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The T Cell Activation Factor NF-ATc Positively Regulates HIV-1 Replication and Gene Expression in T Cells

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Summary

Clinical deterioration in human immunodeficiency virus type 1 (HIV-1) infection is associated with increased levels of viral replication and burden in the peripheral blood and lymphoid organs. T cell activation and ensuing cellular gene activation can be critical for HIV-1 replication. The hypothesis that the nuclear factor of activated T cells (NF-AT) may influence HIV-1 replication is therefore compelling given the tight correlation of HIV-1 transcriptional induction to T cell activation. We report that certain NF-AT(Rel) family members productively bind the κB regulatory elements, synergize with NF-κB and Tat in transcriptional activation of HIV-1, and enhance HIV-1 replication in T cells. These results link regulatory factors critical to T cell commitment directly to HIV-1 replication.

Introduction

It has been reported that efficient replication of human immunodeficiency virus type 1 (HIV-1) can be observed at all stages of HIV-1 infection, irrespective of patient status as either asymptomatic or symptomatic (Bagnarelli et al., 1992; Michael et al., 1992; Embretson et al., 1993; Pantaleo et al., 1993; Piatak et al., 1993; Paul, 1995). However, detailed observations indicate that HIV-1 RNA expression and HIV-1 RNA/DNA ratios dramatically increase during disease progression (Bagnarelli et al., 1992; Michael et al., 1992; Piatak et al., 1993). This suggests that the level of HIV-1 RNA expression in T cells, together with viral transcriptional activity, correlates with and influences the progression of HIV-1 disease.

HIV-1 gene expression is controlled by an interplay of viral and host regulatory proteins such as Tat, Rev, Nef, and NF-κB (Cullen, 1991; Haseltine, 1991). The interaction between these viral and cellular proteins acting through the HIV-1 long terminal repeat (LTR) is thought to regulate virus replication, including having effects on the pathogenesis and progression of acquired immunodeficiency syndrome (AIDS). Among the transcriptional elements driving HIV-1 expression and regulation, the κB enhancer regulatory elements at nucleotides −104 to −81 in the HIV-1 LTR are considered the most critical *cis*-acting elements. Deletion and site-directed mutation of the κB regulatory elements can abolish transcriptional activation induced by T cell activation using certain inducers (Nabel and Baltimore, 1987; Siekevitz et al., 1987; Tong et al., 1987; Kawakami et al., 1988; Markovitz et al., 1990). Therefore it has been long suggested that NF-κB, which binds to these elements, is a critical transcription factor for HIV-1 gene regulation in T cells (Nabel and Baltimore, 1987).

The nuclear factor of activated T cells (NF-AT) is an immediate-early activation factor that plays a necessary early role in T cell activation and commitment processes through its control of interleukin-2 (IL-2) gene activation (Crabtree, 1989). NF-AT activity has been defined as a complex family of transcriptional regulators composed of a monomeric NF-AT(Rel) component cross-coupled to a basic-leucine zipper–containing dimer. The NF-AT Rel components cloned to date are NF-ATp (McCaffrey et al., 1993), NF-ATc (Northrop et al., 1994), NF-AT3 (Hoey et al., 1995), and NF-ATx (Masuda et al., 1995). The basic-leucine zipper components are thought to be Jun/Fos as activator protein 1 (AP-1) (McCaffrey et al., 1993). NF-AT binding activity has been defined as either an NF-AT Rel component binding alone (whether as a monomer NF-AT(Rel) or as a dimer is not entirely clear) or as an NF-AT Rel component in a complex with AP-1 (Jain et al., 1993; Nolan, 1994; Jain et al., 1995).

Speculation that NF-AT may be involved in HIV-1 regulation has led previous investigators to study potential roles for NF-AT in HIV-1 gene expression (Shaw et al., 1988; Greene, 1990; Lu et al., 1990; Tong et al., 1990). Despite intense efforts, no causal link has previously been established between NF-AT and the activation of HIV-1 gene expression. The first studies on potential NF-AT involvement in HIV-1 began with the recognition of IL-2 promoter homologies in the HIV-1 LTR, including the presence of putative NF-AT binding sites in the negative regulatory element (NRE). Footprinting studies of the DNA region immediately upstream of the κB enhancer motifs indicated the presence of NF-AT binding sites (Shaw et al., 1988). Analysis of the functions of these upstream NF-AT sites concluded that no activity dependent on NF-AT could be correlated with the presence of these motifs (Markovitz et al., 1992). These and other studies resulted in the conclusion that NF-AT was not involved in HIV-1 activation through this region (Lu et al., 1990; Gruters et al., 1991; Lu et al., 1991; Markovitz et al., 1992), because deletion and site-directed mutation of the NF-AT binding sites at nucleotides −255 to −217 in the HIV-1 LTR had no measurable influence on T cell activation–dependent transcriptional activity (Lu et al., 1990; Markovitz et al., 1992). Despite this negative functional data, the hypothesis that NF-AT plays a role in HIV-1 gene regulation in activated T cells remains attractive, given the role of NF-AT in T cell activation and the tight correlation of HIV-1 transcriptional induction to T cell activation.

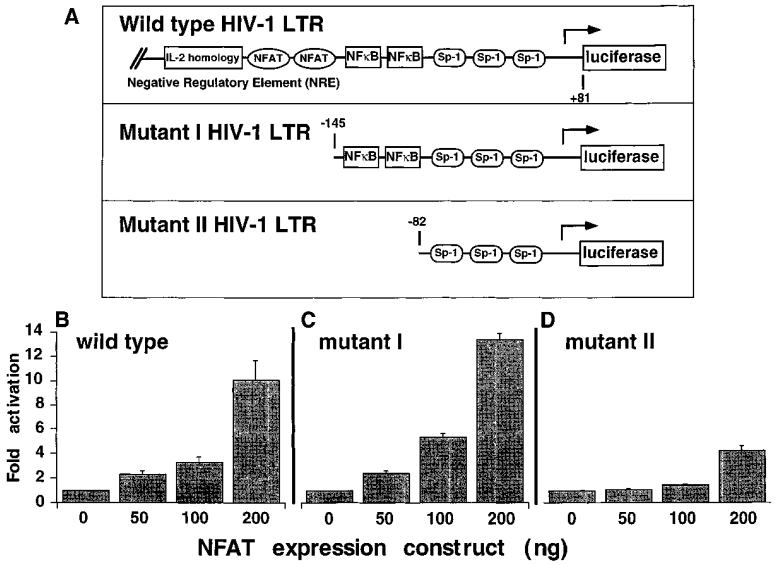

Figure 1. NF-AT Enhances Promoter Activity of HIV-1 LTR through the κB Regulatory Element

(A) Construction of HIV-1 LTR reporter plasmids. The wild- type and 5′ deletion mutants of HIV-1 LTR were linked to the structural sequence of the firefly luciferase gene. The NF-ATc cDNA coding region was cloned into the retroviral expression plasmid pBabeS. The indicated amounts of pBabeS NF-ATc and pBabeS β-galactosidase as the internal control plasmid were transfected into 293 cells with (B) wild-type, (C) mutant I, and (D) mutant II HIV-1 LTR reporter plasmids, respectively. The experiments were repeated three times and the results plotted with error bars; values shown are the average ± SD. Reporter plasmid transfected alone is shown in each graph, is assigned a value of 1, and is then used to calculate the fold activation. Transfection efficiencies were normalized to cotransfected *lacZ* plasmid driven by the Moloney LTR, known to contain no κB regulatory elements.

T cell activation is critical for the human immune system to mount an effective response to pathogenic invasion. Paradoxically, it is T cell activation itself that contributes to virus replication and the progressive immune dysfunction associated with HIV-1 infection, inducing events within cells that enhance HIV-1 transcription (Greene et al., 1986; Zagury et al., 1986). To understand more fully the T cell aspects of AIDS pathology, we reexamined the role(s) of NF-AT in the connections between events that link T cell commitment processes, intracellular signaling, and aspects of the HIV-1 life cycle. We demonstrate evidence for a fundamental role for NF-AT in HIV-1 replication processes in T cells.

Results

Induction of Transcriptional Activity by NF-ATc Maps to the HIV-1 Enhancer

To examine whether NF-AT can enhance the promoter activity of the HIV-1 LTR, we performed cotransfections using cloned NF-ATc (Northrop et al., 1994) and HIV-1 LTR reporter plasmids (Figure 1). NF-ATc enhanced the activity of the HIV-1 LTR more than 10-fold over that of cells transfected with reporter plasmid alone (basal) in a dose-dependent manner (Figure 1B). However, when background luminescence measured from nontransfected cells was subtracted from the basal signal, stimulation of transcription was greater than 45-fold and, in addition, the dose dependency of the NF-κB p65 subunit was the same as that for NF-ATc (data not shown). Thus, under these standard conditions, NF-ATc and p65 transactivate to equivalent levels.

Using truncated HIV-1 LTRs, we began mapping the region targeted for transcriptional activation by NF-ATc (Figure 1A). Previous footprinting experiments have suggested that NF-AT holoprotein can bind sites from −255 to −217 in the NRE of the HIV-1 LTR (Shaw et al., 1988), but it has been demonstrated by several groups that these sites do not physiologically influence HIV-1 gene activation (Lu et al., 1990; Markovitz et al., 1992). Figure 1C shows that deletion of the NRE did not decrease NF-AT activation of the HIV-1 LTR, suggesting that the activation we observed after NF-ATc transfections occurs through other sites. When the region between nucleotides −145 and −82 in HIV-1 LTR is deleted, the transcriptional activity of NF-ATc is markedly reduced (Figure 1D). It is noteworthy that the κB regulatory elements are located here (Nabel and Baltimore, 1987; Siekevitz et al., 1987; Tong et al., 1987; Markovitz et al., 1990).

To rule out indirect mechanisms by which NF-AT activates NF-κB to activate the HIV-1 enhancer, we tested a reporter construct with three tandem repeats of the κB element of the mouse immunoglobulin κ chain. (Fujita et al., 1992). This construct, p55-IgκLuc, was significantly activated by NF-κB but not by NF-ATc (Table 1); thus, NF-ATc does not indirectly act on κB elements through NF-κB activation. This experiment also demonstrates that NF-ATc does not have activity through all types of NF-κB motifs. Although NF-ATc contains a Rel domain, as determined both by evolutionary comparisons (Nolan, 1994) and by crystallography (Wolfe et al., 1997), it is incapable as a monomer of activating through a trimeric Ig-κB site driving a minimal promoter. Thus, NF-AT(Rel) binding likely follows a more complex set of

Table 1. NF-AT Does Not Directly or Indirectly Activate Transcription through Classic Igκ NF-κB-Binding Sites

	Fold Activation in Luciferase Activity	
	NF-κB	NF-AT
0 ng	1.0 ± 0	1.0 ± 0
50 ng	7.1 ± 2.0	1.0 ± 0.1
100 ng	48.6 ± 1.1	1.3 ± 0.1
200 ng	398.5 ± 36.0	2.1 ± 0.1

The indicated amounts of NF-κB p50/p65 and NF-AT were transfected into 293 cells with p55-IgκLuc reporter plasmid. Other conditions are the same as those described in the legend to Figure 1.

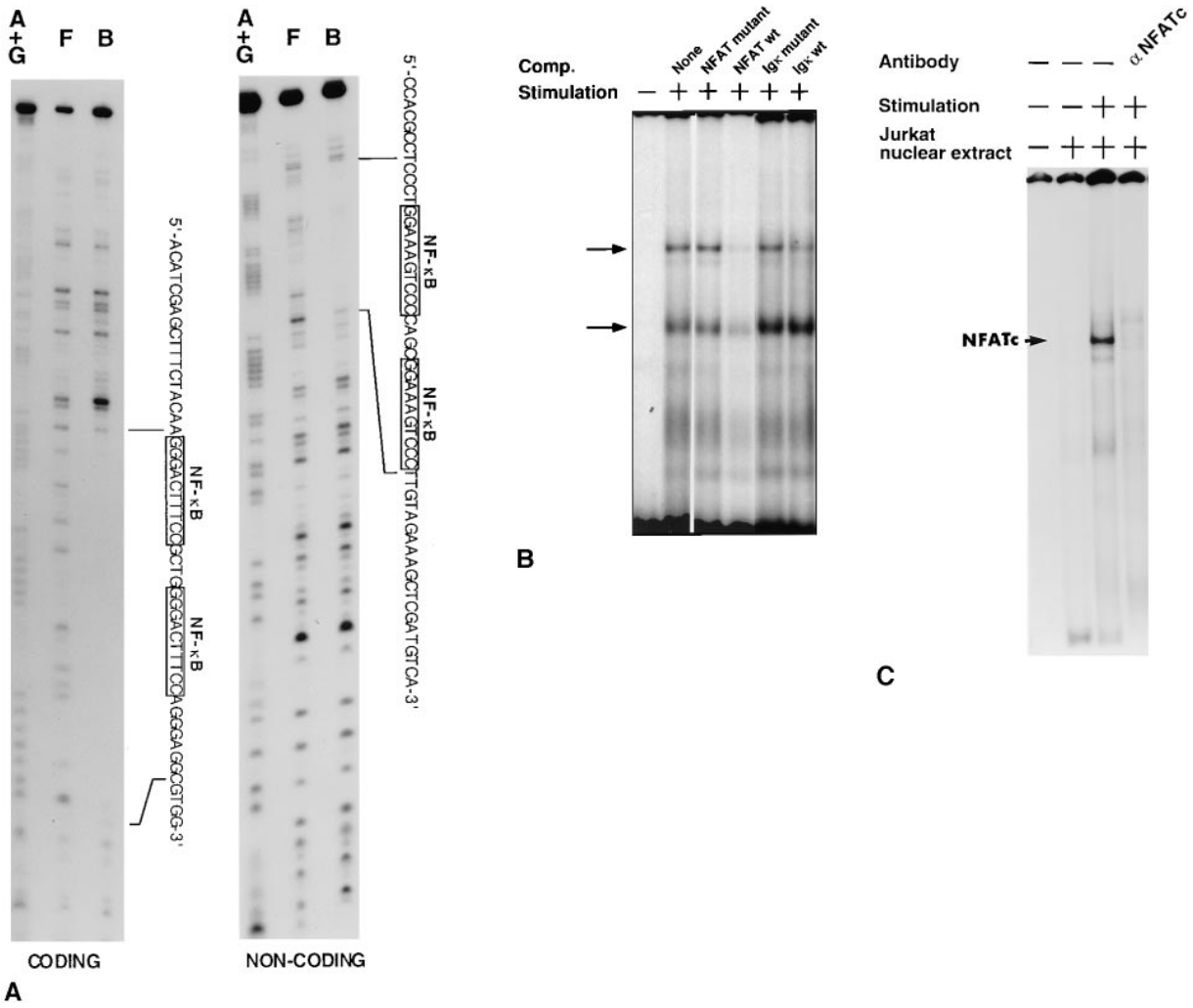

Figure 2. NF-ATc Binds the κB Regulatory Element in the HIV-1 LTR

(A) DNase I footprinting of NF-ATc. The probe containing the nucleotides −159 to −69 of the HIV-1 LTR was end-labeled on either the coding or the noncoding strand and partially digested with DNase I in the absence of (lanes F) or in the presence of (lanes B) purified NF-ATc (60 ng). Nucleotide sequence markers prepared from the same fragment using the A+G reaction of Maxam-Gilbert were also electrophoresed (lanes A+G).

(B) The inducible complexes are eliminated by NF-AT– specific competitors (Comp.). EMSA was performed using the κB regulatory element (nucleotides −108 to −78 in HIV-1 LTR) with crude nuclear extracts from Jurkat cells with or without stimulation with 20 ng/ml PMA and 2 μg/ml PHA for 3 hr. Nuclear extracts were preincubated with the indicated oligonucleotide competitors before the addition of radiolabeled probe. NF-AT WT (mouse IL-2), TCGAGCCCAAAGAGGAAAATTTGTTTCATG; NF-AT mutant, TCGAGCCCAAAGACCTTAATTTGTTTCATACAG; Igκ WT (mouse immunoglobulin κ chain), GTCTCGGGACTTTCCATCTC; Igκ κB mutant, GTCTCCCACTTTAAGAGAG.

(C) The inducible complexes are recognized by NF-ATc– specific antibody. Nuclear extracts were preincubated with 7A6 NF-ATc–specific monoclonal antibody before the addition of radiolabeled probe. Other conditions are the same as those described in the legend to Figure 2B.

DNA recognition rules, possibly to accommodate corecognition of the enhancer region by both NF-ATc and NF- κB (see below).

NF-ATc Binds to the κB Enhancer and Flanking DNA Regions

To confirm that NF-ATc binds to the HIV-1 enhancer region between nucleotides −145 and −82 in the LTR, deoxyribonuclease I (DNase I) footprinting was performed using purified NF-ATc. Clear DNase I protection by purified NF-ATc was observed to encompass completely the DNA region from nucleotides −104 to −74 on the coding strand and from nucleotides −104 to −77 on the noncoding strand (Figure 2A). A similar DNase I footprint was obtained using NF-ATp (data not shown). Some protection of flanking region DNA outside the κB elements was observed, further suggesting that additional sequence elements might be required for binding of NF-ATp/c and activation of the HIV-1 promoter.

We next examined whether nuclear extracts prepared from Jurkat T cells contained an NF-AT–specific activity that bound to the κB enhancer of HIV-1 by preparing nuclear extracts using a biochemical protocol that extracts nuclear-localized NF-AT complexes (Flanagan et al., 1991). We observed two induced complexes in phorbol myristate acetate (PMA)– and phytohemagglutinin

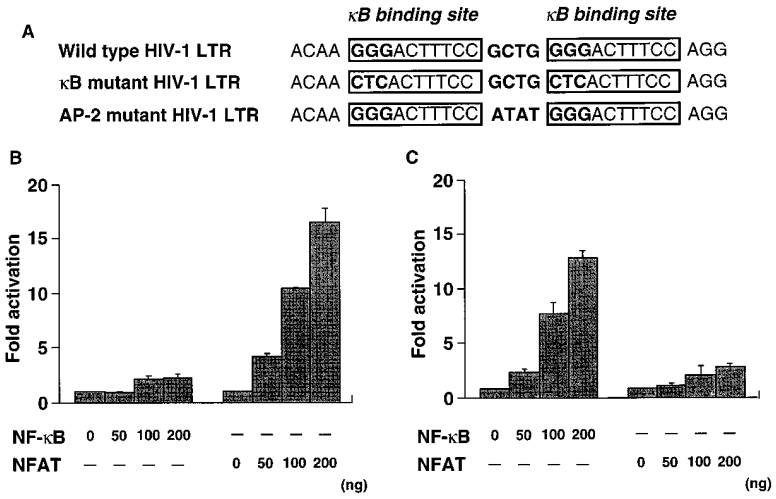

Figure 3. NF-AT and NF-κB Activation of HIV-1 Have Distinct but Overlapping DNA Motif Requirements

(A) Nucleotide sequence of the κB regulatory elements in HIV-1 LTR. Shown are wild-type sequences of the κB regulatory motif, the enhancer region mutant at residues critical to NF-κB, and the region mutant in the spacer region, shown previously (Perkins et al., 1994) to prevent binding of AP-2 but to have no effect on NF-κB binding. The indicated amounts of NF-κB, p50/p65, and NF-AT were transfected into 293 cells with κB mutant (B) and AP-2 mutant (C) HIV-1 LTR reporter plasmids, respectively. Other conditions are the same as those described in the legend to Figure 1.

(PHA)–stimulated Jurkat nuclear extracts. Electromobility shift assays (EMSA) were performed using cold oligonucleotides to NF-AT– or NF-κB–binding sites as specific competitors. Wild-type NF-AT oligonucleotide competes for the upper and lower complexes, whereas wild-type Igκ oligonucleotide competes for the upper complex alone. NF-AT and NF-κB site oligonucleotides mutant at residues known to prevent binding of their respective proteins did not inhibit the binding of any complexes in these gel shifts (Figure 2B). To confirm that these complexes induced by PMA and PHA include NF-AT, supershift gel shift assays were performed using NF-ATc specific antibody (7A6). The upper and lower complexes were completely supershifted by this antibody (Figure 2C). This NF-ATc antibody is mouse monoclonal antibody prepared against a non-Rel region of NF-ATc. This antibody does not cross-react with NF-κB and does not lead to shifting of naked radiolabeled probe DNA (data not shown). Thus, the data support the hypothesis that nuclear extracts prepared from activated T cells contain complexes that are composed at least in part by NF-ATc.

NF-ATc and NF-κB Recognize Distinct DNA Sequences within and Proximal to the κB Regulatory Elements

NF-ATc enhances transcriptional activity through regions called the κB regulatory elements in the HIV-1 LTR, but it had no effect on classic NF-κB–binding sites driving a minimal promoter (Figure 1 and Table 1). Moreover, the protected region of NF-ATc DNase I footprinting extends outside the NF-κB–binding sites in the HIV-1 LTR (Figure 2A). This suggests there are sequence requirements for NF-AT binding that are independent of residues comprising the canonical NF-κB sites. To examine this possibility, we performed cotransfections using HIV-1 LTRs mutant at the κB motifs (Nabel and Baltimore, 1987) or the 4 bp spacer region between the duplicated κB motifs that have been suggested to bind AP-2 (Perkins et al., 1994) (Figure 3A). When the κB sites were mutated, transfected NF-κB was not transcriptionally active on the mutated HIV-1 LTR, as expected. On the other hand, NF-ATc had significant transcriptional activity that increased in a dose-dependent manner (Figure 3B). In contrast, when the spacer region between the κB elements was mutated, the opposite effect occurred: NF-κB showed dose-dependent transcriptional activity, whereas the NF-ATc transcriptional activity was abolished (Figure 3C). These data show that although NF-ATc can bind to elements containing certain κB-like motifs in their core sequences (McCaffrey et al., 1992; Nolan, 1994), NF-ATc binding to the κB regulatory elements of HIV-1 has subtle requirements that distinguish its binding from those of the NF-κB class of Rel factors. This conclusion is supported by recent crystallographic data of the NF-ATc DNA-binding domain (Wolfe et al., 1997).

Synergistic Transcriptional Activation of NF-ATc and NF-κB through the κB Regulatory Element

NF-ATc and NF-κB recognize overlapping but distinct DNA sequences in the κB enhancer elements of the HIV-1 LTR. To examine further whether NF-ATc and NF-κB can interact transcriptionally at the HIV-1 enhancer, we analyzed HIV-1 transcriptional activity with cotransfected combinations of expression vectors for NF-κB subunits p50, p65, and NF-ATc. A strong synergistic effect was observed when NF-ATc was cotransfected with NF-κB heterodimer (p50 plus p65) or the NF-κB (p65) subunit on reporter plasmids driven by either the wild-type or mutant I HIV-1 LTR. No synergistic effect was observed between p50 and NF-ATc (Figures 4A and 4B). However, when the κB enhancer was deleted, no synergistic effect was observed with any of the combinations of transfected proteins (Figure 4C). NF-κB has been shown to synergize with other proteins at the HIV-1 promoter, such as AP-1 components (Stein et al., 1993), but the synergy of NF-ATc with NF-κB is greater than that of AP-1 components with either NF-ATc or NF-κB, and AP-1 also did not influence the synergistic interaction of NF-ATc and NF-κB (data not shown).

T Cell Signals That Activate NF-ATc Specifically Induce HIV-1 Gene Activity in a Tat-Stimulated Manner

The HIV-1 regulatory protein Tat is a potent transactivator of HIV-1 gene expression (Arya et al., 1985), there

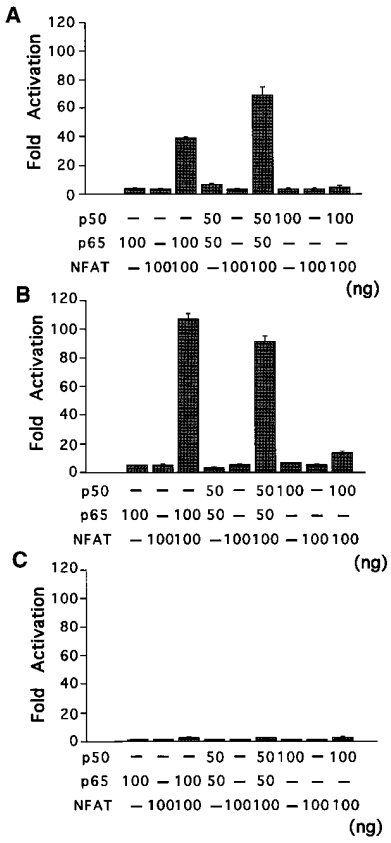

Figure 4. Synergistic Transcriptional Activation of NF-ATc and NF-κB

The HIV-1 LTR reporter plasmid, wild- type (A), mutant I (B), and mutant II (C) were transfected into 293 cells with the indicated amounts of p50, p65, and NF-ATc expression vectors. Other conditions are the same as those described in the legend to Figure 1.

Table 2. NF-AT Participates in Tat-Induced Activation of HIV-1 Promoter Activity

A		Fold Activation in Luciferase Activity	
		− Tat	+ Tat (25 ng)
None		1.0 ± 0	34.4 ± 1.9
NF-AT	50 ng	2.3 ± 0.1	195.7 + 3.2
	75 ng	7.2 ± 0.1	466.2 ± 41.8
	100 ng	12.7 ± 0.6	623.4 ± 19.4
NF-κB	50 ng	3.3 ± 0.3	167.0 ± 6.5
	75 ng	8.2 ± 0.4	237.5 ± 7.3
	100 ng	14.8 ± 1.1	448.8 ± 2.3
NF-AT + NF-κB	50 ng	12.0 ± 0.6	341.6 ± 3.8
	75 ng	45.8 ± 2.4	1098.9 ± 88.2
	100 ng	99.1 ± 5.0	1659.3 ± 23.7

B	Fold Activation in Luciferase Activity	
Stimulation	Jurkat	Jurkat + Tat
None	1.0 ± 0	25.5 ± 2.6
CsA	1.0 ± 0.2	19.2 ± 3.6
PHA	2.0 ± 0.1	80.8 ± 1.1
PHA + CsA	1.2 ± 0.1	36.2 ± 2.5
TNFα	3.5 ± 0.6	81.5 ± 0.8
TNFα + CsA	3.3 ± 0.2	77.3 ± 3.7
PHA + TNFα	6.8 ± 0.8	298.7 ± 13.7
PHA + TNFα + CsA	5.6 ± 0.2	180.8 ± 12.7

(A) Wild-type HIV-1 LTR luciferase reporter plasmid was transfected into 293 cells with the indicated amounts of *tat*, NF-κB (p50/p65), and NF-AT expression vectors. Other conditions are the same as those described in the legend to Figure 1.
(B) Wild-type HIV-1 LTR luciferase reporter plasmid was transfected with or without *tat* expression vector into Jurkat cells. Cells were treated for 3 hr with various agents (PHA 2 μg/ml, TNFα 10 ng/ml, and CsA 500 ng/ml) prior to measurement of luciferase activity.

being general agreement that Tat is physiologically critical for efficient HIV-1 replication (Fisher et al., 1986). To examine whether NF-AT can influence Tat activity in vivo, we cotransfected combinations of Tat, NF-κB, and NF-ATc expression vectors with wild-type HIV-1 LTR reporter plasmid. A strong synergistic transcriptional effect on the HIV-1 promoter was observed when Tat and NF-ATc were cotransfected. Furthermore, the synergistic effect of Tat, NF-κB, and NF-ATc in combination is much stronger than the synergistic effects of either Tat plus NF-κB or the effects of Tat plus NF-ATc (Table 2A). To examine whether cellular NF-AT potentiates gene expression induced by Tat in T cells, we cotransfected a Tat expression vector with wild-type HIV-1 LTR reporter plasmid into Jurkat cells. These transfected cells were stimulated by PHA or tumor necrosis factor α (TNFα); either condition alone, or the conditions in combination, are known to increase HIV-1 replication (Zagury et al., 1986; Poli et al., 1990). PHA induction mimics certain activation events by the T cell receptor, stimulating T cell activation through Ca^{2+}-dependent signaling pathways, leading to NF-AT activation (Crabtree, 1989). In contrast, TNFα is an important activator of NF-κB (Schutze et al., 1992) and is not known to activate NF-AT or Ca^{2+}-dependent signaling in T cells. To examine the influence of NF-AT in Tat-induced HIV-1 gene activation, cyclosporin A (CsA), an inhibitor of Ca^{2+}-dependent signaling in T cells, was used as a specific pharmacologic inhibitor of NF-AT (Flanagan et al., 1991). Cotransfection of wild-type HIV-1 LTR reporter plasmid with Tat expression vector enhanced luciferase activity about 25-fold. When Jurkat cells expressing Tat were stimulated with either PHA or TNFα, luciferase activities increased about 80-fold in each case. Although CsA inhibited Tat-expressing, PHA-induced HIV-1 LTR transcriptional activity, this agent had almost no influence on TNFα-induced Tat-dependent activation. Furthermore, costimulation with PHA and TNFα resulted in synergistic effects on transcriptional activation of HIV-1, and this activity was also inhibited by CsA (Table 2B).

We verified the activation status of NF-ATc and NF-κB under our stimulatory conditions by Western blot analysis. NF-ATc was induced by PHA but not by TNFα. In contrast, NF-κB was induced by TNFα but not by PHA; CsA affected NF-ATc phosphorylation status but did not influence NF-κB activation (Figure 5), correlating with the data in Table 2B.

NF-ATc Enhances HIV-1 Replication in T Cells

Finally, to examine whether NF-AT influences HIV-1 replication, experiments were performed using a replication-competent HIV-1 isolate. First, using recombinant retroviral vectors, we prepared a cell line that stably overexpressed NF-ATc (SupT1:NF-ATc-Lyt2) and a control cell line (SupT1:control-Lyt2) to isolate the effects

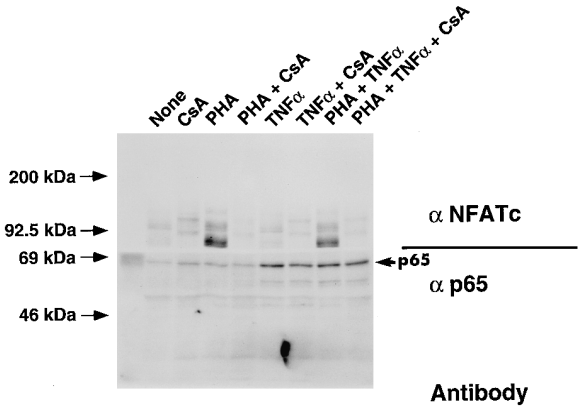

Figure 5. NF-ATc and NF-κB p65 Are Separately Induced by PHA and TNFα, Respectively, in Jurkat T Cell Lines

Jurkat cells were treated for 3 hr with agents (PHA 2 μg/ml, TNFα 10 ng/ml, and CsA 500 ng/ml). Nuclear extracts were boiled with SDS-PAGE sample buffer and analyzed by Western blot using an anti-NF-ATc monoclonal antibody, 7A6 (top), or an anti p65 polyclonal antibody (bottom).

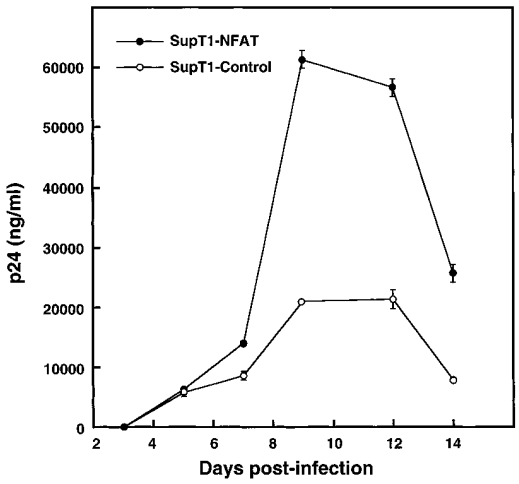

Figure 6. NF-ATc Is a Positive Regulatory Factor for HIV-1 Replication

SupT1:NF-ATc-Lyt2 and SupT1:control-Lyt2 cells were challenged with HIV-1 (NL4–3) at a dose of 400 $TCID_{50}$ per 5×10^4 cells. $p24^{gag}$ levels in culture supernatants were assayed from triple wells on the indicated days after infection. p24 levels were normalized for cell number using an XTT assay. Data are presented as the average ± SD per 10^6 cells. Similar results were observed in four independent experiments.

of NF-ATc from contributions by other cellular factors. Both cell lines grew at equal rates over several weeks, suggesting that there is no growth advantage or disadvantage when NF-ATc is overexpressed. Western blot analysis showed that NF-ATc overexpression leads to some NF-ATc nuclear translocation in unstimulated cells (data not shown). HIV-1, strain NL4–3 (Adachi et al., 1986), was infected into the modified SupT1 cells at a viral titer of 400 $TCID_{50}$ (50% tissue culture infective dose) per 5×10^4 target cells. Each time point was taken in triplicate, and the results were normalized for cell number. Over a time course of 14 days we measured HIV-1 replication activity using a p24 enzyme-linked immunosorbent assay (ELISA). Five days after infection, virus replication was detected at similar levels for SupT1:NF-ATc-Lyt2 and SupT1:control-Lyt2 cells. Syncytia formation was observed beginning at this time point. Seven days after infection there was a significant increase in viral replication in the NF-ATc overexpressing cells over that observed in the control cells (Figure 6). By day 9, a high percentage of cells were observed as syncytia in both the NF-ATc– overexpressing cells and the NF-ATc control cells. At this time point and at days 12 and 14 there was a consistently higher level of virus production as read by p24 levels in the NF-ATc–overexpressing cells as compared to the control vector cells (Figure 6). The results were similar in four independent experiments. After 14 days, most cells were dead and the experiment was terminated. To rule out the possibility that NF-ATc activated NF-κB to induce HIV-1 replication, transfection of p55-IgκLuc reporter plasmid into SupT1:control-Lyt2 and SupT1:NF-ATc-Lyt2 cells confirmed that NF-ATc overexpression does not directly or indirectly activate NF-κB (data not shown).

Discussion

In this study we have demonstrated that NF-ATc can serve as a positive activator of HIV-1 transcription and replication of wild-type HIV-1. NF-ATp/c bound to the HIV-1 κB enhancer motifs (nucleotides -104 to −81), a region formerly thought to bind only NF-κB, and its binding correlated to positive transcriptional activation of the promoter. Although investigators have indicated that NF-AT binds to sites upstream from the κB motifs (Shaw et al., 1988), others have noted that these NF-AT sites have no biological function (Lu et al., 1990; Tong et al., 1990; Lu et al., 1991; Markovitz et al., 1992) in T cells in the context of the HIV-1 enhancer/promoter. The data presented in this report suggest that previous work focusing on these upstream sites was insufficient to conclude a lack of NF-AT involvement in HIV-1 regulation.

Consistent with our finding that NF-ATc activates the HIV-1 LTR, other investigations have shown clear indications of calcium-dependent signaling that activate through the HIV-1 κB enhancer regions. Many early studies of HIV-1 gene activation used PHA as the activator of Ca^{2+} signaling pathways. Although activation of HIV-1 through PHA/Ca^{2+} pathways has previously been suggested to be dependent entirely on NF-κB protein activity, Ca^{2+} signaling is a hallmark of NF-AT activation in T cells (Crabtree, 1989; Hodge et al., 1995; Lee et al., 1995; Masuda et al., 1995; Rooney et al., 1995; Hodge et al., 1996; Luo et al., 1996). It is noteworthy, and it supports the conclusions of this report, that CsA completely abolished a PHA-induced binding activity to the κB regulatory elements and transcriptional induction of the HIV-1 LTR when cells were induced with PHA; CsA had almost no influence on PMA-induced complexes or LTR activation (Schmidt et al., 1990). This is consistent with the fact that CsA can inhibit PHA-mediated NF-AT nuclear translocation and transcriptional activity but not PMA-mediated NF-κB induction. These data are explained by postulating roles for either NF-AT and NF-κB in HIV-1 gene regulation, depending on the inducing

signal received by the T cell. Notably, Franza and colleagues (1987) reported that a 110 kDa protein could be recognized binding to the tandem κB enhancer motifs from the whole-cell extracts of H9, $CD4^+$ human T lymphoblast line. Although other proteins in that study, HIVEN86 and a series of 50 kDa proteins, were later identified as human c-Rel (Ballard et al., 1990) and the NF-κB subunit p50 (Kawakami et al., 1988), the 110 kDa protein has not been identified so far. Since, by SDS–polyacrylamide gel electrophoresis (SDS-PAGE), NF-AT homologs (c and p) both have molecular masses of approximately 120 kDa (McCaffrey et al., 1993; Northrop et al., 1994), it is possible that the 110 kDa protein is NF-AT.

Previous studies have concluded that because HIV-CAT (chloramphenicol acetyl transferase) activity was dramatically enhanced by Tat in PHA plus PMA–stimulated Jurkat T cells, this activation is due to contributions by interaction of NF-κB and Tat (Nabel and Baltimore, 1987). Since activation of T cells with PHA and PMA is a classic method by which NF-AT is activated, and since NF-AT proteins bind at the tandem κB regulatory elements as evidenced from the nuclear extract of PHA plus PMA–stimulated Jurkat cells (Figures 2B and 2C), NF-AT is as equally positioned and as appropriately regulated as NF-κB to contribute to Tat-induced HIV-1 gene activation in T cells. Indeed, the coordinate binding of NF-AT and p65-containing NF-κB complexes to one or the other of the duplicated κB motifs in the HIV-1 enhancer region might allow synergistic interactions of these proteins with Tat bound to TAR, greatly enhancing subsequent transcriptional activation of HIV-1 gene.

Although there have been indications that NF-AT might bind certain NF-κB–like motifs, there have been no reports of NF-AT transcriptional activity through such elements (McCaffrey et al., 1992; Jain et al., 1995). NF-ATc had no transcriptional activity, directly or indirectly, through triplicate consensus κB elements driving a minimal promoter (Table 1). The same monomeric κB present as tandem repeats in the HIV-1 enhancer were potently responsive to NF-ATc. This suggests that the κB regulatory elements in the context of HIV-1 LTR sequences likely represent a special class of κB motifs capable of being bound by either NF-κB or NF-AT classes of Rel proteins. Thus, duplicated NF-κB binding sites in the HIV-1 LTR might confer binding of multiple, discrete, proteins from the NF-κB and NF-AT Rel subfamilies of regulators, giving rise to the potential for synergistic transactivation. Supporting this possibility are data showing that mutation of either one of the two κB regulatory elements (Böhnlein et al., 1988), or of different sequences inserted between the duplicated κB binding sites (Perkins et al., 1994), leads to a dramatic reduction in the transactivation of the HIV-1 LTR. The differential mode of DNA recognition by NF-ATc suggested by crystallographic studies supports our findings that NF-ATc recognizes distinct DNA elements from NF- κB in the κB enhancer elements.

Mutational analysis of the regions within and surrounding the HIV-1 κB elements support the contention that NF-ATc and NF-κB binding and activation requirements overlap but remain distinguishable. Mutation of the spacer region abolished NF-ATc activation, suggesting that NF-ATc requires nucleotides within this region for activation of the HIV-1 enhancer. Mutation of the κB elements at the GGG residues at the 5′ end of the motif had no effect on HIV-1 activation after cotransfection of reporter genes and NF-ATc into 293 cells. However, full physiological activation of HIV-1 in T cells might require the presence of both NF-κB and NF-AT at the HIV-1 LTR. It is noteworthy that infection with HIV-2 (which has only a single κB element in its enhancer) results in a longer asymptomatic phase in infected individuals (Markovitz et al., 1990). It will be interesting to correlate the presence of NF-κB and NF-AT binding determinants to LTR promoter activation using HIV-2 and other HIV-1 subtypes that contain variation in the enhancer regions. In addition, this analysis might suggest that variations in promoter activity among different strains of HIV-1 might correlate to different pathologic outcomes or adaptation to the use of different transcription factors present in target cells that HIV-1 strains infect.

Finally, it has previously been reported that CsA inhibits HIV-1 replication (Karpas et al., 1992; Thali et al., 1994). Studies by Franke and colleagues (1994) and Thali and colleagues (1994) concluded that CsA inhibits HIV-1 by blocking the interactions between HIV-1 Gag polyprotein and cyclophilin A as HIV-1 virions are being assembled to leave the cell, resulting in immature virion production. Questions, however, remain as to whether this is the only mechanism by which CsA effects the HIV-1 life cycle. Since HIV-1 transcription and virion production are inhibited by CsA treatment (Schmidt et al., 1990; Karpas et al., 1992), at least one inhibitory effect of CsA acts prior to any potential effect CsA can have on Gag interaction with cyclophilin A. It is reasonable to conclude that this transcriptional inhibition can be brought about by the action of CsA on NF-AT activation in T cells, given the results in our current report.

We have demonstrated that immediate-early T cell activation proteins, such as NF-ATc and NF-κB, can be exploited by HIV-1 to enhance its replication. Since NF-AT and NF-κB proteins are distantly related in their Rel DNA-binding region (Nolan, 1994; Northrop et al., 1994; Luo et al., 1996), and since each can bind to the HIV-1 κB enhancer region after induction by alternative signaling pathway in lymphocytes, these transcription factors would be advantageous for HIV-1 utilization during T cell signaling-dependent activation. Therefore, the findings here suggest that closer examination of immediate-early T cell events, such as NF-AT activation (Hodge et al., 1995; Lee et al., 1995; Masuda et al., 1995; Rooney et al., 1995; Hodge et al., 1996; Luo et al., 1996), that lead to induction of HIV-1 gene activation might promote the development of T cell–specific therapies that limit the pathogenic progression of AIDS.

Experimental Procedures

Plasmid Construction

NF-ATc, p50, p65 β-galactosidase, and Tat expression vectors (pBabeS NF-ATc, pBabeS p50, pBabeS p65, pBabeS β-galactosidase, and pBabeMN Tat) were constructed by inserting the coding sequences of the respective cDNAs into retrovirus expression vectors, pBabeS or pBabeMN. pBabeS is derived from pBabePuro,

contains changes in the multicloning site, and has been deleted for the SV40-*neo* region of pBabePuro (Morgenstern and Land, 1990). The 5′ LTR and ψ-packaging signals of pBabe MN were driving from the MFG vector (Dranoff et al., 1993). pBabeMN IRES-Lyt2α′ was derived by ligating IRES-Lyt2α′ (a kind gift from P. Achacoso, Stanford University) downstream of the multicloning site of pBabeMN. Lyt2α′ is the splice variant of murine CD8 that lacks a cytoplasmic tail. The IRES (internal ribosome entry site) functionality allows multiple proteins to be driven off of a single promoter. In this manner, NF-ATc expression from the retroviral promoter can be directly correlated to Lyt2α′ expression.

Luciferase Assays

293 cells were cultured in DMEM containing 10% fetal calf serum (FCS). 293 cells were seeded at 5×10^4 cells per well in 24-well plate. Twenty-four hours later, cells were transfected with 10 ng of the reporter plasmid, the indicated amounts of expression vectors, and 50 ng of pBabeS β-galactosidase by the calcium phosphate coprecipitation technique. The total amount of DNA was held constant by using pUC 18 carrier DNA. Cells were harvested 48 hr after transfection and measured for luciferase activity as described (de Wet et al., 1987). β-Galactosidase activity was assayed by standard methods. Jurkat cells were cultured in RPMI 1640 containing 10% FCS. Cells (5×10^6) were transfected with 5 μg of reporter plasmid and 2 μg of *tat* expression vector or pUC 18 carrier DNA by the diethylaminoethyl-dextran method. Thirty-six hours after transfection, cells were treated as indicated and cell extracts were measured for luciferase activity.

DNase I Footprinting

The procedure for DNase I footprinting has been described (Glass et al., 1988). The NF-ATc and NF-ATp proteins (a kind gift from T. Hoey, Tularik, Inc.) (Hoey et al., 1995) were purified from an bacterial source as overexpressed proteins. NF-ATc consisted of a 309–amino acid domain (amino acids 408–716 according to the numbering scheme of Northrop et al. [1994]).

EMSA

Nuclear extract was prepared and EMSA was performed as described previously (Flanagan et al., 1991). Oligonucleotide competitors were preincubated for 15 min with extracts at room temperature before the addition of radiolabeled probe. When supershift analysis was performed, nuclear extract was preincubated with the antibody for 1 hr on ice.

Western Blot Analysis

Nuclear extracts were separated by 7.5% SDS-PAGE, transferred onto membrane filter, analyzed by Western blot using an anti-NF-ATc monoclonal antibody (7A6) (Northrop et al., 1994) followed by horseradish peroxidase–conjugated rabbit anti-mouse IgG1 or an anti-p65 polyclonal antibody (Santa Cruz sc-372) followed by horseradish peroxidase–conjugated goat anti-rabbit IgG and detected by an enhanced chemiluminescence system (Amersham).

Preparation of T Cells Stably Expressing NF-ATc

Stable cell lines expressing NF-ATc and control were made by a transient retrovirus production method (Pear et al., 1993). Producer cells were transfected with 3 μg of pBabeMN NF-ATc-IRES Lyt2α′ or pBabeMN IRES-Lyt2 by the calcium phosphate coprecipitation technique. Forty-eight hours after transfection, recombinant retroviral supernatents were collected from the producer cells and centrifuged at 500 × g for 5 min to remove cells and cell debris. 1×10^6 SupT1 target cells were suspended in 1 ml of retroviral supernatent containing 5 μg/ml polybrene and were seeded in 24-well plates. The plate was centrifuged at 2500 rpm for 90 min at 32°C. After 24 hr, viral supernatent was removed and cells resuspended in fresh medium. Infected cells were stained by anti-mouse-Lyt2 (murine CD8) antibody 53–6.7 conjugated to fluorescein (a kind gift from L. A. Herzenberg, Stanford University) and sorted with a fluorescence-activated cell sorter by standard gating of dead cells with propidium iodide and forward scatter gates. At the first analysis, approximately 30% of SupT1 cells were stably expressing the Lyt2α′ marker. After the second round of sorting the cell population was 95% positive for Lyt2 expression. Subsequent analysis showed that cells carrying the NF-ATc retroviral vector were strongly positive for expression of NF-ATc.

HIV Infection and p24 Assay

SupT1:control-Lyt2 or SupT1:NF-ATc-Lyt2 cells were plated in triplicates in 48-well plates (5×10^4/well) in RPMI 1640 medium containing 10% FCS. HIV-1 infection was performed by incubating cells with NL4–3 (400 $TCID_{50}$/well in 0.5 ml of culture medium) at 37°C for 4 hr. Medium was replaced with 1 ml of fresh medium. Supernatant was collected at the indicated days after infection and replaced with fresh medium. An ELISA assay kit (Abbott Laboratories) was used to determine the $p24^{gag}$ levels as a measure of HIV-1 replication.

XTT Assay

XTT assay was performed as described previously (Jellinger et al., 1997). Fifty microliters of a solution containing 1.5 μM 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) (Polysciences, Warrington, Pennsylvania) and 125 μM phenazine methosulfate (Sigma, St. Louis, Missouri) was added to 150 μl of cell samples in flat-bottomed 96-well plates. The plate was sealed with adhesive plate sealer and the contents of the plate were mixed. After incubation for 4 hr at 37°C, the plate was placed in a V-Max spectrophotomer (Molecular Devices, Menlo Park, California). The optical densities of each well were read at a test wavelength of 450 nm and reference wavelength of 650 nm. The standard linearity of the assay extended from 250 to 10,000 cells. Results from XTT assays were used to normalize p24 assays at different time points of the assay.

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