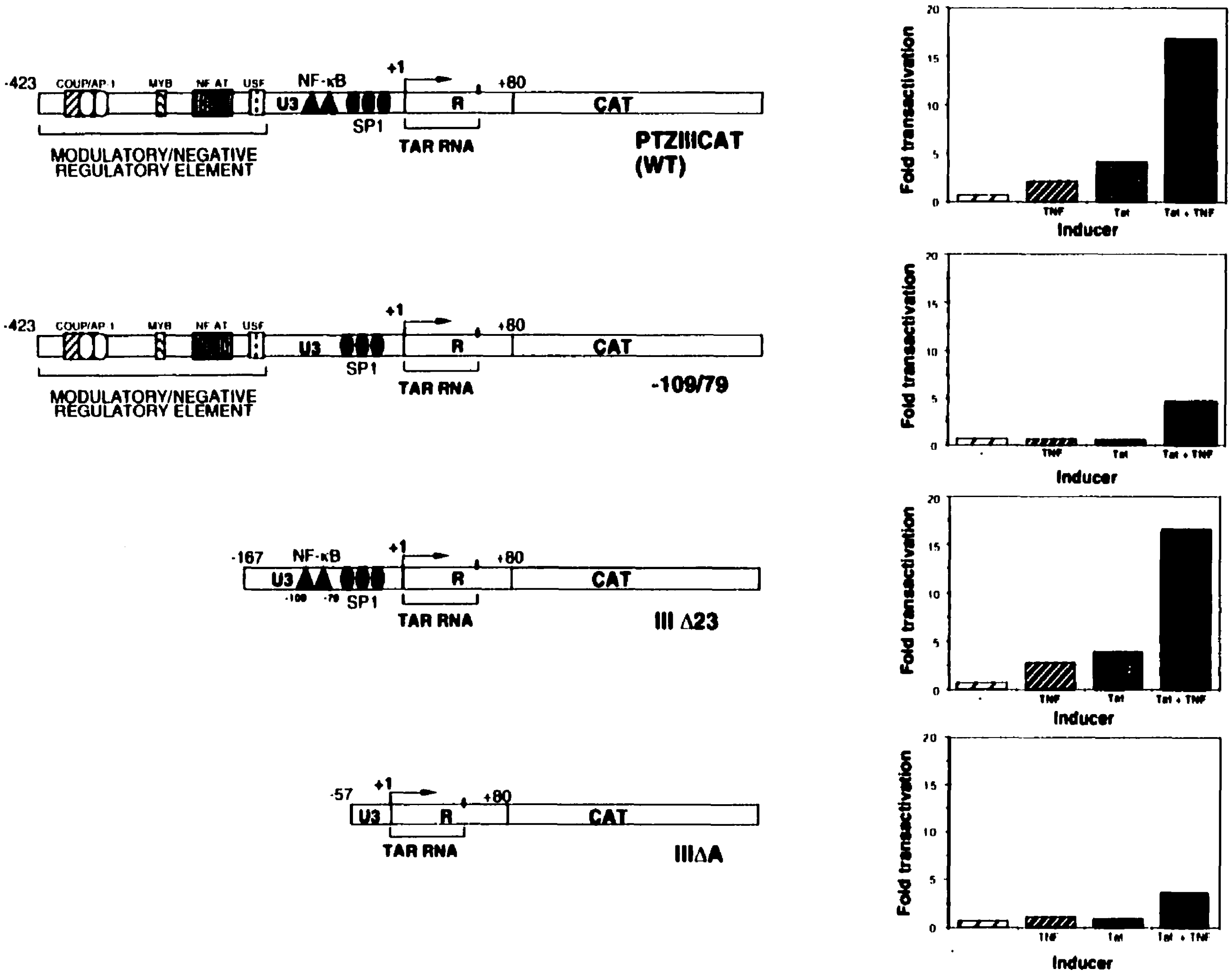
-
+Tat
+TNF
+Tat/TNF
+Tat(RQ)
+Tat(RQ)/TNF
wt HIV LTR
ΔB
B-ACU

a requirement for Tat-TAR interactions in the synergistic transactivation of the HIV-1 LTR.

Tat-TNFα Synergism Requires Intact NF-κB Sites. To characterize the region of the HIV-1 LTR involved in synergistic activation, HIV-1 LTR deletion mutants were transfected into Jurkat cells and stimulated with Tat, TNFα or Tat plus TNFα. As expected, the intact LTR-CAT construct (plasmid ptzIIICAT) was strongly inducible by both activators (Figure 8), with an 18 fold induction with Tat-TNFα. In contrast, the -109/-79 plasmid, a construct lacking the NF-κB sites, had only baseline level of activity and was not transactivated by TNFα. Surprisingly, this construct was not inducible by wt Tat expression plasmid, even though it contained an intact TAR element. Nevertheless, the combination of Tat and TNFα was able to stimulate the -109/-79 plasmid about 5-fold. The IIIΔ23 construct which was deleted for the upstream modulatory sequences of the HIV-1 LTR (upstream of -167) was stimulated by both activators as efficiently as the wild LTR-CAT plasmid, indicating that the upstream elements in the -423 to -167 domain of the LTR did not play a complementary role in Tat-TNFα activation. The plasmid IIIΔA was deleted for all of the LTR sequences upstream of -57 and was not activated significantly by either inducer alone, although a residual 4-fold induction of the IIIΔA plasmid was observed with Tat-TNFα. This experiment indicates that strong synergistic activation of the HIV-1 LTR by the combination of Tat and TNFα required intact NF-κB sites. The fact that some activation could occur in the absence of the enhancer element (in constructs -109/79 and IIIA) suggests that TNFα may be able to potentiate Tat activity at the TAR element independently of the NF-κB sites.

Stability of the IκBα Mutants. Recent experiments have defined specific sites of inducer mediated and constitutive phosphorylation in the IκBα regulatory protein (summarized in Figure 2). In particular, mutation of the N-terminal phosphorylation sites

Figure 8. Maximum Tat-TNFα synergism requires intact NF-κB sites. Jurkat T cells were co-transfected with 5μg of ptzIIICAT, -109/-79, IIIΔ23, or IIIΔA in the absence or presence of pSVexTat (2μg). TNFα was added at 32h after transfection and incubated for an additional 16h. Whole cell extracts were prepared, normalized for total protein, and assayed for CAT activity (50μg for 30min).



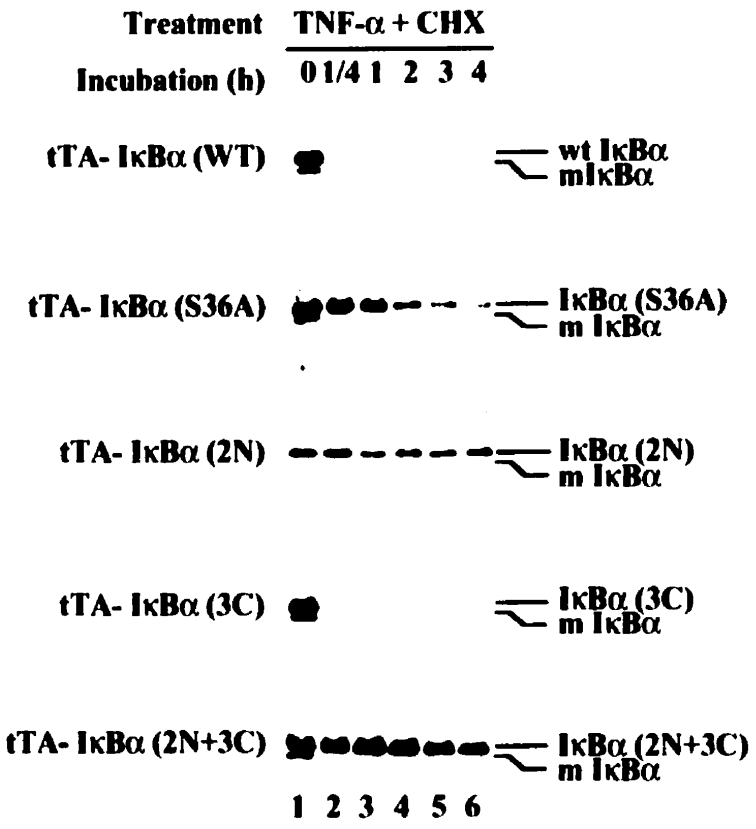
-423
COUP/AP-1
MYB
NF AT
USF
NF-κB
U3
SP1
+1
+80
R
CAT
TAR RNA
MODULATORY/NEGATIVE REGULATORY ELEMENT
PTZIIICAT (WT)
-109/79
-167
-100
-70
III Δ23
-57
IIIΔA
Fold transactivation
0
5
10
15
20
TNF
Tat
Tat + TNF
Inducer

at Ser-32 and Ser-36 in the signal response domain of IκBα prevented inducer mediated phosphorylation and subsequent proteasome-dependent degradation of IκBα (53, 54, 72). Also, triple point mutation of IκBα in the C-terminal residues S283, T291 and T299 abrogated constitutive phosphorylation *in vivo* by casein kinase II and increased the intrinsic stability of IκBα, but did not affect inducer mediated degradation of IκBα (250). IκBα expression plasmids were generated that produced IκBα proteins singly mutated in Ser-32, Ser-36, in both S32 and S36 [IκBα (2N)], in the three C-terminal sites [IκBα (3C)] and in all five regulatory phosphorylation sites [IκBα (2N+3C)]. The inducer mediated turnover of these proteins was analyzed in stably transfected NIH 3T3 cells (26, 250) at different times after treatment with cycloheximide and TNFα (Figure 9A). Immunoblot analysis using an IκBα specific antibody was able to distinguish between the endogenous murine IκBα and the exogenously expressed human IκBα (Figure 9A, lane 1). Cycloheximide was added to eliminate the complicating effects of *de novo* synthesis of IκBα after induction. The endogenous murine IκBα, human wt IκBα and IκBα (3C) all degraded rapidly in response to TNFα addition with a T1/2 of much less than 15 min (Figure 9A, lanes 2-6), indicating that triple mutation of S283, T291 and T299 did not affect inducer mediated degradation of IκBα. In contrast, both S36A (Figure 9A) and S32A mutations (data not shown) stabilized IκBα; both point mutations increased the T1/2 of IκBα to approximately 90-120 min (Figure 9A, lanes 2-6). Double point mutated IκBα (2N) and IκBα (2N+3C) were extremely stable in the presence of TNFα and cycloheximide, with a T1/2 of greater than 4h (Figure 9B). This experiment demonstrates the increased stability of point mutated IκBα molecules and suggests that both IκBα (2N) and IκBα (2N+3C) should be stable transdominant mutants of the NF-κB response.

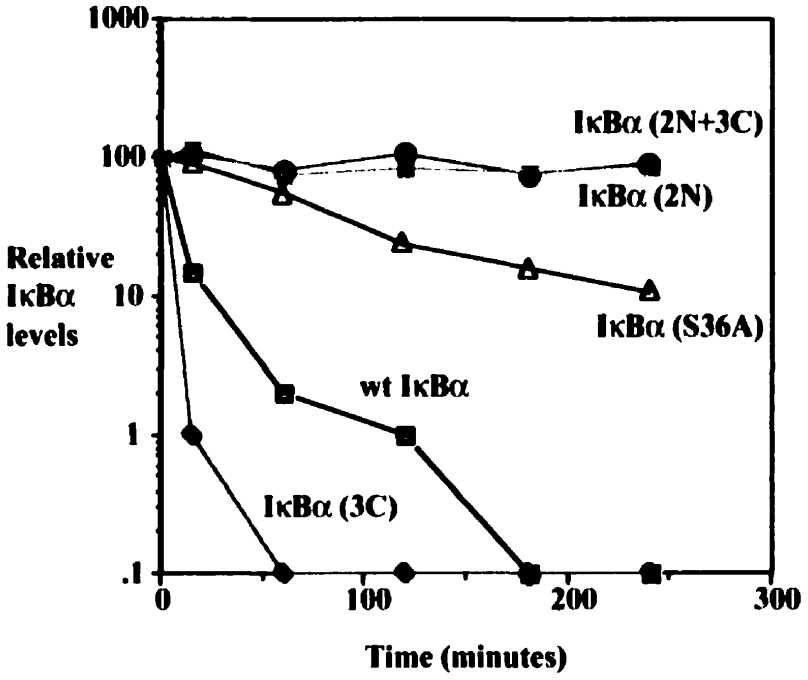
Inhibition of Tat-TNFα Activation of the HIV-1 LTR by IκBα Transdominant Mutants. To examine the effect of wt and mutated forms of IκBα on Tat-TNFα synergistic activation of HIV-1 LTR directed gene expression, the different forms of

Figure 9. Inducer mediated degradation of IκBα. tTA-IκBα (wt), tTA-IκBα (2N), tTA-IκBα (S36A), tTA-IκBα (3C) and tTA-IκBα (2N+3C) cells were treated with TNFα (5ng/ml) and cycloheximide (50μg/ml) for 0 (lane 1), 15 minutes (lane 2), 1h (lane 3), 2h (lane 4), 3h (lane 5) or 4 h (lane 6). (A) Endogenous murine and exogenous human IκBα were detected in whole cell extracts (15 μg) by immunoblotting using affinity purified AR20 antibody. (B) Levels of IκBα were quantified by laser densitometry and represented graphically.

A


Treatment
TNF-α + CHX
Incubation (h)
0 1/4 1 2 3 4
tTA- IκBα (WT)
wt IκBα
mIκBα
tTA- IκBα (S36A)
IκBα (S36A)
m IκBα
tTA- IκBα (2N)
IκBα (2N)
m IκBα
tTA- IκBα (3C)
IκBα (3C)
m IκBα
tTA- IκBα (2N+3C)
IκBα (2N+3C)
m IκBα
1 2 3 4 5 6

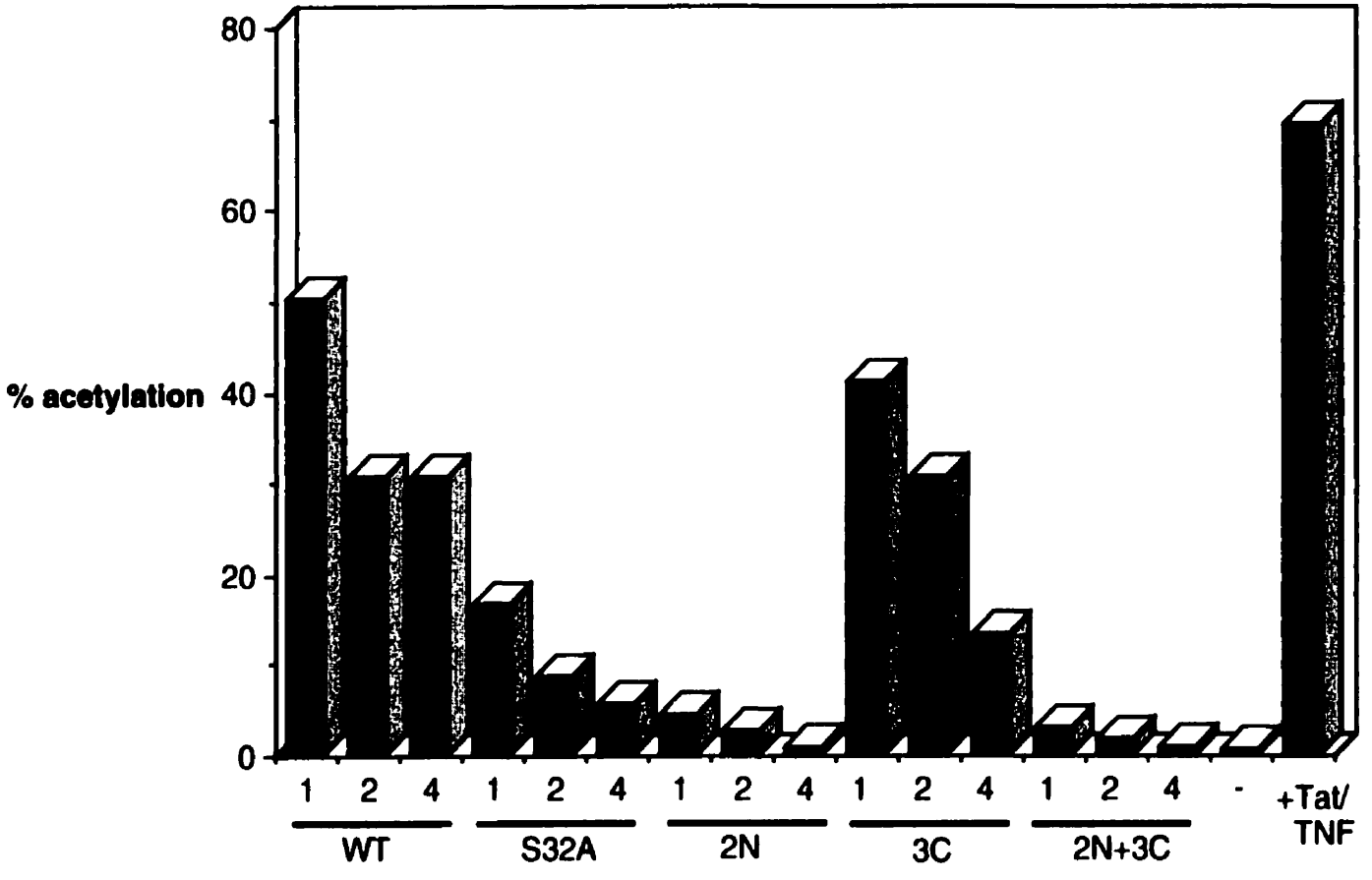
B


1000
100
10
1
.1
Relative IκBα levels
IκBα (2N+3C)
IκBα (2N)
IκBα (S36A)
wt IκBα
IκBα (3C)
0
100
200
300
Time (minutes)

IκBα were co-transfected into Jurkat cells together with the reporter construct and the wt Tat expression plasmid. Expression of both wild type IκBα and IκBα (3C) reduced Tat-TNFα activation in a dose dependent manner from a level of about 70-fold transactivation to 15-30 fold induction (Figure 10). However, neither wt IκBα nor IκBα (3C) completely inhibited HIV-1 LTR mediated expression. Expression of the S32 mutant of IκBα (or S36) dramatically reduced Tat-TNFα activation to 10-20 fold stimulation. Strikingly, the IκBα (2N) and IκBα (2N+3C) mutants were able to eliminate Tat-TNFα transactivation at low concentrations of inhibitory plasmid; 1 μg of IκBα (2N) or IκBα (2N+3C) reduced LTR-directed reporter gene expression down to only 5-fold stimulation. At higher concentrations, Tat-TNFα transactivation of HIV-1 LTR driven gene expression was completely suppressed by the transdominant IκBα mutants (Figure 10).

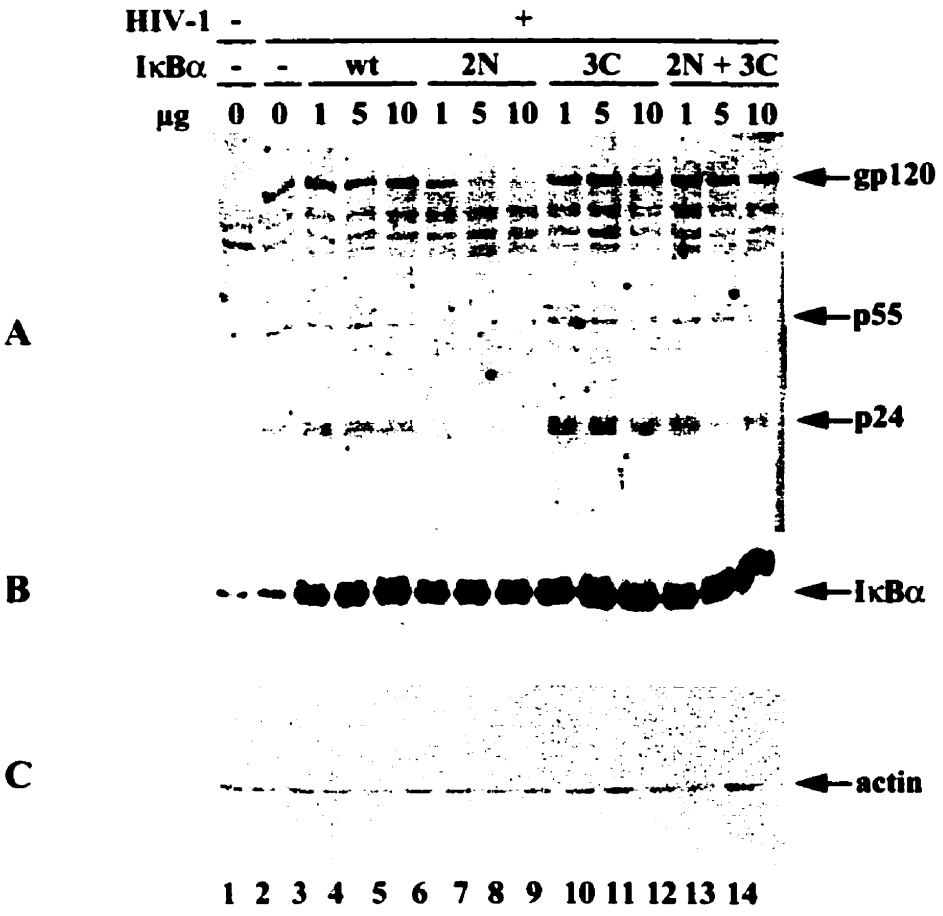
Inhibition of HIV-1 protein and RNA synthesis in a single cycle infection model. To examine the ability of the IκBα mutant proteins to interfere with HIV-1 multiplication, HIV-1 proviral DNA (pSVC21 BH10) was transfected together with the increasing amounts of wt IκBα or mutant IκBα-expressing plasmid into Cos-1 cells. This single cycle infection model permits a single round of virus multiplication and release of infectious HIV-1 but re-infection does not occur because of the absence of the CD4 receptor on Cos-1 cells. At three days post-transfection, high levels of transfected IκBα accumulated in Cos-1 cells (Figure 11B, lane 3-14) compared to the endogenous level of primate IκBα (Figure 11B, lanes 1 and 2). Using a human antiserum that recognizes HIV-1 structural proteins, a dramatic reduction in the amounts of viral specific p24 core antigen, p55 precursor for virion core proteins, and gp120 envelope glycoprotein was observed in cells expressing IκBα (2N) (Figure 11A, lanes 6-8). In other experiments using the single cycle infection model, we found that wt IκBα, IκBα (3C) and IκBα (2N+3C) were also effective in blocking a round of HIV-1 replication, but IκBα (2N) was consistently more effective in a dose dependent manner. Analysis of the intracellular

Figure 10. Transdominant IκBα mutants inhibit Tat-TNFα activation of the HIV LTR. In Jurkat cells, pTZIIICAT (2μg) was co-transfected with pSVexTat and different amounts (1, 2, 4 μg) of pSVK-wt IκBα, pSVK-IκBα (S32A), pSVK-IκBα (2N), pSVK-IκBα (3C) , pSVK-IκBα (2N+3C), and treated with TNFα (5ng/ml) for 16h beginning at 32h post-transfection. The level of HIV-1 LTR driven reporter gene expression was determined by CAT assay on the whole protein extracts of the cells. Positive control (+Tat/TNFα) was obtained by co-transfection with HIV-1 LTR CAT (2μg) and pSVexTat (2μg), and induction with TNFα (5ng/ml) for 16h. The negative control (lane -) was obtained by transfection with the HIV-1 LTR reporter construct only. The results represent the average of three independent experiments.


% acetylation
80
60
40
20
0
1 2 4 1 2 4 1 2 4 1 2 4 1 2 4 - +Tat/ TNF
WT
S32A
2N
3C
2N+3C

accumulation of viral mRNA also confirmed that IκBα and the transdominant negative forms of IκBα differentially inhibited HIV-1 multiplication (Figure 12). Expression of IκBα inhibited HIV-1 proviral transcription in a dose dependent manner (Figure 12A, lanes 3-14). In particular, IκBα(2N) was the strongest inhibitor of HIV-1 transcript accumulation (Figure 12A, lanes 6-8). Complementary results were obtained when the level of p24 antigen release into the supernatant was measured by an enzyme-linked immunosorption assay (ELISA)-based viral antigen capture assay (Figure 11D). Together these results indicate that IκBα(2N) inhibited HIV-1 transcript levels, intracellular viral protein accumulation and release of virions into the supernatant. Surprisingly, additional mutations within the C-terminal phosphorylation sites in IκBα (2N+3C) reduced the inhibitory capacity relative to IκBα(2N) (Figure 11A, lanes 12-14 and Figure 12A, lanes 12-14), suggesting an important role for the intact C-terminal PEST domain in the inhibition of HIV-1 multiplication.

Figure 11. Inhibition of viral protein expression by trans-dominant IκBα mutants. Cos-1 cells were transfected with HIV-1 proviral DNA (pSVC21 BH10: 10 μg) and viral protein expression was inhibited by co-transfecting either 1 μg (lane 3, 6, 9, and 12), 5 μg (lane 4, 7, 10 and 13) or 10 μg (lane 5, 8, 11, and 14) of plasmid expressing wt IκBα (lane 3-5), IκBα (2N) (lane 6-8), IκBα (3C) (lane 9-11) or IκBα (2N+3C) (lane 12-14). Three days after transfection, cells were collected and analysed by immunoblotting for expression of HIV proteins (A), IκBα (B) and β-actin (C). Bands corresponding to the viral envelope glycoprotein gp120, the p55 polyprotein precursor for virion core proteins , and the viral capsid protein p24 are indicated. D) The release of HIV-1 p24 antigen into the supernatant of infected cells was measured by p24 ELISA; the results represent the average of two independent experiments.


HIV-1 - +
IκBα - - wt 2N 3C 2N + 3C
μg 0 0 1 5 10 1 5 10 1 5 10 1 5 10
gp120
p55
p24
A
B
IκBα
C
actin
1 2 3 4 5 6 7 8 9 10 11 12 13 14

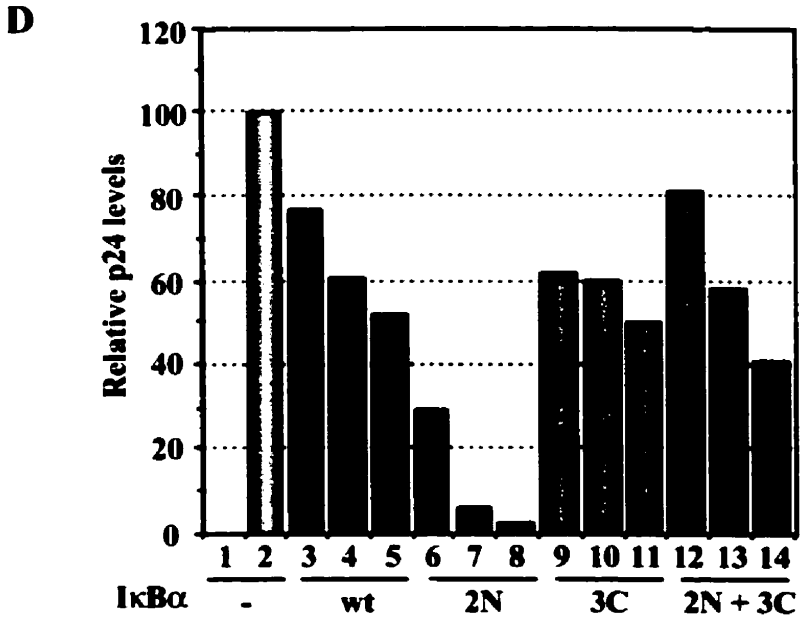
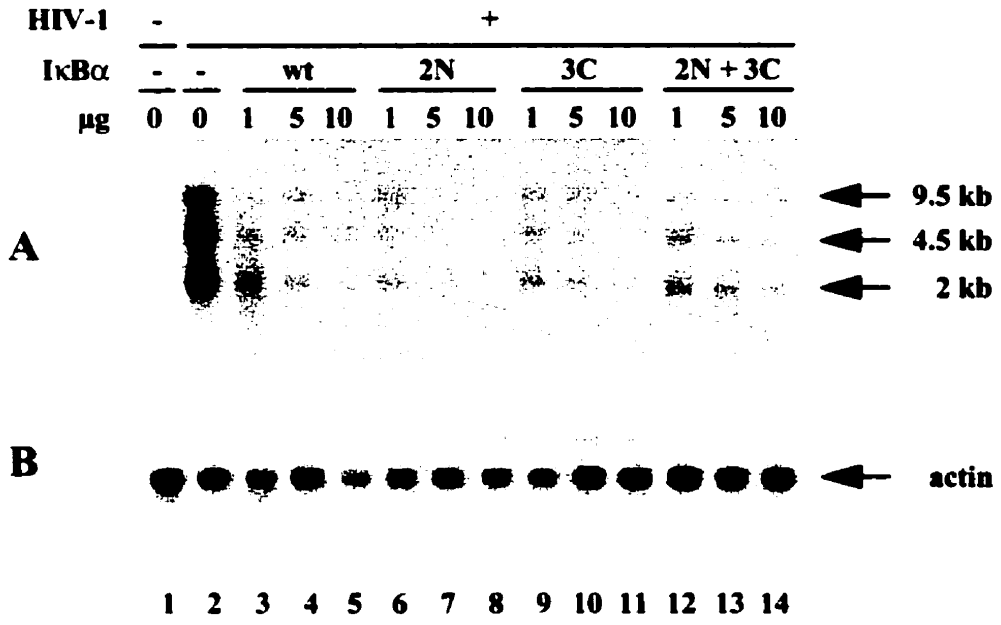

D
Relative p24 levels
120
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14
IκBα - wt 2N 3C 2N + 3C

Figure 12. Inhibition of viral transcription by trans-dominant IκBα mutants. Cos-1 cells were transfected with HIV-1 proviral DNA (pSVC21 BH10: 10 μg) and either 1 μg (lane 3, 6, 9, and 12), 5 μg (lane 4, 7, 10 and 13) or 10 μg (lane 5, 8, 11, and 14) of plasmids expressing wt IκBα (lane 3-5), IκBα (2N) (lane 6-8), IκBα (3C) (lane 9-11) or IκBα (2N+3C) (lane 12-14). Three days after transfection cells were collected and RNA was extracted. (A) Viral transcripts were detected by northern blot analysis; the HIV-1 proviral cDNA probes were the 2 and 2.2 kbp Hind-III fragments derived from pSVC21 BH10. Positions of the 9 kb, 4.5 kb and 2 kb transcripts are indicated. (B) The blot in (A) was stripped and reprobed with a β-actin probe to illustrate the relative amount of RNA present in each lane. The β-actin cDNA used as probe was the 1 kbp Pst-1 fragment derived from pβ–actin plasmid.


HIV-1 - +
IκBα - - wt 2N 3C 2N + 3C
μg 0 0 1 5 10 1 5 10 1 5 10 1 5 10
A
9.5 kb
4.5 kb
2 kb
B
actin
1 2 3 4 5 6 7 8 9 10 11 12 13 14

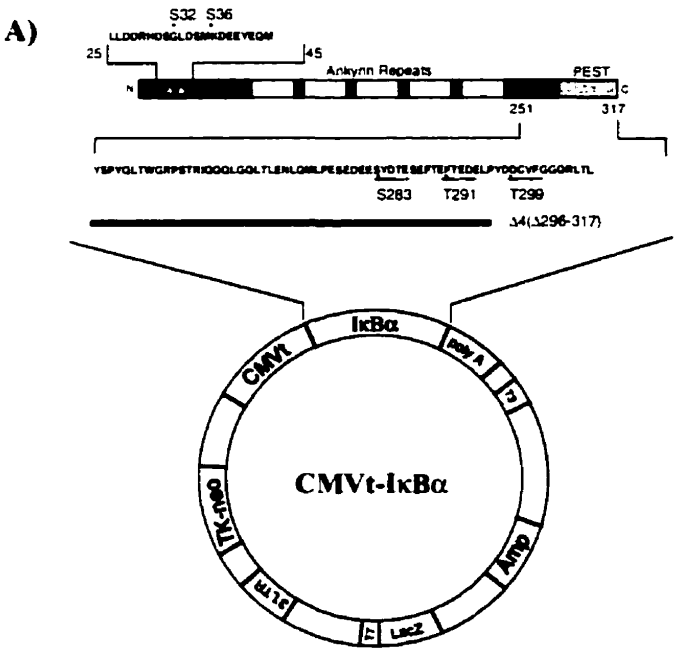
CHAPTER IV

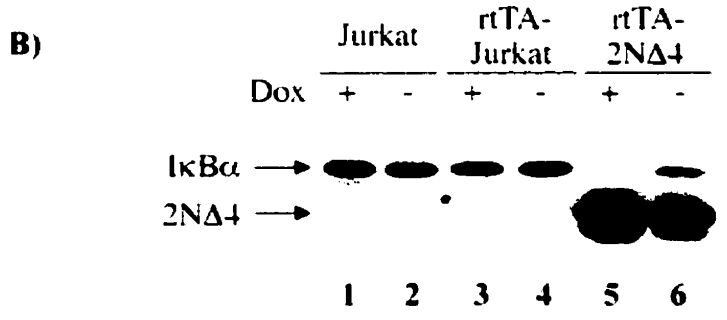
INDUCIBLE EXPRESSION OF IκBα REPRESSOR MUTANTS INTERFERES WITH NF-κB ACTIVITY AND HIV-1 REPLICATION IN JURKAT T CELLS

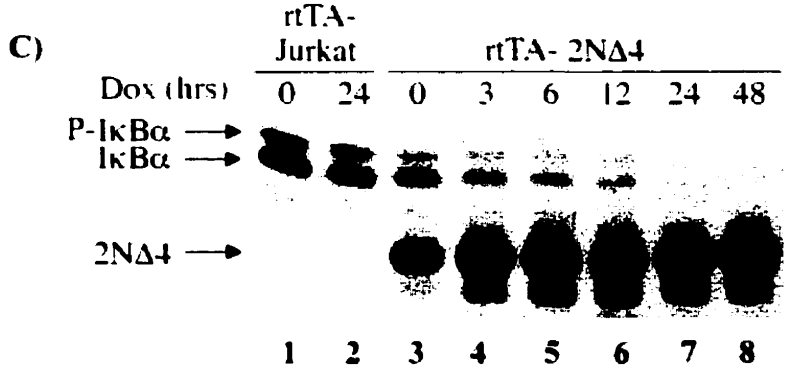
Inhibition of HIV-1 gene expression by transdominant repressors of IκBα (TD-IκBα) in a single cycle infection using COS-1 cells (25) lead us to examine the effect of TD-IκBα on *de novo* HIV-1 infection in Jurkat T cells which are more physiologically relevant. Jurkat T cell lines that inducibly express TD-IκBα were generated and examined for the effect of doxycycline (Dox)-inducible expression of TD-IκBα on NF-κB DNA binding activity, NF-κB dependent gene expression and *de novo* HIV-1 multiplication. The time course of *de novo* HIV-1 infection in TD-IκBα-expressing Jurkat cells was altered by Dox induction of TD-IκBα, resulting in a dramatic transcriptional inhibition of HIV-1 multiplication, as measured by p24 antigen, reverse transcriptase and viral RNA analyses.

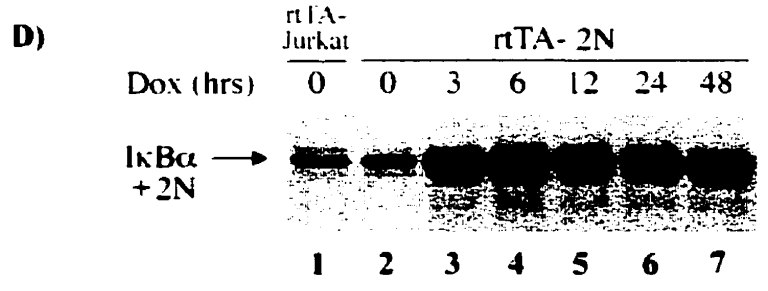
Dox inducible expression of TD-IκBα down regulates endogenous IκBα expression. In order to examine the consequences of overexpression of TD-IκBα on NF-κB dependent gene expression and virus multiplication, Jurkat T cell lines were generated that inducibly expressed two forms of TD-IκBα. One form was IκBα (2N) and the other form of IκBα (2NΔ4) also contained the S32A, S36A mutations like IκBα (2N), as well as a 22 amino acid deletion of the C-terminal portion of IκBα (26, 250), a region of the PEST domain that is dispensable with regard to binding of NF-κB subunits and IκBα degradation (Figure 13A). Starting with Jurkat T cells selected for expression of the reverse tetracycline transactivator protein rtTA (146), expressed under the control of the CMVt autoregulatory promoter (Figure 13A), we isolated pools and clones of rtTA-Jurkat cells expressing IκBα (2N) and IκBα (2NΔ4). The first pool of IκBα-2NΔ4 cells was disappointing because of the high level of leakiness of the transgene (Figure 13B, lane 6), compared to endogenous IκBα (Figure 13B, lanes 1-4). However, analysis of IκBα expression revealed an interesting modulation of endogenous IκBα protein level when IκBα (2NΔ4) was Dox-induced for 24h. The level of endogenous IκBα was decreased about four-fold in Jurkat cells expressing IκBα (2NΔ4) compared to the IκBα levels in either Jurkat or rtTA-Jurkat cells (Figure 13B, lanes 1-4 and lane 6). Dox induction

Figure 13. Schematic summary of the TD-IκBα-expressing CMVt-IκBα vectors and inducibility of TD-IκBα-expressing Jurkat cells. (A) The five ankyrin repeats of IκBα are indicated by the open boxes. Two phosphorylation sites at Ser-32 and Ser-36 (indicated by triangles) in the N-terminal region of IκBα required for inducer mediated degradation of IκBα. Within the highly acidic C-terminal region, several potential casein kinase II (CKII) phosphorylation sites are clustered around S-283, T-291 and T-299. IκBα-2NΔ4 contains a 22aa carboxy terminal deletion of IκBα-2N in which Ser-32 and Ser-36 are mutated to Ala. (B) Jurkat, rtTA-Jurkat and rtTA-IκBα-2NΔ4 Jurkat cells were collected at 24h after doxycycline (Dox) addition and the effect of Dox on IκBα expression was examined by immunoblotting using the IκBα MAD10B antibody. (C) rtTA-IκBα-2NΔ4 cells were incubated with Dox for different intervals (0-48h) and then treated with calpain inhibitor (100μM) for 30 min and subsequently induced with TNF (10ng/ml) for 5 min; expression of IκBα was analyzed by immunoblotting using the IκBα MAD10B antibody. (D) rtTA-IκBα-2N Jurkat cells were harvested after 0 to 48h Dox addition and similarly analyzed by immunoblotting for IκBα expression. Phosphorylated IκBα (P-IκBα), endogenous IκBα (IκBα), and IκBα-2NΔ4 (2NΔ4) are indicated by arrows.


A)
S32 S36
25
45
Ankyrin Repeats
PEST
N
C
251
317
S283
T291
T299
Δ4(Δ296-317)
IκBα
CMVt
Poly A
T7
CMVt-IκBα
Amp
LacZ


B)
Jurkat
rtTA-Jurkat
rtTA-2NΔ4
Dox + - + - + -
IκBα
2NΔ4
1 2 3 4 5 6


C)
rtTA-Jurkat
rtTA- 2NΔ4
Dox (hrs) 0 24 0 3 6 12 24 48
P-IκBα
IκBα
2NΔ4
1 2 3 4 5 6 7 8


D)
rtTA-Jurkat
rtTA- 2N
Dox (hrs) 0 0 3 6 12 24 48
IκBα
+2N
1 2 3 4 5 6 7

resulted in five fold increase in IκBα (2NΔ4) expression after 24h and an almost complete inhibition of endogenous IκBα expression (Figure 13B, lane 5). To characterize this inhibition further, a representative clone of IκBα (2NΔ4) Jurkat cells was Dox induced for 3h-48h and then treated with TNFα (10 ng/ml) for 5 min as an inducer of IκBα phosphorylation, following a 30 min pretreatment with calpain inhibitor I (100 μM) to block inducer mediated degradation of IκBα (24, 26). The different forms of IκBα were resolved by 15% SDS-PAGE; both endogenous IκBα and the slower migrating phosphorylated form of IκBα (P-IκBα) were detected by immunoblotting (Figure 13C, lanes 1 and 2). In the IκBα (2NΔ4) Jurkat clone without Dox addition, the three forms of IκBα were detected (Figure 13C, lane 3); Dox addition for 3 to 6h resulted in a 3-5 fold increase in IκBα (2NΔ4) (Figure 13C, lanes 4-6), and a progressive decrease in the endogenous IκBα forms such that by 24 and 48h after Dox addition, endogenous IκBα was undetectable (Figure 13C, lanes 4-8). Because of the S32A, S36A mutation in IκBα (2NΔ4), this form of IκBα was not phosphorylated as a consequence of TNFα addition (53, 54, 416). Additionally, a clone of IκBα (2N) Jurkat cells was analyzed that demonstrated properties similar to the IκBα (2NΔ4) Jurkat cells, although it was not possible to distinguish the endogenous from the transfected IκBα forms (Figure 13D, lanes 1,2). With this clone, the level of leakiness of the TD-IκBα was significantly lower than that observed with IκBα (2NΔ4), since the amounts of IκBα in rtTA-Jurkat and IκBα (2N) Jurkat in the absence of Dox were similar (Figure 13D, lanes 1,2). Nevertheless, Dox induction resulted in a 20-25 fold increase in the overall level of IκBα in these cells (Figure 13D, lanes 2-7). Based on these results, we have isolated pools and clones of Jurkat cells inducibly expressing TD-IκBα under the control of the Tet-responsive promoter. Furthermore, Dox induction of TD-IκBα resulted in the inhibition of endogenous IκBα, consistent with the fact that the IκBα gene is tightly regulated by an NF-κB dependent mechanism (228).

Growth of TD-IκBα expressing cells and induction of apoptosis. All stably transfected TD-IκBα expressing clones grew well in 10% serum and growth was not dramatically retarded as a consequence of Dox addition or in the case of IκBα (2N) or IκBα (2NΔ4) Jurkat cells, by upregulation of TD-IκBα expression (doubling time of 50h ± 4h). Based on recent observations that NF-κB may play a protective role in apoptotic cell death (28, 423, 433), the response of control and TD-IκBα-expressing Jurkat cells to TNFα-induced apoptosis was examined by TUNEL assay. At 16h after TNFα (10 ng/ml) treatment in Dox induced cells, 65-80 percent of the TD-IκBα–expressing cells were undergoing apoptotic cell death whereas in Dox-induced TD-IκBα-expressing cells without TNFα treatment, less than 2 percent of the cells were apoptotic (Table 2). Similarly, TNFα treatment alone resulted in 2 percent apoptosis in TD-IκBα-expressing cells and 6 percent apoptotic cells in rtTA-Neo Jurkat cells (Table 2). Dox induction of TD-IκBα expression resulted in an increased sensitivity to TNFα-induced apoptosis, as detected by DNA fragmentation analysis at 8 or 16h after TNFα addition in the TD-IκBα-expressing cells but not in the control rtTA-Neo cells (data not shown). It appears that Dox induction of TD-IκBα expression, does not lead to apoptosis *per se*, but rather dramatically sensitizes Jurkat cells to TNFα-induced apoptosis. This observation may explain the observed selection against cells that constitutively express TD-IκBα (see below).

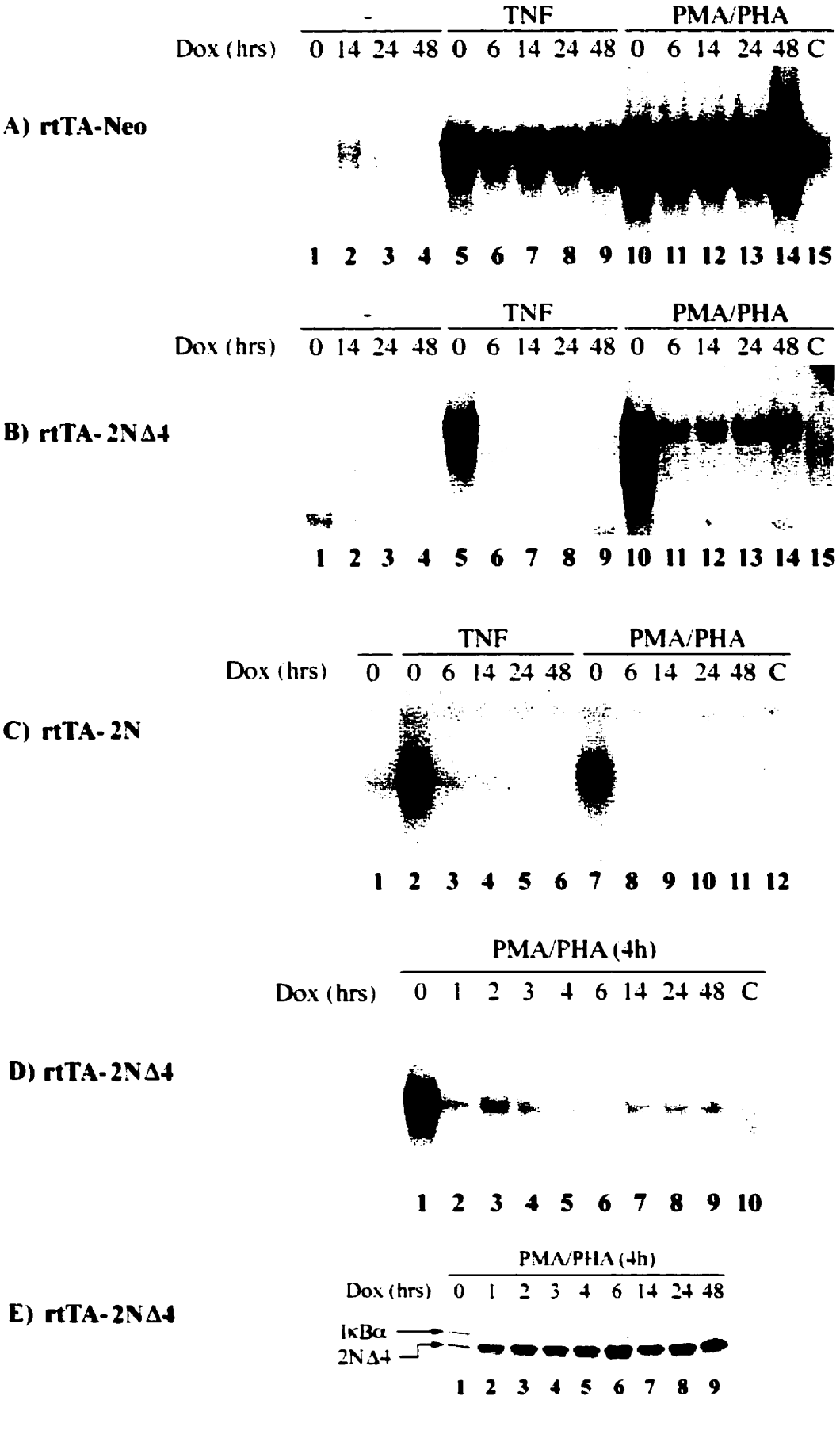
Inhibition of NF-κB DNA binding activity and gene expression by TD-IκBα. The induction of NF-κB DNA binding activity in control and TD-IκBα-expressing cells was examined following treatment of cells with several different inducers (Figures 14). Treatment of rtTA-neo-Jurkat, a control cell line stably transfected with the empty neo CMVt BL (Figure 14A) or rtTA-Jurkat (data not shown) with TNFα or PMA/PHA for 4h resulted in a strong induction of NF-κB DNA binding activity, irrespective of Dox treatment for 6 to 48h (Figure 14A, lanes 5-14). In contrast, TNFα induction of NF-κB activity in IκBα (2NΔ4) Jurkat cells (Figure 14B, lanes 5, 6) was completely blocked by

Table 2. TD-IκBα sensitizes Jurkat cells to TNFα-induced apoptosis. rtTA-Neo-, IκBα-2N-, and IκBα-2NΔ4-expressing Jurkat cells were treated with Dox (1μg/ml) for 24h and then induced with TNFα (10ng/ml) for 16h. Cells were fixed and stained for TUNEL (Boehringer Mannheim) and with Hoechst 33258. The percentage of total cells staining positive by TUNEL assay was determined by counting 200-400 cells per sample. The values are presented as a percentage of positive cells by TUNEL divided by the total number of Hoechst 33258 stained nuclei.

TNFα Induced Apoptosis in TD IκBα-expressing Jurkat cells

Treatment		Cell lines		
Dox	TNFα	rtTA-Neo	IκBα-2N	IκBα-2NΔ4
-	-	<2	<2	<2
+	-	<2	<2	<2
-	+	6.9	2.3	2.5
+	+	6.4	65.9	79.1

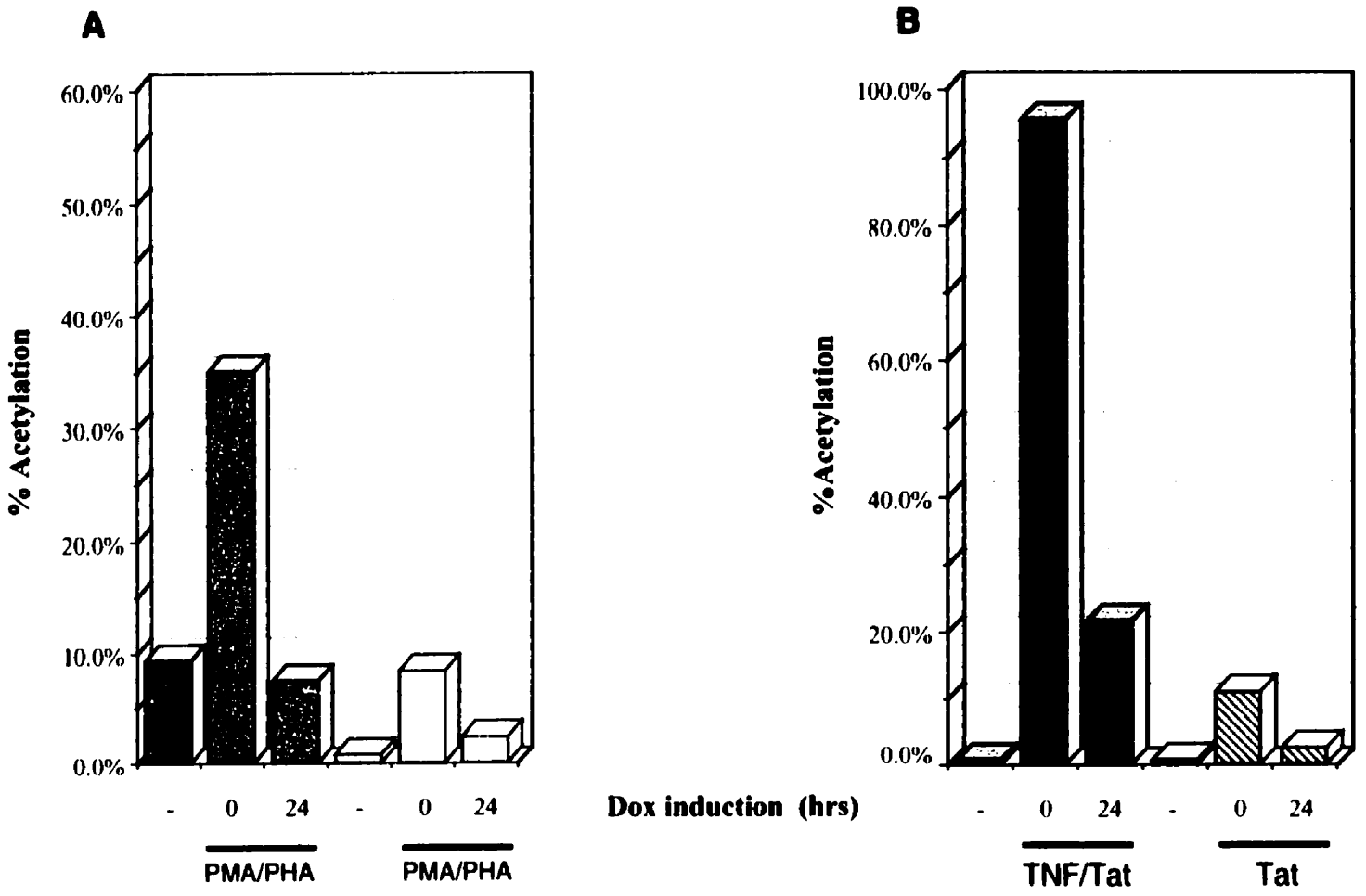
Figure 14. NF-κB DNA binding activity in control and TD-IκBα–expressing Jurkat T cells treated with TNF or PMA/PHA. rtTA-Neo (A), IκBα-2NΔ4 (B, D, E), rtTA-2N (C) Jurkat cells were incubated with Dox for different times as indicated in the Figure and subsequently induced by TNF (10ng/ml) or PMA/PHA (100ng/ml and 1ug/ml, respectively) for 4h. EMSA was performed on nuclear extracts (5μg) prepared from cells. IκBα immunoblot analysis (E) was performed on whole cell extracts (20μg) prepared from IκBα-2NΔ4 cells induced by Dox for different times and subsequently treated with PMA/PHA for 4h. Endogenous IκBα and transfected IκBα-2NΔ4 are indicated by arrows. Control lanes (C) in panels A,B,C,and D represent the competition of NF-κB-DNA complex formation using a 125-fold excess of unlabeled PRDII probe.


-
TNF
PMA/PHA
Dox (hrs)
0 14 24 48 0 6 14 24 48 0 6 14 24 48 C
A) rtTA-Neo
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
-
TNF
PMA/PHA
Dox (hrs)
0 14 24 48 0 6 14 24 48 0 6 14 24 48 C
B) rtTA-2NΔ4
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
TNF
PMA/PHA
Dox (hrs)
0 0 6 14 24 48 0 6 14 24 48 C
C) rtTA-2N
1 2 3 4 5 6 7 8 9 10 11 12
PMA/PHA (4h)
Dox (hrs)
0 1 2 3 4 6 14 24 48 C
D) rtTA-2NΔ4
1 2 3 4 5 6 7 8 9 10
PMA/PHA (4h)
Dox (hrs)
0 1 2 3 4 6 14 24 48
IκBα
2NΔ4
E) rtTA-2NΔ4
1 2 3 4 5 6 7 8 9

the induction of IκBα (2NΔ4) for 6h or longer (Figure 14B, lanes 6-9); the strong induction of NF-κB binding activity by PMA/PHA was also more than 95 percent inhibited by Dox activation of IκBα (2NΔ4) (Figure 14B, lanes 10-14). Similarly, Dox induction of IκBα (2N) also inhibited completely the induction of NF-κB binding activity by TNFα (Figure 14C, lanes 2-6) and PMA/PHA (Figure 14C, lanes 7-12). In a subsequent experiment, the kinetics of activation of IκBα (2NΔ4) and the inhibition of NF-κB binding activity were examined (Figure 14D and E). Surprisingly, as early as 1h after Dox addition, expression of IκBα (2NΔ4) was upregulated (Figure 14E, lanes 1 and 2) and NF-κB DNA binding activity was inhibited (Figure 14D, lanes 1 and 2). Interestingly, comparison of the levels of IκBα (2NΔ4) and endogenous IκBα revealed that constitutive expression of IκBα (2NΔ4) was dramatically decreased with time in culture (compare lane 6 in Figure 13B and lane 1 in Figure 14E), whereas the cells remained highly inducible in response to Dox addition (Figure 14E, lanes 2-9). TD-IκBα expressing Jurkat cells were also resistant to induction of NF-κB binding activity by double stranded RNA (poly I:C) treatment (data not shown). Thus, overexpression of TD-IκBα blocks the induction of NF-κB DNA binding activity by multiple inducers. Also, continued growth in culture selects for Jurkat cells that display low constitutive expression of TD-IκBα, possibly due to an increased sensitivity of TD-IκBα expressing cells to apoptosis.

To complement the observation that overexpression of TD-IκBα inhibited NF-κB binding activity, the effect of TD-IκBα on NF-κB dependent reporter gene expression was also examined. A CAT reporter gene driven by the SV40 minimal promoter, linked to the HIV-1 -109 to -79 region of the LTR, was transfected into IκBα (2NΔ4) expressing Jurkat cells together with a control plasmid mutated in the two NF-κB binding sites of the HIV-1 LTR (363). As shown in Figure 15A, treatment of these cells at 24h after transfection with PMA/PHA (24h) resulted in a 4 fold stimulation of reporter gene activity above background level with the wild type but not mutated promoter; Dox addition

Figure 15. Inhibition of Tat-TNF, Tat, or PMA/PHA induced HIV-1 LTR and HIV-enhancer activation by IκBα mutants. (A) rtTA-IκBα-2NΔ4 Jurkat cells were transfected with HIV-enh (□) or HIV-enh mut CAT (□) reporter plasmid (10μg); at 24h after transfection cells were treated with PMA/PHA or PMA/PHA + Dox for an additional 24h. (B) rtTA-IκBα-2NΔ4 Jurkat cells were transfected with pTZIIICAT (10μg) and in some cases with pSVexTat (10μg); at 24h after transfection some cells were treated with TNFα (10ng/ml) alone, with TNFα + Dox (■) or with Dox alone (▨). The level of HIV-1 LTR driven reporter gene expression was determined by CAT assay on the whole cell extracts (200μg), assayed for 2h. The negative control (lane -) was obtained by transfection with the HIV-1 LTR reporter construct only without TNF induction.

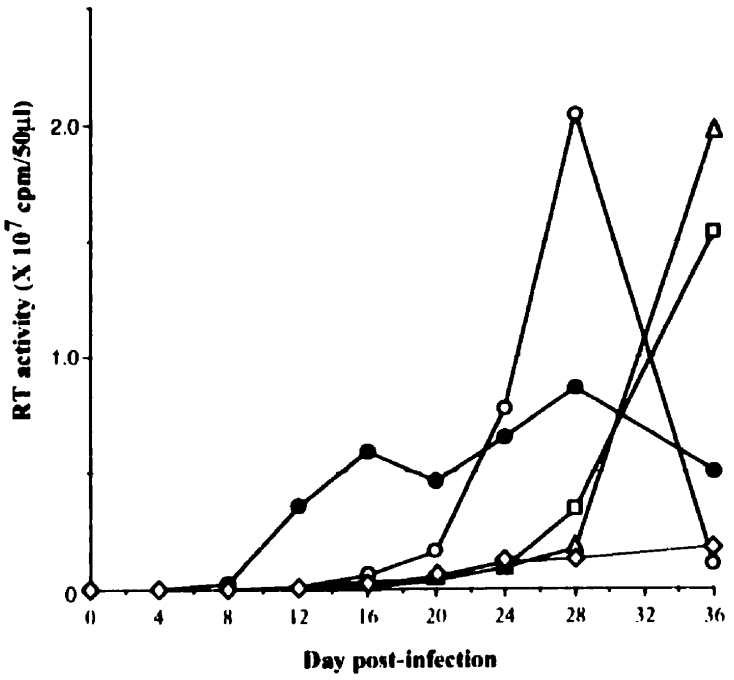

A
60.0%
50.0%
40.0%
30.0%
20.0%
10.0%
0.0%
% Acetylation
- 0 24 - 0 24
PMA/PHA
PMA/PHA
B
100.0%
80.0%
60.0%
40.0%
20.0%
0.0%
%Acetylation
- 0 24 - 0 24
TNF/Tat
Tat
Dox induction (hrs)

simultaneously with PMA/PHA inhibited NF-κB dependent induction of reporter gene activity. The HIV-1 LTR-CAT reporter plasmid was also transfected into TD-IκBα expressing Jurkat cells and the effect of TD-IκBα induction on the Tat-TNFα synergistic activation of the HIV-1 LTR was examined (25) (Figure 15B). In this experiment, Tat-TNFα activation of the LTR represented an 80-fold activation of gene activity that was inhibited more than 5-fold with Dox-induced TD-IκBα expression; the residual LTR-reporter gene activity likely represents NF-κB independent activation of the HIV-LTR. Co-expression of Tat protein also stimulated the HIV-1 LTR about 10-fold; somewhat surprisingly, expression of the TD-IκBα also inhibited induction of the HIV-1 LTR mediated by Tat alone (Figure 15B).

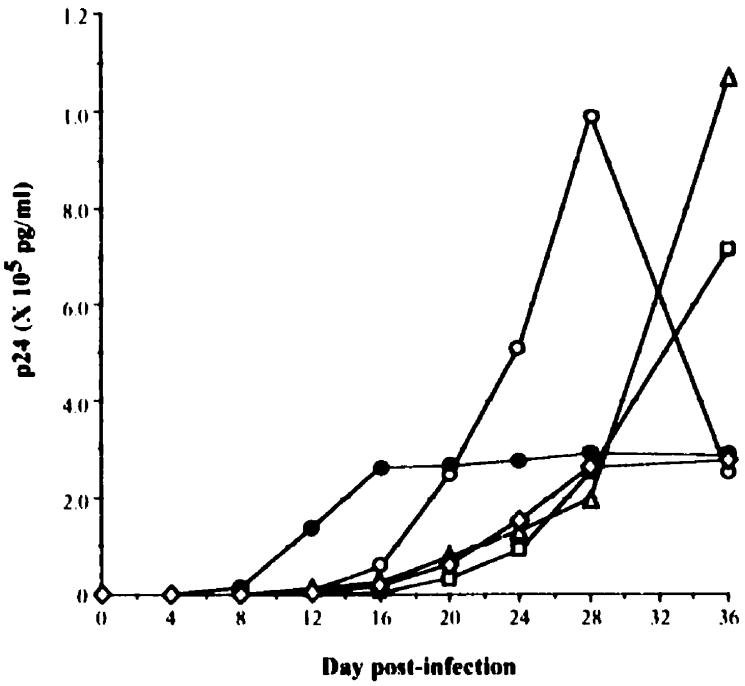
Inhibition of *de novo* HIV-1 multiplication in TD-IκBα expressing Jurkat cells. Given the involvement of NF-κB in the early transcriptional control of HIV-1 LTR gene expression (reviewed in (365), the impact of TD-IκBα expression on the course of *de novo* HIV-1 protein synthesis and virus production was next examined. Control and TD-IκBα-expressing Jurkat cells after preincubation with or without Dox for 24h were infected with the HIV-1 strain III B (derived from the molecular clone HXB2D) at an MOI of 0.01 pfu/ml and HIV-1 infection was monitored by RT assay, p24 ELISA, and p24 antigen accumulation over periods varying from 16 days to 36 days depending on the infection. Both RT assay (Figure 16A) and p24 ELISA analysis (Figure 16B) demonstrated that continuous Dox induced expression of IκBα-2NΔ4 resulted in dramatic delay in the onset of HIV-1 multiplication. In control rtTA-Neo-Jurkat cells, RT and p24 expression were detected as early as 8 days post-infection (p.i.), whereas in IκBα-2NΔ4 cells infection was first detected at day 12; addition of Dox at different times during the infection delayed the onset of detectable HIV-1 multiplication until 16-20 days (Figure 16A and B). The most effective inhibitory regimen was Dox addition 24h before infection (day -1) and subsequent Dox addition to the medium at days 8, 18, and 24. One addition of Dox at day

Figure 16. Suppression of HIV-1 infection in IκBα–2NΔ4 Jurkat cells. rtTA-Neo and rtTA-IκBα-2NΔ4 Jurkat cells were infected with HIV-IIIB for 2h at an MOI of 0.01 pfu/ml. Cells were then grown for 36 days and HIV-1 infection was monitored by A) RT assay (200µl) and (B) p24 ELISA (50µl). Symbols: ● rtTA-Neo + Dox; ○ rtTA-2NΔ4 - Dox; △ rtTA -2NΔ4 - Dox (+Dox12, Dox addition 12 days after infection); □ rtTA-2NΔ4 + Dox (Dox addition 24h before infection); ◇ rtTA-2NΔ4 + Dox (continuous addition of Dox -1, 8, 18, 24 after infection).

A)


RT activity (X 10^7 cpm/50µl)
2.0
1.0
0
0
4
8
12
16
20
24
28
32
36
Day post-infection

B)

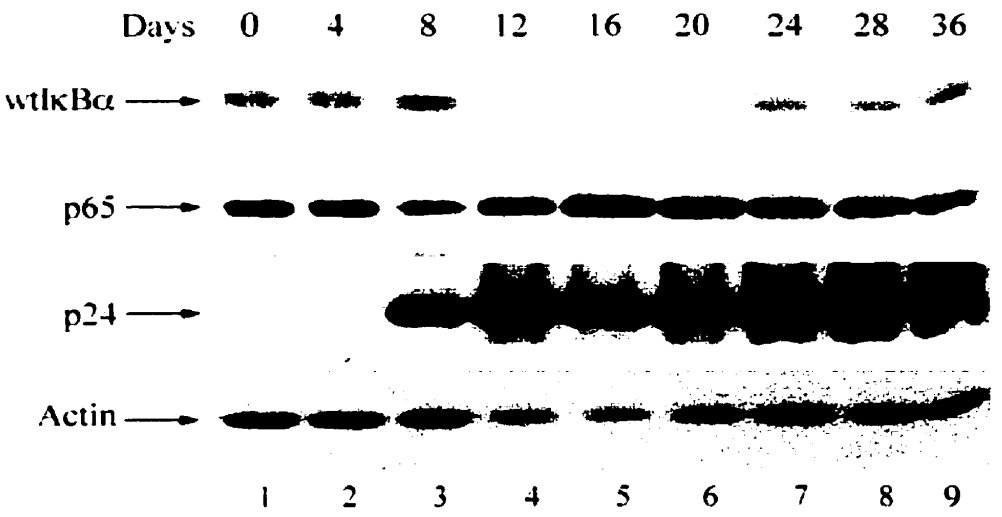

p24 (X 10^5 pg/ml)
1.2
1.0
8.0
6.0
4.0
2.0
0
0
4
8
12
16
20
24
28
32
36
Day post-infection

-1 or addition of Dox at day 12 also effectively blocked the onset of HIV-1 replication. However, with these treatments, breakthrough of HIV-1 multiplication was observed at day 36, as detected by RT and p24 ELISA. On the other hand, intermittent replenishment of Dox in the medium dramatically repressed HIV-1 RT and p24 expression throughout the course of infection (Figure 16A and B).

The intracellular accumulation of p24 antigen was also monitored during the course of *de novo* HIV-1 infection, together with the expression of IκBα and β-actin (Figure 17). p24 accumulation was weakly detected as early as 4 day p.i. (Figure 17A, lane 2); high level expression was detected thereafter throughout the course of infection (Figure 17A, lanes 3-9) in HIV-1 infected rtTA-Neo-Jurkat cells. Also with the analysis of HIV-1 replication in Jurkat cells, a decrease in the level of IκBα was detected during the exponential phase of virus multiplication at days 12 to 20 (Figure 17A, lanes 4-6), reflecting the HIV-1 mediated degradation of IκBα, an effect that contributes to constitutive NF-κB DNA binding activity in HIV-1 infected cells (12, 96, 275, 363, 364). In the IκBα-2NΔ4 expressing cells, the appearance of p24 antigen was delayed until day 12 after infection and peaked at day 20-24 p.i. (Figure 17B, lanes 4-7). In the TD-IκBα-expressing cells, the decrease in IκBα was also delayed until later times after infection. Again, the basal level of IκBα transgene was significantly reduced relative to endogenous IκBα, reflecting the ongoing selection of low background expressing cells (Figure 14E). However, addition of Dox to the infected cell culture at day 12 p.i. resulted in a dramatic increase in IκBα-2NΔ4 expression (Figure 17C, lanes 4 and 5); this time of addition was not sufficient to inhibit the appearance of p24 antigen at day 12 (Figure 17C, lane 4). However, induction of TD-IκBα completely inhibited endogenous IκBα expression by day 16 (Figure 17C, lane 5) and partially blocked the subsequent intracellular accumulation of p24 antigen (compare Figure 17B and 5C, lanes 5-9). Addition of Dox to TD-IκBα expressing Jurkat cells 24h before infection (Figure 17D) or intermittent Dox addition at days -1, 8, 18, and 24 (Figure 17E) delayed the onset of detectable p24 antigen until day

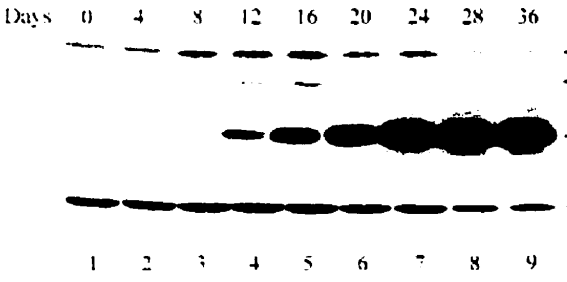
Figure 17. Inhibition of viral p24 protein expression by IκBα-2NΔ4 mutant. rtTA-Neo and rtTA-IκBα-2NΔ4 Jurkat cells were incubated in the presence or absence of Dox and then infected with HIV-IIIB for 2h at an MOI of 0.01 pfu/ml. Cells were collected every fourth day for 36 days and analyzed by immunoblotting. rtTA-Neo Jurkat cells (panel A) and rtTA-IκBα-2NΔ4Jurkat cells (panel D and E) were treated with Dox for 24 hours prior to infection or not treated (panel B). rtTA-IκBα-2NΔ4Jurkat cells (panels B and C) were infected without Dox pretreatment, but Dox was added 12 days after the infection (panel C). Dox was also intermittently added at 8, 18, and 24 days after infection in panel E. Endogenous IκBα (wtIκBα), IκBα-2NΔ4 (2NΔ4), RelA(p65), viral p24 and actin proteins were detected by immunoblotting as described in Material and Methods and are indicated by arrows.

A) rtTA-Neo Jurkat

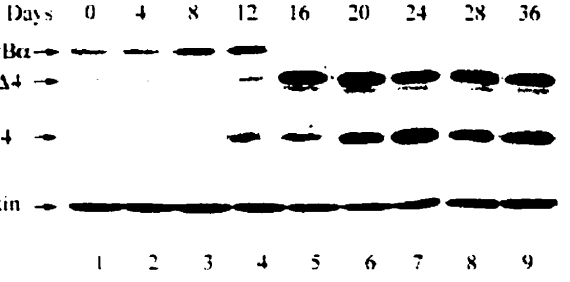


rtTA-2NΔ4 Jurkat

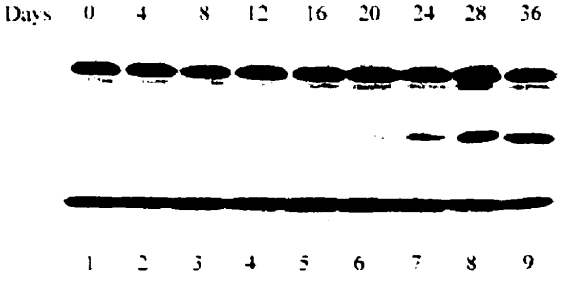
B) -Dox



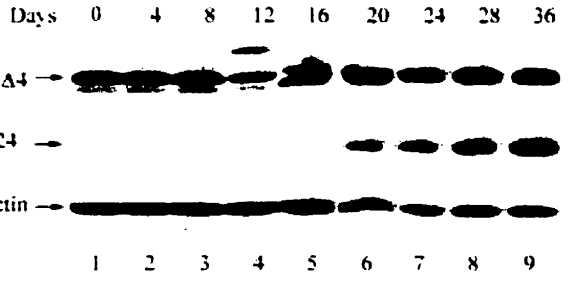
C) −Dox (day 12)



D) +Dox (day 0)



E) −Dox (day 0, 8, 18, 24)

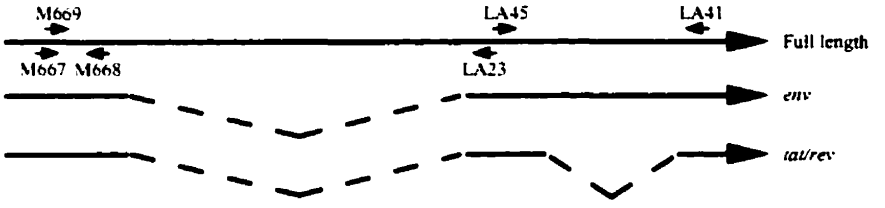


20 p.i. (Figure 17D and E, lane 6). In contrast to the results obtained by RT and p24 ELISA assays, both one time addition and intermittent addition of Dox delayed the accumulation of intracellular p24 antigen to an equivalent extent. Overall, Dox induction of TD-IκBα expression resulted in a 10-20 fold decrease in the production of p24 antigen (Figure 17), as well as inhibition of HIV-1 virion release (Figure 16). Similar results were obtained using the IκBα-2N expressing cells (data not shown), thus demonstrating a dramatic inhibitory effect of TD-IκBα expression on the course of *de novo* HIV-1 infection in Jurkat T cells.

Decreased levels of HIV-1 mRNA in TD-IκBα expressing Jurkat cells. In order to link the inhibition of HIV-1 multiplication with NF-κB inhibitory effects of TD-IκBα, the effects of TD-IκBα induction on HIV-1 induced NF-κB binding activity was evaluated by mobility shift analysis. Virus induced activation of NF-κB was blocked by TD-IκBα expression (data not shown), thus complementing the results described in Figure 14, and confirming that TD-IκBα interfered with HIV-induced NF-κB binding. To further characterize the block in HIV-1 multiplication, accumulation of HIV-1 viral RNA species was evaluated by semi-quantitative RT-PCR, using the primers and procedures described previously (468). The primer pair M667/M668 detected total viral RNA with fragment of 161 bp; M669/LA23 identified a fragment of 214 bp which represented singly spliced (env) and doubly spliced tat/rev RNA; and LA41/LA45 amplified a fragment of 123 bp corresponding to doubly spliced tat/rev RNA (Figure 18A). Viral RNA species in rtTA-Neo-Jurkat and rtTA-2NΔ4-Jurkat cells were detected at different times after *de novo* HIV infection in the presence or absence of Dox-induced TD-IκBα (Figure 18B). In rtTA-Neo Jurkat cells, HIV mRNA transcripts were detected as early as 2-4 days after infection (Figure 18B, lanes 2, 3, 7 and 8) and their levels increased at days 6 and 10 post-infection (Figure 18B, lanes 4, 5, 9 and 10), regardless of Dox addition. In rtTA-2NΔ4 Jurkat cells, without Dox addition, a low level of full length viral RNA was

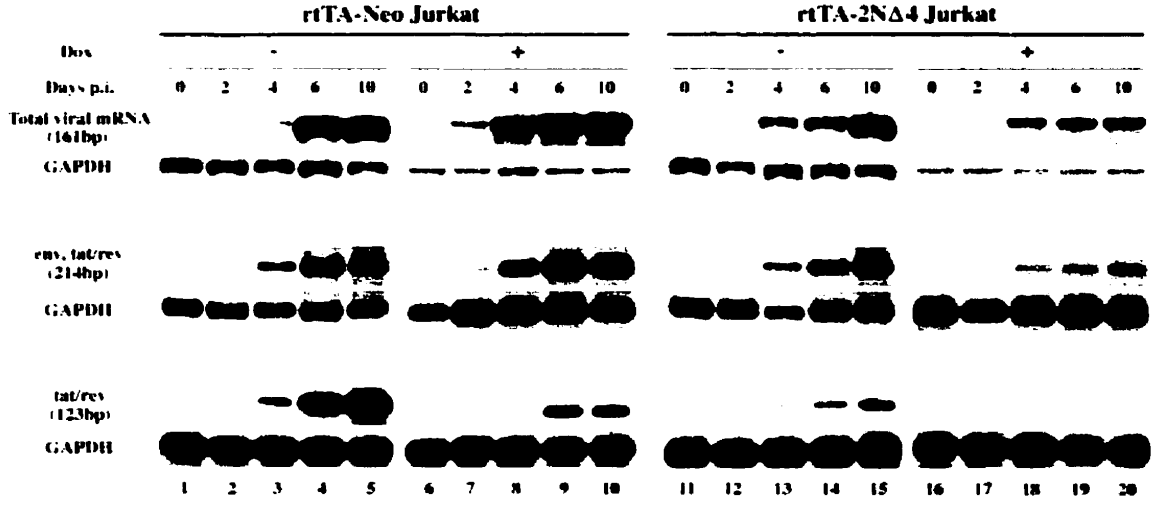
Figure 18. Suppression of HIV-1 transcription in IκBα-2NΔ4 expressing Jurkat cells. (A) A schematic representation of full-length and spliced mRNAs (*env, tat/rev*) of HIV-1 with specific primers used for RT-PCR analysis. M667/M668 detects both full-length and spliced mRNAs and generate a 161bp fragment. The primer pair M669/LA23 detects only spliced mRNA (*env, tat/rev*) and produces a 214bp fragment. *tat/rev* mRNA is detected by LA41/LA45 primer pair and a 123bp fragment is generated following PCR. (B) RT-PCR analysis of HIV-1 RNA. rtTA-Neo and rtTA-IκBα-2NΔ4 Jurkat cells were incubated in the presence or absence of Dox and then infected with HIV-IIIB for 2h at an MOI of 0.01 pfu/ml. Cells were collected 2, 4, 6 and 10 days after infection and total RNA (2 μg) was analyzed by RT-PCR. All results are normalized to the levels of GAPDH.

A)



M667 M668 (161bp) : Full length, *env*, *tat/rev*
M669 LA23 (214bp) : *env*, *tat/rev*
LA41 LA45 (123bp) : *tat/rev*

B)



detected at day 2 (Figure 18B, lane 12, upper panel) and by day 4 unspliced and spliced viral RNA was detected (Figure 18B, lane 13) and accumulated thereafter at days 6 and 10 (Figure 18B, lanes 14 and 15). However, with Dox induction of TD-IκBα, viral RNA levels were reduced (Figure 18B, lanes 17-20). Full length RNA was detected at day 2 (Figure 18B, lane 17, upper panel) but the overall levels of unspliced and spliced RNA were decreased at days 6 and 10, relative to the levels detected in the absence of Dox or in rtTA-Neo cells. Strikingly, doubly spliced tat/rev mRNA was not detected in rtTA-2NΔ4 Jurkat cells treated with Dox (Figure 18B, lanes 18-20, lower panel). These results demonstrate a transcriptional inhibitory effect of TD-IκBα expression on HIV mRNA accumulation in Jurkat cells engineered to express TD-IκBα.

CHAPTER V

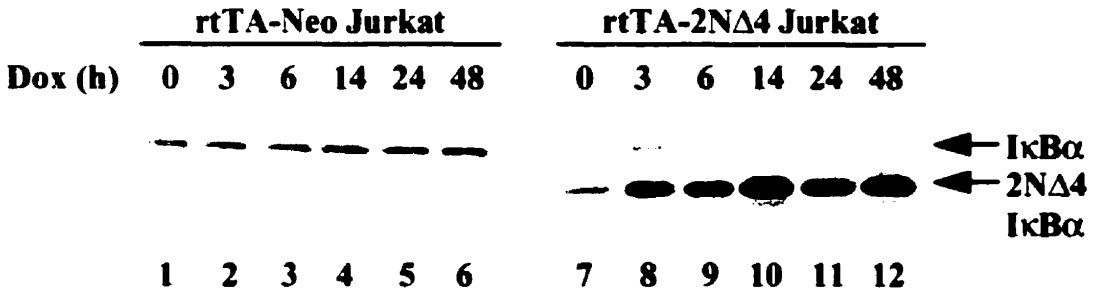
IDENTIFICATION BY *In Vivo* GENOMIC FOOTPRINTING OF AN NF-κB/Sp1 TRANSCRIPTIONAL SWITCH THAT REGULATES THE IκBα PROMOTER

In Jurkat T cells that inducibly express TD-IκBα (222), endogenous IκBα protein expression was blocked by TD-IκBα. We now demonstrate that inducer dependent induction of the IκBα gene transcription was blocked by TD-IκBα expression at the transcriptional level. To further analyze the autoregulatory control of IκBα expression, dimethylsulfate genomic footprinting was used to determine the pattern of protein-DNA interactions at the IκBα locus in stimulated Jurkat T cells and TD-IκBα expressing cells. These studies permit the first *in vivo* characterization of IκBα transcriptional autoregulation by NF-κB and identify the promoter proximal NF-κB/Sp1 transcriptional switch as an essential component in the regulation of the IκBα promoter.

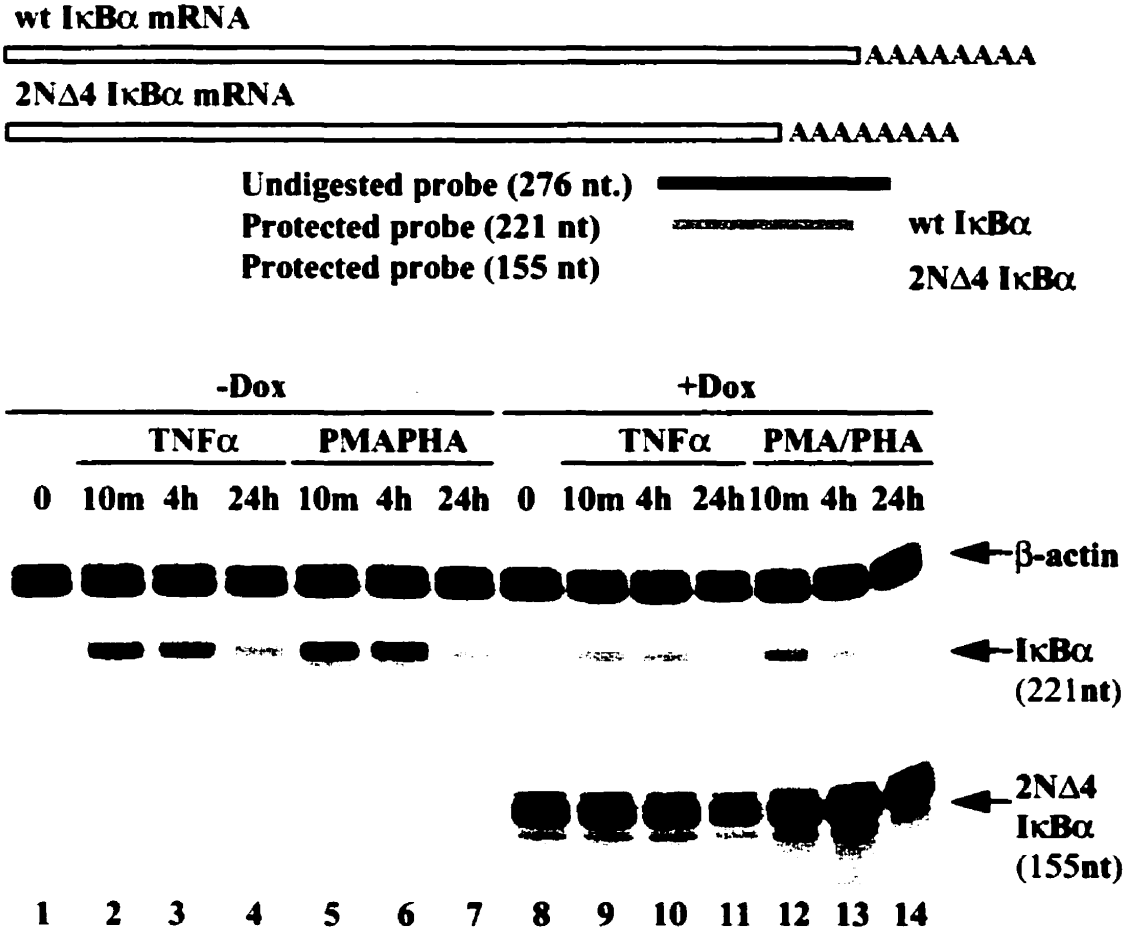
Tet-induced TD-IκBα blocks expression of IκBα gene. As shown previously (222), endogenous IκBα protein expression was abolished after TD-IκBα induction for 24h (Figure 19A, lane 7-11). To determine whether IκBα gene transcription was downregulated by TD-IκBα, rtTA-IκBα (2NΔ4) Jurkat cells were treated with TNFα or PMA in the presence or absence of Dox and endogenous IκBα and TD-IκBα mRNA were analyzed by RNase protection analysis with a 276 nt 3' cDNA probe that specifically recognized the C-terminus of endogenous IκBα mRNA as well as the truncated IκBα (2NΔ4) mRNA (Figure 19B). After 10 min of TNFα or PMA/PHA induction, 20 and 40 fold increases in IκBα mRNA were detected, respectively (Figure 19B, lanes 2 and 5); subsequently the level of IκBα mRNA declined with time in rtTA-IκBα (2NΔ4) Jurkat cells (Figure 19B, lanes 4 and 7). In TD-IκBα expressing cells, endogenous IκBα mRNA induction was decreased 5-fold by Dox addition, whereas high level expression of the IκBα (2NΔ4) transgene was easily detected by RNase protection (Figure 19B, lanes 8-14). These results indicate that Dox-induced TD-IκBα expression interfered with the induced but not the basal level of endogenous IκBα mRNA transcription.

Figure 19. Tet-induced TD-IκBα inhibits endogenous IκBα expression. (A) rtTA-Neo (lanes 1-6) and rtTA-IκBα(2NΔ4) Jurkat cells (lanes 7-12) were incubated with Dox (1 μg/ml) for different times (0, 10 min, 4h, 48h). Endogenous IκBα (top arrow) and TD-IκBα (bottom arrow) were detected by immunoblotting using MAD10B antibody. (B) Schematic representation of C-terminal IκBα probe used in RNase protection analysis. rtTA-IκBα(2NΔ4) Jurkat cells were treated with TNFα (10 ng/ml, lanes 2-4, 9-11) or PMA (50 ng/ml) plus PHA (1μg/ml) (lanes 5-7, 12-14) for different times (0, 10 min, 4h, 24h) in the absence (lanes 1-7) or presence (lanes 8-14) of Dox (1 μg/ml, 24h). Endogenous IκBα and TD-IκBα mRNA were detected using the 276nt 3'cDNA probe by RNase protection assay. Arrows indicate β-actin, IκBα (221nt band) and IκBα(2NΔ4) (155nt band) from top to bottom. The results shown are representative of at least three independent experiments.

A


rtTA-Neo Jurkat
rtTA-2NΔ4 Jurkat
Dox (h)
0 3 6 14 24 48
0 3 6 14 24 48
IκBα
2NΔ4 IκBα
1 2 3 4 5 6
7 8 9 10 11 12

B


wt IκBα mRNA
AAAAAAAA
2NΔ4 IκBα mRNA
AAAAAAAA
Undigested probe (276 nt.)
Protected probe (221 nt)
Protected probe (155 nt)
wt IκBα
2NΔ4 IκBα
-Dox
+Dox
TNFα
PMAPHA
TNFα
PMA/PHA
0 10m 4h 24h 10m 4h 24h 0 10m 4h 24h 10m 4h 24h
β-actin
IκBα
(221nt)
2NΔ4
IκBα
(155nt)
1 2 3 4 5 6 7 8 9 10 11 12 13 14

TD-IκBα inhibits NF-κB binding to κB1 site of the IκBα promoter. Using Jurkat T cells inducibly expressing TD-IκBα (222), the effect of TD-IκBα on TNFα and PMA induced NF-κB binding activity was studied by EMSA using the κB1 site of the IκBα promoter as a probe. Treatment of rtTA-Neo Jurkat with TNFα or PMA/PHA resulted in a strong induction of NF-κB binding activity, irrespective of Dox treatment (Figure 20AB, top panel, lanes 1-4, 6-9). In contrast, TNFα or PMA/PHA induced NF-κB binding activity in rtTA-IκBα (2NΔ4) Jurkat cells was completely blocked by Dox induction of TD-IκBα (Figure 20AB, bottom panel, lanes 6-9), while NF-κB binding activity was observed in rtTA-IκBα (2NΔ4) Jurkat cells without Dox-treatment (Figure 20AB, bottom panel, lanes 1-4). To identify the subunit composition of the NF-κB complexes, supershift analysis of extracts from rtTA-Neo Jurkat cells treated with PMA for 4h was performed using anti-p65, -p50, -p52 and -c-rel antibodies. Complexes were shifted with p65 or p50 antibodies but not with p52 or c-rel antibodies (data not shown). Therefore, NF-κB DNA binding activity was completely blocked by expressing TD-IκBα.

p65 is tightly bound to TD-IκBα. Co-immunoprecipitation studies were performed with anti-p65 (Figure 21AB) and anti-IκBα antibodies (Figure 21C) to determine whether TD-IκBα could associate with p65 *in vivo* during the course of TNFα or PMA/PHA induction. In unstimulated rtTA-Neo Jurkat cells, p65 complexed with IκBα in the presence or absence of Dox (Figure 21AB, lanes 1 and 5). In unstimulated rtTA-IκBα (2NΔ4) Jurkat cells in the absence of Dox pretreatment, p65 was associated with both endogenous IκBα and TD-IκBα (Figure 21AB, lane 10), while in Dox-treated TD-IκBα expressing cells, p65 was mainly associated with TD-IκBα (Figure 21AB, lane 14), due to downregulation of endogenous IκBα expression. TNFα or PMA/PHA stimulation induced degradation of IκBα which reappeared after 60 min in control Jurkat cells (Figure 21AB, lanes 2-4, 6-8) in the presence or absence of Dox; in rtTA-IκBα (2NΔ4) Jurkat cells in the absence of Dox the same loss and reappearance of endogenous IκBα was observed (Figure 21AB,

Figure 20. TD-IκBα blocks NF-κB binding to κB1 site of the IκBα promoter. (A) Nuclear extracts from rtTA-Neo (top panel) and rtTA-IκBα(2NΔ4) Jurkat cells (lower panel) treated with TNFα (10 ng/ml) for the indicated times in the absence (lanes 1-5) or presence (lanes 6-9) of Dox pretreatment (1 μg/ml, 24h) were subjected to an EMSA using the κB1 probe. (B) Nuclear extracts from rtTA-Neo (top panel) and rtTA-IκBα(2NΔ4) Jurkat cells (lower panel) treated with PMA (50 ng/ml) / PHA (1μg/ml) for the indicated times in the absence (lanes 1-5) or presence (lanes 6-9) of Dox pretreatment (1 μg/ml, 24h) were subjected to an EMSA using the κB1 probe. Control lanes C (lane 5 in A and B) represent the competition of NF-κB-DNA complex formation using a 125-fold excess of unlabeled κB1 probe.

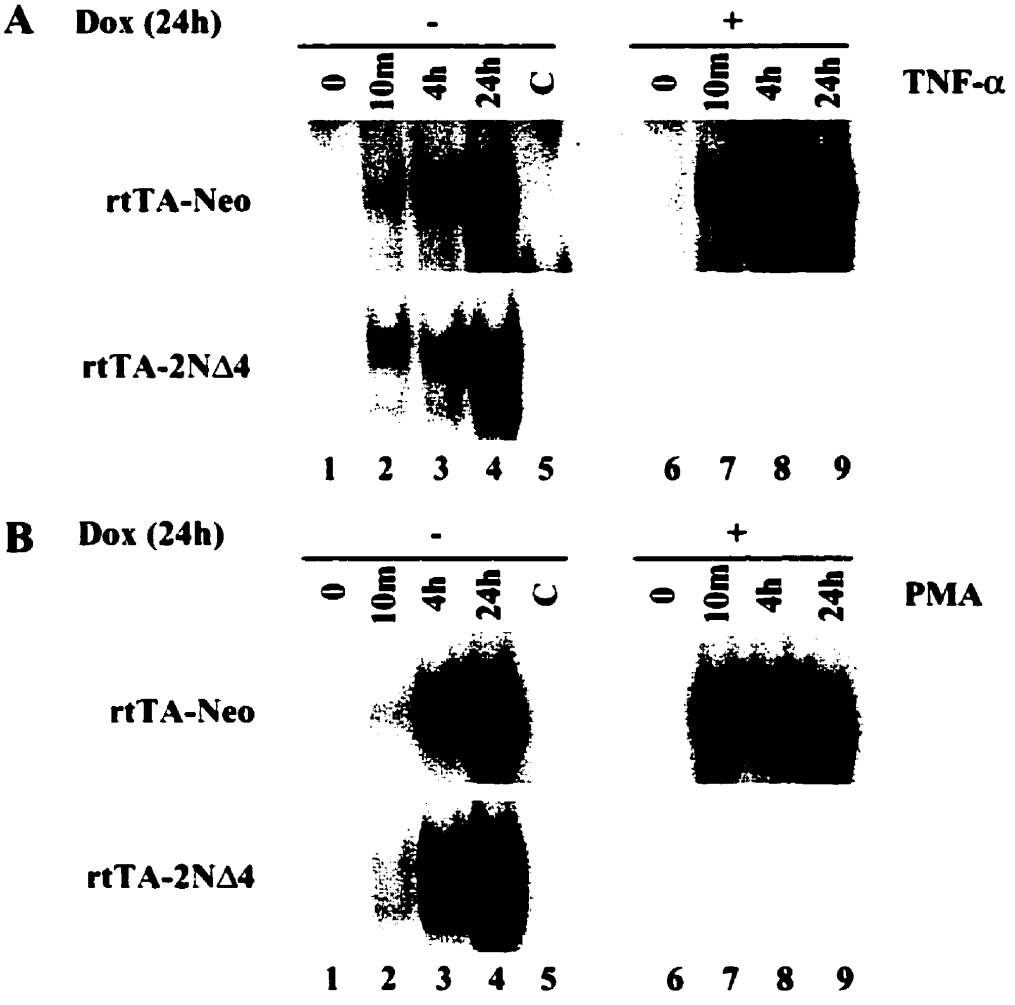
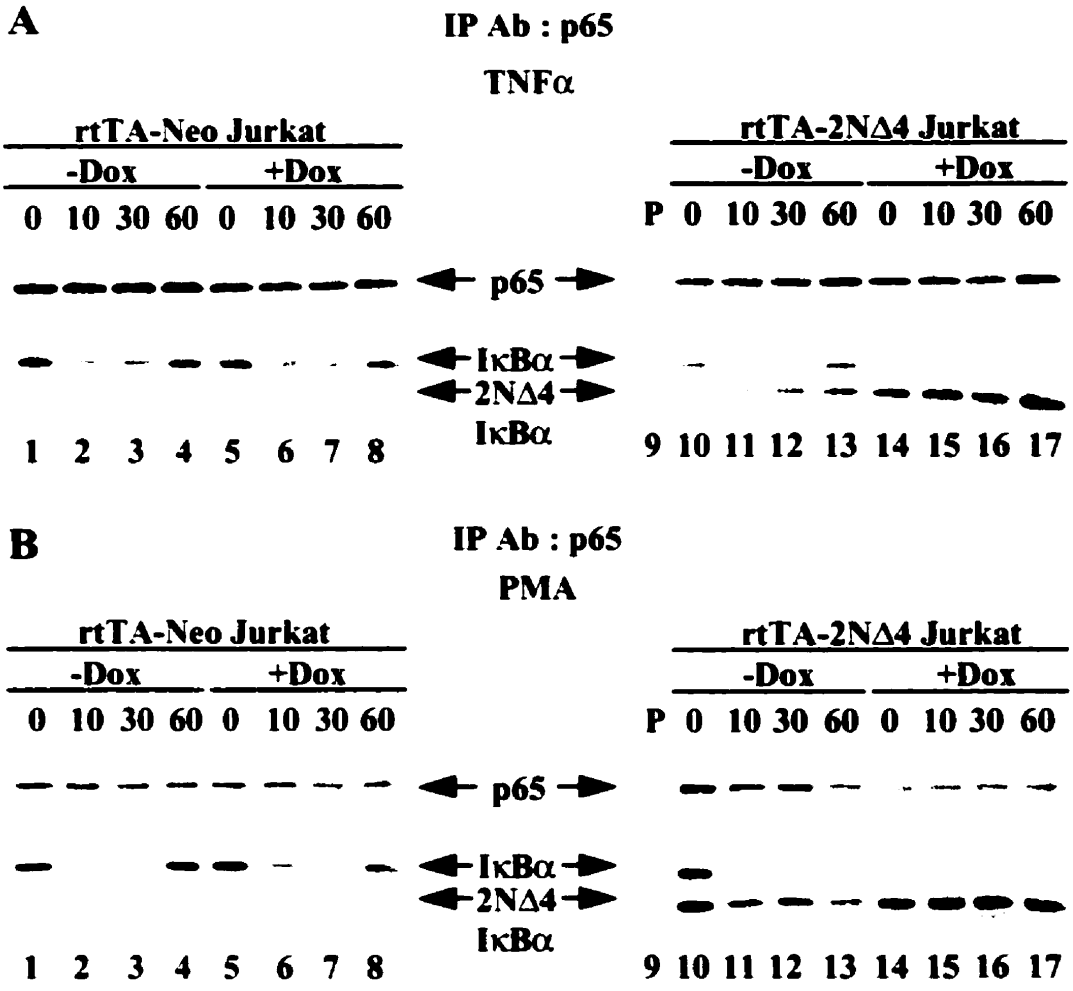

A
Dox (24h)
-
+
0
10m
4h
24h
C
0
10m
4h
24h
TNF-α
rtTA-Neo
rtTA-2NΔ4
1 2 3 4 5 6 7 8 9
B
Dox (24h)
-
+
0
10m
4h
24h
C
0
10m
4h
24h
PMA
rtTA-Neo
rtTA-2NΔ4
1 2 3 4 5 6 7 8 9

Figure 21. TD-IκBα is tightly bound to RelA(p65). rtTA-Neo (lanes 1-8) and rtTA-IκBα(2NΔ4) (lanes 9-17) Jurkat cells were treated with TNFα (10 ng/ml) (A) or PMA (50 ng/ml) / PHA (1μg/ml) (B) for various times in the absence (lanes 1-4, 9-13) or presence (lanes 5-8, 14-17) of doxycycline (1 μg/ml) for 24h. Cell lysates were immunoprecipitated with p65 specific antibody and immunoblot analysis was performed using the anti-IκBα and anti-p65 antibodies. Arrows indicate p65, IκBα and IκBα(2NΔ4) from top to bottom. Control lane P (lane 9) represents an immunoprecipitation with rabbit preimmune sera. (C) rtTA-IκBα(2NΔ4) Jurkat cells were treated with PMA (50ng/ml) / PHA (1μg/ml) for various times in the absence (lanes 1-5) or presence (lanes 6-9) of Dox (1 μg/ml. 24h). Cell lysates were immunoprecipitated with IκBα specific antibody. Western blot analysis was done using the anti-p65 and anti-IκBα antibodies. Arrows indicate p65, IκBα and IκBα(2NΔ4) from top to bottom. Control lane P (lane 1) represents an immunoprecipitation with mouse preimmune sera.

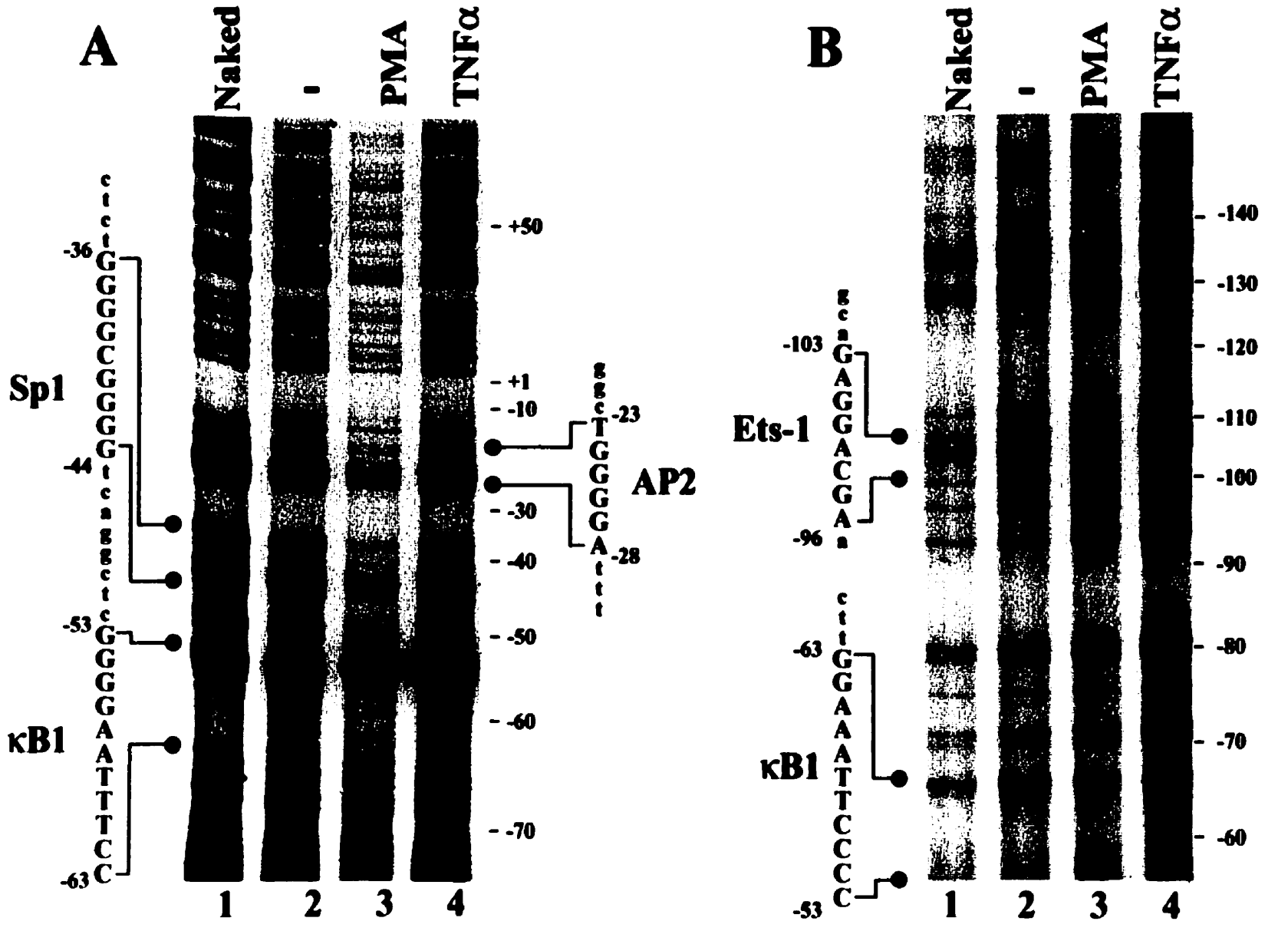

A
IP Ab : p65
TNFα
rtTA-Neo Jurkat
-Dox
+Dox
0 10 30 60 0 10 30 60
rtTA-2NΔ4 Jurkat
-Dox
+Dox
P 0 10 30 60 0 10 30 60
p65
IκBα
2NΔ4
IκBα
1 2 3 4 5 6 7 8
9 10 11 12 13 14 15 16 17
B
IP Ab : p65
PMA
rtTA-Neo Jurkat
-Dox
+Dox
0 10 30 60 0 10 30 60
rtTA-2NΔ4 Jurkat
-Dox
+Dox
P 0 10 30 60 0 10 30 60
p65
IκBα
2NΔ4
IκBα
1 2 3 4 5 6 7 8
9 10 11 12 13 14 15 16 17
C
IP Ab : IκBα
PMA
rtTA-2NΔ4 Jurkat
-Dox
+Dox
P 0 10 30 60 0 10 30 60
p65
IκBα
2NΔ4 IκBα
1 2 3 4 5 6 7 8 9

lanes 11-13). In contrast, in TD-IκBα expressing Jurkat cells, immunoprecipitation with anti-p65 antibody resulted in the co-immunoprecipitation of predominantly IκBα (2NΔ4) (Figure 21AB, lanes 14-17), indicating that the degradation-resistant TD-IκBα was tightly associated with p65 throughout induction. The reciprocal immunoprecipitation with anti-IκBα antibody was also performed (Figure 21C). Both endogenous and TD-IκBα were immunoprecipitated from rtTA-IκBα (2NΔ4) expressing cells in the absence of Dox addition together with p65 (Figure 21C, lanes 2-5), whereas following Dox treatment, only TD-IκBα and associated p65 were identified (Figure 21C, lanes 7-9). Therefore, inhibition of NF-κB DNA binding activity and endogenous IκBα transcription in TD-IκBα inducible cells is due to the tight association between the NF-κB transactivator p65 and TD-IκBα which is resistant to inducer-mediated degradation.

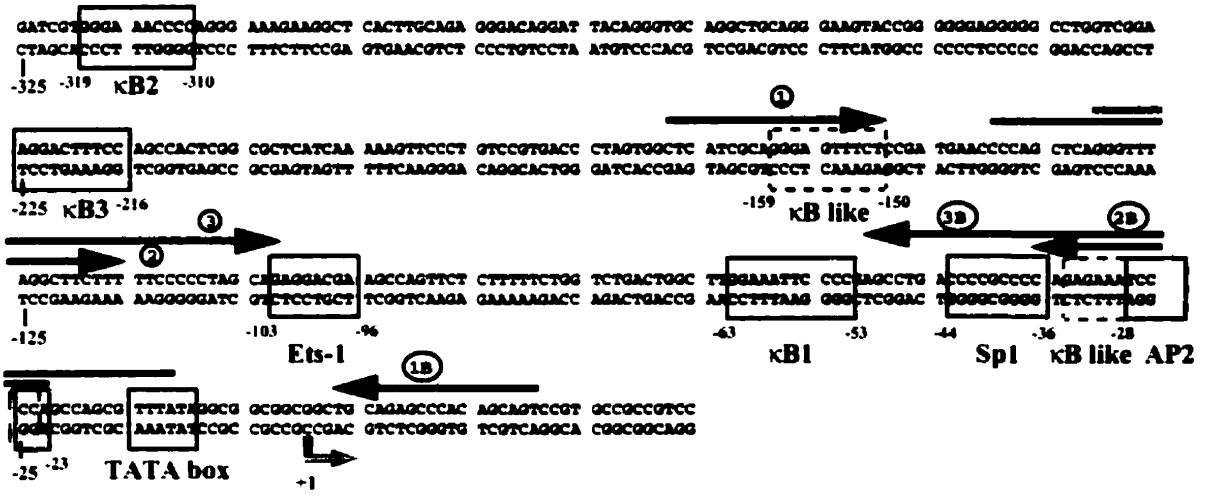
***In vivo* genomic footprinting of the IκBα promoter.** In order to analyze the inducibility of IκBα gene transcription, genomic footprinting analysis was performed in Jurkat and IκBα (2NΔ4) expressing Jurkat cells. Following stimulation by either TNFα or PMA/PHA, living cells were submitted to DMS (dimethyl sulfate) treatment which methylates G residues and to a lesser extent A residues; genomic DNA was then extracted and submitted to piperidine treatment to cleave methylated residues. Then, piperidine-cleaved DNA was amplified by ligation-mediated PCR using specific primers for IκBα promoter as detailed in Figure 22C. A G specific sequence ladder was also generated as reference and analyzed by sequencing.

Footprinting primers were initially designed to analyze *in vivo* protein-DNA interactions occurring in the proximal -10 to -170 region of the IκBα promoter (primers 1, 2, 3 for the non-coding strand, and 1B, 2B, 3B for the coding strand, Figure 22C). In resting Jurkat T cells, the proximal G residues of the κB1 site at -53 to -56 were cleaved and easily detected by comparison with the G ladder revealing no induction in the absence of

Figure 22. ***In vivo*** **footprinting of the proximal IκBα gene promoter in Jurkat T cells.** (A) Non-coding strand and (B) coding strand analysis. Naked DNA was treated *in vitro* by DMS (lane 1). Cells were either non-stimulated (lane 2), treated by PMA/PHA (lane 3) or by TNFα (Lane 4) for 40 min and then were treated with DMS. Genomic DNA was extracted and treated with piperidine. All DNA samples were amplified by LM-PCR and visualized on Long-Ranger™ sequencing gel. (C) Sequence of the IκBα promoter. Major consensus sites for protein binding such as the NF-κB sites and sites of Ets-1, Sp1 and AP2 are enclosed in boxes. The mRNA start site and TATA box are also shown. Arrows correspond to primers used in genomic footprinting. Primers 1, 2, and 3 were designed to characterize the non-coding strand and primers 1B, 2B, and 3B for the coding strand.


A
Naked
-
PMA
TNFα
Sp1
κB1
AP2
-36
-44
-53
-63
+50
+1
-10
-23
-30
-28
-40
-50
-60
-70
1
2
3
4
B
Naked
-
PMA
TNFα
Ets-1
κB1
-103
-96
-63
-53
-140
-130
-120
-110
-100
-90
-80
-70
-60
1
2
3
4

C


-325
-319
κB2
-310
-225
κB3
-216
-159
κB like
-150
-125
-103
-96
Ets-1
-63
-53
κB1
-44
-36
-28
Sp1
κB like
AP2
-25
-23
TATA box
+1

stimulation (Figure 22A, lanes 1 and 2). In the absence of stimulation, an interaction was detected at the Sp1 site located between -44 to -36 bp, adjacent to the κB1 site (Figure 22A, lanes 1 and 2). In response to PMA/PHA or TNFα induction for 40 min, the κB1 site was strongly protected by protein-DNA interactions, resulting in very limited cleavage of the -54G, -55G and -56G residues and a hypersensitive cleavage at the -53G residue (Figure 22A, lanes 3 and 4). Similarly, modifications of the pattern of the Sp1 site were observed; with naked DNA or DNA from unstimulated cells, cleavage of -44G, -43G, -42G and -41G was detected as well as -39G, -37G, -36G (Figure 22A, lanes 1 and 2), while following induction, the G residues of the Sp1 site were protected with the exception of the -42G residue which was hypersensitive to cleavage (Figure 22A, lanes 3 and 4).

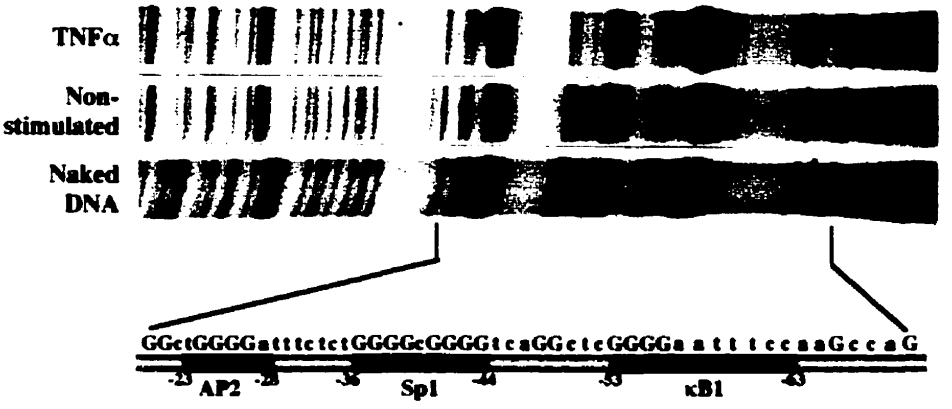
Changes in the coding strand methylation pattern of the κB1 site were also detected using specific primers, although PCR amplification was difficult to obtain because of the GC rich nature of the region, allowing only the detection of κB1 and Ets but not Sp1 sites (see Figure 22C). The two G residues of the κB1 site located at -63 and -62 were methylated on naked DNA (Figure 22B, lane 1 and 2). Following stimulation of Jurkat T cells with PMA/PHA or TNFα, only the -63G residue was methylated and cleaved, whereas the -62G residue was protected from methylation (Figure 22B, lanes 3 and 4), thus demonstrating protection of the κB1 site on both coding and non-coding strands. Two other potential binding sites in the -10 to -110 region - AP2 and Ets-1 - were identified *in vivo* (Figure 22 AB); hypermethylation of the -24G and -25G residues was observed at the AP2 site in both unstimulated and stimulated cells (Figure 22A, compare lane 1 to lanes 2-4). Similarly, methylation of the -99A residue at the Ets-1 site appeared slightly enhanced in resting and stimulated cells (data not shown); for both sites induction specific changes in promoter occupancy were not detected.

Scanning analysis of the methylation pattern of the [-20 to -70] IκBα promoter region. Since the modifications at the Sp1 site were more discrete than those detected at the κB1 site, changes in the methylation pattern of the [-20 to -70] region of IκBα promoter which includes the AP2, Sp1 and κB1 sites were analyzed by densitometry scanning (Figure 23). A representative autoradiography is presented in Figure 23A. Comparison of non-stimulated and naked DNA patterns revealed increased methylation of the -42G residue and a slight decrease in -41G methylation at the Sp1 site whereas no significant differences in methylation were observed for the bordering -20G and -66G residues, indicating an even methylation pattern in the scanned region (Figure 23B). These data indicate that the Sp1 site was constitutively occupied in resting Jurkat T-cells. Similarly, hypermethylation of -24 and -25G residues in non-stimulated Jurkat cells suggests a constitutive binding at the AP2 site. In contrast, no binding was detected on the κB1 site in unstimulated conditions. Following stimulation by either TNFα, as shown in Figure 23C, or PMA/PHA (data not shown), protection of the Sp1 site was modified as observed by a strong decrease in methylation at residues -36, -37, -39, -41, -43 and -44 G; only the -42G residue remained methylated. As clearly seen in Figure 2A, inducible binding at the κB1 site is characterized by decreased methylation of the -54, -55 and -56 G residues as well as a very strong increase in -53G methylation, detected as a broad peak by densitometric scanning (Figure 23C). Interestingly, methylation of -48 and -49G residues which are located between Sp1 and κB1 sites was also significantly decreased after stimulation, indicating that the inducible changes at the κB1 and Sp1 sites affect the whole region delimited by these two sites (Figure 23C). In contrast, no significant modifications of -66G and -21G residues were observed after stimulation, thus restricting the inducible region to the Sp1 and κB1 sites.

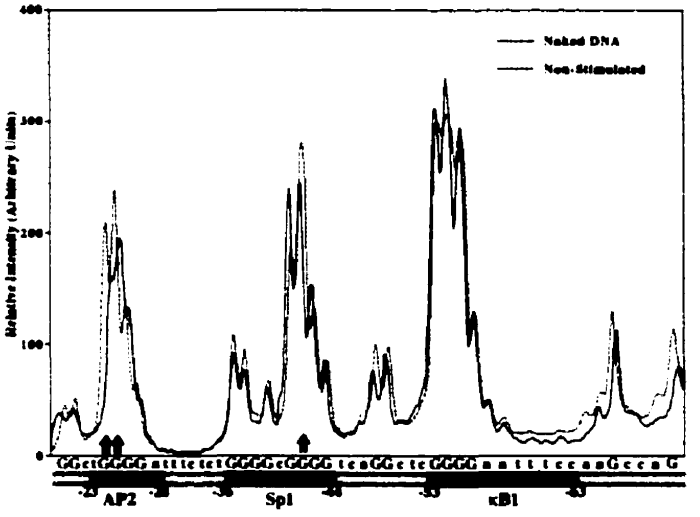
Together, these results demonstrate the *in vivo* occupancy of the -63 to -53 κB1 site of the IκBα promoter after stimulation with either PMA/PHA or TNFα and also indicate that the

Figure 23. Scanning analyses of *In vivo* footprinting of the proximal IκBα gene promoter in Jurkat T cells. Methylation patterns observed on the [-20 to -70] region of non-coding strand of IκBα promoter with naked DNA, or from non-stimulated or TNFα stimulated cells for 4 h, presented in (A) were analysed by densitometry scanning using Scan Jet 4c Hewlett Packard scanner and NIH Image 1.60 software. Comparison of profiles obtained with naked DNA versus DNA from resting Jurkat T cells (B) or with DNA from resting cells versus TNFα stimulated cells (C) correspond to profiles obtained in at least three independent experiments. Open arrows represent constitutive modifications whereas filled arrows correspond to inducible changes. Arrows pointing up or down represent increased or decreased methylation on G residues. The sequence of the scanned region, where the methylated G residues are in capital letters, is indicated below each graph.

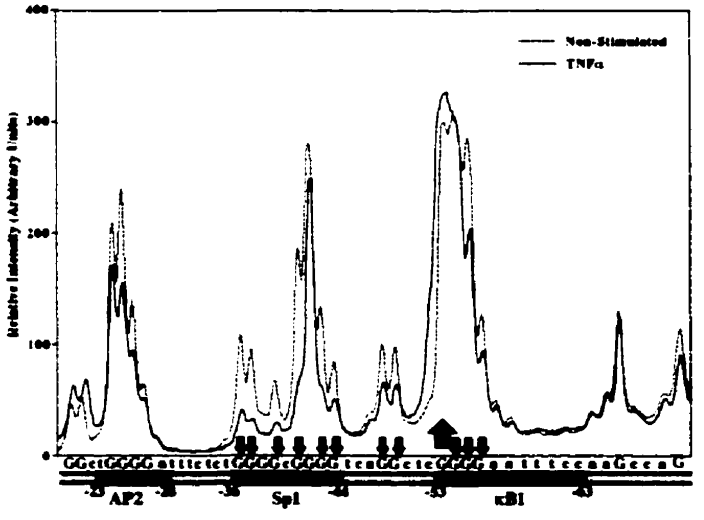
A


TNFα
Non-stimulated
Naked DNA
GGctGGGGatttctctGGGGcGGGGtcaGGctcGGGGa a t t t c c a a G c c a G
-23 AP2 -28 -36 Sp1 -44 -55 κB1 -63

B


Naked DNA
Non-Stimulated
Relative Intensity (Arbitrary Units)
400
300
200
100
0
GGctGGGGatttctctGGGGcGGGGtcaGGctcGGGGa a t t t c c a a G c c a G
-23 AP2 -28 -36 Sp1 -44 -55 κB1 -63

C

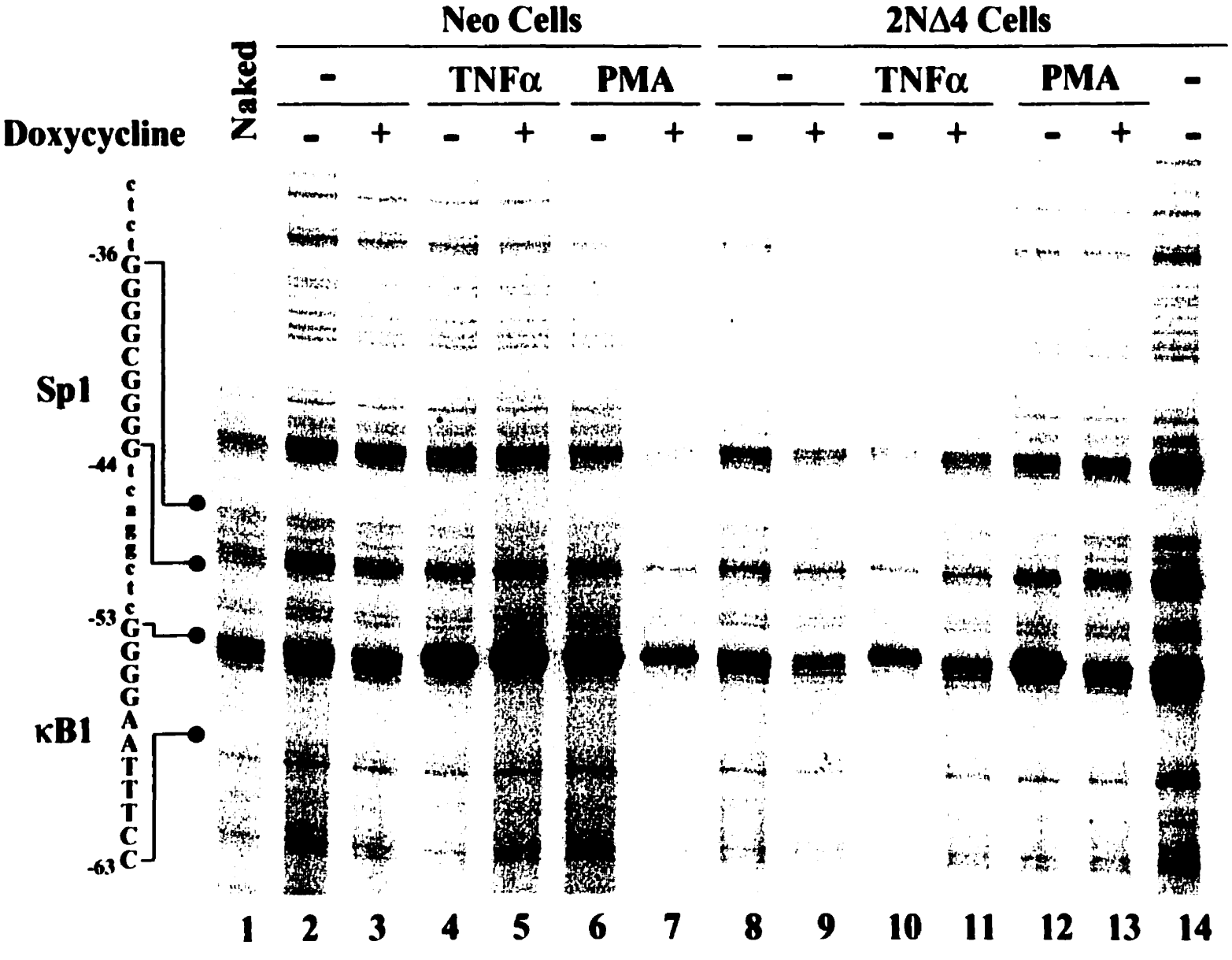

Non-Stimulated
TNFα
Relative Intensity (Arbitrary Units)
400
300
200
100
0
-23 AP2 -28 -36 Sp1 -44 -55 κB1 -63

Sp1 site located 10 bp downstream from the κB1 site may play a role in the inducible transcription of the IκBα promoter.

Footprinting analysis of the IκBα upstream promoter. The potential role of other upstream NF-κB sites that may play a role in IκBα inducibility (185, 228), including κB2 and κB3 sites - was also evaluated. No significant modification of the patterns was observed in the region corresponding to the κB2 site at -319 to -310 or in the putative κB site at -34 to -24, located downstream of κB1. However, the A residue at position -222 in the κB3 site appeared methylated in unstimulated cells as well as in TNFα induced cells, suggesting constitutive protein binding to this site (data not shown). These data indicate that although other κB sites in the IκBα promoter are recognized *in vitro* by NF-κB complexes (185, 228) and *in vivo* by constitutive binding complexes, they are not modified *in vivo* after stimulation of Jurkat T-cells. Only the κB1 site appears to be targeted by inducible NF-κB binding proteins as detected by *in vivo* genomic footprinting (Figure 22).

NF-κB protection of the κB1 site is blocked in TD-IκBα expressing cells. Next, control Jurkat (Neo) and TD-IκBα (2NΔ4) expressing cells were treated with TNFα or PMA/PHA for 40 min, following 24h of Dox induction (Figure 24) and then subjected to genomic footprint analysis using non-coding specific primers 1, 2 and 3. In control cells, the 4G ladder was easily identified (Figure 24, lanes 1-3), and following TNFα or PMA/PHA addition, the characteristic hypersensitive cleavage of -53G was detected (Figure 24, lanes 4-7). In IκBα (2NΔ4) expressing cells, TD-IκBα induction resulted in complete inhibition of inducible binding complexes to the κB1 site in a Dox-dependent manner; TNFα or PMA/PHA stimulation in the absence of Dox-induced TD-IκBα resulted in a footprint at the κB1 site that was indistinguishable from that of stimulated Jurkat cells (Figure 24, lanes 4-7,10,12, and 14), whereas in the presence of Dox-induced TD-IκBα,

Figure 24. ***In vivo*** **footprint of the IκBα gene promoter in rtTA-Neo and Tet-inducible TD-IκBα expressing cells.** Non-coding strand analysis was performed with rtTA-Neo (lanes 2 to 7) or rtTA-IκBα(2NΔ4) Jurkat cells (lanes 8 to 14), either unstimulated (lanes 2, 3, 8, 9 and 14), stimulated by TNFα (10 ng/ml, lanes 4, 5, 9 and 10) or stimulated by PMA/PHA (respectively 50 ng/ml and 1 μg/ml, lanes 6, 7, 12 and 13) for 40 min. Naked DNA was methylated *in vitro* (lanes 1). Where indicated (+), cells were pretreated by doxycycline (1 μg/ml, 24h).

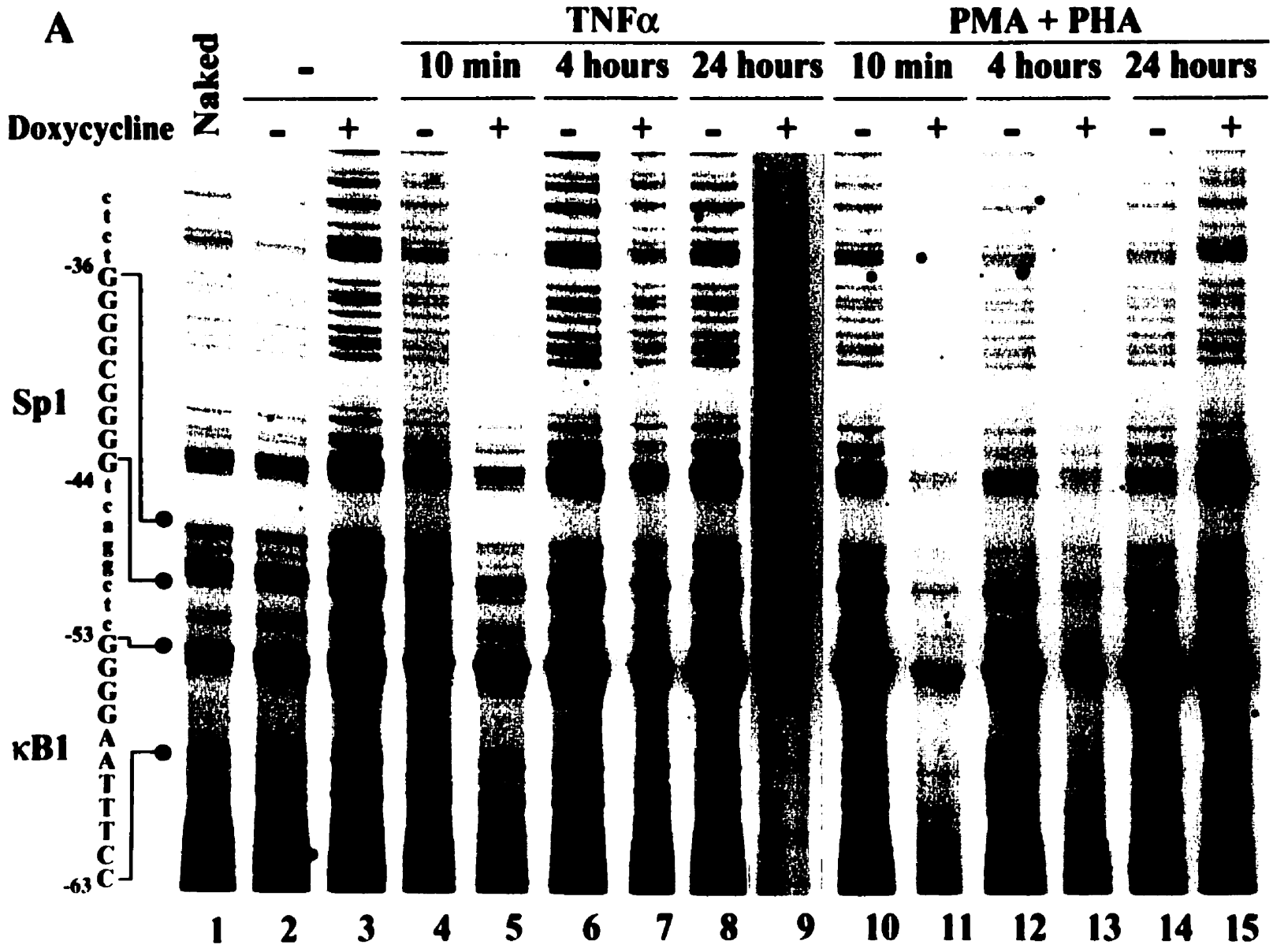

Neo Cells
2NΔ4 Cells
Naked
-
TNFα
PMA
-
TNFα
PMA
-
Doxycycline
- + - + - + - + - + - + - +
Sp1
κB1
-36
-44
-53
-63
1 2 3 4 5 6 7 8 9 10 11 12 13 14

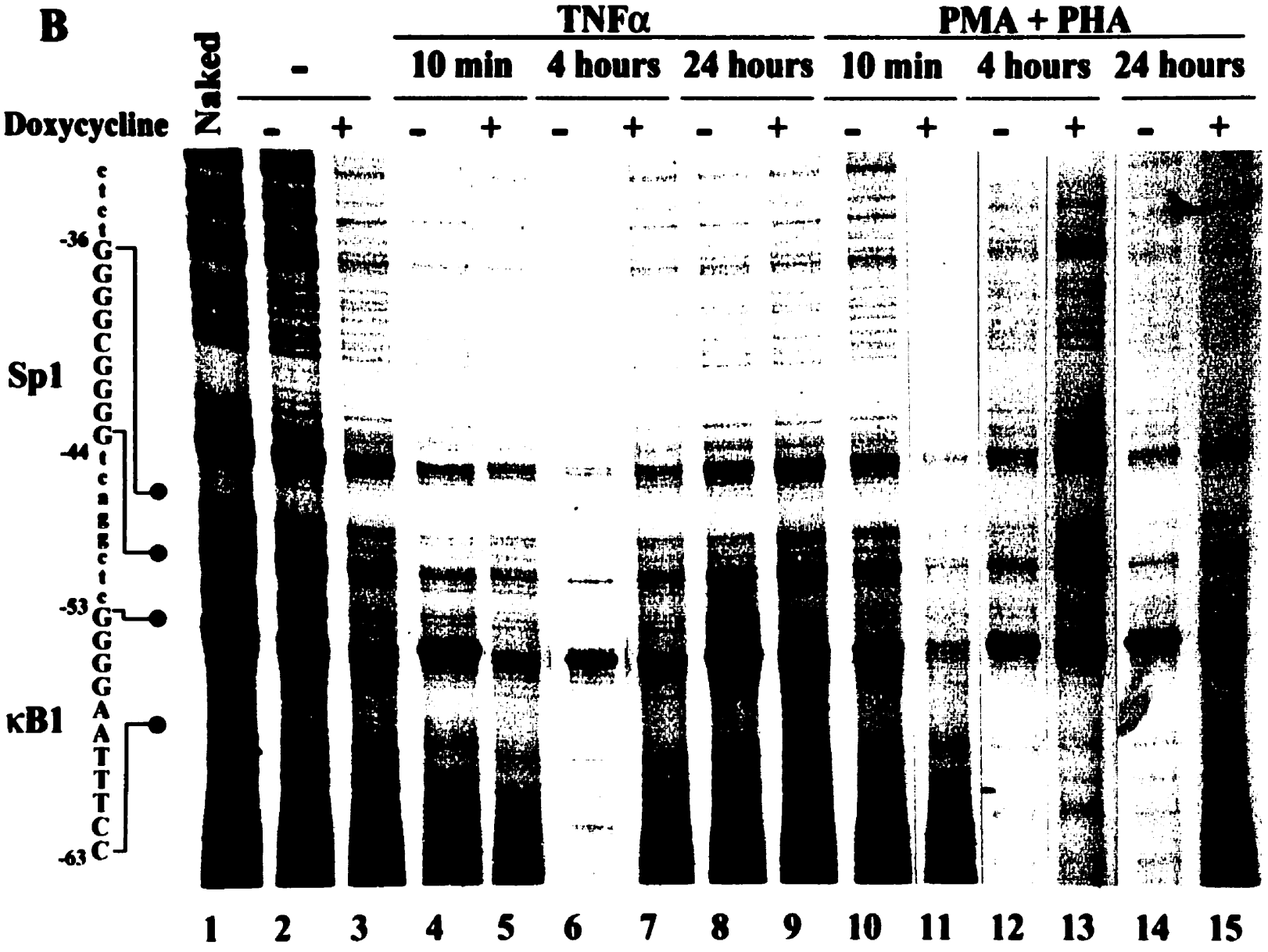
the observed footprint pattern resembled unstimulated control Jurkat cells (Figure 24, lanes 2, 3, 8, 9, 11 and 13). Although less clearly resolved, the pattern of methylation and cleavage of the adjacent Sp1 site was also sensitive to Dox induction. For example, in TD-IκBα inducible cells, PMA/PHA treatment resulted in protection of the Sp1 site with the exception of -42G (Figure 24, lane 12), whereas Dox-induction resulted in protection of the Sp1 site, characteristic of unstimulated control Jurkat cells (Figure 24, lane 13, compare with lanes 2, 3 and 8). These results further indicate that binding of complexes to the Sp1 site may be coordinately regulated by the adjacent κB1 site in a TD-IκBα inducible manner.

Prolonged NF-κB binding and temporal switch in the composition of NF-κB complexes at κB1 site. To determine the kinetics of the *in vivo* occupancy of κB1 and Sp1 sites of IκBα promoter, control Neo (Figure 25A) and TD-IκBα expressing cells (Figure 25B) were analyzed at different times after stimulation by TNFα or PMA/PHA. Surprisingly, the same protection of the κB1 site was observed from 10 min to 24h following TNFα or PMA/PHA treatment in both cell types (Figure 25A, lanes 4-15, Figure 25B lanes 4, 6, 8, 10, 12 and 14). In TD-IκBα expressing cells pretreated with Dox, the pattern of methylation and cleavage of the κB1 site remained characteristic of unstimulated cells (Figure 25B, lanes 5, 7, 9, 11, 13 and 15), regardless of the time of TNFα or PMA/PHA stimulation. Prolonged protection of the adjacent Sp1 site was also observed from 10 min to 24h in stimulated control or TD-IκBα expressing cells (Figure 25A, lanes 4, 5, 7, 10, 12, 14, 15, Figure 25B, lanes 4, 8, 12). Again, Dox induction of TD-IκBα expression resulted in a methylation pattern at the Sp1 site that was characteristic of unstimulated cells (Figure 25B, lanes 5, 7, 9, 13).

To identify the subunit composition of the NF-κB complexes during IκBα induction, EMSA supershift analysis of extracts from rtTA-Neo Jurkat cells treated with PMA/PHA

Figure 25. Kinetics of protein-DNA interactions on the proximal IκBα promoter. Non-coding strand analysis of (A) rtTA-Neo Jurkat T cells and (B) rtTA-IκBα(2NΔ4) Jurkat T cells. For both panels, naked DNA was methylated *in vitro* (lane 1). Cells were either unstimulated (lanes 2 and 3), stimulated by TNFα (10 ng/ml, lanes 4 to 9) or by PMA/PHA (respectively 50 ng/ml and 1 μg/ml, lanes 10 to 15). The time of cell harvesting following TNFα or PMA/PHA stimulation is indicated above the lanes. Where indicated (+), cells were pretreated by doxycycline (1 μg/ml, 24h).


A
Naked
-
TNFα
PMA + PHA
10 min
4 hours
24 hours
10 min
4 hours
24 hours
Doxycycline
- + - + - + - + - + - + - + - +
Sp1
κB1
-36
-44
-53
-63
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15


B
Naked
-
TNFα
10 min
4 hours
24 hours
PMA + PHA
10 min
4 hours
24 hours
Doxycycline
- + - + - + - + - + - + - + - +
Sp1
κB1
-36
-44
-53
-63
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

for 10 min, 4h and 24h was performed using [-66 to -51] κB1 probe and anti-p65, -p50 and -c-Rel antibodies. All PMA/PHA-induced complexes were shifted with anti-p50 antibodies regardless of the time of stimulation (Figure 26, lanes 5-7). Interestingly, at early times of induction - 10 min and 4h - , majority of the NF-κB complex detected contained p65 subunit (Figure 26, lanes 8-10), whereas at later times - 24h - c-Rel constituted the main partner of p50 subunit (Figure 26, lanes 11-13). Thus, induction of IκBα gene transcription appears to require the p50-p65 NF-κB heterodimer during activation, while downregulation may be due in part to p50-c-Rel heterodimer binding to κB1 site. This temporal switch in NF-κB complexes composition is likely to be responsible for the prolonged protection of the κB1 site observed by *in vivo* genomic footprinting.

p65 and Sp1 bind together to the κB1/Sp1 site of IκBα promoter. To assess Sp1 binding to the NF-κB/Sp1 region, EMSA analysis was performed using probes encompassing the [-44 to -36] Sp1 site, the [-63 to -36] κB1 site or both κB1 and Sp1 sites (κB1/Sp1 probe from the [-65 to -34] region of the IκBα promoter). PMA treatment of Jurkat cells resulted in a 10-fold increase in the intensity of the Sp1-binding complex (Figure 27A, upper panel, lanes 1 and 2); complex formation was blocked in competition reactions by both Sp1 and κB1/Sp1 binding sites (Figure 27A, upper panel, lanes 3 and 7), but not by the κB1 site alone (Figure 27A, lane 6). This complex was further identified as an Sp1 binding activity by its competition in the presence of the consensus Sp1 sequence, whereas only partial inhibition was observed when a mutated Sp1 sequence was used as competitor (Figure 27A, upper panel, lanes 4 and 5). As control, no inhibition of NF-κB binding to κB1 was detected in the presence of the Sp1 site of the IκBα promoter, consensus Sp1 or mutated Sp1 sequences (Figure 27A, middle panel, lanes 2-5). Identification of the complexes detected by κB1/Sp1 probe was further determined by supershift analysis (Figure 27B). As expected, anti-p50 and anti-p65

Figure 26. Switch in the composition of NF-κB complexes bound to κB1 site of the IκBα promoter. Supershift analysis was performed with nuclear extracts from rtTA-Neo Jurkat cells treated with PMA/PHA for different times indicated above each lane, using anti-p65 (lanes 5-7), anti-p50 (lanes 8-10) and anti-c-Rel (lanes 11-13) antibodies with an κB1 probe ^{32}P-γATP labeled. Arrows indicate the positions of complexes corresponding to p50/p65 and p50/c-Rel heterodimers, respectively.

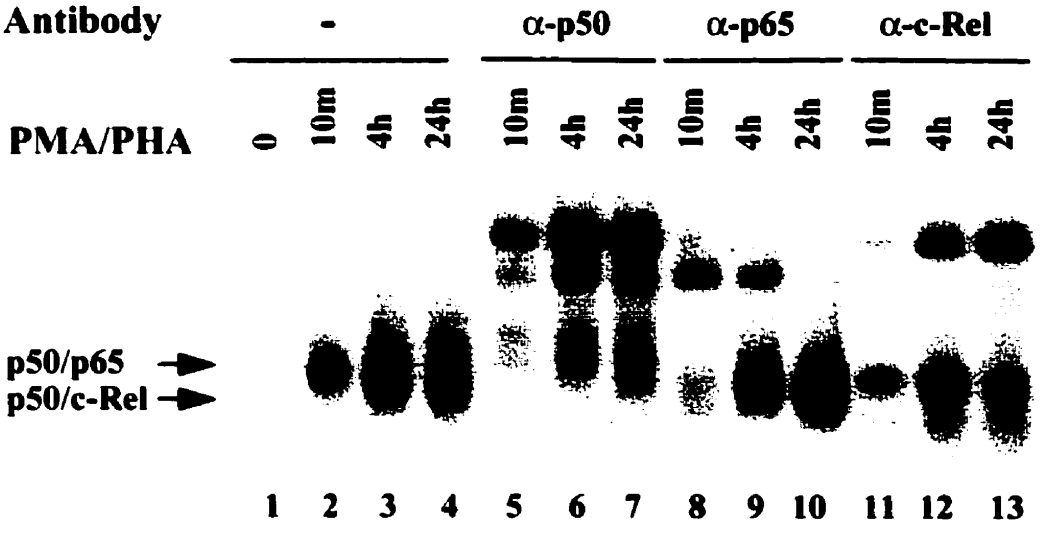
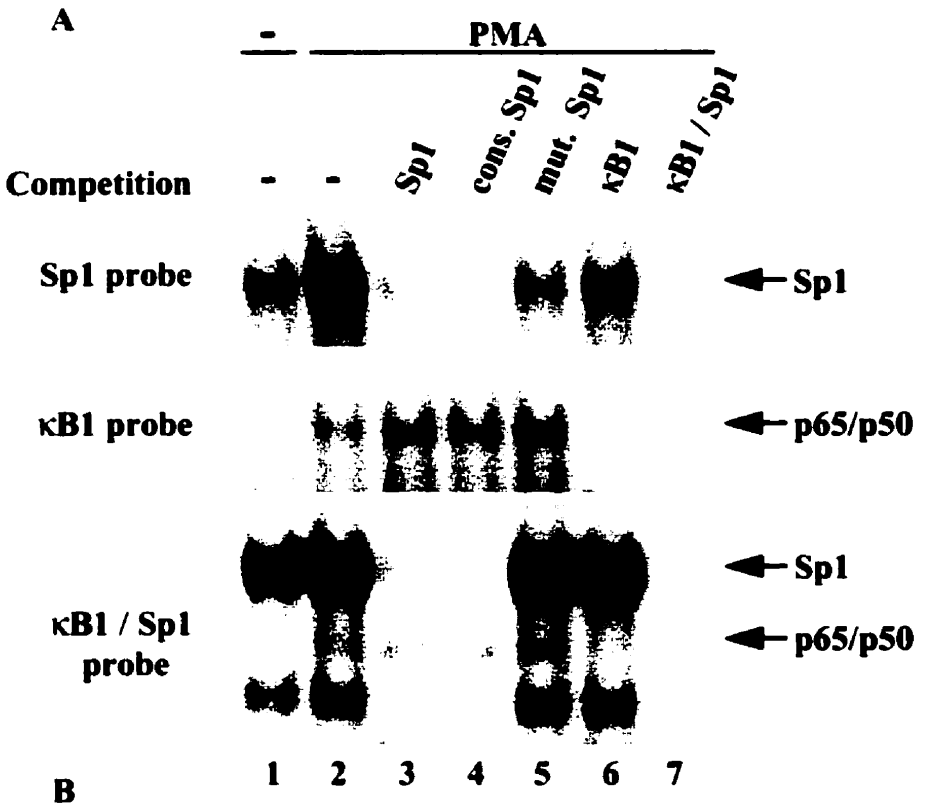

Antibody
-
α-p50
α-p65
α-c-Rel
PMA/PHA
0
10m
4h
24h
10m
4h
24h
10m
4h
24h
10m
4h
24h
p50/p65
p50/c-Rel
1 2 3 4 5 6 7 8 9 10 11 12 13

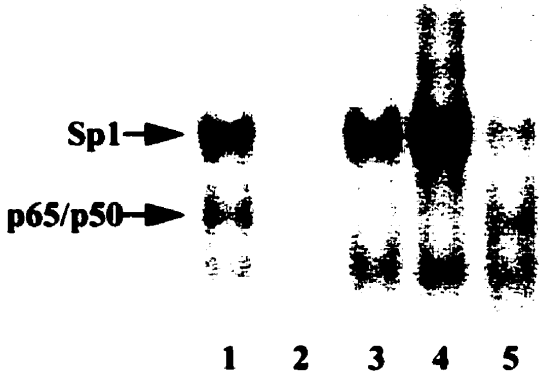
Figure 27. NF-κB and Sp1 bind to the -63 to -36 region of the IκBα promoter. (A) EMSA analysis was performed with different radiolabeled oligonucleotide probes: Sp1 (upper panel), κB1 (middle panel) and κB1/Sp1 (lower panel). Nuclear extracts prepared from Jurkat cells were either unstimulated (lane 1) or treated with PMA (50 ng/ml) for 2h (lanes 2 to 7). Competition was performed in the presence of a 125-fold excess of unlabeled oligunucleotide: Sp1 site of IκBα promoter (lane 3), Sp1 consensus (cons. Sp1, lane 4), mutant Sp1 (mut. Sp1, lane 5), κB1 (lane 6) and κB1/Sp1 (lane 7). To facilitate detection of simultaneous binding of NF-κB and Sp1, EMSA buffer conditions were modified as described in the Materials and Methods and the amount of extract used in the binding reactions was varied between 150ng and 3μg; the binding reactions shown in this figure used 150ng of Jurkat nuclear extract and 500ng of poly dI:dC. (B) Complex composition was analyzed by supershift analysis. PMA-induced nuclear extracts were incubated with anti-p65 (lane 3), anti-p50 (lane 4) and anti-Sp1 (lane 5) antibodies.

A



B

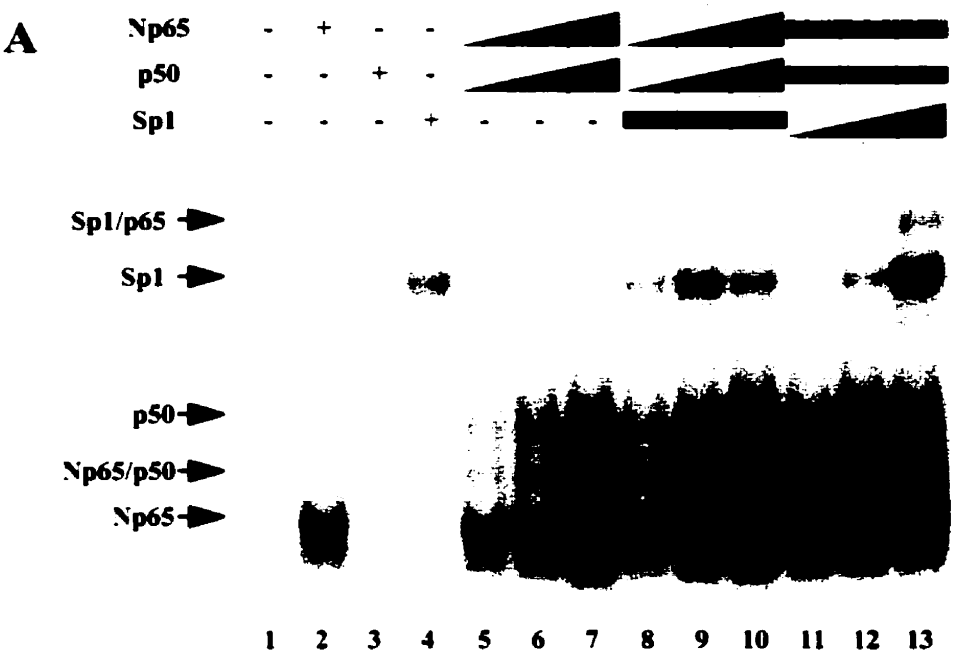
Antibodies	-	-	p65	p50	Sp1
Competition	-	+	-	-	-

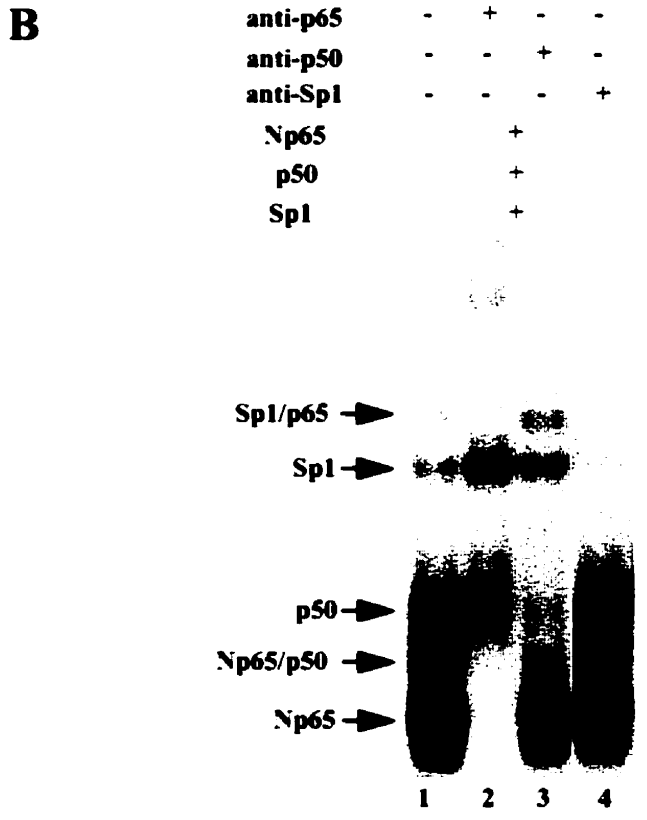


antibodies abolished p65/p50 binding to κB1/Sp1 probe, whereas anti-Sp1 antibodies reacted against Sp1-containing complex (Figure 27B, lanes 3, 4 and 5). The faster migrating band observed with κB1/Sp1 probe (Figure 27A, lanes 1, 2, 5 and 6) is likely to be a degradation product of Sp1-containing complex generated during nuclear protein extraction; this band is competed by Sp1-related oligonucleotides (Figure 27A, lanes 3, 4 and 7) but is not affected by anti-Sp1 antibodies (Figure 27B, lane 5). This EMSA analysis failed to reveal a complex formed by both endogenous Sp1 and NF-κB bound to the κB1/Sp1 probe.

To determine whether Sp1 and p65/p50 bind to their recognition sites (κB1/Sp1) cooperatively in the IκBα promoter, an EMSA was performed with different amounts of recombinant p65/p50 or Sp1 using the κB1/Sp1 probe from the [-65 to -34] region of the IκBα promoter. In the absence of Sp1, p65 and p50 bound to the κB1/Sp1 probe in a dose-dependent manner (Figure 28A, lanes 2, 3, 5-7). In the presence of the same amount of Sp1, no cooperative binding was observed between Sp1 and p65-p50 with increasing amounts of p65-p50 (Figure 28A, lanes 8, 9 and 10). However, at high concentration of p65/p50, an additional complex of slower mobility was observed that also appeared with increasing concentrations of Sp1 when a fixed amount of p65 and p50 was used (Figure 28A, 11-13). This complex was composed of p65 and Sp1 since incubation with specific antibodies eliminated complex formation (Figure 28B). Similarly, anti-p50 antibody removed the p50 containing complexes (Figure 28B, lane 3) while anti-p65 antibody shifted the complexes containing p65 (Figure 28B, lane 2). Anti-Sp1 antibody also disrupted complex formation by removing either Sp1 binding alone or Sp1/p65 heterodimers (Figure 28B, lane 4). Therefore, while Sp1 and p65/p50 do not bind cooperatively to the κB1/Sp1 site, both Sp1 and p65 bind together to the κB1/Sp1 sites of IκBα promoter. The discrepancy between EMSA performed with Jurkat nuclear extracts and recombinant proteins regarding the formation of NF-κB/Sp1 complex could

Figure 28. NF-κB and Sp1 can co-occupy κB1/Sp1 site of IκBα promoter. (A) EMSA was performed with recombinant p65 (GST-Np65), p50 (GST-p50) and Sp1 (rhSp1) proteins using radiolabeled κB1/Sp1 site ^{32}P-γATP labeled. Each recombinant p65 (2 ng), p50 (2.73 ng) and Sp1 (3ng) were used alone in lanes 2-4. Increasing amounts of p65 (0.5, 1 and 2 ng) combined with increasing amounts of p50 (0.65, 1.3 and 2.73 ng) were incubated in the absence (lanes 5-7) or in the presence (lanes 8-10) of Sp1 (3 ng). Increasing amounts of Sp1 (1, 3 and 5 ng) were also tested with the same amount of p65 (1 ng) and p50 (1.3 ng) in lanes 11-13. Shifted complexes are indicated by arrows. (B) Combinations of recombinant p65 (2 ng), p50 (2.73 ng) and Sp1 (3 ng) proteins were incubated with radiolabeled κB1/Sp1 oligonucleotides in the presence or absence of specific antibodies for p65, p50 and Sp1 proteins. The composition of the complexes was analyzed by supershift analysis using anti-p65, anti-p50 or anti-Sp1 antibodies (lanes 2-4).


A
Np65
p50
Sp1
Sp1/p65
Sp1
p50
Np65/p50
Np65
1 2 3 4 5 6 7 8 9 10 11 12 13

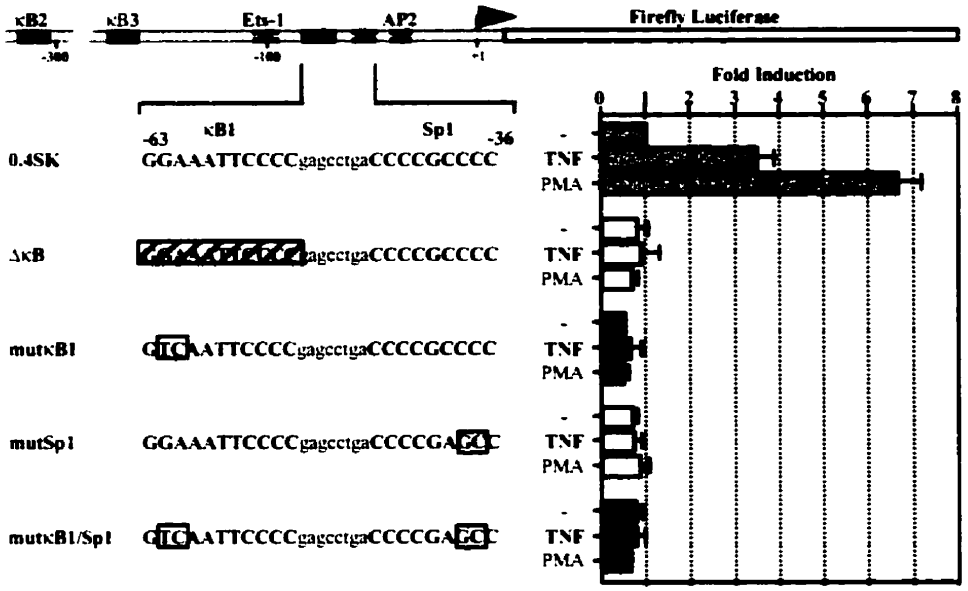

B
anti-p65
anti-p50
anti-Sp1
Np65
p50
Sp1
Sp1/p65
Sp1
p50
Np65/p50
Np65
1 2 3 4

be due to limiting amount of one of the component in nuclear extract, thus excluding detection of this complex. This hypothesis is consistent with the weakness of the Sp1/p65 complex detected in the presence of high amount of recombinant proteins, compared to the p50- and/or p65-containing complexes (Figure 27A, lane 13).

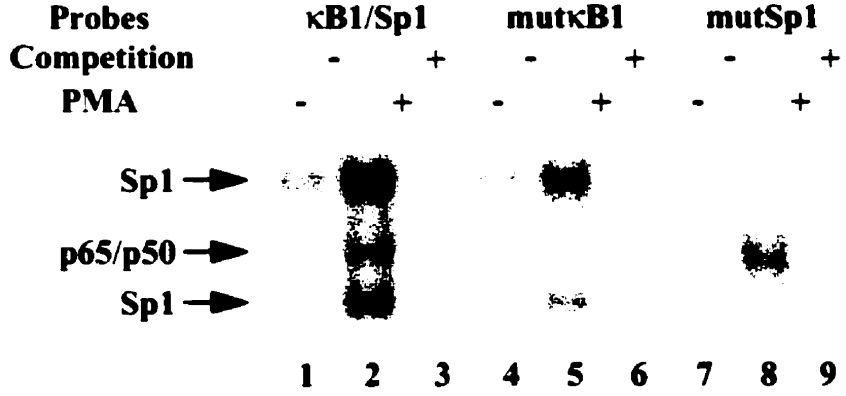
IκBα gene expression is dependent on both NF-κB and Sp1 binding. The functional role of the NF-κB and Sp1 sites in IκBα gene transcription was next examined in Jurkat cells by transient co-transfection with luciferase reporter constructs driven by the wild type IκBα promoter (0.4SK) (228) or mutated versions of the IκBα promoter (Figure 29). Treatment of transfected Jurkat cells with TNFα or PMA/PHA resulted in a 4- and 10-fold stimulation of gene activity, respectively. Deletion or point mutation of the κB1 site of the IκBα promoter (ΔκB and mutκB1) abrogated TNFα and PMA/PHA induced gene activation relative to the wild type promoter (Figure 29A). Strikingly, point mutation of the Sp1 site (mutSp1) also dramatically decreased induction of IκBα gene expression and also slightly decreased basal level promoter activity. As expected, mutation of both κB1 and Sp1 sites also completely inhibited gene activity. As shown in Figure 29B, EMSA analysis demonstrated that impairment of transactivation was due to lack of NF-κB binding (Figure 29B, lanes 4-6) or Sp1 binding (Figure 29B, lanes 7-9) to κB1/Sp1 sites. From these results, both κB1 and Sp1 sites are required for full induction of IκBα promoter. To further analyze whether activation requires direct contact between NF-κB and Sp1, mutant luciferase reporter plasmids containing deletions or insertions between κB1 and Sp1 sites were tested (Figure 29C). Interestingly, the introduction of 5 nucleotides or 9 nucleotides between the κB1 and Sp1 sites which alters the helical relationship between the two sites decreased but did not eliminate IκBα inducibility (Figure 29C). When eight nucleotides (Δ8) between κB1 and Sp1 sites were deleted, transcriptional inducibility was nearly completely abolished. Together, these results indicate that both κB1 and Sp1 sites are required for full induction of IκBα promoter.

Figure 29. Both κB and Sp1 are required for full TNFα or PMA induced IκBα promoter activity. (A) Jurkat cells were transfected with 1 μg of luciferase reporter plasmid containing wild type (0.4SK), mutant κB1 (ΔκB, mutκB1), mutant Sp1 (mutSp1) or mutant κB1/Sp1 (mutκB1/Sp1) IκBα promoter. Twenty four hours after transfection, cells were treated with TNFα (10 ng/ml) or PMA (50 ng/ml) or left untreated for an additional 16h. Transfection efficiency was normalized by *Renila* luciferase (See Materials and Methods). The experiments were performed in triplicate and the average fold induction was calculated. (B) EMSA analysis was performed with different radiolabeled oligonucleotide probes : κB1/Sp1, mutated κB1/Sp1 (mutκB1) and κB1/mutated Sp1 (mutSp1) using uninduced or PMA-induced Jurkat nuclear extract (50 ng/ml PMA for 2h). (C) Luciferase assays were performed as shown in (A) using 1μg of reporter plasmids containing eight nucleotide deletion (Δ8), five nucleotide addition (+5) and nine nucleotide addition (+9) between κB1 and Sp1 sites of IκBα promoter. Transfection efficiency was normalized by *Renila* luciferase (See Materials and Methods). The experiments were performed in triplicate and the average fold induction was calculated.

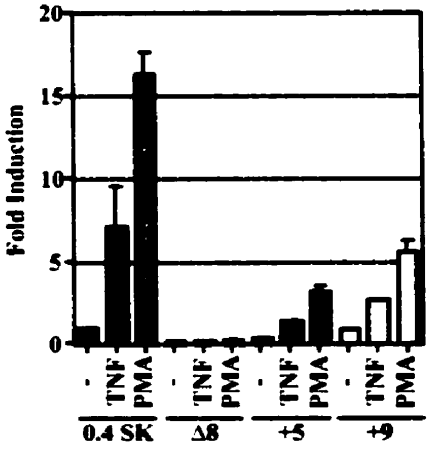
A


κB2
κB3
Ets-1
AP2
Firefly Luciferase
-300
-100
+1
Fold Induction
0 1 2 3 4 5 6 7 8
-63
κB1
Sp1
-36
0.4SK
GGAAATTCCCCgagcctgaCCCCGCCCC
ΔκB
gagcctgaCCCCGCCCC
mutκB1
GTCAATTCCCCgagcctgaCCCCGCCCC
mutSp1
GGAAATTCCCCgagcctgaCCCCGATGC
mutκB1/Sp1
GTCAATTCCCCgagcctgaCCCCGATGC
TNF
PMA

B


Probes
κB1/Sp1
mutκB1
mutSp1
Competition
PMA
Sp1
p65/p50
Sp1
1 2 3 4 5 6 7 8 9

C


Fold Induction
20
15
10
5
0
TNF
PMA
0.4 SK
Δ8
+5
+9

CHAPTER VI

GENERAL DISCUSSION

CONTROL OF NF-κB REGULATED GENE EXPRESSION AND HIV-1 REPLICATION BY MUTANTS OF IκBα

1.0 SUMMARY OF RESULTS

NF-κB plays an important role in stimulation of HIV-1 transcription after T-cell activation, and the HIV-1 LTR is the target of several signal transduction pathways involving both cellular and HIV-1 Tat protein. NF-κB binding sites in HIV-1 LTR have been shown to be indispensable for LTR-driven gene expression in the absence or presence of the HIV-1 Tat protein (160, 165, 258, 293, 411).. Therefore, activators of NF-κB such as TNFα and PMA, have been shown to induce HIV-1 LTR-mediated gene expression and viral replication (1, 160, 336-338). Synergistic activation of HIV-1 LTR-driven gene expression was observed by the concerted action of NF-κB and Tat (42, 43, 258, 446). Therefore, antagonistic effects on these two regulatory factors would adversely affect the level of HIV-1 LTR-mediated activation of viral genes and of viral replication.

1.1 Inhibition of HIV-1 Gene Expression and Virus Multiplication by Transdominant Mutants of IκBα

Different forms of IκBα mutated in distinct regulatory phosphorylation sites, were examined for the effects on the Tat-TNFα synergistic activation of the HIV-1 LTR in Jurkat cells. Transactivation of the HIV-1 LTR was dependent upon both functional Tat-TAR interaction and the presence of NF-κB binding sites in the -100 enhancer region of the HIV-1 LTR. Co-expression of wt IκBα or mutants of IκBα inhibited Tat-TNFα synergism in a dose dependent manner. Interestingly, the transdominant mutants IκBα (2N) or IκBα (2N+3C) were at least five times more effective than wt IκBα in inhibiting HIV-1 LTR directed gene expression. Moreover, IκBα (2N) but surprisingly not IκBα (2N+3C) was more effective in blocking HIV-1 protein and RNA synthesis in a single cycle infection model than wt IκBα or IκBα (3C). The observation that mutations in the C-terminal PEST domain of IκBα decreased the inhibitory potential of IκBα(2N) is

surprising and indicates that an intact C-terminus is required for maximal inhibition of HIV-1 multiplication by IκBα(2N). This effect of the C-terminal domain was not apparent in assays measuring inhibition of LTR transactivation and may thus reflect a distinct functional activity for the IκBα C-terminus.

To further characterize the impact of TD-IκBα on the course of *de novo* HIV-1 infection, Jurkat T cell clones were engineered to inducibly express two transdominant repressor forms of IκBα under the control of a Tet-responsive promoter and first, the effects of inducible expression of TD-IκBα on NF-κB activation were tested. Dox inducible expression of IκBα-2N and IκBα-2NΔ4 was detected as early as 1h after Dox addition and blocked the induction of NF-κB DNA binding by multiple inducers including TNFα, PMA/PHA and dsRNA; expression of TD-IκBα also repressed the expression of the endogenous IκBα gene, consistent with the observation that the IκBα gene contains multiple NF-κB binding sites in its promoter and is tightly regulated by NF-κB (228); NF-κB dependent reporter gene activity was also inhibited following the induction of TD-IκBα. Finally, induced expression of the TD-IκBα successfully inhibited *de novo* HIV-1 infection in Jurkat cells as measured by RT assay, p24 antigen accumulation and viral RNA analysis by RT-PCR. During these studies, the levels of CD4 and fusin were also measured by flow cytometry to determine if TD-IκBα expression repressed cell surface expression of the HIV-1 co-receptors (113); no modulation of either CD4 or fusin levels was observed after 48-96h of Dox-induced TD-IκBα expression (data not shown). Thus, HIV-1 infection was not inhibited at the level of virus attachment and entry.

Our results are complementary to a number of recent experiments which have addressed the role of NF-κB transcription factors in HIV-1 regulated gene expression (42, 43, 258, 446). Westendorp *et al.* demonstrated that HIV-1 Tat protein amplified the activity of TNFα with regard to TNFα-induced activation of NF-κB and TNFα mediated cytotoxicity

via the formation of reactive oxygen intermediates (446). Tat suppressed the expression of Mn-dependent superoxide dismutase (Mn-SOD) which normally functions as part of the cellular response to oxidative stress, thus shifting the cellular redox state towards pro-oxidative conditions. Under these conditions, higher levels of NF-κB binding activity that contribute to stimulation of HIV-1 LTR directed gene expression were observed (data not shown). Under conditions of maximal Tat-TNFα synergism, IκBα was nonetheless able to interfere with NF-κB induction, by sequestering NF-κB in the cytoplasm in a concentration dependent manner (data not shown). IκBα molecules mutated in the N-terminal signal response phosphorylation sites S32 and/or S36 did not undergo rapid inducer mediated degradation (Figure 9) and were at least five times more effective in blocking LTR-directed gene expression. Our experiments furthermore extend a recent study demonstrating that IκB molecules inhibited Tat-mediated transactivation of the HIV-LTR (155).

Biswas *et al.* showed that Tat protein provided a low level of activation of the viral LTR, even in absence of a functional TAR element (42, 43), thus confirming the earlier described TAR-independent mode of Tat action (1, 17, 411). The TAR-independent mode of Tat action was proposed to occur through the transcriptional activation of TNFα which would in turn stimulate NF-κB binding activity (43, 446). These observations are reminiscent of a study demonstrating that interleukin-2 (IL-2) secretion was upregulated at the transcriptional level by the addition of extracellular Tat to activated T cells. The response element in the IL-2 promoter also mapped to the NF-κB site at positions -206 to -195 (447). As was observed in our study, Biswas *et al* found that mutations in the NF-κB motifs decreased Tat activation dramatically, indicating that maximal stimulation of the LTR directed gene expression required Tat-TNFα cooperation (43).

A systematic comparison of HIV-1 LTR activity in human CD4+ primary T cells and a transformed lymphoblastoid cell line J-Jhan demonstrated strikingly different requirements for maximal LTR activation (1). In unstimulated CD4 T lymphocytes, a low basal level of LTR activity was detected, whereas in the lymphoblastoid cell line a high spontaneous level of LTR activity was found that was essentially independent of the NF-κB responsive elements. In contrast, in primary lymphocytes there was an absolute dependence upon the NF-κB sites for initiation and Tat-mediated amplification of HIV-1 transcription. These results are in keeping with differences in the permissiveness to HIV replication of primary versus established cell types. In lymphoblastoid cell lines, HIV infection resulted in active replication in the absence of other stimuli, whereas in primary T cells, replication was undetectable and was dependent upon T cell activation for triggering of viral replication (1). In our study we also found that HIV LTR-directed gene activity required both Tat-TAR transactivation and NF-κB induction, indicating that our Jurkat cell model may reflect more closely the quiescent state of primary T cells, rather than the activated state of other lymphoblastoid cell lines.

NF-κB sites in the HIV-1 LTR were recently demonstrated to be important in enhancing the growth of primary HIV-1 isolates; nonetheless, the virus was still able to grow slowly in the absence of NF-κB sites (70). Similarly, in our study, conditional inhibition of NF-κB in Jurkat T cells by an IκBα repressor significantly interfered with virus multiplication, but could not suppress HIV-1 growth indefinitely. Although the above studies suggest a transcriptional role for IκBα in the inhibition of HIV-1 LTR gene expression, IκBα may act at a distinct level in the HIV-1 life cycle - at the post-transcriptional level of Rev function (452, 453). HIV Rev contains an RNA binding domain, required for interaction with Rev response element (RRE) present in HIV-1 RNA, and an effector domain required for RNA-bound Rev to function in export. The Rev effector domain contains a nuclear export signal (NES) and interacts with the

recently identified nuclear NES receptor, CRM1 (119, 127, 315, 405). IκBα also contains two putative NES elements at the N- and C-terminus, which are from amino acid 45 to 55 and from 265 to 277 respectively, matching the NES consensus within Rev which could be functionally replaced with the IκBα-derived NES (9, 124, 193). Furthermore, newly synthesized IκBα can localize to the nucleus, dissociate NF-κB-DNA complexes and translocate back to the cytoplasm (8, 9, 193, 466). Thus, an additional function of IκBα is to serve as a shuttle protein to export NF-κB from the nucleus. Nuclear export of IκBα has been shown to be mediated by CRM1 (193, 352). One possible explanation for the inhibitory activity of TD-IκBα in HIV-1 infection is that the stable form of IκBα competes effectively with HIV Rev for the nuclear export pathway utilized by CRM1.

HIV-1 infection also causes constitutive activation of NF-κB DNA-binding activity in infected cells (365). A direct temporal correlation exists between HIV infection and the appearance of NF-κB DNA-binding activity in myeloid cells (12, 96, 275, 325, 363, 364), which may in turn prime or stimulate cytokine release (113). Cytokine release from HIV-1 infected cells may contribute to the elevated levels of TNFα, IL-1, IL-6, TGFβ and IFNγ, present in the sera of AIDS patients in late stage disease. In addition, elevated IFNα/β activity is also present in the sera of AIDS patients in late stage disease and serves as a marker for poor prognosis (365). Preliminary data shows that TD-IκBα also interferes with TNFα- and PMA-induced inflammatory cytokines (data not shown), therefore suggesting the possibility of inhibitory effects of TD-IκBα on HIV-1 infection at the level of expression of HIV-1-induced inflammatory cytokines.

Recently, NF-κB activation was shown to play a protective function in the response to TNFα-, ionizing irradiation- and daunorubicin-induced apoptosis (28, 222, 423, 433). Since apoptosis has been suggested to be one of the major mechanisms of CD4+ T cell depletion in HIV-1 infected patients (6, 18, 217, 413) and TNFα plasma levels correlate with disease

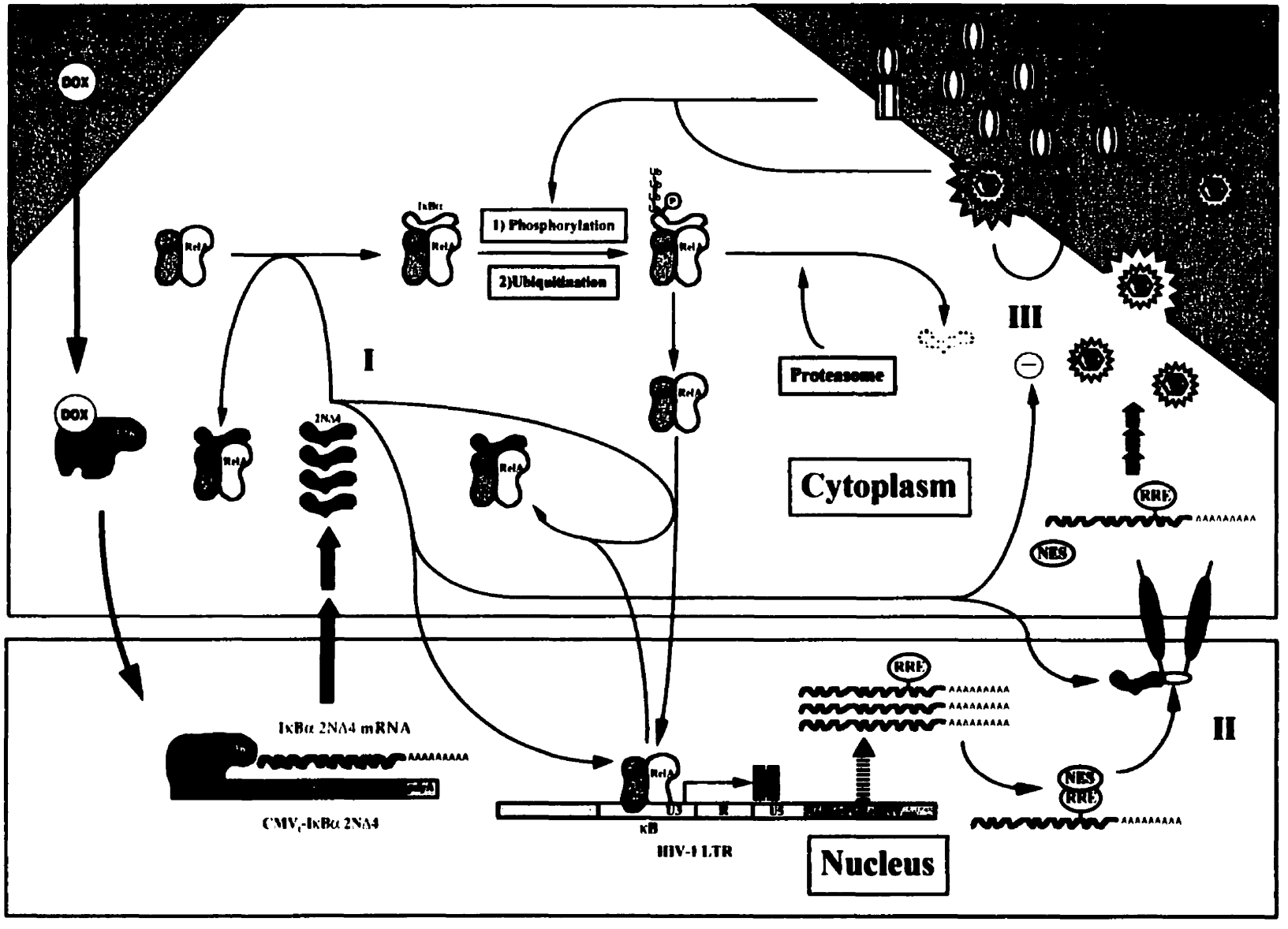
progression (365), the role of NF-κB in HIV-1 induced apoptosis was examined in HIV-1 infected myeloid cells and was also shown to protect HIV-1 infected myeloid cells from apoptosis (94). These studies demonstrated that constitutive NF-κB activation is required to counteract a persistent apoptotic signal resulting from HIV-1 infection; thus, a previously unrecognized role for constitutive NF-κB activation in HIV-1 infected cells is to protect from virus-mediated apoptotic cell death. Similarly, activation of TD-IκBα sensitized Jurkat cells to TNFα-induced cell death (Table 2). However, HIV-1 induced apoptosis was not enhanced by TD-IκBα in Jurkat T cells (data not shown) and, recently, Quinto *et al.* have shown that potent and stable attenuation of recombinant HIV-1 virus engineered to express TD-IκBα was not due to an increase in HIV-1 induced apoptosis (343). Further studies are required to see whether a protective role of NF-κB on HIV-1 induced apoptosis in T cells is also valid as observed in myeloid cells (94).

Thus, given the multiple functions of the NF-κB transcription factors and IκBα inhibitory proteins, Dox-induced TD-IκBα expression may interfere with HIV-1 multiplication at several levels (Figure 30): LTR-mediated transcription, Rev-mediated export of viral RNA, inhibition of HIV-1 induced pro-inflammatory cytokines, and increased sensitivity of HIV-1 infected cells to apoptosis. A transcriptional role for IκBα in the inhibition of HIV-1 LTR driven gene expression was well characterized in our studies.

1.2 Identification by *In Vivo* Genomic Footprinting of an NF-κB/Sp1 Transcriptional Switch that Regulates the IκBα Promoter

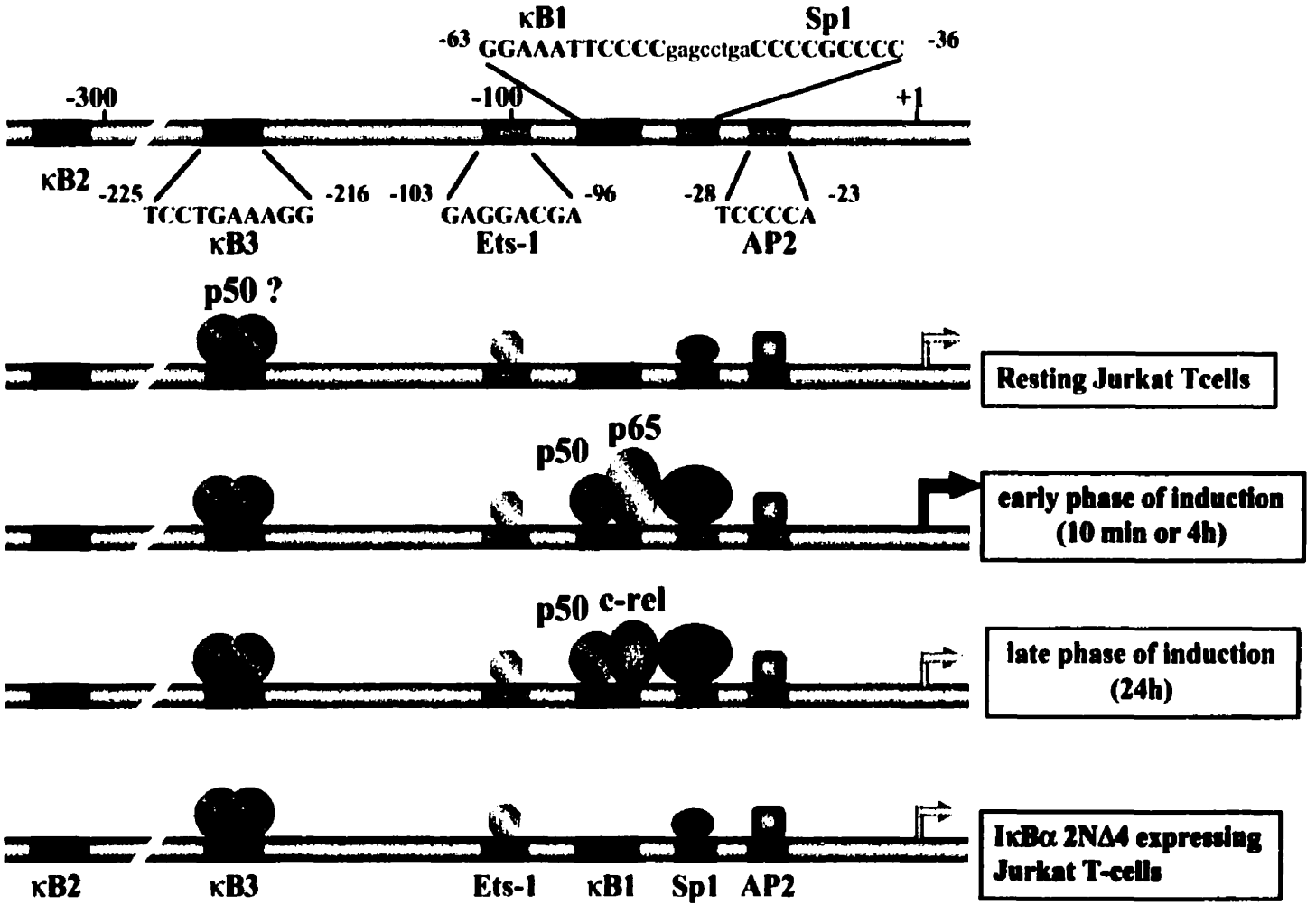
TD-IκBα expression was shown to inhibit endogenous IκBα at the protein level as well as to interfere with NF-κB binding and HIV-1 multiplication in Jurkat cells (222). This led us to study further the autoregulatory control of IκBα transcription by using an *in vivo* genomic footprinting analysis of the IκBα promoter. We demonstrated that induction of

Figure 30. Potential mechanisms of TD-IκBα mutants-mediated inhibition of HIV-1 multiplication. TD-IκBα would be capable of sequestering of newly synthesized NF-κB in uninduced cells as well as inhibition of released NF-κB in the cytoplasm, and removal of DNA bound NF-κB in the nucleus in induced cells by TNFα or virus (I), therefore inhibiting HIV-1 LTR transcription. TD-IκBα is also able to compete with HIV-1 rev protein for the usage of nuclear export pathway which occurs through CRM1 (II). It is also possible that production of virus-induced and NF-κB regulated cytokine such as TNFα can be inhibited by TD-IκBα (III).


DOX
IκBα
RelA
1) Phosphorylation
2)Ubiquitination
Proteasome
I
III
II
2NA4
Cytoplasm
RRE
NES
AAAAAAAAA
IκBα 2NA4 mRNA
CMV-IκBα 2NA4
κB
U3
R
U5
HIV-1 LTR
Nucleus

endogenous IκBα after TNFα or PMA/PHA treatment is suppressed by TD-IκBα at the transcriptional level. Tet-induced TD-IκBα expression blocked NF-κB binding activity at the proximal -63 to -53 κB1 site of the IκBα promoter. This inhibition was shown to be due to the tight association between transactivator p65 and TD-IκBα as revealed by co-immunoprecipitation studies. Similar data were recently obtained in thymocytes isolated from transgenic mice expressing either non-degradable IκBα or IκBβ (11, 48, 168). *In vivo* genomic footprinting revealed multiple protein-DNA interactions in the region of the IκBα promoter between -250 to +100 bp in Jurkat T cells; protection of Sp1, AP2, Ets-1 and κB3 sites in unstimulated cells, indicate that these sites participate in basal level IκBα transcription. In response to stimulation of Jurkat T cells by PMA/PHA or TNFα, changes in methylation of the κB1 site and the adjacent Sp1 site were observed, whereas no inducible changes were detected at κB3 or the other sites (data not shown). The protection observed at κB1 and Sp1 sites was sustained from 10 min to 24h, and together with the EMSA results demonstrated that binding of p50-p65 heterodimer correlated with transcriptional induction of the IκBα gene; at later times, the switch in composition of the NF-κB complexes to predominantly p50-c-Rel heterodimers correlated with transcriptional downregulation. Deletion and point mutagenesis of the IκBα promoter demonstrated that both the κB1 and Sp1 sites were absolutely required for IκBα promoter induction, whereas only Sp1 was involved in basal transcription of this promoter. These results further demonstrated a strict spacing requirement between κB1 and Sp1 sites for full activation of the IκBα promoter. Together, these studies allowed us to propose a model for IκBα transcriptional regulation in Jurkat T cells summarized in Figure 31. In this model, downregulation of IκBα transcription, occurring at later times of induction, is associated with a switch in the composition of NF-κB complexes bound to the κB1 site, from p50-p65 to p50-c-Rel heterodimers. This mechanism is in agreement with inhibition of p65-mediated transcription of the HIV-1 LTR and IL-2Rα promoters by c-Rel (105). Similarly, c-Rel protein, shown to be induced by PMA or TNFα with delayed

Figure 31. Schematic representation of the protein-DNA interactions regulating the IκBα promoter. IκBα promoter organization, including κB1 to κB3 as well as Sp1, Ets-1 and AP2 binding sites, is described in the upper part of the figure. In resting Jurkat T cells, protection are observed at the κB3, Ets-1, Sp1 and AP2 sites which are likely responsible for basal IκBα transcription. At early time of induction of cells by PMA/PHA or TNFα, the κB1 site (-63 to -53) is occupied by p65/p50 heterodimers. At later time, a switch in complex composition to p50-c-Rel heterodimer is correlated with downregulation of IκBα transcription. NF-κB specific binding and IκBα gene transcription is blocked by Tet-induced expression of TD-IκBα, via sequestration of p65 in the cytoplasm. Protection observed at the adjacent Sp1 site (-44 to -36) is also modulated in response to stimulation and is likewise modified by Tet-induced TD-IκBα expression. Binding to the Sp1 site may be related to the occupancy of the κB1 site by inducible NF-κB complexes. No changes were observed on κB3, Ets-1, and AP2 sites during cell activation.


κB1
Sp1
-63 GGAAATTCCCCgagcctgaCCCCGCCCC -36
-300
-100
+1
κB2 -225 TCCTGAAAGG -216
κB3
-103 GAGGACGA -96
Ets-1
-28 TCCCCA -23
AP2
p50 ?
Resting Jurkat Tcells
p50 p65
early phase of induction
(10 min or 4h)
p50 c-rel
late phase of induction
(24h)
κB2 κB3 Ets-1 κB1 Sp1 AP2
IκBα 2NΔ4 expressing
Jurkat T-cells

kinetics compared to p65 (105), may also inhibit IκBα transcription by competition with p50/p65 for occupancy of the κB1 binding site (Figure 31).

The EMSA and genomic footprinting data are consistent with inhibition of IκBα promoter activity identified by deletion of the κB1 site (228); the κB2 and κB3 sites appear to play no role in the inducibility of the IκBα promoter at least in Jurkat T cells stimulated by PMA/PHA or TNFα. Our results are also in agreement with the mutagenesis analysis performed by Ito *et al.* (185), showing a predominant role for the κB1 site. This analysis had also suggested that full activation of the IκBα promoter also required another κB-like site located downstream of κB1 between nucleotides -34 to -24, as well as the upstream κB2 site. In the present study, no inducible *in vivo* protein-DNA interactions were observed at either of these sites. Although a role for κB2 and κB3 sites cannot be excluded, our *in vivo* data clearly demonstrate that κB1 and Sp1 sites play the major role in the inducibility of the IκBα promoter in Jurkat T cells. Furthermore, *in vivo* genomic footprinting experiments performed with the U937 promonocytic cell line revealed the same *in vivo* protection pattern as observed with Jurkat T cells - only the Sp1 site was protected before stimulation and both Sp1 and κB1 sites were targeted by inducible complexes after induction (data not shown). Thus, a common mechanism of IκBα regulation involving the NF-κB/Sp1 transcriptional switch is likely active in multiple cell types, including T cells and monocytes/macrophages.

Many genes regulated by NF-κB also contain adjacent Sp1 sites and direct interaction between NF-κB proteins and Sp1 has been demonstrated (329); a recent study also identified *in vitro* binding of Sp1 to the κB sites located on promoters such as the IL-6 and P-selectin (171). We have demonstrated binding of p50-c-Rel and p50-p65 heterodimers to the κB1 site in response to cell induction, as well as binding of Sp1 to its own site in IκBα promoter. Furthermore, the Sp1 protection observed was reversed with

TD-IκBα activation (Figure 31). This coordinate change suggests that the binding of NF-κB inducible complexes to the κB1 site may also facilitate an increased Sp1 binding affinity to the adjacent Sp1 site. Interestingly, the residues at position -48 and -49, located between the NF-κB and Sp1 sites became more sensitive to methylation/cleavage, suggesting that the protein-DNA conformation of the entire NF-κB/Sp1 region is modified after stimulation (Figure 23).

Sp1 binding to its consensus site prior to stimulation indicates that it may contribute to basal transcription of the IκBα gene. Interestingly, a different methylation and cleavage pattern was observed at the Sp1 site after stimulation. Direct Sp1 conformational changes and/or alterations in Sp1 binding affinity may be induced after stimulation via Sp1 post-translational modification. Sp1 has been described as a zinc finger phosphoprotein which upon cell activation undergoes specific phosphorylations and dephosphorylations that regulate its DNA binding activity and its interactions with other proteins (10, 355, 377). Following cell stimulation, Sp1 can be phosphorylated by casein kinase II, protein kinase A and by a recently described 60 kDa kinase, activated in response to Neu differentiation factors (NDFs) (5). The inducible change at the Sp1 binding site in response to Jurkat T cells stimulation may reflect such Sp1 modifications leading to increased binding on DNA.

The critical role of the κB1 site and the adjacent Sp1 site in the inducibility of the IκBα promoter is further supported by the conservation of these two sites in the murine and porcine homologs of the IκBα promoter (74, 90). Moreover, not only are the exact sequences conserved, but also the 10 bp spacing between both sites is maintained between species. This distance, corresponding to one helical turn of DNA, may permit a physical interaction between proteins bound to the Sp1 site and the p65-p50 complex on the same face of chromatin *in vivo*, as shown for the HIV-1 LTR promoter (329).

Although no cooperativity in the binding of NF-κB and Sp1 was observed by EMSA, transfection studies using hybrid IκBα promoters in which nucleotides between κB1 and Sp1 sites were inserted or deleted, revealed a strict spacing requirement for maximal inducibility of IκBα promoter. Deletion of the 8 nucleotides between both sites or insertion of 5 or 9 nucleotides significantly lowered IκBα promoter inducibility. The fact that addition of an half helical turn, or a complete helical turn led to a similar decrease in IκBα gene inducibility, argues against a direct physical interaction between NF-κB and Sp1, and rather suggests a requirement for the interaction of NF-κB and/or Sp1 with basal transcription factors such as TAFs or with the transcription machinery for maximal induction of IκBα promoter. Sp1 has been found in a multiprotein complex containing CBP/p300, although a direct interaction has not been shown, and the NF-κB p65 subunit is able to interact with the N- and C-terminal region of the coactivator p300, resulting in gene activation of E-selectin and VCAM-1 (138, 317, 330). Further studies are required to characterize the association of NF-κB and Sp1 with TAFs, TBP or CBP/p300, and their role in IκBα regulation.

As shown above by luciferase assays as well as others (31, 74, 185, 228) basal expression of IκBα gene is not regulated by NF-κB. However, we observed a gradual disappearance of endogenous IκBα upon an inducible expression of TD-IκBα. Therefore, RNase protection assay was performed using total RNA isolated from TD-IκBα expressing cells treated with Dox up to 72h. There was no or very little decrease in the amount of endogenous IκBα mRNA (data not shown). Similar results by Beg *et al.* indicated that no difference was observed in the basal levels of IκBα RNA expression in both wt and *p65*$^{-/-}$ embryonic fibroblasts yet, IκBα protein levels were decreased in *p65*$^{-/-}$ embryonic fibroblasts (31). This may be due to a decreased stability of IκBα in the absence of p65 as observed in a number of studies (26, 348, 389, 407) As shown by co-immunoprecipitation studies (Figure 21), endogenous p65 was mainly associated with

TD-IκBα after 24h of Dox treatment, due to an increased amount of TD-IκBα. These experiments revealed the presence of TD-IκBα in latent NF-κB complex containing the transactivating subunit p65. Unbound endogenous IκBα thus is rapidly degraded in the free form and a decreased steady-state levels of endogenous IκBα become undetectable by Western Blot Analysis. Similar data was obtained in thymocytes isolated from transgenic mice expressing IκBα (ΔN), another constitutive repressor of IκBα lacking N-terminal signal response domain (48, 53). In contrast, decreased levels of endogenous IκBα were not observed in thymocytes which were also isolated from transgenic mice expressing similar transdominant mutants of IκBα (mIκBα) (168). However, this may be due to different levels of transgene expression. In our Jurkat T cell model, there was significantly more TD-IκBα than endogenous IκBα and TD-IκBα can be further increased by Dox treatment while almost equal levels of mIκBα were observed compared to endogenous IκBα in thymocytes expressing mIκBα (2, 168, 222). Thymocytes isolated from these mice similarly responded to NF-κB inducers as analyzed by Western Blot analysis, co-immunoprecipitation and DNA binding assays. Therefore, our *in vitro* Jurkat T cell system can reflect the *in vivo* transgenic mice model on NF-κB/IκB signaling studies.

2.0 FUTURE DIRECTIONS

The experiments presented in this thesis analyze the effects of TD-IκBα on HIV-1 replication. Both transient and stable transfection of TD-IκBα efficiently inhibited HIV-1 multiplication in a single cycle infection model and in *de novo* HIV-1 infection. This inhibitory effect of TD-IκBα resulted in part from cytoplasmic sequestration of NF-κB which has been shown to be an important host transcription factor for HIV-1 LTR activity.

1) As shown in our experiments, inhibition of Tat-mediated HIV-1 LTR transcription by TD-IκBα has also been verified by others (157). Therefore, it would be interesting to see how TD-IκBα inhibits Tat-mediated HIV-1 LTR transcriptional activity. One possible explanation for the inhibitory effect of TD-IκBα on Tat-mediated HIV-1 LTR transcription can be made based upon the observation that NF-κB p65 interacts with p300/CBP which has been shown to activate Tat by acetylation (138, 209, 330, 476). However, Tat has also been shown to interact with p300/CBP (34, 175, 271, 272). Therefore, it is not clear whether acetylation of Tat is performed by p300/CBP that is associated with Tat or by p300/CBP that is associated with p65. To differentiate these two possible mechanisms, one can use GAL4 luciferase reporter in which only GAL4 DNA binding sites are introduced to upstream of TAR element without the upstream regulatory elements. These two elements would be sufficient because intact Tat- or Tat-TNFα synergistic activation was observed using IIIΔ23 construct which contains only the enhancer and the TAR elements (Figure 8). The question above would be answered by introducing GAL4-p300/CBP or GAL4 DNA binding domain (negative control) along with HIV-1 Tat and GAL4-TAR hybrid luciferase.

Some research has suggested that Tat can also induce NF-κB activation (47, 84, 97, 220). This effect of Tat on NF-κB activation was shown by extracellular, not intracellular, Tat. In addition, we performed the reporter assay using NF-κB specific luciferase (HIVenh/pGL3) and observed no effect of intracellular Tat introduced by a transient transfection (data not shown). Whether extracellular Tat-mediated NF-κB activation occurs through signal transduction involving an unidentified HIV-1 Tat receptor is not currently known. Experiments are in progress to assess the effect of exogenous Tat on NF-κB activation using the inducible TD-IκBα expressing Jurkat cells. Tat has also been involved in some other signal transduction pathways involving PKC and PI3K (84, 284, 296). Interestingly, these kinases have recently been shown to be involved in IKK

activation (197, 224, 310, 356, 378). Therefore, once we get the system in which extracellular Tat activates NF-κB, it would be also interesting to see the involvement of the recently identified IKKs as well as upstream kinases on this extracellular Tat-mediated NF-κB activation by using transdominant mutants of these kinases. It has also been suggested that Tat-mediated NF-κB activation was proposed to occur through the transcriptional activation of TNFα gene expression by Tat which would in turn activate NF-κB via autocrine or paracrine effect of TNFα (43, 446).

2) Unexpected results were obtained with IκBα (2N+3C) in a single cycle infection. IκBα (2N) was significantly more effective in inhibiting HIV-1 multiplication than IκBα (2N+3C) (Figure 11 and 12). The only difference between these two mutants were at the C-terminal region. This many suggest the importance of the C-terminal region on posttranscriptional inhibitory effect on HIV-1 because both mutants could effectively inhibit HIV-1 LTR transcription induced by Tat and TNFα (Figure 10). The posttranscriptional inhibitory effect of IκBα on HIV-1 rev function was previously suggested by Dr. Gary Nabel's group (452, 453). This inhibitory effect of IκBα may be through the competition with HIV-1 rev protein for the usage of nuclear export pathway which occurs through CRM1 (193, 352). First of all, we have to further verify whether IκBα (2N+3C) mutants are less effective in blocking HIV-1 replication compared IκBα (2N) in cell types other than Cos-1 cells. Therefore, we tried to generate Jurkat T cells that are inducibly expressing wt and mutants of IκBα. Unfortunately, only three cell lines (rtTA-Neo, 2N and 2NΔ4) were obtained which were used in this thesis. Because the generation of stable cell line is time-consuming and labor-intensive, we started to use the protein transduction method (431). It has been demonstrated that not only Jurkat T cells can be transduced but also many different cell types (294, 388). Introduction of these proteins to Jurkat cells and their subsequent HIV-1 infection would tell us whether the C-terminal region of IκBα is also important in inhibiting HIV-1 multiplication in these cells.

It would then be interesting to analyze the inhibitory effect of TD-IκBα on HIV-1 Rev function at the posttranscriptional level through the nuclear export pathways.

3) A number of cytokine genes such as IL-2, Granulocyte colony-stimulating factor (G-CSF), TNFα, TNFβ, Lymphotoxin β (LT-β) and β-interferon (IFN-β) are regulated by NF-κB and HIV-1 infection causes constitutive activation of NF-κB DNA binding activity (16, 24, 287, 319, 365). Therefore, it would be interesting to analyze the effect of TD-IκBα on HIV-1-induced cytokine gene expression. In addition, preliminary data shows that the inducible expression of TD-IκBα inhibited TNFα- and PMA-induced cytokine expression (data not shown).

4) There are a number of inhibitors of NF-κB activity that act at the following levels: 1) through induction of IκBα expression, 2) interference with NF-κB nuclear transport 3) inhibition of NF-κB DNA binding, 4) suppression of transactivational potential of NF-κB (110). For example, cyclopentenone prostaglandins (cyPGs) have been shown to inhibit NF-κB activation by preventing phosphorylation and degradation of IκBα (360). Recently, it has been shown to inhibit IKKβ activity through PPAR-γ independent mechanism (67, 361). Current problems with these inhibitors are that some of them can affect signaling pathways other than the NF-κB signaling pathway. Also, the molecular mechanisms of action of these inhibitors is not well defined. However, TD-IκBα specifically inhibits NF-κB activity. On the other hand, overexpression of non-degradable IκBα or IκBβ has caused problems such as spontaneous development of squamous cell carcinoma and malfunction of immune cells (48, 168, 425). Therefore, an ideal solution is to express TD-IκBα conditionally. As our study demonstrated, inducible expression of TD-IκBα inhibited HIV-1 replication, possibly at multiple different stages of viral life cycle (Figure 30).

A recent publication demonstrated that the HIV-1 protease expressed in HIV-1 infected cells cleaved a modified pro-caspase 3 (431). Upon cleavage by HIV-1 protease, caspase 3 became active and executed an apoptotic process, which lead to the elimination of infected cells only. Pro-caspase 3 in uninfected cells was not active, leaving uninfected cells unharmed. Modeling after this study, it will be interesting to introduce HIV-1 proteolytic cleavage sites into the N-terminal region of IκBα after the signal response domain. We propose that upon HIV-1 infection, this N-terminal region along with the signal response domain will be cleaved away by HIV-1 protease, creating N-terminally deleted IκBα (ΔN-IκBα) which behaves like TD-IκBα. Therefore, the HIV-1 protease induced TD-IκBα should eventually inhibit HIV-1 multiplication. In uninfected cells, modified IκBα containing HIV-1 protease cleavage sites will behave like wt IκBα. Therefore, these cells should function normally.

3.0 CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

NF-κB plays an important role not only in immune response, but also in viral pathogenesis of HIV-1. HIV-1 LTR expression involves a complex interaction between HIV-1 Tat protein and host transcription factors such as NF-κB. Therefore, the effect of TD-IκBα on Tat/TNFα synergistic activation of HIV-1 LTR was first analyzed. Use of Jurkat cell line inducibly expressing TD-IκBα permitted a study on the effect of TD-IκBα on *de novo* HIV-1 infection. We further examined the autoregulation of NF-κB signaling by analyzing IκBα promoter using *in vivo* genomic footprinting.

1) Transactivation of the HIV-1 LTR by Tat and TNFα is dependent on both functional Tat-TAR interaction and the presence of NF-κB binding sites. Coexpression of wt IκBα or mutants of IκBα inhibits Tat-TNFα synergism in a dose-dependent manner. HIV-1 protein and RNA synthesis is inhibited by TD-IκBα in a single cycle infection model and this occurs at the transcriptional level.

2) Jurkat cells are sensitized to TNFα induced apoptosis by inducible expression of TD-IκBα. This may result from the inhibition of anti-apoptotic role of NF-κB by TD-IκBα. NF-κB DNA binding activity and LTR transactivation is reduced by TD-IκBα. Inducible expression of TD-IκBα inhibits *de novo* HIV-1 infection at the transcriptional level.

3) Inducible expression of TD-IκBα suppress endogenous wt IκBα expression, which was due to tight interaction between TD-IκBα and NF-κB which prevented NF-κB binding activity. *In vivo* genomic footprinting revealed stimulus-responsive protein-DNA binding to the κB1 and Sp1 sites in the IκBα promoter which was inhibited by inducible expression of TD-IκBα. With time after stimulation, decreased levels of transcriptionally active p65/p50 and increased c-Rel/p50 heterodimers were detected at the κB1 site. Both

κB1 and Sp1 sites were required for IκBα promoter induction and a strict spacing between two sites was necessary for full activation.

CHAPTER VII

REFERENCES

1. **Alcami, J., T. Lain de Lera, L. Folgueira, M.-A. Pedraza, J.-M. Jacque, F. Bachelerie, A. R. Noriega, R. T. Hay, D. Harrich, R. B. Gaynor, J.-L. Virelizier, and F. Arenzana-Seisdedos.** 1995. Absolute dependence on κB responsive elements for initiation and Tat-mediated amplification of HIV transcription in blood CD4 T lymphocytes. EMBO Journal **14:**1552-1560.

2. **Algarte, M., H. Kwon, P. Genin, and J. Hiscott.** 1999. Identification by in vivo genomic footprinting of a transcriptional switch containing NF-kappaB and Sp1 that regulates the IkappaBalpha promoter. Mol Cell Biol **19:**6140-53.

3. **Algarté, M., P. Lécine, R. Costello, A. Plet, D. Olive, and J. Imbert.** 1995. In vivo regulation of interleukin-2 receptor alpha gene transcription by the coordinated binding of constitutive and inducible factors in human primary T cells. EMBO Journal **16:**5060-5072.

4. **Alkalay, I., A. Yaron, A. Hatzubai, A. Orian, A. Ciechanover, and Y. Ben-Neriah.** 1995. Stimulation-dependent IκBα phosphorylation marks the NF-κB inhibitor for degradation *via* the ubiquitin-proteasome pathway. Proceedings of the National Academy of Sciences USA **92:**10599-10603.

5. **Alroy, I., L. Soussan, R. Seger, and Y. Yarden.** 1999. Neu Differentiation factor stimulates phosphorylation and activation of the Sp1 transcription factor . Molecular Biology of the Cell **19:**1961-1972.

6. **Ameisen, J., J. Estaquier, T. Idziorek, and F. DeBels.** 1995. The relevance of apoptosis to AIDS pathogenesis. Trends in Cell Biology **5:**27-32.

7. **Aoki, T., Y. Sano, T. Yamamoto, and J. Inoue.** 1996. The ankyrin repeats but not the PEST-like sequences are required for signal-dependent degradation of IκBα. Oncogene **12:**1159-1164.

8. **Arenzana-Seisdedos, F., J. Thompson, M. S. Rodriguez, F. Bachelerie, D. Thomas, and R. T. Hay.** 1995. Inducible nuclear expression of newly synthesized IκBα negatively regulates DNA-binding and the transcriptional activities of NF-κB. Mol.Cell.Biol. **15:**2689-2696.

9. **Arenzana-Seisdedos, F., P. Turpin, M. Rodriguez, D. Thomas, R. T. Hay, J.-L. Virelizier, and C. Dargemont.** 1997. Nuclear localization of IκBα promotes active transport of NF-κB from the nucleus to the cytoplasm. Journal of Cell Science **110**:369-378.

10. **Armstrong, S. A., D. A. Barry, R. W. Leggett, and C. R. Mueller.** 1997. Casein kinase II-mediated phosphorylation of the C terminus of Sp1 decreases its DNA binding activity. Journal of Biological Chemistry **272**:13489-13495.

11. **Attar, R. M., H. MacDonald-Bravo, C. Raventos-Suarez, S. K. Durham, and R. Bravo.** 1998. Expression of constitutively active IκBβ in T cells of transgenic mice: peristent NF-κB activity is required for T-cell immune responses. Molecular and Cellular Biology **18**:477-487.

12. **Bachelerie, F., J. Alcami, F. Arenzana-Seisdedos, and J.-L. Virelizier.** 1991. HIV enhancer activity perpetuated by NF-κB induction on infection of monocytes. Nature **350**:709-712.

13. **Baeuerle, P. A.** 1998. IκB-NF-κB structures: At the interface of inflammation Control. Cell **95**:729-731.

14. **Baeuerle, P. A., and D. Baltimore.** 1988. IκB: a specific inhibitor of the NF-κB transcription factor. Science **242**:540-546.

15. **Baeuerle, P. A., and D. Baltimore.** 1996. NF-κB: Ten years after. Cell **87**:13-20.

16. **Baeuerle, P. A., and T. Henkel.** 1994. Function and activation of NF-κB in the immune system. Annual Review of Immunology **12**:141-179.

17. **Bagasra, O., K. Khalili, T. Seshamma, J. Taylor, and R. Pomerantz.** 1992. TAR-independent replication of human immunodeficiency virus type 1 in glial cells. Journal of Virology **66**:7522-7528.

18. **Baichwal, V. R., and P. A. Baeuerle.** 1997. Apoptosis: Activate NF-κB or die? Current Biology **7**:R94-R96.

19. **Baldi, L., K. Brown, G. Franzoso, and U. Siebenlist.** 1996. Critical role for lysines 21 and 22 in signal-induced, ubiquitin-mediated proteolysis of IκBα. Journal of Biological Chemistry **271:**376-379.

20. **Baldwin, A. S. J.** 1996. The NF-κB and IκB proteins: new discoveries and insights. Annual Review of Immunology **14:**649-681.

21. **Ballard, D. W., W. H. Walker, S. Doerre, S. Sista, J. A. Molitor, E. P. Dixon, N. J. Peffer, M. Hanninck, and W. C. Greene.** 1990. The v-rel oncogene encodes a κB enhancer binding protein that inhibits NF-κB function. Cell **63:**803-814.

22. **Barroga, C. F., J. K. Stevenson, E. M. Schwarz, and I. M. Verma.** 1995. Constitutive phosphorylation of IκBα by casein kinase II. Proceedings of the National Academy of Sciences USA **92:**7637-7641.

23. **Baud, V., Z. G. Liu, B. Bennett, N. Suzuki, Y. Xia, and M. Karin.** 1999. Signaling by proinflammatory cytokines: oligomerization of TRAF2 and TRAF6 is sufficient for JNK and IKK activation and target gene induction via an amino terminal effector domain. Genes & Development **13:**1297-1308.

24. **Beauparlant, P., and J. Hiscott.** 1996. Biological and biochemical inhibitors of the NF-κB/Rel proteins and cytokine synthesis. Cytokine and Growth Factor Reviews **7:**175-190.

25. **Beauparlant, P., H. Kwon, M. Clarke, R. Lin, N. Sonenberg, M. Wainberg, and J. Hiscott.** 1996. Transdominant mutants of IκBα block Tat-TNF synergistic activation of HIV-1 expression and virus multiplication. Journal of Virology **70:**5777-5785.

26. **Beauparlant, P., R. Lin, and J. Hiscott.** 1996. The role of the C-terminal domain of IκBα in protein degradation and stability. Journal of Biological Chemistry **271:**10690-10696.

27. **Beg, A. A., and S. Baldwin, Jr.** 1993. The IκB proteins: multifunctional regulators of Rel/NF-κB transcription factors. Genes & Development **7:**2064-2070.

28. **Beg, A. A., and D. Baltimore.** 1996. An essential role for NF-κB in preventing TNF-α-induced cell death. Science **274:**782-784.

29. **Beg, A. A., S. M. Ruben, R. I. Scheinman, S. Haskill, C. A. Rosen, and A. S. Baldwin, Jr.** 1992. IκB interacts with the nuclear localization sequence of the subunits of NF-κB: a mechanism for cytoplasmic retention. Genes & Development **6:**1899-1913.

30. **Beg, A. A., W. C. Sha, R. T. Bronson, and D. Baltimore.** 1995. Constitutive NF-κB activation, enhanced granulopoiesis, and neonatal lethality in IκBα-deficient mice. Genes & Development **9:**2736-2746.

31. **Beg, A. A., W. C. Sha, R. T. Bronson, S. Ghosh, and D. Baltimore.** 1995. Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-κB. Nature **376:**167-170.

32. **Belich, M. P., A. Salmeron, L. H. Johnston, and S. C. Ley.** 1999. TPL-2 kinase regulates the proteolysis of the NF-κB inhibitory protein NF-κB1 p105. Nature **397:**363-368.

33. **Bender, K., M. Gottlicher, S. Whiteside, H. J. Rahmsdorf, and P. Herrlich.** 1998. Sequential DNA damage-independent and -dependent activation of NF- kappaB by UV. Embo J **17:**5170-81.

34. **Benkirane, M., R. F. Chun, H. Xiao, V. V. Ogryzko, B. H. Howard, Y. Nakatani, and K. T. Jeang.** 1998. Activation of integrated provirus requires histone acetyltransferase. p300 and P/CAF are coactivators for HIV-1 Tat. J Biol Chem **273:**24898-905.

35. **Beraud, C., W. J. Henzel, and P. A. Baeuerle.** 1999. Involvement of regulatory and catalytic subunits of phosphoinositide 3- kinase in NF-kappaB activation. Proc Natl Acad Sci U S A **96:**429-34.

36. **Bergmann, M., L. Hart, M. Lindsay, P. J. Barnes, and R. Newton.** 1998. IkappaBalpha degradation and nuclear factor-kappaB DNA binding are insufficient for interleukin-1beta and tumor necrosis factor-alpha- induced kappaB-dependent

transcription. Requirement for an additional activation pathway. J Biol Chem **273:**6607-10.

37. **Berkhout, B., A. Gatignol, A. B. Rabson, and K.-T. Jeang.** 1990. TAR-independent activation of the HIV-1 LTR: evidence that Tat requires specific regions of the promoter. Cell **62:**757-767.

38. **Berkhout, B., and K. T. Jeang.** 1992. Functional roles for the TATA promoter and enhancers in basal and Tat-induced expression of the human immunodeficiency virus type 1 long terminal repeat. Journal of Virology **66:**139-149.

39. **Berkhout, B., R. H. Silverman, and K. T. Jeang.** 1989. Tat trans-activates the human immunodeficiency virus through a nascent RNA target. Cell **59:**273-82.

40. **Betts, J. C., and G. J. Nabel.** 1996. Differential regulation of NF-kappaB2(p100) processing and control by amino-terminal sequences. Mol Cell Biol **16:**6363-71.

41. **Bird, T. A., K. Schooley, S. K. Dower, H. Hagen, and G. D. Virca.** 1997. Activation of nuclear transcription factor NF-kappaB by interleukin-1 is accompanied by casein kinase II-mediated phosphorylation of the p65 subunit. J Biol Chem **272:**32606-12.

42. **Biswas, D. K., C. M. Ahlers, B. J. Desube, and A. B. Pardee.** 1993. Cooperative inhibition of NF-κB and Tat-induced superactivation of human immunodeficiency virus type 1 long terminal repeat. Proceedings of the National Academy of Sciences USA **90:**11044-11048.

43. **Biswas, D. K., T. R. Salas, F. Wang, C. M. Ahlers, B. J. Dezube, and A. B. Pardee.** 1995. A Tat-induced auto-up-regulatory loop for superactivation of the human immunodeficiency virus type 1 promoter. Journal of Virology **69:**7437-7444.

44. **Bitar, R., P. Beauparlant, R. Lin, P. Pitha, and J. Hiscott.** 1995. Retrovirus-mediated transfer of nuclear factor-κB subunit genes modulates IκBα and interferon β expression. Cell Growth and Differentiation **6:**965-976.

45. **Blank, V., P. Kourilsky, and A. Israël.** 1991. Cytoplasmic retention, DNA binding and processing of the NF-κB p50 precursor are controlled by a small region in its C-terminus. EMBO Journal **10:**4159-4167.

46. **Blank, V., P. Kourilsky, and A. Israël.** 1992. NF-κB and related proteins: Rel/dorsal homologies meet ankyrin-like repeats. Trends in Biochemical Sciences **17:**135-140.

47. **Blazquez, M. V., A. Macho, C. Ortiz, C. Lucena, M. Lopez-Cabrera, F. Sanchez-Madrid, and E. Munoz.** 1999. Extracellular HIV type 1 Tat protein induces CD69 expression through NF- kappaB activation: possible correlation with cell surface Tat-binding proteins. AIDS Res Hum Retroviruses **15:**1209-18.

48. **Boothby, M., A. L. Mora, D. Scherer, J. Brockman, and D. W. Ballard.** 1997. Perturbation of the T lymphocyte lineage in transgenic mice expressing a constitutive repressor of NF-κB. Journal of Experimental Medicine **185:**1897-1907.

49. **Bours, V., V. Azarenko, E. Dejardin, and U. Siebenlist.** 1994. Human RelB (I-Rel) functions as a κB site-dependent transactivating member of the family of Rel-related proteins. Oncogene **9:**1699-1702.

50. **Bours, V., P. R. Burd, K. Brown, J. Villalobos, S. Park, R.-P. Ryseck, R. Bravo, K. Kelly, and U. Siebenlist.** 1992. A novel mitogen-inducible gene product related to p50/p105-NF-κB participates in transactivation through a κB site. Molecular and Cellular Biology **12:**685-695.

51. **Bours, V., G. Franzoso, V. Azarenko, S. Park, T. Kanno, K. Brown, and U. Siebenlist.** 1993. The oncoprotein Bcl-3 directly transactivates through κB motifs via association with DNA-binding p50B homodimers. Cell **72:**729-739.

52. **Bours, V., J. Villalobos, P. R. Burd, K. Kelly, and U. Siebenlist.** 1990. Cloning of a mitogen-inducible gene encoding a κB DNA-binding protein with homology to the *rel* oncogene and to cell-cycle motifs. Nature **348:**76-80.

53. **Brockman, J. A., D. C. Scherer, T. A. McKinsey, S. M. Hall, X. Qi, W. Y. Lee, and D. W. Ballard.** 1995. Coupling of a signal response domain in IκBα to multiple pathways for NF-kB activation. Mol.Cell.Biol. **15:**2809-2818.

54. **Brown, K., S. Gerstberger, L. Carlson, G. Franzoso, and U. Siebenlist.** 1995. Control of IκBα proteolysis by site-specific, signal-induced phosphorylation. Science **267:**1485-1488.

55. **Brown, K., S. Park, T. Kanno, G. Franzoso, and U. Siebenlist.** 1993. Mutual regulation of the transcriptional activator NF-κB and its inhibitor, IκBα. Proceedings of the National Academy of Sciences USA **90:**2532-2536.

56. **Brownell, D., N. Mittereder, and N. R. Rice.** 1989. A human *rel* proto-oncogene cDNA can containing an Alu fragment as a potential coding exon. Oncogene **4:**935-942.

57. **Bull, P., K. L. Morley, M. F. Hoekstra, T. Hunter, and I. M. Verma.** 1990. The mouse c-Rel protein has an N-terminal regulatory domain and a C-terminal transcriptional transactivation domain. Molecular and Cellular Biology **10:**5473-5485.

58. **Buonaguro, L., F. M. Buonaguro, G. Giraldo, and B. Ensoli.** 1994. The human immunodeficiency virus type 1 Tat protein transactivates tumor necrosis factor beta gene expression through a TAR-like structure. J Virol **68:**2677-82.

59. **Burkly, L., C. Hession, L. Ogata, C. Reilly, L. A. Marconi, D. Olson, R. Tizard, R. Cate, and D. Lo.** 1995. Expression of RelB is required for the development of thymic medulla and dendritic cells. Nature **373:**531-536.

60. **Butscher, W. G., C. Powers, M. Olive, C. Vinson, and K. Gardner.** 1998. Coordinate transactivation of the interleukin-2 CD28 response element by c-Rel and ATF-1/CREB2. J Biol Chem **273:**552-60.

61. **Caamano, J. H., P. Perez, S. A. Lira, and R. Bravo.** 1996. Constitutive expression of Bcl-3 in thymocytes increases the DNA binding of NF-kappaB1 (p50) homodimers in vivo. Mol Cell Biol **16:**1342-8.

62. **Caamano, J. H., C. A. Rizzo, S. K. Durham, D. S. Barton, C. Raventos-Suarez, C. M. Snapper, and R. Bravo.** 1998. Nuclear factor (NF)-kappa B2 (p100/p52) is required for normal splenic microarchitecture and B cell-mediated immune responses. J Exp Med **187:**185-96.

63. **Cao, Z., W. J. Henzel, and X. Gao.** 1996. IRAK: a kinase associated with the interleukin-1 receptor. Science **271:**1128-31.

64. **Cao, Z., J. Xiong, M. Takeuchi, T. Kurama, and D. V. Goeddel.** 1996. TRAF6 is a signal transducer for interleukin-1. Nature **383:**443-6.

65. **Carrasco, D., R. P. Ryseck, and R. Bravo.** 1993. Expression of relB transcripts during lymphoid organ development: specific expression in dendritic antigen-presenting cells. Development **118:**1221-31.

66. **Casolaro, V., S. N. Georas, Z. Song, I. D. Zubkoff, S. A. Abdulkadir, D. Thanos, and S. J. Ono.** 1995. Inhibition of NF-AT-dependent transcription by NF-kappa B: implications for differential gene expression in T helper cell subsets. Proc Natl Acad Sci U S A **92:**11623-7.

67. **Castrillo, A., M. J. Diaz-Guerra, S. Hortelano, P. Martin-Sanz, and L. Bosca.** 2000. Inhibition of IkappaB kinase and IkappaB phosphorylation by 15-deoxy-Delta(12,14)-prostaglandin J(2) in activated murine macrophages [In Process Citation]. Mol Cell Biol **20:**1692-8.

68. **Chan, H., D. P. Bartos, and L. B. Owen-Schaub.** 1999. Activation dependent transcriptional regulation of the human Fas promoter requires NF-κB p50-p65 recruitment. Molecular and Cellular Biology **19:**2098-2108.

69. **Chang, C.-C., J. Zhang, L. Lombardi, A. Neri, and R. Dalla-Favera.** 1994. Mechanism of expression and role in transcriptional control of the proto-oncogene NFKB-2/LYT-10. Oncogene **9:**923-933.

70. **Chen, B. J., M. B. Feinberg, and D. Baltimore.** 1997. The κB sites in the human immunodeficiency virus type 1 long terminal repeat enhance virus replication yet are not absolutely required for virus growth. Journal of Virology **71:**5495-5504.

71. **Chen, J. H.** 1985. The proto-oncogene c-ets is preferentially expressed in lymphoid cells. Mol Cell Biol **5:**2993-3000.

72. **Chen, Z., J. Hagler, V. J. Palombella, F. Melandri, D. Scherer, D. Ballard, and T. Maniatis.** 1995. Signal-induced site-specific phosphorylation targets IκBα to the ubiquitin-proteasome pathway. Genes & Development **9:**1586-1597.

73. **Chen, Z., L. Parent, and T. Maniatis.** 1996. Site-specific phosphorylation of IκBα by a novel ubiquitination-dependent protein kinase activity. Cell **84:**853-862.

74. **Chiao, P. J., S. Miyamoto, and I. M. Verma.** 1994. Autoregulation of IκBα activity. Proceedings of the National Academy of Sciences USA **91:**28-32.

75. **Chu, C. H., T. McKinsey, L. Liu, J. Gentry, M. Malim, and D. W. Ballard.** 1997. Suppression of tumor necrosis factor-induced cell death by inhibitor of apoptosis c-IAP2 is under NF-κB control. Proceedings of the National Academy of Sciences USA **94:**10057-10062.

76. **Chu, W. M., D. Ostertag, Z. W. Li, L. Chang, Y. Chen, Y. Hu, B. Williams, J. Perrault, and M. Karin.** 1999. JNK2 and IKKbeta are required for activating the innate response to viral infection. Immunity **11:**721-31.

77. **Chu, Z.-L., T. A. McKinsey, L. Liu, X. Qi, and D. Ballard.** 1996. Basal phosphorylation of the PEST domain in IκBβ regulates its functional interaction with the c-*rel* proto-oncogene product. Molecular and Cellular Biology **16:**5974-5984.

78. **Chu, Z. L., Y. A. Shin, J. M. Yang, J. A. DiDonato, and D. W. Ballard.** 1999. IKKγ mediates the interaction of cellular IκB kinases with the tax transforming protein of HTLV-1. Journal of Biological Chemistry **274:**15297-15300.

79. **Chun, R. F., and K. T. Jeang.** 1996. Requirements for RNA polymerase II carboxyl-terminal domain for activated transcription of human retroviruses human T-cell lymphotropic virus I and HIV-1. J Biol Chem **271:**27888-94.

80. **Cogswell, P. C., M. W. Mayo, and A. S. Baldwin, Jr.** 1997. Involvement of Egr-1/RelA synergy in distinguishing T cell activation from tumor necrosis factor-alpha-induced NF-kappa B1 transcription. J Exp Med **185:**491-7.

81. **Cogswell, P. C., R. I. Scheinman, and A. S. Baldwin, Jr.** 1993. Promoter of the human NF-κB p50/p105 gene. Journal of Immunology **150:**2794-2804.

82. **Cohen, E. A., E. F. Terwillinger, J. G. Sodroski, and W. A. Haseltine.** 1988. Identification of a protein encoded by the *vpu* gene of HIV-1. Nature **334:**532-534.

83. **Cohen, L., W. J. Henzel, and P. A. Baeuerle.** 1998. IKAP is a scaffold protein of the IκB kinase complex. Nature **395:**292-296.

84. **Conant, K., M. Ma, A. Nath, and E. O. Major.** 1996. Extracellular human immunodeficiency virus type 1 Tat protein is associated with an increase in both NF-κB binding and protein kinase C activity in primary human astrocytes. Journal of Virology **70:**1384-1389.

85. **Connelly, M. A., and K. B. Marcu.** 1995. CHUK, a new member of the helix-loop-helix and leucine zipper families of interacting proteins, contains a serine-threonine kinase catalytic domain. . Cell.Mol.Biol.Res. **41:**537-549.

86. **Cujec, T. P., H. Okamoto, K. Fujinaga, J. Meyer, H. Chamberlin, D. O. Morgan, and B. M. Peterlin.** 1997. The HIV transactivator TAT binds to the CDK-activating kinase and activates the phosphorylation of the carboxy-terminal domain of RNA polymerase II. Genes Dev **11:**2645-57.

87. **Cullen, B. R.** 1993. Does HIV-1 Tat induce a change in viral initiation rights? Cell **73:**417-420.

88. **D'Addario, M., M. A. Wainberg, and J. Hiscott.** 1992. Activation of cytokine genes in HIV-1 infected myelomonoblastic cells by phorbol ester and tumor necrosis factor. Journal of Immunology **148:**1222-1229.

89. **Davis, N., S. Ghosh, D. L. Simmons, P. Tempst, H.-C. Liou, D. Baltimore, and H. R. Bose, Jr.** 1991. Rel-associated pp40: an inhibitor of the rel family of transcription factors. Science **253:**1268-1271.

90. **de Martin, R., B. Vanhove, Q. Cheng, E. Hofer, V. Csizmadia, H. Winkler, and F. H. Bach.** 1993. Cytokine-inducible expression in endothelial cells of an IκBα-like gene is regulated by NF-κB. EMBO Journal **12:**2773-2779.

91. **Dechend, R., F. Hirano, K. Lehmann, V. Heissmeyer, S. Ansieau, G. G. Wulczyn, C. Scheidereit, and A. Leutz.** 1999. The Bcl-3 oncoprotein acts as a bridging factor between NF-κB/Rel and nuclear co-regulators. Oncogene **18:**3316-3323.

92. **Delhase, M., M. Hayakawa, Y. Chen, and M. Karin.** 1999. Positive and negative regulation of the IκB kinase activity through IKKβ subunit phosphorylation. Science **284:**309-313.

93. **Delling, U., S. Roy, M. Sumner-Smith, R. Barnett, L. Reid, C. A. Rosen, and N. Sonenberg.** 1991. The number of positively charged amino acids in the basic domain of Tat is critical for trans-activation and complex formation with TAR RNA. Proceedings of the National Academy of Sciences USA **88:**6234-6238.

94. **DeLuca, C., H. J. Kwon, N. Pelletier, M. A. Wainberg, and J. Hiscott.** 1998. NF-κB protects HIV-1 infected myeloid cells from apoptosis. Virology **244:**27-38.

95. **DeLuca, C., L. Petropoulos, D. Zmeureanu, and J. Hiscott.** 1999. Nuclear IκBβ maintains persistent NF-κB activation in HIV-1-infected myeloid cells. Journal of Biological Chemistry **274:**13010-13016.

96. **DeLuca, C., A. Roulston, A. Koromilas, M. A. Wainberg, and J. Hiscott.** 1996. Chronic human immunodeficiency virus type I infection of myeloid cells disrupts the autoregulatory control of the NF-κB/Rel pathway via enhanced IκBα degradation. Journal of Virology **70:**5183-5193.

97. **Demarchi, F., F. d'Adda di Fagagna, A. Falaschi, and M. Giacca.** 1996. Activation of transcription factor NF-κB by the Tat protein of Human Immunodeficiency virus type 1. Journal of Virology **70:**4427-4437.

98. **Diaz-Meco, M. T., I. Dominguez, L. Sanz, P. Dent, J. Lozano, M. M. Municio, E. Berra, R. T. Hay, T. W. Sturgill, and J. Moscat.** 1994. V PKC induces phosphorylation and inactivation of IκBα *in vitro*. EMBO Journal **13:**2842-2848.

99. **Didonato, J., F. Mercurio, C. Rosette, J. Wu-Li, H. Suyang, S. Ghosh, and M. Karin.** 1996. Mapping of the inducible IκB phosphorylation sites that signal its ubiquitination and degradation. Molecular and Cellular Biology **16:**1295-1304.

100. **DiDonato, J. A., M. Hayakawa, D. M. Rothwarf, E. Zandi, and M. Karin.** 1997. A cytokine-responsive IκB kinase that activates the transcription factor NF-κB. Nature **388:**548-554.

101. **Diehl, J., W. Tong, G. Sun, and M. Hannink.** 1995. TNFa-dependent activation of a RelA homodimer in astrocytes: increased phosphorylation of RelA and MAD-3 precede activation of RelA. Journal of Biological Chemistry **270:**2703-2707.

102. **Dingwall, C., I. Ernberg, M. J. Gait, S. M. Green, S. Heaphy, J. Karn, A. D. Lowe, M. Singh, and M. A. Skinner.** 1990. HIV-1 tat protein stimulates transcription by binding to a U-rich bulge in the stem of the TAR RNA structure. Embo J **9:**4145-53.

103. **Dobrzanski, P., R. P. Ryseck, and R. Bravo.** 1993. Both N- and C-terminal domains of RelB are required for full transactivation: role of the N-terminal leucine zipper-like motif. Mol Cell Biol **13:**1572-82.

104. **Dobrzanski, P., R. P. Ryseck, and R. Bravo.** 1994. Differential interactions of Rel-NF-kappa B complexes with I kappa B alpha determine pools of constitutive and inducible NF-kappa B activity. Embo J **13:**4608-16.

105. **Doerre, S., P. Sista, S.-C. Sun, D. W. Ballard, and W. C. Greene.** 1993. The *c-rel* protooncogene product represses NF-κB p65-mediated transcriptional activation of the long terminal repeat of type 1 human immunodeficiency virus. Proceedings of the National Academy of Sciences USA **90:**1023-1027.

106. **Doi, T. S., M. W. Marino, T. Takahashi, T. Yoshida, T. Sakakura, L. J. Old, and Y. Obata.** 1999. Absence of tumor necrosis factor rescues RelA-deficient mice from embryonic lethality. Proc Natl Acad Sci U S A **96:**2994-9.

107. **Duckett, C. S., N. D. Perkins, T. F. Kowalik, R. M. Schmid, E.-S. Huang, A. S. Baldwin, Jr., and G. J. Nabel.** 1993. Dimerization of NF-κB2 with RelA(p65) regulates DNA binding, transcriptional activation, and inhibition by an IκBα (MAD3). Molecular and Cellular Biology **13:**1315-1322.

108. **Ellinger-Ziegelbauer, H., K. Brown, K. Kelly, and U. Siebenlist.** 1997. Direct activation of the stress-activated protein kinase (SAPK) and extracellular signal-regulated protein kinase (ERK) pathways by an inducible mitogen-activated protein Kinase/ERK kinase kinase 3 (MEKK) derivative. J Biol Chem **272:**2668-74.

109. **Ensoli, B., G. Barillari, S. Z. Salahuddin, R. C. Gallo, and F. Wong-Staal.** 1990. Tat protein of HIV-1 stimulates growth of cells derived from Kaposi's sarcoma lesions of AIDS patients. Nature **345:**84-6.

110. **Epinat, J. C., and T. D. Gilmore.** 1999. Diverse agents act at multiple levels to inhibit the Rel/NF-kappaB signal transduction pathway. Oncogene **18:**6896-6909.

111. **Ernst, M. K., L. L. Dunn, and N. R. Rice.** 1995. The PEST-like sequence of IκBα is responsible for inhibition of DNA binding but not for cytoplasmic retention of c-Rel or RelA homodimers. Mol.Cell.Biol. **15:**872-882.

112. **Fan, C.-M., and T. Maniatis.** 1991. Generation of p50 subunit of NF-κB by processing of p105 through an ATP-dependant pathway. Nature **354:**395-398.

113. **Fauci, A. S.** 1996. Host factors and pathogenesis of HIV-induced disease. Nature **384:**529-534.

114. **ferreira, V., N. Tarantino, and M. Korner.** 1998. Discrimination between RelA and RelB Transcriptional Regulation by a Dominant Negative Mutant of IκBα. Journal of Biological Chemistry **273:**592-599.

115. **Finco, T. S., and A. S. Baldwin, Jr.** 1993. κB site-dependant induction of gene expression by diverse inducers of nuclear factor κB requires Raf-1. Journal of Biological Chemistry **268:**17676-17679.

116. **Fischer, U., J. Huber, W. C. Boelens, I. W. Mattaj, and R. Luhrmann.** 1995. The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. Cell **82:**475-483.

117. **Fisher, A. G., E. Collalti, L. Ratner, R. C. Gallo, and F. Wong-Staal.** 1985. A molecular clone of HTLV-III with biological activity. Nature **316:**262-265.

118. **Folks, T. M., K. A. Clouse, J. Justement, A. Rabson, E. Duh, J. H. Kerhl, and A. S. Fauci.** 1989. Tumor necrosis factor alpha induces expression of human immunodeficiency virus in a chronically infected T-cell clone. Proceedings of the National Academy of Sciences USA **86:**2365-2368.

119. **Fornerod, M., M. Ohno, M. Yoshida, and I. W. Mattaj.** 1997. CRM1 is an export receptor for leucine-rich nuclear export signals. Cell **90:**1051-1060.

120. **Franzoso, G., V. Bours, V. Azarenko, S. Park, M. Tomita-Yamaguchi, T. Kanno, K. Brown, and U. Siebenlist.** 1993. The oncoprotein Bcl-3 can facilitate NF-κB-mediated transactivation by removing inhibiting p50 homodimers from select κB sites. EMBO Journal **12:**3893-3901.

121. **Franzoso, G., V. Bours, S. Park, M. Tomita-Yamaguchi, K. Kelly, and U. Siebenlist.** 1992. The candidate oncoprotein Bcl-3 is an antagonist of p50/NF-κB-mediated inhibition. Nature **359:**339-342.

122. **Franzoso, G., L. Carlson, L. Poljak, E. W. Shores, S. Epstein, A. Leonardi, A. Grinberg, T. Tran, T. Scharton-Kersten, M. Anver, P. Love, K. Brown, and U. Siebenlist.** 1998. Mice deficient in nuclear factor (NF)-kappa B/p52 present with defects in humoral responses, germinal center reactions, and splenic microarchitecture. J Exp Med **187:**147-59.

123. **Franzoso, G., L. Carlson, L. Xing, L. Poljak, E. W. Shores, K. D. Brown, A. Leonardi, T. Tran, B. F. Boyce, and U. Siebenlist.** 1997. Requirement for NF-kappaB in osteoclast and B-cell development. Genes Dev **11**:3482-96.

124. **Fritz, C. C., and M. R. Green.** 1996. HIV Rev uses a conserved cellular protein export pathway for the nucleocytoplasmic transport of viral RNAs. Current Biology **6**:848-854.

125. **Fujita, T., G. P. Nolan, S. Ghosh, and D. Baltimore.** 1992. Independent modes of transcriptional activation by the p50 and p65 subunits of NF-κB. Genes and Devel. **6**:775-787.

126. **Fujita, T., G. P. Nolan, H.-C. Liou, M. L. Scott, and D. Baltimore.** 1993. The candidate proto-oncogene bcl-3 encodes a transcriptional coactivator that activates through NF-κB p50 homodimers. Genes & Development **7**:1354-1363.

127. **Fukuda, M., S. Asano, T. Nakamura, M. Adachi, M. Yoshida, M. Yanagida, and E. Nishida.** 1997. CRM1 is responsible for intracellular transport mediated by the nuclear export signal. Nature **390**:308-11.

128. **Ganchi, P. A., S.-C. Sun, W. C. Greene, and D. W. Ballard.** 1992. IκB/MAD-3 masks the nuclear localization signal of NF-κB p65 and requires the transactivation domain to inhibit NF-κB p65 DNA binding. Molecular Biology of the Cell **3**:1339-1352.

129. **Garber, M. E., P. Wei, V. N. KewalRamani, T. P. Mayall, C. H. Herrmann, A. P. Rice, D. R. Littman, and K. A. Jones.** 1998. The interaction between HIV-1 Tat and human cyclin T1 requires zinc and a critical cysteine residue that is not conserved in the murine CycT1 protein. Genes Dev **12**:3512-27.

130. **Garcia, J. A., F. K. Wu, R. Mitsuyasu, and R. Gaynor.** 1987. Interactions of cellular proteins involved in the transcriptional regulation of the human immunodeficiency virus. EMBO Journal **6**:3761-3770.

131. **Garcia-Martinez, L. F., G. Mavankal, J. M. Neveu, W. S. Lane, D. Ivanov, and R. B. Gaynor.** 1997. Purification of a Tat-associated kinase reveals a TFIIH complex that modulates HIV-1 transcription. Embo J **16**:2836-50.

132. **Garoufalis, E., I. Kwan, R. Lin, A. Mustafa, N. Pepin, A. Roulston, J. Lacoste, and J. Hiscott.** 1994. Viral induction of the human interferon beta promoter: modulation of transcription by NF-κB/rel proteins and interferon regulatory factors. Journal of Virology **68:**4707-4715.

133. **Garrity, P. A., and B. Wold.** 1992. Effects of different DNA polymerases in ligation-mediated PCR: enhanced genomic sequencing and *in vivo* footprinting. Proceedings of the National Academy of Sciences USA **89:**1021-1025.

134. **Gatignol, A., K.-T. Jeang.** 2000. Tat as a transcriptional activator and a potential therapeutic target for HIV-1., p. (in Press). *In* K.-T. Jeang (ed.), HIV: Molecular mechanism and clinical applications, vol. 48. in Advances in Pharmacology.

135. **Gaynor, R.** 1992. Cellular transcription factors involved in the regulation of HIV-1 gene expression. AIDS **6:**347-363.

136. **Gerondakis, S., M. Grossmann, Y. Nakamura, T. Pohl, and R. Grumont.** 1999. Genetic approaches in mice to understand Rel/NF-kappaB and IkappaB function: transgenics and knockouts. Oncogene **18:**6888-6895.

137. **Gerondakis, S., N. Morrice, I. B. Richardson, R. Wettenhall, J. Fecondo, and R. J. Grumont.** 1993. The activity of a 70 kilodalton IκB molecule identical to the carboxyl terminus of the p105 NF-κB precursor is modulated by protein kinase A. Cell Growth and Differentiation **4:**617-627.

138. **Gerritsen, M. E., A. J. Williams, A. S. Neish, S. Moore, Y. Shi, and T. Collins.** 1997. CREB-binding protein/p300 are transcriptional coactivators of p65. Proceedings of the National Academy of Sciences USA **7:**2927-2932.

139. **Ghosh, S., and D. Baltimore.** 1990. Activation *in vitro* of NF-κB by phosphorylation of its inhibitor IκB. . Nature **344:**678-682.

140. **Ghosh, S., A. M. Gifford, L. R. Riviere, P. Tempst, G. P. Nolan, and D. Baltimore.** 1990. Cloning of the p50 DNA binding subunit of NF-κB: homology to rel and dorsal. Cell **62:**1019-1029.

141. **Gill, G., E. Pascal, Z. H. Tseng, and R. Tjian.** 1994. A glutamine-rich hydrophobic patch in transcription factor Sp1 contacts the dTAFII110 component of the Drosophila TFIID complex and mediates transcriptional activation. Proc Natl Acad Sci U S A **91:**192-6.

142. **Gilmore, T. D.** 1999. Multiple mutations contribute to the oncogenicity of the retroviral oncoprotein v-Rel. Oncogene **18:**6925-6937.

143. **Gilmore, T. D., and H. M. Temin.** 1988. V-rel oncoproteins in the nucleus and in the cytoplasm transform chicken spleen cells. Journal of Virology **62:**703-714.

144. **Gold, M. O., X. Yang, C. H. Herrmann, and A. P. Rice.** 1998. PITALRE, the catalytic subunit of TAK, is required for human immunodeficiency virus Tat transactivation in vivo. J Virol **72:**4448-53.

145. **Good, L., and S.-C. Sun.** 1996. Persistent activation of NF-κB/Rel by human T-cell leukemia virus type 1 Tax involves degradation of IκBβ. Journal of Virology **70:**2730-2735.

146. **Gossen, M., S. Freundlieb, G. Bender, G. Müller, W. Hillen, and H. Bujard.** 1995. Transcriptional activation by tetracyclines in mammalian cells. Science **268:**1766-1769.

147. **Greenfeder, S. A., P. Nunes, L. Kwee, M. Labow, R. A. Chizzonite, and G. Ju.** 1995. Molecular cloning and characterization of a second subunit of the interleukin 1 receptor complex. J Biol Chem **270:**13757-65.

148. **Griffin, G. E., K. Leung, T. M. Folks, S. Kunkel, and G. Nabel.** 1989. Activation of HIV gene expression during monocyte differentiation by induction of NF-κB. Nature **339:**70-73.

149. **Grimm, S., M. K. Bauer, P. A. Baeuerle, and K. Schulze-Osthoff.** 1996. Bcl-2 down-regulates the activity of transcription factor NF-κB induced upon apoptosis. J.Cell Biol. **134:**13-23.

150. **Grumont, R. J., and S. Gerondakis.** 1994. The subunit composition of NF-kappa B complexes changes during B-cell development. Cell Growth Differ **5:**1321-31.

151. **Grumont, R. J., I. B. Richardson, C. Gaff, and S. Gerondakis.** 1993. rel/NF-κB nuclear complexes that bind κB sites in the murine c-*rel* promoter are required for constitutive c-*rel* transcription in B-cells. Cell Growth & Differentiation **4:**731-743.

152. **Grumont, R. J., I. J. Rourke, and S. Gerondakis.** 1999. Rel-dependent induction of A1 transcription is required to protect B cells from antigen receptor ligation-induced apoptosis. Genes & Development **13:**400-411.

153. **Grumont, R. J., I. J. Rourke, L. A. O'Reilly, A. Strasser, K. Miyake, W. Sha, and S. Gerondakis.** 1998. B lymphocytes differentially use the Rel and nuclear factor kappaB1 (NF- kappaB1) transcription factors to regulate cell cycle progression and apoptosis in quiescent and mitogen-activated cells. J Exp Med **187:**663-74.

154. **Hannink, M., and H. M. Temin.** 1990. Structure and autoregulation of the *c-rel* promoter. Oncogene **5:**1843-1850.

155. **Harhaj, E., J. Blaney, S. Millhouse, and S.-C. Sun.** 1996. Differential effects of IκB molecules on Tat-mediated transactivation of HIV-1 LTR. Virology **216:**284-287.

156. **Harhaj, E. W., S. B. Maggirwar, L. Good, and S.-C. Sun.** 1996. CD28 mediates a potent costimulatory signal for rapid degradation of IκBβ which is associated with accelerated activation of various NF-κB/Rel heterodimers. Mol.Cell.Biol. **16:**6736-6743.

157. **Harhaj, E. W., S. B. Maggirwar, and S. C. Sun.** 1996. Inhibition of p105 processing by NF-kappaB proteins in transiently transfected cells. Oncogene **12:**2385-92.

158. **Harhaj, E. W., and S. C. Sun.** 1999. IKKgamma serves as a docking subunit of the IkappaB kinase (IKK) and mediates interaction of IKK with the human T-cell leukemia virus Tax protein. J Biol Chem **274:**22911-4.

159. **Harhaj, E. W., and S. C. Sun.** 1997. The serine/threonine phosphatase inhibitor calyculin A induces rapid degradation of IκBβ. Requirements of both the N- and C-terminal sequences. Journal of Biological Chemistry **272:**5409-5412.

160. **Harrich, D., J. Garcia, R. Mitsuyasu, and R. Gaynor.** 1990. TAR independent activation of the human immunodeficiency virus in phorbol ester stimulated T-lymphocytes. EMBO Journal **9:**4417-4424.

161. **Harrich, D., J. Garcia, F. Wu, R. Mitsuyasu, J. Gonzalez, and R. Gaynor.** 1989. Role of Sp1 binding domains in *in vivo* transcriptional regulation of the human immunodeficiency virus type 1 long terminal repeat. Journal of Virology **63:**2585-2591.

162. **Haskill, S., A. A. Beg, S. M. Tompkins, J. S. Morris, A. D. Yurochko, A. Sampson-Johannes, K. Mondal, P. Ralph, and A. S. Baldwin, Jr.** 1991. Characterization of an immediate-early gene induced in adherent monocytes that encodes IκB-like activity. Cell **65:**1281-1289.

163. **Hatada, E. N., M. Naumann, and C. Scheidereit.** 1993. Common structural constituents confer IκB activity to NF-κB p105 and IκB/MAD-3. EMBO Journal **12:**2781-2788.

164. **Hatada, E. N., A. Nieters, F. G. Wulczyn, M. Naumann, R. Meyer, G. Nucifora, T. W. McKeithan, and C. Scheidereit.** 1992. The ankyrin repeat domains of the NF-κB precursor p105 and the proto-oncogene *bcl-3* act as specific inhibitors of NF-κB DNA binding. Proceedings of the National Academy of Sciences USA **89:**2489-2493.

165. **Hazan, U., D. Thomas, J. Alcami, F. Bachelerie, N. Israël, H. Yssel, J.-L. Virelizier, and F. Arenzana-Seisdedos.** 1990. Stimulation of a human T-cell clone with anti-CD3 or tumor necrosis factor induces NF-kappa B translocation but not human immunodeficiency virus 1 enhancer-dependent transcription. Proceedings of the National Academy of Sciences USA **87:**7861-7865.

166. **Heissmeyer, V., D. Krappmann, F. G. Wulczyn, and C. Scheidereit.** 1999. NF-kappaB p105 is a target of IkappaB kinases and controls signal induction of Bcl-3-p50 complexes. Embo J **18:**4766-78.

167. **Henkel, T., U. Zabel, K. van Zee, J. M. Müller, E. Fanning, and P. A. Baeuerle.** 1992. Intramolecular masking of the nuclear location signal and dimerization domain in the precursor for the p50 NF-κB subunit. Cell **68:**1121-1133.

168. **Hettmann, T., J. Didonato, M. Karin, and J. M. Leiden.** 1999. An essential role for NF-κB in promoting double positive thymocyte apoptosis. Journal of Experimental Medicine **189:**145-157.

169. **Heusch, M., L. Lin, R. Geleziunas, and W. C. Greene.** 1999. The generation of nfkb2 p52: mechanism and efficiency. Oncogene **18:**6201-8.

170. **Hirano, F., M. Chung, H. Tanaka, N. M. Maruyama, N., I. Makino, D. Moore, and C. Scheidereit.** 1998. Alternative splicing variants of IkappaB beta establish differential NF-kappaB signal responsiveness in human cells. Molecular and Cellular Biology **18:**2596-2607.

171. **Hirano, F., H. Tanaka, Y. Hirano, M. Hiramoto, H. Handa, I. Makino, and C. Scheidereit.** 1998. Functional Interference of Sp1 and NF-κB through the Same DNA Binding Site. Mol.Cell.Biol. **18:**1266-1274.

172. **Hirano, M., S. Osada, T. Aoki, S. Hirai, M. Hosaka, J. Inoue, and S. Ohno.** 1996. MEK kinase is involved in tumor necrosis factor alpha-induced NF-kappaB activation and degradation of IkappaB-alpha. J Biol Chem **271:**13234-8.

173. **Hiscott, J., P. Beauparlant, P. Crepieux, C. DeLuca, H. J. Kwon, R. Lin, and L. Petropoulos.** 1997. Cellular and viral protein interactions regulating IκBα activity during human retrovirus infection. Journal of Leukocyte Biology **62:**82-92.

174. **Hiscott, J., J. Marois, J. Garoufalis, M. D'Addario, A. Roulston, I. Kwan, N. Pepin, J. Lacoste, H. Nguyen, G. Bensi, and M. Fenton.** 1993. Characterization of a functional NF-κB site in the human interleukin 1β promoter: evidence for a positive autoregulatory loop. Molecular and Cellular Biology **13:**6231-6240.

175. **Hottiger, M. O., and G. J. Nabel.** 1998. Interaction of human immunodeficiency virus type 1 Tat with the transcriptional coactivators p300 and CREB binding protein. J Virol **72:**8252-6.

176. **Howcroft, T. K., K. Strebel, M. A. Martin, and D. S. Singer.** 1993. Repression of MHC class I gene promoter activity by two-exon Tat of HIV. Science **260:**1320-2.

177. **Hsu, H., J. Huang, H. B. Shu, V. Baichwal, and D. V. Goeddel.** 1996. TNF-dependent recruitment of the protein kinase RIP to the TNF receptor- 1 signaling complex. Immunity **4:**387-96.

178. **Hsu, H., H. Shu, M. Pan, and D. V. Goeddel.** 1996. TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. Cell **84:**299-308.

179. **Hu, Y., V. Baud, M. Delhase, P. Zhang, T. Deerinck, M. Ellisman, R. Johnson, and M. Karin.** 1999. Abnormal morphogenesis but intact IKK activation in mice lacking IKKα subunit of IκB kinase. Science **284:**316-320.

180. **Huxford, T., D.-B. Huang, S. Malek, and G. Ghosh.** 1998. The crystal structure of the IκBα/NF-κB complex reveals mechanisms of NF-κB inactivation. Cell **95:**759-770.

181. **Imbert, V., R. A. Rupec, A. Livolsi, H. L. Pahl, E. B.-M. Traenckner, C. Mueller-Dieckmann, D. Farahifar, B. Rossi, P. Auberger, P. A. Baeuerle, and J.-F. Peyron.** 1996. Tyrosine phosphorylation of IκB-α activates NF-κB without proteolytic degradation of IκB-α. Cell **86:**787-798.

182. **Inoue, J.-I., T. Takahara, T. Akizawa, and O. Hino.** 1993. Bcl-3, a member of the IκB proteins, has distinct specificity towards the Rel family of proteins. Oncogene **8:**2067-2073.

183. **Inoue, J. I., L. D. Kerr, A. Kakizuka, and I. Verma.** 1992. IκBγ, a 70 kd protein identical to the C-terminal half of p110 NF-κB: a new member of the IκB family. Cell **68:**1109-1120.

184. **Inoue, J. I., L. D. Kerr, L. J. Ransone, E. Bengal, T. Hunter, and I. M. Verma.** 1991. *c-rel* activates but *v-rel* suppresses transcription from κB sites. Proceedings of the National Academy of Sciences USA **88:**3715-3719.

185. **Ito, C. Y., A. G. Kazantsev, and A. S. J. Baldwin.** 1994. Three NF-κB sites in the IκB-α promoter are required for induction of gene expression by TNFα. Nucleic Acids Research **22:**3787-3792.

186. **Jacobs, M. D., and S. C. Harrison.** 1998. Structure of an IκBα/NF-κB complex. Cell **95:**749-758.

187. **Jaffray, E., K. M. Wood, and R. T. Hay.** 1995. Domain organization of IκBα and the sites of interaction with NF-κB p65. Mol.Cell.Biol. **15:**2166-2172.

188. **Janknecht, R., and T. Hunter.** 1996. Versatile molecular glue. Transcriptional control. Curr Biol **6:**951-4.

189. **Jeang, K.-T., P. R. Shank, and A. Kumar.** 1988. Transcriptional activation of homologous viral long terminal repeats by the human immunodeficiency virus type 1 or the human T-cell leukemia virus type I tat proteins occurs in the absence of *de novo* protein synthesis. Proc.Natl.Acad.Sci. **85:**8291-8295.

190. **Jeang, K. T., and A. Gatignol.** 1994. Comparison of regulatory features among primate lentiviruses. Curr Top Microbiol Immunol **188:**123-44.

191. **Jeremias, I., I. Herr, T. Boehler, and K. M. Debatin.** 1998. TRAIL/Apo-2-ligand-induced apoptosis in human T cells. Eur J Immunol **28:**143-52.

192. **Jin, D. Y., V. Giordano, K. V. Kibler, H. Nakano, and K. T. Jeang.** 1999. Role of adaptor function in oncoprotein-mediated activation of NF-κB. HTLV-1 Tax interacts directly with IKKγ. Journal of Biological Chemistry **274:**17402-17405.

193. **Johnson, C., D. Van Antwerp, and T. J. Hope.** 1999. An N-terminal nuclear export signal is required for the nucleocytoplasmic shuttling of IkappaBalpha. Embo J **18:**6682-93.

194. **Jones, K. A.** 1997. Taking a new TAK on tat transactivation [comment]. Genes Dev **11:**2593-9.

195. **Jones, K. A., and B. M. Peterlin.** 1994. Control of RNA initiation and elongation at the HIV-1 promoter. Annual Review of Biochemistry **63:**717-743.

196. **Kamens, J., P. Richardson, G. Mosialos, R. Brent, and T. D. Gilmore.** 1990. Oncogenic transformation by vRel requires an amino-terminal activation domain. Mol.Cell.Biol. **10:**2840-2847.

197. **Kane, L. P., V. S. Shapiro, D. Stokoe, and A. Weiss.** 1999. Induction of NF-kappaB by the Akt/PKB kinase. Curr Biol **9:**601-4.

198. **Kanno, M., C. Fromental, A. Staub, F. Ruffenach, I. Davidson, and P. Chambon.** 1989. The SV40 TC-II (kappa B) and the related H-2Kb enhansons exhibit different cell type specific and inducible proto-enhancer activities, but the SV40 core sequence and the AP-2 binding site have no enhanson properties. EMBO Journal **8:**4205-4214.

199. **Kashanchi, F., G. Piras, M. F. Radnovich, J. F. Duvall, A. Fattaey, C. M. Chiang, R. Roeder, and J. N. Brady.** 1994. Direct interaction of human TFIID with the HIV-1 transactivator Tat. . Nature **367:**295-299.

200. **Kasibhatla, S., T. Brunner, L. Genestier, F. Echeverri, A. Mahboubi, and D. R. Green.** 1998. DNA damaging agents induce expression of Fas ligand and subsequent apoptosis in T lymphocytes via the activation of NF-kappa B and AP-1. Mol Cell **1:**543-51.

201. **Kato, H., M. Horikoshi, and R. G. Roeder.** 1991. Repression of HIV-1 transcription by a cellular protein. Science **251:**1476-1479.

202. **Kauffmann-Zeh, A., P. Rodriguez-Viciana, E. Ulrich, C. Gilbert, P. Coffer, J. Downward, and G. Evan.** 1997. Suppression of c-Myc-induced apoptosis by Ras signalling through PI(3)K and PKB. Nature **385:**544-8.

203. **Kelliher, M. A., S. Grimm, Y. Ishida, F. Kuo, B. Z. Stanger, and P. Leder.** 1998. The death domain kinase RIP mediates the TNF-induced NF-kappaB signal. Immunity **8:**297-303.

204. **Kennedy, S. G., A. J. Wagner, S. D. Conzen, J. Jordan, A. Bellacosa, P. N. Tsichlis, and N. Hay.** 1997. The PI 3-kinase/Akt signaling pathway delivers an anti-apoptotic signal. Genes Dev **11**:701-13.

205. **Kerr, L. D., C. S. Duckett, P. Wamsley, Q. Zhang, P. Chiao, G. Nabel, T. W. McKeithan, P. A. Baeuerle, and I. M. Verma.** 1992. The proto-oncogene *BCL-3* encodes an IκB protein. Genes and Devel. **6**:2352-2363.

206. **Kerr, L. D., L. J. Ransone, P. Wamlsey, M. J. Schmitt, T. G. Boyer, Q. Zhou, A. J. Berk, and I. M. Verma.** 1993. Association between proto-oncogene Rel and TATA-binding protein mediates transcriptional activation by NF-κB. Nature **365**:412-419.

207. **Khwaja, A.** 1999. Akt is more than just a Bad kinase [news; comment]. Nature **401**:33-4.

208. **Kieran, M., V. Blank, F. Logeat, J. Vandekerckhove, F. Lottspeich, O. Le Bail, M. B. Urban, P. Kourilsky, P. A. Baeuerle, and A. Israël.** 1990. The DNA-binding subunit of NF-κB is identical to factor KBF1 and homologous to the *rel* oncogene product. Cell **62**:1007-1018.

209. **Kiernan, R. E., C. Vanhulle, L. Schiltz, E. Adam, H. Xiao, F. Maudoux, C. Calomme, A. Burny, Y. Nakatani, K. T. Jeang, M. Benkirane, and C. Van Lint.** 1999. HIV-1 tat transcriptional activity is regulated by acetylation. Embo J **18**:6106-18.

210. **Kim, J. Y., F. Gonzalez-Scarano, S. L. Zeichner, and J. C. Alwine.** 1993. Replication of type 1 human immunodeficiency viruses containing linker substitution mutations in the -201 to -130 region of the long terminal repeat. J Virol **67**:1658-62.

211. **Kirillov, A., B. Kistler, R. Mostoslavsky, H. Cedar, T. Wirth, and Y. Bergman.** 1996. A role for nuclear NF-kappaB in B-cell-specific demethylation of the Igkappa locus. Nat Genet **13**:435-41.

212. **Kistler, B., B. Baumann, Y. Bergman, and T. Wirth.** 1997. RelB is a key player for both kappa B-dependent transcription and demethylation in B cells. Immunobiology **198**:24-34.

213. **Klaver, B., and B. Berkhout.** 1994. Evolution of a disrupted TAR RNA hairpin structure in the HIV-1 virus. Embo J **13:**2650-9.

214. **Klement, J. F., N. R. Rice, B. D. Car, S. J. Abbondanzo, G. D. Powers, P. H. Bhatt, C. H. Chen, C. A. Rosen, and C. L. Stewart.** 1996. IκBα deficiency results in a sustained NF-κB response and severe widespread dermatitis in mice. Molecular and Cellular Biology **16:**2341-2349.

215. **Kolesnitchenko, V., L. King, A. Riva, Y. Tani, S. J. Korsmeyer, and D. I. Cohen.** 1997. A major human immunodeficiency virus type 1-initiated killing pathway distinct from apoptosis. J Virol **71:**9753-63.

216. **Kontgen, F., R. J. Grumont, A. Strasser, D. Metcalf, R. Li, D. Tarlington, and S. Gerondakis.** 1995. Mice lacking the c-*rel* proto-oncogene exhibit defects in lymphocyte proliferation, humoral immunity and interleukin-2 expression. Genes & Development **9:**1965-1977.

217. **Kornbluth, R. S.** 1995. Significance of T cell apoptosis for macrophages in HIV infection. Journal of Leukocyte Biology **56:**247-256.

218. **Krappmann, D., F. G. Wulczyn, and C. Scheidereit.** 1996. Different mechanisms control signal-induced degradation and basal turnover of the NF-κB inhibitor IκBα *in vivo*. EMBO Journal **15:**6716-6726.

219. **Kretzschmar, M., M. Meisterernst, C. Scheidereit, G. Li, and R. G. Roeder.** 1992. Transcriptional regulation of the HIV-1 promoter by NF-kappa B in vitro. Genes Dev **6:**761-74.

220. **Kumar, A., S. Dhawan, A. Mukhopadhyay, and B. B. Aggarwal.** 1999. Human immunodeficiency virus-1-tat induces matrix metalloproteinase-9 in monocytes through protein tyrosine phosphatase-mediated activation of nuclear transcription factor NF-kappaB. FEBS Lett **462:**140-4.

221. **Kumar, A., J. Haque, J. Lacoste, J. Hiscott, and B. R. G. Williams.** 1994. Double-stranded RNA-dependent protein kinase activates transcription factor NF-κB by

phosphorylating IκB. Proceedings of the National Academy of Sciences USA **91:**6288-6292.

222. **Kwon, H., N. Pelletier, C. DeLuca, P. Genin, S. Cisternas, R. Lin, M. A. Wainberg, and J. Hiscott.** 1998. Inducible expression of IκBα repressor mutants interferes with NF-κB activity and HIV-1 replication in Jurkat T cells. Journal of Biological Chemistry **273:**7431-7440.

223. **La rosa, F. A., J. W. Pierce, and G. E. Sonenshein.** 1994. Differential regulation of the c-*myc* oncongene promoter by the NF-κB rel family of transcription factors. Molecular and Cellular Biology **14:**1039-1044.

224. **Lallena, M.-J., M. T. Diaz-Meco, G. Bren, C. V. Payá, and J. Moscat.** 1999. Activation of IκB kinase by protein kinase C isoforms. Mol.Cell.Biol. **19:**2180-2188.

225. **Landt, O., H. P. Grunert, and U. Hahn.** 1990. A general method for rapid site-directed mutagenesis using the polymerase chain reaction. Gene **96:**125-128.

226. **Lange-Carter, C. A., C. M. Pleiman, A. M. Gardner, K. J. Blumer, and G. L. Johnson.** 1993. A divergence in the MAP kinase regulatory network defined by MEK kinase and Raf. Science **260:**315-9.

227. **Laspia, M. F., A. P. Rice, and M. B. Mathew.** 1989. HIV-1 Tat protein increases transcriptional initiation and stabilizes elongation. Cell **59:**283-292.

228. **Le Bail, O., R. Schmidt-Ullrich, and A. Israël.** 1993. Promoter analysis of the gene encoding the IκB-α/MAD 3 inhibitor of NF-κB: positive regulation by members of the rel/NF-κB family. EMBO Journal **12:**5043-5049.

229. **Ledebur, H. C., and T. P. Parks.** 1995. Transcriptional regulation of the intercellular adhesion molecule-1 gene by inflammatory cytokines in human endothelial cells. Essential roles of a variant NF-kappa B site and p65 homodimers. J Biol Chem **270:**933-43.

230. **Lee, F. S., J. Hagler, Z. J. Chen, and T. Maniatis.** 1997. Activation of the IκBα kinase complex by MEKK1, a kinase of the JNK pathway. Cell **88:**213-222.

231. **Lee, F. S., R. T. Peters, L. C. Dang, and T. Maniatis.** 1998. MEKK1 activates both IkappaB kinase alpha and IkappaB kinase beta. Proc Natl Acad Sci U S A **95:**9319-24.

232. **Lee, J. W., H. S. Choi, J. Gyuris, R. Brent, and D. D. Moore.** 1995. Two classes of proteins dependent on either the presence or absence of thyroid hormone for interaction with the thyroid hormone receptor. Mol Endocrinol **9:**243-54.

233. **Lee, M. H., K. Sano, F. E. Morales, and D. T. Imagawa.** 1987. Sensitive reverse transcriptase assay to detect and quantitate human immunodificiency virus. Journal of Clinical Microbiology **25:**1717-1721.

234. **Lee, S. Y., A. Reichlin, A. Santana, K. A. Sokol, M. C. Nussenzweig, and Y. Choi.** 1997. TRAF2 is essential for JNK but not NF-kappaB activation and regulates lymphocyte proliferation and survival. Immunity **7:**703-13.

235. **Leprince, D., M. Duterque-Coquillaud, R. P. Li, C. Henry, A. Flourens, B. Debuire, and D. Stehelin.** 1988. Alternative splicing within the chicken c-ets-1 locus: implications for transduction within the E26 retrovirus of the c-ets proto-oncogene. J Virol **62:**3233-41.

236. **Lernbecher, T., B. Kistler, and T. Wirth.** 1994. Two distinct mechanisms contribute to the constitutive activation of RelB in lymphoid cells. Embo J **13:**4060-9.

237. **Lernbecher, T., U. Müller, and T. Wirth.** 1993. Distinct NF-κB/rel transcription factors are responsible for tissue-specific and inducible gene activation. Nature **365:**767-770.

238. **Li, C.-C., M. Korner, D. K. Ferris, E. Chen, R.-M. Dai, and D. L. Longo.** 1994. NF-κB/Rel family members are physically associated phosphoprotein. Biochem.J. **303:**499-506.

239. **Li, C. C., R. M. Dai, E. Chen, and D. L. Longo.** 1994. Phosphorylation of NF-KB1-p50 is involved in NF-kappa B activation and stable DNA binding. J Biol Chem **269:**30089-92.

240. **Li, C. J., D. J. Friedman, C. Wang, V. Metelev, and A. B. Pardee.** 1995. Induction of apoptosis in unifected lymphocytes by HIV-1 Tat protein. Science **268:**429-431.

241. **Li, J., G. W. Peet, S. S. Pullen, J. Schembri-King, T. C. Warren, K. B. Marcu, M. R. Kehry, R. Barton, and S. Jakes.** 1998. Recombinant IkappaB kinases alpha and beta are direct kinases of Ikappa Balpha. J Biol Chem **273:**30736-41.

242. **Li, N., and M. Karin.** 1998. Ionizing radiation and short wavelength UV activate NF-kappaB through two distinct mechanisms. Proc Natl Acad Sci U S A **95:**13012-7.

243. **Li, Q., Q. Lu, J. Y. Hwang, D. Buscher, K. F. Lee, J. C. Izpisua-Belmonte, and I. M. Verma.** 1999. IKK1-deficient mice exhibit abnomal development of skin and skeleton. Genes & Development **13:**1322-1328.

244. **Li, Q., D. Van Antwerp, F. Mercurio, K. F. Lee, and I. M. Verma.** 1999. Severe liver degeration in mice lacking the IKK2 gene. Science **284:**321-325.

245. **Li, Z., and G. J. Nabel.** 1997. A new member of the I kappaB protein family, I kappaB epsilon, inhibits RelA (p65)-mediated NF-kappaB transcription. Mol Cell Biol **17:**6184-90.

246. **Li, Z. W., W. Chu, Y. Hu, M. Delhase, T. Deerinck, M. Ellisman, R. Johnson, and M. Karin.** 1999. The IKKβ subunit of IKK is essential for NF-κB activation and prevention of apoptosis. Journal of Experimental Medicine **189:**1839-1845.

247. **Lin, K. I., S. H. Lee, R. Narayanan, J. M. Baraban, J. M. Hardwick, and R. R. Ratan.** 1995. Thiol agents and Bcl-2 identify an alphavirus-induced apoptotic pathway that requires activation of the transcription factor NF-kappa B. Journal of Cellular Biochemistry **131:**1149-1161.

248. **Lin, L., G. N. DeMartino, and W. C. Greene.** 1998. Cotranslational biogenesis of NF-kappaB p50 by the 26S proteasome. Cell **92:**819-28.

249. **Lin, L., and S. Ghosh.** 1996. A glycine-rich region in NF-kappaB p105 functions as a processing signal for the generation of the p50 subunit. Mol Cell Biol **16:**2248-54.

250. **Lin, R., P. Beauparlant, C. Makris, S. Meloche, and J. Hiscott.** 1996. Phosphorylation of IκBα in the C-terminal PEST domain by casein kinase II affects intrinsic protein stability. Mol.Cell.Biol. **16:**1401-1409.

251. **Lin, R., D. Gewert, and J. Hiscott.** 1995. Differential transcriptional activation *in vitro* by NF-κB/Rel proteins. Journal of Biological Chemistry **270:**3123-3131.

252. **Lin, X., E. T. J. Cunningham, Y. Mu, R. Geleziunas, and W. C. Greene.** 1999. The proto-oncogene Cot kinase participates in CD3/CD28 induction of NF-κB acting through the NIK and IκB kinases. Immunity **10:**271-280.

253. **Lin, X., Y. Mu, E. T. Cunningham, Jr., K. B. Marcu, R. Geleziunas, and W. C. Greene.** 1998. Molecular determinants of NF-kappaB-inducing kinase action. Mol Cell Biol **18:**5899-907.

254. **Ling, L., Z. Cao, and D. V. Goeddel.** 1998. NF-κB-inducing kinase activates IKK-α by phosphorylation of Ser-176. Proceedings of the National Academy of Sciences USA **95:**3792-3797.

255. **Link, E., L. D. Kerr, R. Schreck, U. Zabel, I. Verma, and P. A. Baeuerle.** 1992. Purified IκB-β is inactivated upon dephosphorylation. Journal of Biological Chemistry **267:**239-246.

256. **Liou, H.-C., G. P. Nolan, S. Ghosh, T. Fujita, and D. Baltimore.** 1992. The NF-κB p50 precursor, p105, contains an internal IκB-like inhibitor that preferentially inhibits p50. EMBO Journal **11:**3003-3009.

257. **Liou, H.-C., W. C. Sha, M. L. Scott, and D. Baltimore.** 1994. Sequential induction of NF-κB/rel family proteins during B-cell terminal differentiation. Molecular and Cellular Biology **14:**5349-5359.

258. **Liu, J., N. D. Perkins, R. Schmid, and G. Nabel.** 1992. Specific NF-κB subunits act in concert with Tat to stimulate human immunodeficiency virus type 1 transcription. Journal of Virology **66:**3883-3887.

259. **Liu, Z., H. Hsu, D. V. Goeddel, and M. Karin.** 1996. Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-κB activation prevents cell death. Cell **87:**565-576.

260. **Lombardi, L., P. Ciana, C. Cappellini, D. Trecca, L. Guerrini, A. Migliazza, A. T. Maiolo, and A. Neri.** 1995. Structural and functional characterization of the promoter regions of the NFKB2 gene. Nucleic Acids Res **23:**2328-36.

261. **Lu, Y., M. Stenzel, J. Sodroski, and W. Haseltine.** 1989. Effects of long terminal repeat mutations on human immunodeficiency virus type 1 replication. Journal of Virology **63:**4115-4119.

262. **Lu, Y. C., N. Touzjian, M. Stenzel, T. Dorfman, J. Sodroski, and W. A. Haseltine.** 1990. Identification of cis-acting repressive sequences within the negative regulatory element of human immunodeficiency virus type 1. Journal of Virology **64:**5226-5229.

263. **Luque, I., and C. Gelinas.** 1998. Distinct domains of IkappaBalpha regulate c-Rel in the cytoplasm and in the nucleus. Mol Cell Biol **18:**1213-24.

264. **Lux, S. E., K. M. John, and V. Bennett.** 1990. Analysis of cDNA for human erythrocyte ankyrin indicates a repeated structure with homology to tissue-differentiation and cell-cycle control proteins. Nature **344:**36-42.

265. **MacKichan, M. L., F. Logeat, and A. Israel.** 1996. Phosphorylation of p105 PEST sequence via a redox-insensitive pathway up-regulates processing of p50 NF-kappaB. J Biol Chem **271:**6084-91.

266. **Madrid, L. V., C. Y. Wang, D. C. Guttridge, A. J. Schottelius, A. S. Baldwin, Jr., and M. W. Mayo.** 2000. Akt suppresses apoptosis by stimulating the transactivation potential of the RelA/p65 subunit of NF-kappaB [In Process Citation]. Mol Cell Biol **20:**1626-38.

267. **Magnuson, D. S., B. E. Knudsen, J. D. Geiger, R. M. Brownstone, and A. Nath.** 1995. Human immunodeficiency virus type 1 tat activates non-N-methyl-D-aspartate excitatory amino acid receptors and causes neurotoxicity. Ann Neurol **37**:373-80.

268. **Malinin, N. L., M. P. Boldin, A. V. Kovalenko, and D. Wallach.** 1997. MAP3K-related kinase involved in NF-κB induction by TNF, CD95 and IL-1. Nature **385**:540-544.

269. **Mancebo, H. S., G. Lee, J. Flygare, J. Tomassini, P. Luu, Y. Zhu, J. Peng, C. Blau, D. Hazuda, D. Price, and O. Flores.** 1997. P-TEFb kinase is required for HIV Tat transcriptional activation in vivo and in vitro. Genes Dev **11**:2633-44.

270. **Marciniak, R. A., M. A. Garcia-Blanco, and P. A. Sharp.** 1990. Identification and characterization of a HeLa nuclear protein that specifically binds to the trans-activation-responsive TAR element RNA. Proceedings of the National Academy of Sciences USA **87**:3624-3628.

271. **Marzio, G., M. Tyagi, M. I. Gutierrez, and M. Giacca.** 1998. HIV-1 tat transactivator recruits p300 and CREB-binding protein histone acetyltransferases to the viral promoter. Proc Natl Acad Sci U S A **95**:13519-24.

272. **Marzio, G., M. Tyagi, M. I. Gutierrez, and M. Giacca.** 1998. HIV-1 tat transactivator recruits p300 and CREB-binding protein histone acetyltransferases to the viral promoter. Proceedings of the National Academy of Sciences USA **95**:13519-13524.

273. **May, M. J., and S. Ghosh.** 1997. Rel/NF-kappa B and I kappa B proteins: an overview. Semin Cancer Biol **8**:63-73.

274. **McCracken, S., N. Fong, K. Yankulov, S. Ballantyne, G. Pan, J. Greenblatt, S. D. Patterson, M. Wickens, and D. L. Bentley.** 1997. The C-terminal domain of RNA polymerase II couples mRNA processing to transcription. Nature **385**:357-61.

275. **McElhinny, J. A., W. S. MacMorran, G. D. Bren, R. M. Ten, A. Israel, and C. V. Paya.** 1995. Regulation of IκBα and p105 in monocytes and macrophages persistently infected with human immunodeficiency virus. Journal of Virology **69:**1500-1509.

276. **McElhinny, J. A., S. A. Trushin, G. D. Bren, N. Chester, and C. V. Paya.** 1996. Casein kinase II phosphorylates IκBα at S-283, S-289,S-293, and T-291 and is required for its degradation. Molecular and Cellular Biology **16:**899-906.

277. **McKinsey, T. A., J. A. Brockman, D. C. Scherer, S. W. Al-Murrani, P. L. Green, and D. W. Ballard.** 1996. Inactivation of IκBβ by the Tax protein of human T-cell leukemia virus type 1: a potential mechanism for constitutive induction of NF-κB. Mol.Cell.Biol. **16:**2083-2090.

278. **Mellits, K. H., R. T. Hay, and S. Goodbourn.** 1993. Proteolytic degradation of MAD3 (IκBα) and enhanced processing of the NF-κB precursor p105 are obligatory steps in the activation of NF-κB. Nucleic Acids Research **21(22):**5059-5066.

279. **Memet, S., D. Laouini, J. C. Epinat, S. T. Whiteside, B. Goudeau, D. Philpott, S. Kayal, P. J. Sansonetti, P. Berche, J. Kanellopoulos, and A. Israel.** 1999. IkappaBepsilon-deficient mice: reduction of one T cell precursor subspecies and enhanced Ig isotype switching and cytokine synthesis. J Immunol **163:**5994-6005.

280. **Mercurio, F., J. A. DiDonato, C. Rosette, and M. Karin.** 1993. p105 and p98 precursor proteins play an active role in NF-κB mediated signal transduction. Genes & Development **7:**705-718.

281. **Mercurio, F., B. W. Murray, A. Shevshenko, B. L. Bennet, D. B. Young, J. W. Li, G. Pascual, A. Motiwala, H. Zhu, M. Mann, and A. M. Manning.** 1999. I kappa B kinase (IKK)-associated protein 1, a common component of the heterogeneous IKK complex. Mol.Cell.Biol. **19:**1526-1538.

282. **Mercurio, F., H. Zhu, B. W. Murray, A. Shevchenko, B. L. Bennett, J. W. Li, D. B. Young, M. Barbosa, and M. Mann.** 1997. IKK-1 and IKK-2: cytokine-activated IκB kinases essential for NF-κB activation. Science **278:**860-866.

283. **Meyer, C. F., X. Wang, C. Chang, D. Templeton, and T. H. Tan.** 1996. Interaction between c-Rel and the mitogen-activated protein kinase kinase kinase 1 signaling cascade in mediating kappaB enhancer activation. J Biol Chem **271:**8971-6.

284. **Milani, D., M. Mazzoni, P. Borgatti, G. Zauli, L. Cantley, and S. Capitani.** 1996. Extracellular human immunodeficiency virus type-1 Tat protein activates phosphatidylinositol 3-kinase in PC12 neuronal cells. J Biol Chem **271:**22961-4.

285. **Mitchell, P. J., and R. Tjian.** 1989. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science **245:**371-378.

286. **Miyamoto, S., M. Maki, M. J. Schmitt, M. Hatanaka, and I. M. Verma.** 1994. Tumor necrosis factor α-induced phosphorylation of IκBα is a signal for its degradation but not dissociation from NF-κB. Proceedings of the National Academy of Sciences USA **91:**12740-12744.

287. **Miyamoto, S., and I. M. Verma.** 1995. Rel/NF-κB/IκB story. Advances in Cancer Research **66:**255-292.

288. **Miyoshi, J., T. Higashi, H. Mukai, T. Ohuchi, and T. Kakunaga.** 1991. Structure and transforming potential of the human cot oncogene encoding a putative protein kinase. Mol Cell Biol **11:**4088-96.

289. **Molitor, J. A., W. H. Walker, S. Doerre, D. W. Ballard, and W. C. Greene.** 1990. NF-κB: a family of inducible and differentially expressed enhancer-binding proteins in human T - cells. Proceedings of the National Academy of Sciences USA **87:**10028-10032.

290. **Montano, M. A., C. P. Nixon, and M. Essex.** 1998. Dysregulation through the NF-kappaB enhancer and TATA box of the human immunodeficiency virus type 1 subtype E promoter. J Virol **72:**8446-52.

291. **Mueller, P. R., and B. Wold.** 1989. In vivo footprinting of a muscle specific enhancer by ligation mediated PCR. Science **246:**780-786.

292. **Muzio, M., J. Ni, P. Feng, and V. M. Dixit.** 1997. IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL- 1 signaling. Science **278:**1612-5.

293. **Nabel, G., and D. Baltimore.** 1987. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. Nature **326:**711-713.

294. **Nagahara, H., A. M. Vocero-Akbani, E. L. Snyder, A. Ho, D. G. Latham, N. A. Lissy, M. Becker-Hapak, S. A. Ezhevsky, and S. F. Dowdy.** 1998. Transduction of full-length TAT fusion proteins into mammalian cells: TAT-p27Kip1 induces cell migration. Nat Med **4:**1449-52.

295. **Nakano, H., M. Shindo, S. Sakon, S. Nishinaka, M. Mihara, H. Yagita, and K. Okumura.** 1998. Differential regulation of IkappaB kinase alpha and beta by two upstream kinases, NF-kappaB-inducing kinase and mitogen-activated protein kinase/ERK kinase kinase-1. Proc Natl Acad Sci U S A **95:**3537-42.

296. **Nath, A., K. Psooy, C. Martin, B. Knudsen, D. S. Magnuson, N. Haughey, and J. D. Geiger.** 1996. Identification of a human immunodeficiency virus type 1 Tat epitope that is neuroexcitatory and neurotoxic. J Virol **70:**1475-80.

297. **Natoli, G., A. Costanzo, F. Moretti, M. Fulco, C. Balsano, and M. Levrero.** 1997. Tumor necrosis factor (TNF) receptor 1 signaling downstream of TNF receptor-associated factor 2. Nuclear factor kappaB (NFkappaB)-inducing kinase requirement for activation of activating protein 1 and NFkappaB but not of c-Jun N-terminal kinase/stress-activated protein kinase. J Biol Chem **272:**26079-82.

298. **Naumann, M., and C. Scheidereit.** 1994. Activation of NF-κB in vivo is regulated by multiple phosphorylations. EMBO Journal **13:**4597-4607.

299. **Naumann, M., F. G. Wulczyn, and C. Scheidereit.** 1993. The NF-κB precursor p105 and the proto-oncogene product Bcl-3 are IκB molecules and control nuclear translocation of NF-κB. EMBO Journal **12:**213-222.

300. **Nekhai, S., R. R. Shukla, and A. Kumar.** 1997. A human primary T-lymphocyte-derived human immunodeficiency virus type 1 Tat-associated kinase

phosphorylates the C-terminal domain of RNA polymerase II and induces CAK activity. J Virol **71:**7436-41.

301. **Nemoto, S., J. A. DiDonato, and A. Lin.** 1998. Coordinate regulation of IkappaB kinases by mitogen-activated protein kinase kinase kinase 1 and NF-kappaB-inducing kinase. Mol Cell Biol **18:**7336-43.

302. **Neri, A., C.-C. Chang, L. Lombardi, M. Salina, P. Corradini, A. T. Maiolo, R. S. K. Chaganti, and R. Dalla-Favera.** 1991. B cell lymphoma-associated chromosomal translocation involves candidate oncogene *lyt*-10, homologous to NF-κB p50. Cell **67:**1075-1087.

303. **Neumann, M., K. Tsapos, J. A. Scheppler, J. Ross, and B. R. Franza, Jr.** 1992. Identification of complex formation between two intracellular tyrosine kinase substrates: human c-rel and the p105 precursor of p50 NF-κB. Oncogene **7:**2095-2104.

304. **New, D. R., M. Ma, L. G. Epstein, A. Nath, and H. A. Gelbard.** 1997. Human immunodeficiency virus type 1 Tat protein induces death by apoptosis in primary human neuron cultures. J Neurovirol **3:**168-73.

305. **New, D. R., S. B. Maggirwar, L. G. Epstein, S. Dewhurst, and H. A. Gelbard.** 1998. HIV-1 Tat induces neuronal death via tumor necrosis factor-alpha and activation of non-N-methyl-D-aspartate receptors by a NFkappaB- independent mechanism. J Biol Chem **273:**17852-8.

306. **Ninomiya-Tsuji, J., K. Kishimoto, A. Hiyama, J. Inoue, Z. Cao, and K. Matsumoto.** 1999. The kinase TAK1 can activate the NIK-IκB as well as the MAP kinase cascade in the IL-1 signaling pathway. Nature **398:**252-256.

307. **Nolan, G. P., and D. Baltimore.** 1992. The inhibitory ankyrin and activator Rel proteins. Current Opinion in Genetics and Development **2:**211-220.

308. **Nolan, G. P., T. Fujita, K. Bhatia, C. Huppi, H.-C. Liou, M. L. Scott, and D. Baltimore.** 1993. The *bcl-3* proto-oncogene encodes a nuclear IκB-like molecule that preferentially interacts with NF-κB p50 and p52 in a phosphorylation-dependent manner. Molecular and Cellular Biology **13:**3557-3566.

309. **Nolan, G. P., S. Ghosh, H.-C. Liou, P. Tempst, and D. Baltimore.** 1991. DNA binding and IκB inhibition of the cloned p65 subunit of NF-κB, a *rel*-related polypeptide. Cell **64:**961-969.

310. **O'Mahony, A., X. Lin, R. Geleziunas, and W. C. Greene.** 2000. Activation of the heterodimeric IkappaB kinase alpha (IKKalpha)-IKKbeta complex is directional: IKKalpha regulates IKKbeta under both basal and stimulated conditions [In Process Citation]. Mol Cell Biol **20:**1170-8.

311. **Ohno, H., G. Takimoto, and T. W. McKeithan.** 1990. The candidate proto-oncogene *bcl*-3 is related to genes implicated in cell lineage determination and cell-cycle control. Cell **60:**991-997.

312. **Okamoto, H., C. T. Sheline, J. L. Corden, K. A. Jones, and B. M. Peterlin.** 1996. Trans-activation by human immunodeficiency virus Tat protein requires the C-terminal domain of RNA polymerase II. Proc Natl Acad Sci U S A **93:**11575-9.

313. **Okamoto, T., T. Benter, S. F. Josephs, M. R. Sadaie, and F. Wong-Staal.** 1990. Transcriptional activation from the long-terminal repeat of human immunodeficiency virus *in vitro*. Virology **177:**606-614.

314. **Olashaw, N. E., T. F. Kowalik, E. S. Huang, and W. J. Pledger.** 1992. Induction of NF-kappa B-like activity by platelet-derived growth factor in mouse fibroblasts. Mol Biol Cell **3:**1131-9.

315. **Ossareh-Nazari, B., F. Bachelerie, and C. Dargemont.** 1997. Evidence for a role of CRM1 in signal-mediated nuclear protein export. Science **278:**141-144.

316. **Ott, M., S. Emiliani, K. Van Lint, G. Herbein, J. Lovett, N. Chirmule, T. McCloskey, S. Pahwa, and E. Verdin.** 1997. Immune hyperactivation of HIV-1-infected T cells mediated by Tat and the CD28 pathway. Science **275:**1481-1485.

317. **Owen, G. I., J. K. Richer, L. Tung, G. Takimoto, and K. B. Horwitz.** 1998. Progesterone regulates transcription of the p21(WAF1) cyclin-dependent kinase inhibitor gene through Sp1 and CBP/p300. Journal of Biological Chemistry **273:**10696-10701.

318. **Ozes, O. N., L. D. Mayo, J. A. Gustin, S. R. Pfeffer, L. M. Pfeffer, and D. B. Donner.** 1999. NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase [see comments]. Nature **401**:82-5.

319. **Pahl, H. L.** 1999. Activators and target genes of Rel/NF-kappaB transcription factors. Oncogene **18**:6853-6866.

320. **Palombella, V. J., O. J. Rando, A. L. Goldberg, and T. Maniatis.** 1994. The ubiquitin-proteosome pathway is required for processing the NF-κB1 precursor protein and the activation of NF-κB. Cell **78**:773-785.

321. **Pan, J., and R. P. McEver.** 1995. Regulation of the human P-selectin promoter by Bcl-3 and specific homodimeric members of the NF-kappa B/Rel family. J Biol Chem **270**:23077-83.

322. **Parada, C. A., and R. G. Roeder.** 1996. Enhanced processivity of RNA polymerase II triggered by Tat-induced phosphorylation of its carboxy-terminal domain. Nature **384**:375-378.

323. **Parker, S. F., L. K. Felzien, N. D. Perkins, M. J. Imperiale, and G. J. Nabel.** 1997. Distinct domains of adenovirus E1A interact with specific cellular factors to differentially modulate human immunodeficiency virus transcription. J Virol **71**:2004-12.

324. **Patriotis, C., A. Makris, S. E. Bear, and P. N. Tsichlis.** 1993. Tumor progression locus 2 (Tpl-2) encodes a protein kinase involved in the progression of rodent T-cell lymphomas and in T-cell activation. Proc Natl Acad Sci U S A **90**:2251-5.

325. **Paya, C. V., R. M. Ten, C. Bessia, J. Alcami, R. T. Hay, and J.-L. Virelizier.** 1992. NF-κB-dependent induction of the NF-κB p50 subunit gene promoter underlies self-perpetuation of human immunodefiency virus transcription in monocytic cells. Proceedings of the National Academy of Sciences USA **89**:7826-7830.

326. **Pazin, M. J., P. L. Sheridan, K. Cannon, Z. Cao, J. G. Keck, J. T. Kadonaga, and K. A. Jones.** 1996. NF-κB-mediated chromatin reconfiguration and transcriptional activation of the HIV-1 enhancer in vitro. Genes & Development **10**:37-49.

327. **Peng, J., Y. Zhu, J. T. Milton, and D. H. Price.** 1998. Identification of multiple cyclin subunits of human P-TEFb. Genes Dev **12:**755-62.

328. **Pepin, N., A. Roulston, J. Lacoste, R. Lin, and J. Hiscott.** 1994. Subcellular redistribution of HTLV-1-Tax protein by NF-κB/Rel transcription factors. Virology **204:**706-716.

329. **Perkins, N. D., N. L. Edwards, C. S. Duckett, A. B. Agranoff, R. M. Schmid, and G. J. Nabel.** 1993. A cooperative interaction between NF-κB and Sp1 is required for HIV enhancer activation. EMBO Journal **12:**3551-3558.

330. **Perkins, N. D., L. K. Felzien, J. C. Betts, K. Leung, D. H. Beach, and G. J. Nabel.** 1997. Regulation of NF-κB by cyclin-dependent kinases associated with p300 coactivator. Science **275:**523-527.

331. **Perkins, N. D., R. M. Schmid, C. S. Duckett, K. Leung, N. R. Rice, and G. J. Nabel.** 1992. Distinct combinations of NF-κB subunits determine the specificity of transcriptional activation. Proceedings of the National Academy of Sciences USA **89:**1529-1533.

332. **Perona, R., S. Montaner, L. Saniger, I. Sanchez-Perez, R. Bravo, and J. C. Lacal.** 1997. Activation of the nuclear factor-kappaB by Rho, CDC42, and Rac-1 proteins. Genes Dev **11:**463-75.

333. **Petropoulos, L., R. Lin, and J. Hiscott.** 1996. Human T cell leukemia virus type 1 Tax protein increases NF-κB dimer formation and antagonizes the inhibitory activity of the IκBα regulatory protein. Virology **225:**52-64.

334. **Pettit, A. R., C. Quinn, K. P. MacDonald, L. L. Cavanagh, G. Thomas, W. Townsend, M. Handel, and R. Thomas.** 1997. Nuclear localization of RelB is associated with effective antigen- presenting cell function. J Immunol **159:**3681-91.

335. **Plaksin, D., P. A. Baeuerle, and L. Eisenbach.** 1993. KBF1 (p50 NF-κB homodimer) acts as a repressor of H-2K^b gene expression in metastatic tumor cells. Journal of Experimental Medicine **177:**1651-1662.

336. **Poli, G., and A. S. Fauci.** 1993. Cytokine modulation of HIV expression. Seminars in Immunology **5:**165-173.

337. **Poli, G., and A. S. Fauci.** 1992. The effect of cytokines and pharmacologic agents on chronic HIV infection. Aids Research and Human Retroviruses **8:**191-207.

338. **Poli, G., A. Kinter, J. S. Justement, J. H. Kehrl, P. Bressler, S. Stanley, and A. S. Fauci.** 1990. Tumor necrosis factor α functions in an autocrine manner in the induction of human immunodeficiency virus expression. Proceedings of the National Academy of Sciences USA **87:**782-785.

339. **Pugh, B. F., and R. Tjian.** 1991. Transcription from a TATA-less promoter requires a multisubunit TFIID complex. Genes Dev **5:**1935-45.

340. **Puri, R. K., P. Leland, and B. B. Aggarwal.** 1995. Constitutive expression of human immunodeficiency virus type 1 tat gene inhibits interleukin 2 and interleukin 2 receptor expression in a human CD4+ T lymphoid (H9) cell line. AIDS Res Hum Retroviruses **11:**31-40.

341. **Purvis, S. F., D. L. Georges, T. M. Williams, and M. M. Lederman.** 1992. Suppression of interleukin-2 and interleukin-2 receptor expression in Jurkat cells stably expressing the human immunodeficiency virus Tat protein. Cell Immunol **144:**32-42.

342. **Qian, J., V. Bours, J. Manischewitz, R. Blackburn, U. Siebenlist, and H. Golding.** 1994. Chemically selected subclones of the CEM cell line demonstrate resistance to HIV-1 infection resulting from a selective loss of NF- kappa B DNA binding proteins. J Immunol **152:**4183-91.

343. **Quinto, I., M. Mallardo, F. Baldassarre, G. Scala, G. Englund, and K. T. Jeang.** 1999. Potent and stable attenuation of live HIV-1 by gain of a proteolysis-resistant inhibitor of NF-κB (IκBα S32/36A) and the implications for vaccine development. Journal of Biological Chemistry **274:**17567-17572.

344. **Rana, T. M., and K. T. Jeang.** 1999. Biochemical and functional interactions between HIV-1 Tat protein and TAR RNA. Arch Biochem Biophys **365:**175-85.

345. **Read, M. A., M. Z. Whitley, S. Gupta, J. W. Pierce, J. Best, R. J. Davis, and T. Collins.** 1997. Tumor necrosis factor alpha-induced E-selectin expression is activated by the nuclear factor-kappaB and c-JUN N-terminal kinase/p38 mitogen- activated protein kinase pathways. J Biol Chem **272:**2753-61.

346. **Regnier, C. H., H. Y. Song, X. Gao, D. V. Goeddel, Z. Cao, and M. Rothe.** 1997. Identification and characterization of an IkappaB kinase. Cell **90:**373-83.

347. **Reits, E. A. J., A. M. Benham, B. Plougastel, J. Neefjes, and J. Trowsdale.** 1997. Dynamics of proteasome distribution in living cells. Embo J **16:**6087-94.

348. **Rice, N. R., and M. K. Ernst.** 1993. *In vivo* control of NF-κB activation by IκBα. EMBO Journal **12:**4685-4695.

349. **Rice, N. R., M. L. MacKichan, and A. Israel.** 1992. The precursor of NF-κB p50 has IκB-like functions. Cell **71:**243-253.

350. **Richardson, P. M., and T. D. Gilmore.** 1991. v-Rel is an inactive member of the rel family of transcriptional activating proteins. Journal of Virology **65:**3122-3130.

351. **Rodriguez, M. S., I. Michalopoulos, F. Arenzana-Seisdedos, and R. T. Hay.** 1995. Inducible degradation of IκBα *in vitro* and *in vivo* requires the acidic C-terminal domain of the protein. Mol.Cell.Biol. **15:**2413-2419.

352. **Rodriguez, M. S., J. Thompson, R. T. Hay, and C. Dargemont.** 1999. Nuclear retention of IkappaBalpha protects it from signal-induced degradation and inhibits nuclear factor kappaB transcriptional activation. J Biol Chem **274:**9108-15.

353. **Rodriguez, M. S., J. Wright, J. Thompson, D. Thomas, F. Baleux, J. L. Virelizier, R. T. Hay, and F. Arenzana-Seisdedos.** 1996. Identification of lysine residues for signal-induced ubiquitination and degradation of IκBα *in vivo*. Oncogene **12:**2425-2435.

354. **Roff, M., J. Thompson, M. S. Rodriguez, J. M. Jacque, F. Baleux, F. Arenzana-Seisdedos, and R. T. Hay.** 1996. Role of IkappaBalpha ubiquitination in signal-induced activation of NFkappaB in vivo. J Biol Chem **271:**7844-50.

355. **Rohlff, C., S. Ahmad, F. Borellini, J. Lei, and R. I. Glazer.** 1997. Modulation of transcription factor Sp1 by cAMP-dependent protein kinase. Journal of Biological Chemistry **272:**21137-21141.

356. **Romashkova, J. A., and S. S. Makarov.** 1999. NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling [see comments]. Nature **401:**86-90.

357. **Rooke, R., R. Tremblay, and M. A. Wainberg.** 1990. AZT (zidovudine) may act postintegrationally to inhibit generation of HIV-1 progeny virus in chronically infected cells. Virology **176:**205-215.

358. **Rosen, C. A., J. G. Sodroski, and W. A. Haseltine.** 1985. The location of cis-acting regulatory sequences in the human T cell lymphotropic virus type III (HTLV-III/LAV) long terminal repeat. Cell **41:**813-23.

359. **Ross, E. K., A. J. Buckler-White, A. B. Rabson, G. Englund, and M. A. Martin.** 1991. Contribution of NF-κB and Sp1 binding motifs to the replicative capacity of human immunodeficiency virus type 1: Distinct patterns of viral growth are determined by T-cell types. Journal of Virology **65:**4350-4358.

360. **Rossi, A., G. Elia, and M. G. Santoro.** 1997. Inhibition of nuclear factor kappa B by prostaglandin A1: an effect associated with heat shock transcription factor activation. Proc Natl Acad Sci U S A **94:**746-50.

361. **Rossi, A., P. Kapahi, G. Natoli, T. Takahashi, Y. Chen, M. Karin, and M. G. Santoro.** 2000. Anti-inflammatory cyclopentenone prostaglandins are direct inhibitors of IkappaB kinase. Nature **403:**103-8.

362. **Rothwarf, D. M., E. Zandi, G. Natoli, and M. Karin.** 1998. IKK-γ is an essential regulatory subunit of the IκB kinase complex. Nature **395:**297-300.

363. **Roulston, A., P. Beauparlant, N. R. Rice, and J. Hiscott.** 1993. Chronic human immunodeficiency virus type 1 infection stimulates distinct NF-κB/rel DNA binding activities in myelomonoblastic cells. Journal of Virology **67:**5235-5246.

364. **Roulston, A., M. D'Addario, F. Boulerice, S. Caplan, M. Wainberg, and J. Hiscott.** 1992. Induction of monocytic differentiation and NF-κB-like activities by human immunodeficiency virus-1 infection of human myelomonoblastic cells. Journal of Experimental Medicine **175:**751-763.

365. **Roulston, A., R. Lin, P. Beauparlant, M. A. Wainberg, and J. Hiscott.** 1995. Regulation of HIV-1 and cytokine gene expression in myeloid cells by NF-κB/Rel transcription factors. Microbiological Reviews **59:**481-505.

366. **Roy, S., U. Delling, C.-H. Chen, C. A. Rosen, and N. Sonenberg.** 1990. A bulge structure in HIV-1 TAR RNA is required for Tat binding and Tat-mediated *trans*-activation. Genes & Development **4:**1365-1373.

367. **Roy, S., N. T. Parkin, C. Rosen, J. Itovitch, and N. Sonenberg.** 1990. Structural requirement for trans-activation of human immunodeficiency virus type 1 long terminal repeat-directed gene expression by Tat: importance of base pairing, loop sequence, and bulge in the Tat-responsive sequence. Journal of Virology **64:**1402-1406.

368. **Ruben, S. M., P. J. Dillon, R. Schreck, T. Henkel, C. H. Chen, M. Maher, P. A. Baeuerle, and C. A. Rosen.** 1991. Isolation of a *rel*-related human cDNA that potentially encodes the 65-kD subunit of NF-κB. Science **251:**1490-1493.

369. **Ruben, S. M., J. F. Klement, T. A. Coleman, M. Maher, C.-H. Chen, and C. A. Rosen.** 1992. I-rel: a novel *rel*-related protein that inhibits NF-κB transcriptional activity. Genes & Development **6:**745-760.

370. **Ryseck, R.-P., P. Bull, M. Takamiya, V. Bours, U. Siebenlist, P. Dobrzanski, and R. Bravo.** 1992. RelB, a new rel family transcription activator that can interact with p50-NF-κB. Mol.Cell.Biol. **12:**674-684.

371. **Sachdev, S., A. Hoffmann, and M. Hannink.** 1998. Nuclear localization of IκBα is mediated by the second ankyrin repeat: the IκBα ankyrin repeats define a novel class of cis-acting nuclear import sequences. Mol.Cell.Biol. **18:**2524-2534.

372. **Sachdev, S., E. M. Rottjakob, J. A. Diehl, and M. Hannink.** 1995. IκBα-mediated inhibition of nuclear transport and DNA-binding by Rel proteins are separable functions: phosphorylation of C-terminal serine residues of IκBα is specifically required for inhibition of DNA binding. Oncogene **11:**811-823.

373. **Sakurai, H., H. Chiba, H. Miyoshi, T. Sugita, and W. Toriumi.** 1999. IkappaB kinases phosphorylate NF-kappaB p65 subunit on serine 536 in the transactivation domain. J Biol Chem **274:**30353-6.

374. **Sakurai, H., H. Miyoshi, W. Toriumi, and T. Sugita.** 1999. Functional interactions of transforming growth factor beta-activated kinase 1 with IkappaB kinases to stimulate NF-kappaB activation. J Biol Chem **274:**10641-8.

375. **Sakurai, H., N. Shigemori, K. Hasegawa, and T. Sugita.** 1998. TGF-beta-activated kinase 1 stimulates NF-kappa B activation by an NF- kappa B-inducing kinase-independent mechanism. Biochem Biophys Res Commun **243:**545-9.

376. **Sambucetti, L. C., J. M. Cherrington, G. W. Wilkison, and E. S. Mocarski.** 1989. NF-kappa-B activation of the cytomegalovirus enhancer is mediated by a viral transactivator and by T cell stimulation. EMBO Journal **8:**4251-4258.

377. **Sanceau, J., T. Kaisho, T. Hirano, and J. Wietzerbin.** 1995. Triggering of the human interleukin-6 gene by interferon-gamma and tumor necrosis factor-alpha in monocytic cells involves cooperation between interferon regulatory factor-1, NF- kappa B, and Sp1 transcription factors. Journal of Biological Chemistry **17:**27920-27931.

378. **Sanz, L., P. Sanchez, M.-J. Lallena, M. T. Diaz-Meco, and J. Moscot.** 1999. The interaction of p62 with RIP links the atypical PKCs to NF-κB activation. EMBO Journal **18:**3044-3053.

379. **Scheinman, R. I., A. A. Beg, and A. S. Baldwin, Jr.** 1993. NF-κB p100 (lyt-10) is a component of H2TF1 and can function as an IκB-like molecule. Molecular and Cellular Biology **13**:6089-6101.

380. **Scherer, D. C., J. A. Brockman, Z. Chen, T. Maniatis, and D. W. Ballard.** 1995. Signal-induced degradation of IκBα requires site-specific ubiquitination. Proceedings of the National Academy of Sciences USA **92**:11259-11263.

381. **Schmid, R. M., N. D. Perkins, C. S. Duckett, P. C. Andrews, and G. J. Nabel.** 1991. Cloning of an NF-κB subunit which stimulates HIV transcription in synergy with p65. Nature **352**:733-736.

382. **Schmitz, M. L., and P. A. Baeuerle.** 1991. The p65 subunit is responsible for the strong transcription activating potential of NF-κB. EMBO Journal **10**:3805-3817.

383. **Schmitz, M. L., M. A. dos Santos Silva, and P. A. Baeuerle.** 1995. Transactivation domain 2 (TA2) of p65 NF-κB. Similarity to TA1 and phorbol ester-stimulated activity and phosphorylation in intact cells. Journal of Biological Chemistry **270**:15576-15584.

384. **Schmitz, M. L., G. Stelzer, H. Altmann, M. Meisterernst, and P. A. Baeuerle.** 1995. Interaction of the COOH-terminal transactivation domain of p65 NF-kappa B with TATA-binding protein, transcription factor IIB, and coactivators. J Biol Chem **270**:7219-26.

385. **Schouten, G. J., A. C. Vertegaal, S. T. Whiteside, A. Israel, M. Toebes, J. C. Dorsman, A. J. van der Eb, and A. Zantema.** 1997. IκBα is a target for the mitogen-activated 90kDa ribosomal S6 kinase. EMBO Journal **16**:3133-3144.

386. **Schwarz, E., D. Antwerp, and I. Verma.** 1996. Constitutive phosphorylation of IκBα by casein kinase II occurs preferentially at serine 293: requirement for degradation of free IκBα. Molecular and Cellular Biology **16**:3554-3559.

387. **Schwarz, E. M., P. Krimpenfort, A. Berns, and I. M. Verma.** 1997. Immunological defects in mice with a targeted disruption in Bcl-3. Genes Dev **11**:187-97.

388. **Schwarze, S. R., A. Ho, A. Vocero-Akbani, and S. F. Dowdy.** 1999. In vivo protein transduction: delivery of a biologically active protein into the mouse [see comments]. Science **285:**1569-72.

389. **Scott, M. L., T. Fujita, H.-C. Liou, G. P. Nolan, and D. Baltimore.** 1993. The p65 subunit of NF-κB regulates IκB by two distinct mechanisms. Genes & Development **7:**1266-1276.

390. **Selby, M. J., E. S. Bain, P. A. Luciw, and B. M. Peterlin.** 1989. Structure, sequence, and position of the stem-loop in TAR determine transcriptional elongation by Tat through the HIV-1 long terminal repeat. Genes & Development **3:**547-558.

391. **Selby, M. J., and B. M. Peterlin.** 1990. Trans-activation by HIV-1 Tat via a heterologous RNA binding protein. Cell **62:**769-76.

392. **Sha, W. C., H.-C. Liou, E. I. Tuomanen, and D. Baltimore.** 1995. Targeted disruption of the p50 subunit of NF-κB leads to multifocal defects in immune responses. Cell **80:**321-330.

393. **Sheline, C. T., L. H. Milocco, and K. A. Jones.** 1991. Two distinct nuclear transcription factors recognize loop and bulge residues of the HIV-1 TAR RNA hairpin. Genes & Development **5:**2508-2520.

394. **Sheridan, P. L., T. P. Mayall, E. Verdin, and K. A. Jones.** 1997. Histone acetyltransferases regulate HIV-1 enhancer activity in vitro. Genes Dev **11:**3327-40.

395. **Sheridan, P. L., C. T. Sheline, K. Cannon, M. L. Voz, M. J. Pazin, J. T. Kadonaga, and K. A. Jones.** 1995. Activation of the HIV-1 enhancer by the LEF-1 HMG protein on nucleosome- assembled DNA in vitro. Genes Dev **9:**2090-104.

396. **Shibuya, H., K. Yamaguchi, K. Shirakabe, A. Tonegawa, Y. Gotoh, N. Ueno, K. Irie, E. Nishida, and K. Matsumoto.** 1996. TAB1: an activator of the TAK1 MAPKKK in TGF-beta signal transduction. Science **272:**1179-82.

397. **Shinkura, R., K. Kitada, F. Matsuda, K. Tashiro, K. Ikuta, M. Suzuki, K. Kogishi, T. Serikawa, and T. Honjo.** 1999. Alymphoplasia is caused by a point mutation in the mouse gene encoding Nf-kappa b-inducing kinase. Nat Genet **22:**74-7.

398. **Shockett, p., M. Difilippantonio, N. Hellman, and D. G. Schatz.** 1995. A modified tetracycline-regulated system provides autoregulatory, inducible gene expression in cultured cells and transgenic mice. Proceedings of the National Academy of Sciences USA **92:**6522-6526.

399. **Siekevitz, M., S. F. Josephs, M. Dukovich, N. Peffer, F. Wong-Staal, and W. C. Greene.** 1987. Activation of the HIV-LTR by T-cell mitogens and the trans-activator protein of HTLV-1. Science **238:**1575-1578.

400. **Sieweke, M. H., H. Tekotte, U. Jarosch, and T. Graf.** 1998. Cooperative interaction of ets-1 with USF-1 required for HIV-1 enhancer activity in T cells. Embo J **17:**1728-39.

401. **Sif, S., and T. D. Gilmore.** 1994. Interaction of the v-Rel oncoprotein with cellular transcription factor Sp1. J Virol **68:**7131-8.

402. **Simeonidis, S., S. Liang, G. Chen, and D. Thanos.** 1997. Cloning and functional characterization of mouse IkappaBepsilon. Proc Natl Acad Sci U S A **94:**14372-7.

403. **Sizemore, N., S. Leung, and G. R. Stark.** 1999. Activation of phosphatidylinositol 3-kinase in response to interleukin- 1 leads to phosphorylation and activation of the NF-kappaB p65/RelA subunit. Mol Cell Biol **19:**4798-805.

404. **Song, H. Y., C. H. Regnier, C. J. Kirschning, D. V. Goeddel, and M. Rothe.** 1997. Tumor necrosis factor (TNF)-mediated kinase cascades: bifurcation of nuclear factor-κB and c-jun N-terminal kinase (JNK/SAPK) pathways at TNF receptor-associated factor 2. Proceedings of the National Academy of Sciences USA **94:**9792-9796.

405. **Stade, K., C. S. Ford, C. Guthrie, and K. Weis.** 1997. Exportin 1 (Crm1) is an essential nuclear export factor. Cell **90:**1041-1050.

406. **Sun, S.-C., J. Elwood, and W. C. Greene.** 1996. Both amino- and carboxyl-terminal sequences within IκBα regulate its inducible degradation. Molecular and Cellular Biology **16:**1058-1065.

407. **Sun, S.-C., P. A. Ganchi, D. W. Ballard, and W. C. Greene.** 1993. NF-κB controls expression of inhibitor IκBα: evidence for an inducible autoregulatory pathway. Science **259:**1912-1915.

408. **Suyang, H., R. Phillips, I. Douglas, and S. Ghosh.** 1996. Role of unphosphorylated, newly synthesized IκBβ in persistent activation of NF-κB. Molecular and Cellular Biology **16:**5444-5449.

409. **Takeda, K., O. Takeuchi, T. Tsujimura, S. Itami, O. Adachi, T. Kawai, H. Sanjo, K. Yoshikawa, N. Terada, and S. Akira.** 1999. Limb and skin abnormalities in mice lacking IKKalpha [see comments]. Science **284:**313-6.

410. **Tanaka, M., M. E. Fuentes, K. Yamaguchi, M. H. Durin, S. A. Dalrymple, K. L. Hardy, and D. V. Goeddel.** 1999. Embrionic lethality, liver degeneration, and impaired NF-κB activation in IKKβ deficient mice. Immunity **10:**421-429.

411. **Taylor, J. P., R. Pomerantz, O. Bagasra, M. Chowdhury, J. Rappaport, K. Khalili, and S. Amini.** 1992. TAR-independent transactivation by Tat in cells derived from the CNS: a novel mechanism of HIV-1 gene regulation. EMBO Journal **11:**3395-3403.

412. **Ten, R. M., C. V. Paya, N. Israël, O. Le Bail, M.-G. Mattei, J.-L. Virelizier, P. Kourilsky, and A. Israël.** 1992. The characterization of the promoter of the gene encoding the p50 subunit of NF-κB indicates that it participates in its own regulation. EMBO Journal **11:**195-203.

413. **Tewari, M., and V. M. Dixit.** 1996. Recent advances in tumor necrosis factor and CD40 signaling. Current Opinion in Genetics and Development **6:**39-44.

414. **Tewari, M., P. Dobrzanski, K. L. Mohn, D. E. Cressman, J.-C. Hsu, R. Bravo, and R. Taub.** 1992. Rapid induction in regenerating liver of RL/IF-1 (an IκB that

inhibits NF-κB, RelB-p50, and c-Rel-p50) and PHF, a novel κB site-binding complex. Molecular and Cellular Biology **12**:2898-2908.

415. **Thompson, J. E., R. J. Phillips, H. Erdjument-Bromage, P. Tempst, and S. Ghosh.** 1995. IκB-β regulates the persistent response in a biphasic activation of NF-κB. Cell **80**:573-582.

416. **Traenckner, E. B., H. L. Phal, T. Henkel, N. K. Schmidt, S. Wilk, and P. A. Baeuerle.** 1995. Phosphorylation of human IκBα on serines 32 and 36 controls IκBα proteolysis and NF-κB activation in response to diverse stimuli. EMBO Journal **14**:2876-2883.

417. **Tran, K., M. Merika, and D. Thanos.** 1997. Distinct functional properties of IκBα and IκBβ. Molecular and Cellular Biology **17**:5386-5399.

418. **Travis, A., A. Amsterdam, C. Belanger, and R. Grosschedl.** 1991. LEF-1, a gene encoding a lymphoid-specific protein with an HMG domain, regulates T-cell receptorα enhancer function. Genes & Development **5**:880-894.

419. **Turpin, P., R. T. Hay, and C. Dargemont.** 1999. Characterization of IkappaBalpha nuclear import pathway. J Biol Chem **274**:6804-12.

420. **Uhlik, M., L. Good, G. Xiao, E. Harhaj, E. Zandi, M. Karin, and S.-C. Sun.** 1998. NF-κB-inducing kinase and IκB kinase participate in human T-cell leukemia virus 1 Tax-mediated NF-κB activation. Journal of Biological Chemistry **273**:21132-21136.

421. **Urban, M. B., and P. A. Baeuerle.** 1990. The 65-kD subunit of NF-κB is a receptor for IκB and a modulator of DNA-binding specificity. Genes and Devel. **4**:1975-1984.

422. **Vacca, A., M. Farina, M. Maroder, E. Alesse, I. Screpanti, L. Frati, and A. Gulino.** 1994. Human immunodeficiency virus type-1 tat enhances interleukin-2 promoter activity through synergism with phorbol ester and calcium-mediated activation of the NF-AT cis-regulatory motif. Biochem Biophys Res Commun **205**:467-74.

423. **Van Antwerp, D. J., S. J. Martin, T. Kafri, D. R. Green, and I. M. Verma.** 1996. Suppression of TNF-α-induced apoptosis by NF-κB. Science **274:**787-789.

424. **Van Antwerp, D. J., and I. Verma.** 1996. Signal-induced degradation of IκBα : association with NF-κB and the PEST sequence in IκBα are not required. Molecular and Cellular Biology **16:**6037-6045.

425. **van Hogerlinden, M., B. L. Rozell, L. Ahrlund-Richter, and R. Toftgard.** 1999. Squamous cell carcinomas and increased apoptosis in skin with inhibited Rel/nuclear factor-kappaB signaling. Cancer Res **59:**3299-303.

426. **Van Lint, C., S. Emiliani, M. Ott, and E. Verdin.** 1996. Transcriptional activation and chromatin remodeling of the HIV-1 promoter in response to histone acetylation. Embo J **15:**1112-20.

427. **Vanden Berghe, W., S. Plaisance, E. Boone, K. De Bosscher, M. L. Schmitz, W. Fiers, and G. Haegeman.** 1998. p38 and extracellular signal-regulated kinase mitogen-activated protein kinase pathways are required for nuclear factor-kappaB p65 transactivation mediated by tumor necrosis factor. J Biol Chem **273:**3285-90.

428. **Verdin, E., P. Paras, Jr., and C. Van Lint.** 1993. Chromatin disruption in the promoter of human immunodeficiency virus type 1 during transcriptional activation [published erratum appears in EMBO J 1993 Dec;12(12):4900]. Embo J **12:**3249-59.

429. **Verma, I. M., J. K. Stevenson, E. M. Schwarz, D. V. Antwerp, and S. Miyamoto.** 1995. Rel/NF-κB/IκB family: intimate tales of association and dissociation. Genes & Development **9:**2723-2735.

430. **Viscidi, R. P., K. Mayur, H. M. Lederman, and A. D. Frankel.** 1989. Inhibition of antigen-induced lymphocyte proliferation by Tat protein from HIV-1. Science **246:**1606-1608.

431. **Vocero-Akbani, A. M., N. V. Heyden, N. A. Lissy, L. Ratner, and S. F. Dowdy.** 1999. Killing HIV-infected cells by transduction with an HIV protease- activated caspase-3 protein [see comments]. Nat Med **5:**29-33.

432. **Wang, C. Y., D. C. Guttridge, M. W. Mayo, and A. S. Baldwin, Jr.** 1999. NF-kappaB induces expression of the Bcl-2 homologue A1/Bfl-1 to preferentially suppress chemotherapy-induced apoptosis. Mol Cell Biol **19:**5923-9.

433. **Wang, C. Y., M. W. Mayo, and A. S. Baldwin.** 1996. TNF- and cancer therapy-induced apoptosis : potentiation by inhibition of NF-κB. Science **274:**784-787.

434. **Wang, C. Y., M. W. Mayo, R. G. Korneluk, D. V. Goeddel, and A. S. J. Baldwin.** 1998. NF-κB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. Science **281:**1680-1683.

435. **Wang, D., and A. S. Baldwin, Jr.** 1998. Activation of nuclear factor-kappaB-dependent transcription by tumor necrosis factor-alpha is mediated through phosphorylation of RelA/p65 on serine 529. J Biol Chem **273:**29411-6.

436. **Watanabe, N., T. Iwamura, T. Shinoda, and T. Fujita.** 1997. Regulation of NFKB1 proteins by the candidate oncoprotein BCL-3: generation of NF-kappaB homodimers from the cytoplasmic pool of p50- p105 and nuclear translocation. Embo J **16:**3609-20.

437. **Waterman, M. L., W. H. Fischer, and K. A. Jones.** 1991. A thymus-specific member of the HMG protein family regulates the human T-cell receptor Cα enhancer. Genes & Development **5:**656-669.

438. **Wei, P., M. E. Garber, S.-M. Fang, W. H. Fischer, and K. A. Jones.** 1998. A novel CDK9-associated C-type cyclin interacts directly with HIV-1 Tat and mediates its high-affinity. loop-specific binding to TAR RNA. Cell **92:**451-462.

439. **Weih, F., D. Carrasco, and R. Bravo.** 1994. Constitutive and inducible Rel/NF-kappa B activities in mouse thymus and spleen. Oncogene **9:**3289-97.

440. **Weih, F., D. Carrasco, S. K. Durham, D. S. Barton, C. A. Rizzo, R.-P. Ryseck, S. A. Lira, and R. Bravo.** 1995. Multiorgan inflammation and hematopoietic abnormalities in mice with a targeted disruption of RelB, a member of the NF-κB/Rel family. Cell **80:**331-340.

441. **Weil, R., C. Laurent-Winter, and A. Israel.** 1997. Regulation of IκBβ degradation. Similarities to and differences from IκBα. Journal of Biological Chemistry **272:**9942-9949.

442. **Wen, W., J. L. Meinkoth, R. Y. Tsien, and S. S. Taylor.** 1995. Identification of signal for rapid export of proteins from the nucleus. Cell **82:**463-473.

443. **Wesche, H., W. J. Henzel, W. Shillinglaw, S. Li, and Z. Cao.** 1997. MyD88: an adapter that recruits IRAK to the IL-1 receptor complex. Immunity **7:**837-47.

444. **Wesche, H., C. Korherr, M. Kracht, W. Falk, K. Resch, and M. U. Martin.** 1997. The interleukin-1 receptor accessory protein (IL-1RAcP) is essential for IL-1-induced activation of interleukin-1 receptor-associated kinase (IRAK) and stress-activated protein kinases (SAP kinases). J Biol Chem **272:**7727-31.

445. **Westendorp, M. O., R. Frank, C. Ochsenbauer, K. Stricker, J. Dhein, H. Walczak, K.-M. Debatin, and P. H. Krammer.** 1995. Sensitization of T cells to CD95-mediated apoptosis by HIV-1 Tat and gp120. Nature **375:**497-500.

446. **Westendorp, M. O., V. A. Shatrov, K. Schulze-Osthoff, R. Frank, M. Kraft, M. Los, P. H. Krammer, W. Drige, and V. Lehnann.** 1995. HIV-1 Tat potentiates TNF-induced NF-κB activation and cytotoxicity by altering the cellular redox state. EMBO Journal **14 (3):**546-554.

447. **Westendorp, M. O., M.-L. Weber, R. W. Frank, and P. H. Krammer.** 1994. Human immunodeficiency virus type 1 Tat upregulates interleukin-2 secretion in activated T cells. Journal of Virology **68(7):**4177-4185.

448. **Whiteside, S. T., J. C. Epinat, N. R. Rice, and A. Israel.** 1997. I kappa B epilson, a novel member of the IκB family, controls RelA and c-Rel NF-κB activity. EMBO Journal **16:**1413-1426.

449. **Whiteside, S. T., M. K. Ernst, O. LeBail, C. Laurent-Winter, N. Rice, and A. Israel.** 1995. N- and C-terminal sequences control degradation of MAD3/IκBα in response to inducers of NF-κB activity. Molecular and Cellular Biology **15:**5339-5345.

450. **Witte, S., M. Villalba, K. Bi, Y. Liu, N. Isakov, and A. Altman.** 2000. Inhibition of the c-Jun N-terminal kinase/AP-1 and NF-kappaB pathways by PICOT, a novel protein kinase C-interacting protein with a thioredoxin homology domain. J Biol Chem **275:**1902-9.

451. **Woronicz, J. D., X. Gao, Z. Cao, M. Rothe, and D. V. Goeddel.** 1997. IκB kinase-β: NF-κB activation and complex formation with IκB kinase-α and NIK. Science **278:**866-869.

452. **Wu, B.-Y., C. Woffendin, C. S. Duckett, T. Ohno, and G. J. Nabel.** 1995. Regulation of human retroviral latency by the NF-κB/IκB family: Inhibition of human immunodeficiency virus replication by IκB through a Rev-dependent mechanism. Proceedings of the National Academy of Sciences USA **92:**1480-1484.

453. **Wu, B. Y., C. Woffendin, I. MacLachlan, and G. J. Nabel.** 1997. Distinct domains of IκB-alpha inhibit human immunodeficiency virus type 1 replication through NF-κB and Rev. Journal of Virology **71:**3161-3167.

454. **Wu, M., H. Lee, R. E. Bellas, S. L. Schauer, M. Arsura, D. Katz, M. J. FitzGerald, T. L. Rothstein, D. H. Sherr, and G. E. Sonenshein.** 1996. Inhibition of NF-κB/Rel induces apoptosis of murine B cells. EMBO Journal **15:**4682-4690.

455. **Wulczyn, F. G., D. Krappmann, and C. Scheidereit.** 1998. Signal-dependent degradation of IkappaBalpha is mediated by an inducible destruction box that can be transferred to NF-kappaB, bcl-3 or p53. Nucleic Acids Res **26:**1724-30.

456. **Wulczyn, F. G., M. Naumann, and C. Scheidereit.** 1992. Candidate proto-oncogene *bcl-3* encodes a subunit-specific inhibitor of transcription factor NF-κB. Nature **358:**597-599.

457. **Xia, Y., S. Chen, Y. Wang, N. Mackman, G. Ku, D. Lo, and L. Feng.** 1999. RelB modulation of IkappaBalpha stability as a mechanism of transcription suppression of interleukin-1alpha (IL-1alpha), IL-1beta, and tumor necrosis factor alpha in fibroblasts. Mol Cell Biol **19:**7688-96.

458. **Yamaguchi, K., K. Shirakabe, H. Shibuya, K. Irie, I. Oishi, N. Ueno, T. Taniguchi, E. Nishida, and K. Matsumoto.** 1995. Identification of a member of the MAPKKK family as a potential mediator of TGF-beta signal transduction. Science **270:**2008-11.

459. **Yamaoka, S., G. Courtois, C. Bessia, S. T. Whiteside, R. Weil, F. Agou, H. E. Kirk, R. J. Kay, and A. Israel.** 1998. Complementation cloning of NEMO, a component of the IκB kinase complex essential for NF-κB activation. Cell **93:**1231-1240.

460. **Yang, X., M. O. Gold, D. N. Tang, D. E. Lewis, E. Aguilar-Cordova, A. P. Rice, and C. H. Herrmann.** 1997. TAK, an HIV Tat-associated kinase, is a member of the cyclin-dependent family of protein kinases and is induced by activation of peripheral blood lymphocytes and differentiation of promonocytic cell lines. Proc Natl Acad Sci U S A **94:**12331-6.

461. **Yang, X., C. H. Herrmann, and A. P. Rice.** 1996. The human immunodeficiency virus Tat proteins specifically associate with TAK in vivo and require the carboxyl-terminal domain of RNA polymerase II for function. J Virol **70:**4576-84.

462. **Yang, X. J., V. V. Ogryzko, J. Nishikawa, B. H. Howard, and Y. Nakatani.** 1996. A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. Nature **382:**319-24.

463. **Yaron, A., A. Hatzubai, M. Davis, I. Lavon, S. Amit, A. M. Manning, J. S. Andersen, M. Mann, F. Mercurio, and Y. Ben-Neriah.** 1998. Identification of the receptor component of the IκBα-ubiquitin ligase. Nature **396:**590-594.

464. **Yeh, W. C., A. Shahinian, D. Speiser, J. Kraunus, F. Billia, A. Wakeham, J. L. de la Pompa, D. Ferrick, B. Hum, N. Iscove, P. Ohashi, M. Rothe, D. V. Goeddel, and T. W. Mak.** 1997. Early lethality, functional NF-kappaB activation, and increased sensitivity to TNF-induced cell death in TRAF2-deficient mice. Immunity **7:**715-25.

465. **Yin, M.-J., L. B. Christerson, Y. Yamamoto, Y.-T. Kwak, S. Xu, F. Mercurio, M. Barbosa, M. H. Cobb, and R. B. Gaynor.** 1998. HTLV-I Tax protein binds to MEKK1 to stimulate IκB kinase activity and NF-κB activation. Cell **93:**875-884.

466. **Zabel, U., and P. A. Baeuerle.** 1990. Purified human IκB can rapidly dissociate the complex of the NF-κB transcription factor with its cognate DNA. Cell **61**:255-265.

467. **Zabel, U., T. Henkel, M. Silva, and P. Baeuerle.** 1993. Nuclear uptake control of NF-κB by MAD-3, an IκB protein present in the nucleus. EMBO Journal **12**:201-211.

468. **Zack, J. A., S. J. Arrigo, S. R. Weitzmann, A. S. Goo, A. Haislip, and I. S. Y. Chen.** 1990. HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile latent viral structure. Cell **61**:213-222.

469. **Zamanian-Daryoush, M., T. H. Mogensen, J. A. DiDonato, and B. R. Williams.** 2000. NF-kappaB activation by double-stranded-RNA-activated protein kinase (PKR) is mediated through NF-kappaB-inducing kinase and IkappaB kinase. Mol Cell Biol **20**:1278-90.

470. **Zandi, E., Y. Chen, and M. Karin.** 1998. Direct phosphorylation of IkappaB by IKKalpha and IKKbeta: discrimination between free and NF-kappaB-bound substrate. Science **281**:1360-3.

471. **Zandi, E., D. M. Rothwarf, M. Delhase, M. Hayakawa, and M. Karin.** 1997. The IκB kinase complex (IKK) contains two kinase subunits, IKKα and IKKβ, necessary for IκB phosphorylation and NF-κB activation. Cell **91**:243-252.

472. **Zauli, G., D. Gibellini, D. Milani, D. Mazzoni, P. Borgatti, M. La Placa, and S. Capitani.** 1993. HIV-1 Tat protein protects lymphoid, epithelial and neuronal cell lines from death by apoptosis. Cancer Research **53**:4481-4485.

473. **Zhang, Q., J. A. Didonato, M. Karin, and T. W. McKeithan.** 1994. BCL3 encodes a nuclear protein which can alter the subcellular location of NF-kappa B proteins. Mol Cell Biol **14**:3915-26.

474. **Zhao, Q., and F. S. Lee.** 1999. Mitogen-activated protein kinase/ERK kinase kinases 2 and 3 activate nuclear factor-kappaB through IkappaB kinase-alpha and IkappaB kinase- beta. J Biol Chem **274**:8355-8.

475. **Zhong, H., H. Suyang, H. Erdjument-Bromage, P. Tempst, and S. Ghosh.** 1997. The transcriptional activity of NF-κB is regulated by the IκB-associated PKAc subunit through a cyclic AMP-independent mechanism. Cell **89**:413-424.

476. **Zhong, H., R. E. Voll, and S. Ghosh.** 1998. Phosphorylation of NF-kappa B p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300. Mol Cell **1**:661-71.

477. **Zhu, Y., T. Pe'ery, J. Peng, Y. Ramanathan, N. Marshall, T. Marshall, B. Amendt, M. B. Mathews, and D. H. Price.** 1997. Transcription elongation factor P-TEFb is required for HIV-1 Tat transactivation in vitro. Genes Dev. **11**:2622-2632.

478. **Zong, W. X., L. C. Edelstein, C. Chen, J. Bash, and C. Gelinas.** 1999. The prosurvival Bcl-2 homolog Bfl-1/A1 is a direct transcriptional target of NF-kappaB that blocks TNFalpha-induced apoptosis. Genes Dev **13**:382-7.