Identification of Three NFAT Binding Motifs in the 5’-Upstream Region of the Human CD3γ Gene That Differentially Bind NFATc1, NFATc2, and NF-κB p50

Received for publication, June 25, 2002, and in revised form, September 24, 2002 Published, JBC Papers in Press, October 8, 2002, DOI 10.1074/jbc.M206330200

Bassam M. Badran‡, Steven M. Wolinsky§, Arsène Burny‡, and Karen E. Willard-Gallo¶¶

From the ‡Laboratory of Experimental Hematology, Faculty of Medicine, Université Libre de Bruxelles, Brussels B1000, Belgium and the ¶¶Division of Infectious Diseases, Department of Medicine, Northwestern University Medical School, Chicago, Illinois 60611

Human immunodeficiency virus, type 1 (HIV-1) infection of CD4+ T cells progressively abrogates T cell receptor (TCR)-CD3 function and surface expression by specifically interfering with CD3γ gene transcription. Our data show that the loss of CD3γ transcripts begins very early after infection and accumulates to a >90% deficiency before a significant effect on surface receptor density is apparent. Blocking TCR-CD3-directed NFAT activation with cyclosporin A provokes a partial re-expression of CD3γ gene transcripts and surface complexes in a time- and dose-dependent manner. We have identified three NFAT consensus sequences (5′-GGAAA-3′) in the 5′-upstream region of the human CD3γ gene at: −124 to −120 (NFATγ1), −384 to −380 (NFATγ2), and +450 to +454 (NFATγ3) from the first transcription initiation site. Using electrophoretic mobility shift and supershift assays, we show that NFATc2 alone binds to the NFATγ2 motif; however, complexes containing either NFATc2 or NFATc1 plus NF-κB p50 bind to the NFATγ1 and NFATγ3 sites. We further demonstrate that NFATc1 and NF-κB p50 bind in the same protein-DNA complex and that a fourth Ala added to the core sequence (5′-GGAAA-3′) in NFATγ1 and NFATγ3 is critical for their binding. Finally, we have shown that an increase in the binding of nuclear NFATc2, NFATc1, and NF-κB p50 to these three motifs is correlated with a progressive loss of CD3γ transcripts after HIV-1 infection.

T cell receptor (TCR)-CD3 cell surface density has been linked with the ability of the cell to elicit an effective signal, suggesting that T cells regulate their responsiveness to antigen-induced activation by increasing or decreasing the number of cell surface complexes (1–4). The quantity of TCR-CD3 complexes present on the surface at any given time is a result of the balance between receptor internalization, leading to intracellular degradation or recycling to the surface, coupled with the synthesis, processing, and exportation of newly formed receptors (reviewed in Ref. 5). It is currently thought that two pathways regulate antigen-induced TCR-CD3 down-regulation from the cell surface: phosphorylation of the immunoreceptor tyrosine-based activation motifs present in the cytoplasmic tails of CD3ζ, CD3γ, CD3δ, and CD3ε (6, 7) and protein kinase C (PKC)-mediated serine phosphorylation of the di-leucine endocytosis motif in CD3γ (8, 9). A recent study has shown that the di-leucine motif in CD3γ increases ligand-induced receptor internalization and degradation 3- to 10-fold, indicating that this chain plays a major role in TCR-CD3 down-modulation (10).

Defects in TCR-CD3 surface expression and function are increasingly being reported in an expanding range of clinical conditions, including both peripheral blood and tumor-infiltrating T cells in a wide variety of cancer patients (reviewed in Refs. 11 and 12) and after viral infection of CD4+ T cells (13–25). A common denominator for TCR-CD3 down-modulation by the CD4+ T cell tropic viruses that has emerged from in vitro (15, 20–22) and in vivo studies (14, 23–25) is their ability to interfere with expression of one or more of the CD3 genes. We have demonstrated that human immunodeficiency virus (HIV-1 (15, 16) and HIV-2 (20)) infection of the human IL-2-dependent CD4+ T cell line, WE17/10, progressively abrogates TCR-CD3 function and surface expression by specifically interfering with transcription of the CD3γ gene. Our data have shown that, when intracellular conditions favor expression of the viral regulatory genes tat and/or nef in the absence of rev, CD3γ mRNA and TCR-CD3 surface density are down-regulated and TCR-CD3-mediated immune activities are diminished (26). Nef is a multifaceted viral regulatory protein that is capable of a variety of different, independent functions, some of which have been linked with TCR-CD3-controlled events. It has been shown to directly associate with CD3ζ and lead to its down-modulation from the cell surface (27, 28). Nef has also been shown to play a role in the post-transcriptional down-modulation of CD4 via a di-leucine motif in this receptor’s membrane proximal cytoplasmic domain (29). This CD4 domain is strikingly similar to the di-leucine motif in CD3γ (10, 30–32) and thus conditions favoring Nef expression could potentially enhance the activity of the CD3γ di-leucine motif.

The viral transcriptional transactivator protein Tat is also thought to play an important role in the immune suppression observed after infection by activating and suppressing the expression of a variety of cellular immune response genes (33–37). The transcriptional control elements for CD3γ have remained elusive (the 5′-upstream region of this gene lacks a

† To whom correspondence should be addressed: Tel.: 32-2-541-3739; Fax: 32-2-541-3453; E-mail: kwillard@ulb.ac.be.

‡ The abbreviations used are: TCR, T cell receptor; PKC, protein kinase C; HIV-1, -2, human immunodeficiency virus, types 1 and 2; IL, interleukin; LTR, long terminal repeat; CsA, cyclosporin A; EMSA, electrophoretic mobility shift assay; BAPTA/AM, bis(o-aminophenoxy)ethane-N,N,N’N’-tetraacetic acid; PMA, phorbol 12-myristate 13-acetate; PMA+ Iono, PMA with ionomycin; RT, reverse transcriptase.

This paper is available on line at http://www.jbc.org

Vol. 277, No. 49, Issue of December 6, pp. 47136–47148, 2002
Printed in U.S.A.
typical TATA or CAAT box), despite the identification of promoter and enhancer sequences for the other TCR-CD3 genes: TCRα (38, 39), TCRβ (40, 41), TCRγ (42), TCRδ (43), CD3e (44), CD3γ (45, 46), and the highly homologous CD3δ (47–49). However, the recurring defect in CD3γ gene transcripts observed after infection with a wide variety of HIV-1 and HIV-2 isolates suggests that transcription of this cellular gene might be controlled by a mechanism similar to the virus.

The primary function of HIV-1 Tat is to promote transcription by recruiting a kinase complex known as TAK (Tat-associated kinase) to the transactivation response RNA element present at the 5'-ends of all nascent HIV-1 transcripts and subsequently act in concert with cellular transcription factors bound to the long terminal repeat (LTR) (reviewed in Refs. 50 and 51). Among the many regulatory elements in the HIV-1 LTR, there are two adjacent NF-κB and AP-1 motifs (52) that are localized to the HIV-1 LTR promoter-enhancer regions of several activation-associated kinase) to the transactivation response RNA element (53) and activate viral transcription (54). Members of the NFAT family of transcription factors (NFATc1, NFATc2, NFATc3, NFATc4, NFATc5, NFATc6, and NFATc7) are involved in the regulation of gene expression in response to a variety of cellular stimuli, including cytokines, growth factors, and stress signals. These factors are known to bind to specific DNA motifs, such as the NF-κB and AP-1 motifs, and their activity is regulated by phosphorylation and dephosphorylation events.

Several groups have investigated the potential role of NFAT in HIV-1 replication and the interaction between Tat and NFAT and concluded that the NFAT family of proteins may have distinct effects on HIV-1 replication. NFATc2 is thought to negatively regulate the LTR by competing with the NF-κB for its binding sites, whereas NFATc1 has been shown to positively regulate HIV-1 LTR through the NF-κB transcription factor complex (55). Recent studies suggest that virally induced immune suppression may be due to the interaction of Tat with several transcription factors, including Oct, Sp1, and NFAT (57, 58) as well as through indirect effects on the transcriptional activity of NF-κB and AP-1 (59).

The data presented in this paper show that very early after HIV-1 infection in an IL-2-dependent T cell line, the majority (>90%) of CD3γ gene transcripts are lost, and this occurs before significant TCR-CD3 down-modulation from the surface is apparent. Furthermore, treatment with the immunosuppressive drug, cyclosporin A (CsA), which acts by blocking translocation of NFAT proteins to the nucleus, partially reverses this CD3γ transcription defect. We located three NFAT binding motifs (5'-GGGACTTTCC-3') in the 5'-upstream region of the CD3γ gene (NFATc1, NFATc2, and NFATc3) and found that increased nuclear translocation and binding of NFAT family proteins to these three sites parallels the loss of CD3γ gene transcripts. Electrophoretic mobility shift assays (EMSA) show that the NFATc1 and NFATc3 motifs bind complexes containing either NFATc2 or NFATc1 plus NF-κB p50, whereas the NFATc2 motif binds NFATc2 containing complexes only.

EXPERIMENTAL PROCEDURES

Cell Culture Conditions and Reagents—The WE17/10 cell line is a human interleukin 2 (IL-2)-dependent CD3- T cell line (15, 60) that was isolated from a patient with immune deficiency syndrome and is maintained in RPMI 1640 containing 10% fetal bovine serum, 1.25 mM L-glutamine, 0.55 mM L-arginine, 0.24 mM L-aspartate, and 100 units of recombinant human IL-2 per ml (Cetus Corp., Emeryville, CA). WE17/10 cells infected with the HIV-1 isolate LAI (61) or the molecular clone HXB2 (62) were used in previous experiments (15, 60). The human B lymphocyte line, Raji, was obtained from the American Type Culture Collection (Rockville, MD) and was maintained in RPMI 1640 supplemented with 10% fetal bovine serum.

We17/10 cells were treated for 18 h with the calcium channel blockers ETGTA (2.5 μM) and BAPTA/AM (1–10 μM, bis-o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid), the PKC activator PM (30 μg/ml, phorbol 12-myristate 13-acetate), the calcium ionophores A23187 or ionomycin (30 ng/ml), and the protein kinase inhibitors herbizyme A (10–6 M) and staurosporine (1–50 ng/ml). Cells were also treated with the immunosuppressive agent, cyclosporin A (0.1–1.0 μg/ml, CsA) for 1–7 days or stimulated with immobilized anti-CD3 antibody (1–10 μg/ml) for 2–3 days. HIV-1-infected TCR-CD3− cells were pretreated for 1 h with CsA followed by overnight stimulation with PM plus 1% (in the continuous presence of CsA) to achieve the maximum potential induction of NFAT translocation to the nucleus in the presence of the inhibitor.

Flow Cytometry—Cells were analyzed for CD3 expression by flow cytometry as previously described (26). Briefly, cells were labeled with the murine monoclonal antibody OKT3 (directed to CD3ε) in a two-step process (using 1 μg/ml of antibody to ensure saturation binding) followed by the mouse anti-human IgG3 fluorescein-conjugated goat anti-mouse immunoglobulin (BD Biosciences, Erembodegen, Belgium). The labeled cells were fixed in 2% paraformaldehyde, and fluorescence was analyzed on a FACSCalibur (BD Biosciences).

Electrophoretic Mobility Shift Assay—Nuclear extracts were prepared from 2 × 10^9 cells according to a modified version of the method described by Osborn (63). All buffers contained a mixture of protease inhibitors (Complete, Roche Diagnostics, Brussels, Belgium) to minimize proteolysis. The cellular pellet was washed with ice-cold phosphate-buffered saline and then resuspended with 1 ml of ice-cold buffer A (10 mM HEPES buffer, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl). Cells were collected by centrifugation (800 × g for 10 min), resuspended, and incubated for 10 min with 40 μl of ice-cold lysis buffer A containing 0.2% Nonidet P-40 (this step was repeated twice). The pellet (nuclear fraction) was incubated with 30 μl of ice-cold extraction buffer B (20 mM HEPES buffer, pH 7.9, 25% glycerol, 1.5 mM MgCl₂, 420 mM NaCl, 0.2 mM EDTA) for 20 min at 4°C and then centrifuged at 200,000 × g for 10 min at 4°C (the nuclear suspension was treated with 150 μl of buffer D (20 mM HEPES buffer, pH 7.9, 20% glycerol, 50 mM KCl, 0.2 mM EDTA) and stored frozen at −80°C. Protein concentrations were determined by the Bradford method (64).

EMSA were performed as described by Van Lint et al. (65) with some modifications. Single-stranded oligonucleotides were 5′-end-labeled with [γ-32P]ATP (>5000 Ci/mmol, Amersham Biosciences) at Roosendaal, Netherlands) using T4-polynucleotide kinase, annealed, isolated on a polyacrylamide gel, and extracted from the gel using the QIAEX II kit (Westburg, AE Leusden, Netherlands) prior to their use in EMSA experiments. Nuclear extracts (10 μg of protein) were preincubated for 10 min in a reaction mixture containing 10 μg of bovine serum albumin (Sigma-Aldrich, Bornem, Belgium), 1.5 μg of the nonspecific competitor DNA poly(dI-dC) (Amersham Biosciences), 50 μM ZnCl₂, 0.25 mM dithiothreitol, 20 mM Tris-HCl, pH 7.5, 60 mM KCl, 1 mM MgCl₂, 0.1 mM EDTA, and 10% (v/v) glycerol. 15,000 cpm of the 32P-labeled probe was subsequently added, and the mixture (final volume, 20 μl) was incubated for a further 20 min at room temperature before being loaded onto a 6% non-denaturing polyacrylamide gel (1× Tris-glycine-EDTA buffer, migrated at 50 V overnight). The radiolabeled proteins were detected by autoradiography on Biomax MR film (Amersham Biosciences).
RESULTS

Measurement of the Relative Amounts of CD3γ mRNA in Uninfected TCR-CD3\(^+\) and HIV-1-infected Cells with Down-modulated TCR-CD3 Surface Complexes—Our previous work, using dot and Northern blot hybridization analyses, suggested that the specific loss of CD3γ transcripts after HIV-1 and HIV-2 infection does not parallel the down-regulation of TCR-CD3 complexes from the surface at a ratio of 1:1 (15, 20). To better define the relationship between the number of CD3γ gene transcripts and the density of TCR-CD3 complexes on the cell surface, we used quantitative competitive RT-PCR to examine transcript levels in uninfected and HIV-1-infected WE1710 cells. RNA was extracted from cells at different stages in the progression from TCR-CD3\(^+\) → TCR-CD3\(^-\) → TCR-CD3\(^0\) (previously described in Ref. 16; in this report the uninfected cells designated as 100% TCR-CD3\(^+\) are all TCR-CD3\(^+\)); whereas, the HIV-1-infected cells described as 90% TCR-CD3\(^+\) (for example) are 10% TCR-CD3\(^+\) and 90% TCR-CD3\(^0\). cDNAs, reverse-transcribed from the native RNA preparation, were co-amplified with serial dilutions of a competitor specific for the human CD3γ gene (pUC18), which had been engineered to produce a larger PCR product (Fig. 1, upper band) than the cellular CD3γ RNA (Fig. 1, lower band).

Representative results comparing the relative amounts of RT-PCR products from uninfected and HIV-1-infected cells expressing various levels of TCR-CD3 surface receptors are shown in Fig. 1A. In the uninfected 100% TCR-CD3\(^+\) cells, the competitor was initially detected when 3.3 × 10\(^5\) molecules were added to the reaction mixture, followed by a corresponding decrease in native CD3γ transcripts until they no longer detectable in the presence of >6.6 × 10\(^5\) molecules of the competitor. The competitor was detected earlier (at 6.6 × 10\(^5\) molecules) in RNA amplified from 100% TCR-CD3\(^+\) HIV-1-infected cells (the mean fluorescence revealed that these cells were actually 100% TCR-CD3\(^+\) with a receptor density equal to 85% of the uninfected control cells analyzed in parallel; data not shown) and indicated that these TCR-CD3\(^+\) cells had already lost ≥80% of their CD3γ gene transcripts. Amplification of RNA from 90% TCR-CD3\(^+\) HIV-1-infected cells initially detected the competitor at a concentration of 1 × 10\(^5\) molecules, revealing a further decline equivalent to a total loss of ≥90% of CD3γ gene transcripts. This extensive loss of transcripts prior to significant TCR-CD3 surface down-modulation was consistent for cells infected with a wide variety of viral variants. RNA extracted from HIV-1-infected cell lines expressing 60–89% TCR-CD3\(^+\) (64% is shown in Fig. 1A) were competed at essentially the same concentrations as the 90% TCR-CD3\(^+\) cells, most likely due to the limited sensitivity of this series of competitor concentrations once transcript numbers are low. Because the cells have lost more than 90% of their CD3γ gene transcripts before substantial numbers of TCR-CD3\(^+\) cells are detectable, any changes in the remaining transcript levels (only 10% of normal levels) would have a magnified effect on the number of surface receptor complexes. The erosion of CD3γ transcripts (represented graphically in Fig. 1B) continues in 25 and 5% TCR-CD3\(^+\) cells (Fig. 1A) and were completely undetectable in HIV-1-infected TCR-CD3\(^+\) cells and the B cell line Raji (Fig. 1C). Under the same standardized RT-PCR conditions, transcript levels for the highly homologous CD3δ gene were unchanged in all of the RNA preparations (Fig. 1D). These data demonstrate that the loss of CD3γ gene transcripts in HIV-1-infected cells begins very early after infection and that a substantial drop in transcript levels (>90% of the normal number) must occur before a significant effect is observed on receptor surface density.
Cyclosporin A Partially Restores TCR-CD3 Expression on the Surface of HIV-1-infected Cells—We next asked whether activators or inhibitors known to affect various steps in the TCR-CD3 activation pathway could arrest or reverse the loss of CD3γ gene transcripts after infection and thereby partially or completely restore receptor surface expression. Uninfected and HIV-1-infected WE17/10 cells at different stages of receptor down-modulation were treated with the PKC activator, PMA, the calcium ionophores, A23187 and ionomycin (which can down-modulation were treated with the PKC activator, PMA, as well as immobilized anti-CD3 antibody to mimic antigen-induced activation. Cells were also treated with the calcium channel blocker EGTA and its membrane-permeant derivative BAPTA/AM, the tyrosine-protein kinase inhibitor herbimycin A, the PKC inhibitor staurosporine, and the immunosuppressive agent cyclosporin A (CsA).

Cells, treated for various lengths of time and at a variety of different drug concentrations, were screened by flow cytometry for modulation of surface CD3, and representative data are shown in Fig. 2. As expected, activation by PMA, PMA + Iono, or anti-CD3 resulted in further down-modulation of receptors on TCR-CD3\(^\gamma\) uninfected or TCR-CD3\(^\gamma\) HIV-1-infected cells but had no effect on the TCR-CD3\(^\gamma\) -infected cells (Fig. 2, A and B; histograms for anti-CD3 are not shown but were similar to those shown for PMA or PMA + Iono). Staurosporine and herbimycin A had a deleterious effect on cell viability after 48 h, but they had no discernable positive or negative effect on receptor surface density after treatment for 18–24 h where viability was not affected (the histogram profiles shown in Fig. 2, A and B, for staurosporine are identical to those for herbimycin A). Cells treated with BAPTA/AM, but not EGTA, exhibited a slight but consistent down-modulation of TCR-CD3 complexes on both uninfected and HIV-1-infected cells, particularly noticeable as an increased number of cells in the TCR-CD3\(^\gamma\) range (Fig. 2, A and B), but this intracellular calcium chelator also had a deleterious effect on cell growth and viability.

The most consistent positive effect was observed after CsA treatment of HIV-1-infected cells, which partially restored TCR-CD3 complexes on the cell surface of HIV-1-infected cells in a time- and dose-dependent manner. This effect is shown graphically as an increase in the percentage of TCR-CD3\(^\gamma\) cells after treatment with 0.1–1.0 \(\mu\)g of CsA for 3 or 7 days (Fig. 2C), as well as by histograms that illustrate the movement of cells from the negative to positive phenotype after 5 days of treatment with 0.1 \(\mu\)g of CsA (Fig. 2D). CsA also provoked a slight decrease in modulation of CD3 density on the surface of uninfected cells (Fig. 2D), which was augmented with increased time and drug concentrations (Fig. 2C). No cytotoxicity was observed in any of the CsA-treated cell cultures likely due to the fact that WE17/10 cells were grown in the presence of an excess of exogenously added IL-2 (70).

RNA from CsA-treated HIV-1-infected cells (85% TCR-CD3\(^\gamma\)) was analyzed by quantitative competitive RT-PCR (to increase...
FIG. 2. **Treatment with activators and inhibitors of the TCR-CD3-directed pathway.** Histogram overlays showing the distribution of anti-CD3 antibody labeling on uninfected (A) and HIV-1-infected (50% TCR-CD3⁺) (B) WE17/10 cells before and after treatment with 2.5 mM EGTA, 10 μM BAPTA/AM, 10 ng/ml staurosporine A, 10 ng/ml PMA, and 10 ng/ml PMA + 30 ng/ml Iono. C, uninfected and HIV-1-infected cells (34% TCR-CD3⁺) treated for 3 and 7 days with 0.1–1.0 μg/ml CsA. D, uninfected and HIV-1-infected (42% TCR-CD3⁺) cells treated for 5 days with 0.1 μg/ml CsA. E, CD3γ RT-PCR products co-amplified with the CD3γ competitor (as described in Fig. 1) from HIV-1 infected (85% TCR-CD3⁺) cells before (top) and after (bottom) CsA treatment (0.1 μg/ml).
Potential binding sites for NFAT are capitalized sequence of the 5'-upstream region of the human X06026 (71)). We further asked whether alignment of the 5'-upstream sequence of the human CD3γ gene identified by alignment with the HIV-1 LTRs (lanes 1, 2, and NFAT1 motif in Fig. 3B) as a control (lanes 1 and 2). Nuclear extracts from TCR-CD3′-infected cells were competed with a 4- and 20-fold molar excess of the homologous oligonucleotide (lanes 4 and 5), an oligonucleotide containing the NFAT consensus sequence in the IL-2 promoter (lanes 6 and 7), an oligonucleotide containing the IL-2 promoter NFAT consensus sequence mutated to abrogate NFAT binding (lanes 8 and 9), an oligonucleotide containing the NFAT3 sequence mutated from GGAA to CCTT (lanes 10 and 11). Bands A-D indicate the four different protein-DNA complexes that specifically bind to the NFAT1 probe. B, binding of proteins from the same nuclear extracts shown in A to a 32P-labeled Oct-1 probe in an EMSA performed as a control (lanes 1–3).

Identification of Three NFAT Consensus Sequences in the Human CD3γ Gene—Consequent to the up-regulation of CD3γ transcripts observed after cyclosporin A treatment, we asked whether there were any potential NFAT binding motifs in the 5'-upstream sequence of the human CD3γ gene. We identified three NFAT consensus sequences (5'-GGAAA-3') at -124 to -120 (NFAT1), -384 to -380 (NFAT2), and +450 to +454 (NFAT3) from the first transcription initiation site (Fig. 3A, based on the published sequence NCB accession number X06026 (71)). We further asked whether alignment of the 5'-upstream region of CD3γ gene with the 5'-LTRs of HIV-1 (Strain HXB2, NCB accession number K03455) and HIV-2 (Strain BEN, NCB accession number M30502) would expose regions of sequence homology. This analysis revealed that the second motif, NFAT2 (5'-TTTCC-3'), is nested in a region (−412 to −372) that shares sequence similarity with the functional NF-κB cis-acting sequences located upstream of the SP1 binding sites and the TATA promoter in both the HIV-1 and HIV-2 LTRs (Fig. 3B). However, the first NF-κB consensus sequence in the HIV-1 and HIV-2 LTRs varies from the potential site in CD3γ by two nucleotides (GGGACTTC in HIV with GGTGCTTC in CD3γ) of which the first three Gs are thought to be critical for NF-κB binding (72, 73).

Nuclear Protein Complexes Bind to the NFAT1 Motif in CD3γ—An oligonucleotide probe extending from −132 to −113 (underlined in Fig. 3A) was used to examine the in vitro binding of nuclear proteins to the NFAT1 motif by EMSA. Nuclear extracts of unstimulated WE17/10 cells (100% TCR-CD3′), PMA+Iono-stimulated WE17/10 cells (100% TCR-CD3′), and receptor negative HIV-1-infected WE17/10 cells (TCR-CD3′) were analyzed in parallel. At least four bands (Fig. 4, A–D), representing DNA-protein complexes with different electro-
Differential NFAT Binding in the Human CD3γ Chain Gene

Fig. 5. Supershift and super-supershift analysis of NFAT, NF-κB, and AP-1 protein binding to the NFATγ1 probe. A, the 32P-labeled NFATγ1 probe was used in a supershift assay with nuclear extracts from TCR-CD3γ HIV-1-infected cells in the absence of antibodies (lane 1) or in the presence of anti-NFATc1 (lane 2), anti-NFATc2 (lane 3), anti-NF-κB p50 (lane 4), anti-c-Fos (lane 11), and anti-c-Jun (lane 12) antibodies. A super-supershift assay was performed by sequentially adding the anti-NFATc1, anti-NFATc2, or anti-NF-κB p50 antibodies (the order they were added was indicated) to the binding reaction in the following combinations: anti-NF-κB p50 plus anti-NFATc1 (lanes 5 and 8), anti-NF-κB p50 plus anti-NFATc2 (lanes 6 and 9), and anti-NFATc1 plus anti-NFATc2 (lanes 7 and 10). B, binding to the 32P-labeled NFATγ1 probe was examined in a supershift assay using nuclear extracts from TCR-CD3γ HIV-1-infected WE17/10 cells untreated (lane 1) or treated with CsA (0.1 μg/ml) and PMA + Iono (each 30 ng/ml) in the absence of antibodies (lane 2) or in the presence of anti-NF-κB p50 (lane 3), anti-NFATc1 (lane 4), anti-NFATc2 (lane 5) antibodies.

The specificity of the complexes bound to the NFATγ1 probe was further investigated by competition experiments using the homologous oligonucleotide (NFATγ1; lanes 4 and 5), an oligonucleotide containing the NFAT consensus sequence in the human IL-2 promoter (74, 75) (NFAT-IL-2; lanes 6 and 7) or versions of NFAT-IL-2γ1 and NFATγ1 mutated to abrogate binding (76) (GGAA → CCTT; NFAT-IL-2mut; lanes 8 and 9; NFATγ1mut; lanes 10 and 11). The homologous and the NFAT IL-2γ1 probes efficiently compete for binding, whereas the NFAT IL-2mut and the NFATγ1mut probes were unable to compete. Furthermore, oligonucleotides containing the HIV-1 LTR NF-κB consensus sequence, either wild type (Fig. 3B) or mutated (72) (GGG → CTC, known to abrogate NF-κB but not NFAT binding), both efficiently compete for binding (data not shown). These experiments indicate that the nuclear protein complexes binding to the NFATγ1 probe in PMA + Iono-induced and HIV-1-infected cells are specific for the NFAT but not the NF-κB consensus sequence.

The Nuclear Protein Complexes Bound to NFATγ1 Contain NFATc1, NFATc2, and NF-κB p50—Identification of some of the proteins present in the complexes bound to the NFATγ1 probe was achieved using antibodies to the NFAT family members, NFATc1 and NFATc2, the NF-κB family members, p50, p65, c-Rel, Rel B, and p52, and the AP-1 family members, c-Fos and c-Jun, with nuclear extracts from TCR-CD3γ HIV-1-infected cells in a supershift assay (Fig. 5A). Antibodies specific for NFATc1 (lane 2), NFATc2 (lane 3), and NF-κB p50 (lane 4) all supershifted a DNA-protein complex, whereas antibodies to c-Jun (lane 11), c-Fos (lane 12), p65, c-Rel, Rel B, and p52 do not (the latter four were identical to c-Jun and c-Fos and are not shown). The A complex can be supershifted with either the
or infected cells bind to the NFAT different complexes present in nuclear extracts from activated antibody in some of the A complexes. Thus, as many as four activated or infected cells, one containing NFATc1 and NF-/H9260Bies (Fig. 5

Alternatively, NF-/p50 could be present but inaccessible to the antibody in some of the A complexes. Thus, as many as four different complexes present in nuclear extracts from activated or infected cells bind to the NFAT1 probe, including: two abundant complexes that contain NFATc2 (bands B and C) but not NFATc1 or NF-/p50 p50 and two low concentration complexes (band A) devoid of NFATc2, one which contains NFATc1 and NF-/p50 p50 and the other either NFATc1 alone or NFATc1 and an inaccessible NF-/p50 p50.

Uninfected TCR3γ and HIV-1-infected TCR3γ cells were treated with CsA and then stimulated with PMA+Iono to achieve the maximum potential induction of nuclear NFAT in the presence of CsA. In all cases, there was a >90% inhibition of nuclear protein binding to the NFAT1 probe in EMSA binding studies (data not shown), which is in agreement with the ability of CsA to block T cell activation (77). These extracts were also used in a supershift assay with the NFAT1 probe and anti-NFATc1, anti-NFATc2, and anti-NF-/p50 p50 antibodies (Fig. 5B). Binding of the NFAT1 and NF-/p50 p50 containing A complex was totally inhibited by CsA treatment (overexposure of the gels did not detect the A complex either in the presence or absence of the anti-NFATc1 and anti-p50 antibodies). The NFATc2-containing B and C complexes were both largely inhibited by CsA, and although a faint B complex could be detected in longer exposures, the normally weaker C complex was readily detectable in lower exposures of the gels (Fig. 5B). This shift in the relative abundance of these two complexes after treatment with CsA suggests that the higher molecular weight B complex is more sensitive to CsA than the lower molecular weight complex and may thus contain a second CsA-sensitive component.

The Quantity of Nuclear NFATc1, NFATc2, and NF-/p50 Is Negatively Correlated with TCR-CD3 Surface Expression in HIV-1-infected Cells—The relationship between the presence of NFATc1, NFATc2, and NF-/p50 in the nucleus and the concentration of CD3γ gene transcripts was assessed by examining differential binding to the NFAT1 probe of nuclear extracts during the progression of HIV-1-infected cells from TCR3γ→TCR3γ→TCR3γ (Fig. 6A). Characteristically, only low levels of the NFATc2-containing complexes (bands C and D) were detectable in the uninfected and unstimulated 100% TCR3γ cells (lane 1). Alternatively, increased binding of the NFATc1/NF-/p50 p50-containing complex (band A) and NFATc2-containing complexes (bands B and C) to NFAT1 occurs in parallel with a decrease in surface TCR3γ expression from 98% (lane 2) to 87% (lane 3) to 39% (lane 4) to 0% (lane 5) of normal receptor levels. A nonspecific band (indicated as NS) was also detectable in these nuclear extracts, but this band could neither be supershifted with the anti-NFATc1, anti-NFATc2, or anti-p50 antibodies nor could it be competed for with the homologous oligonucleotide (data not shown). This
escalation in binding to the NFATc2 probe is specific, because similar amounts of the constitutively expressed Oct-1 protein from each extract bound to an Oct-1 sequence-specific probe (Fig. 6B). These results suggest that a correlation exists between the quantity of NFATc2, and to a lesser extent, NFATc1 and NF-kB p50, in the nucleus and down-modulation of CD3γ transcripts and TCR-CD3 complexes after HIV-1 infection.

Differential Binding of NFATc1, NFATc2, and NF-kB p50 to the NFATγ, NFATδ, and NFATvδ Motifs—We next asked whether members of the NFAT and/or NF-kB protein families could also bind to the NFATγ and/or the NFATvδ motifs. EMSA experiments using the NFATγ probe (−392 to −372, underlined in Fig. 3) and extracts from unstimulated cells, PMA+iono-stimulated cells, and TCR-CD3− HIV-1-infected cells bound in a similar pattern to NFATγ (Fig. 4) except that only the NFATc2-containing complexes (bands B, C and D) but not the NFATc1/NF-kB p50-containing complex (band A) were bound (data not shown). Alternatively, binding to the NFATγ probe (+447 to +466, underlined in Fig. 3) was identical to NFATγ with all four of the complexes bound (A–D; data not shown). An experiment using the NFATδ and NFATvδ probes in competition with the homologous or the NFATc2 and NFAT-IL-2 wild type and mutated probes revealed that the binding of bands A–D to these three sequences was highly specific (data not shown). The differential binding of NFATc1, NFATc2, and NF-kB p50 to the NFATγ, NFATδ, and NFATvδ sequences was confirmed in a supershift assay using antibodies to the NFAT, AP-1, and NF-kB family members and nuclear extracts from TCR-CD3− HIV-1-infected cells (Fig. 7). Only the anti-NFATc2 antibody specifically shifted the complex bound to the NFATvδ probe (Fig. 7A, lane 2, bands B and C), whereas no band shift was observed with antibodies to NFATc1 (lane 3), to the NF-kB proteins p50 (lane 4), p65, c-Rel, Rel B, or p52 (data for the latter four antibodies are identical to p50 and are not shown) or to the AP-1 proteins c-Jun (lane 5) and c-Fos (lane 6). This experiment revealed that NFATc2 but not NFATc1, AP-1, or NF-kB family proteins bind to the NFATγ sequence, despite its homology with the NF-kB region in the HIV-1 LTR. On the contrary, a supershift assay using the NFATγ probe (Fig. 7B) was qualitatively similar to the NFATγ probe, with supershifted complexes observed for the anti-NFATc1 (band A, lane 2), anti-NFATc2 (bands B and C, lane 3), and anti-NF-kB p50 antibodies (band A, lane 4). We compared the relative binding of the NFATc1 plus NF-kB p50- and NFATc2-containing complexes to the NFATγ, NFATvδ, and NFATv3 motifs (Fig. 7C) and found that NFATγ binds significantly more of these protein complexes compared with NFATc2 and NFATvδ, with binding to the NFATγ probe the weakest among the three motifs. Furthermore, there did not appear to be cooperative recruitment of c-Jun and c-Fos in any of the complexes bound to NFATγ, NFATvδ, and NFATv3.

Sequence Variation Is Responsible for the Differential Binding of NFATc1, NFATc2, and NF-kB p50 to the NFATγ, NFATδ, and NFATvδ Motifs—In an effort to understand the basis for the qualitative and quantitative differences in binding to the NFATγ, NFATδ, and NFATvδ motifs, a series of mutant probes were constructed and used in EMSA experiments (the mutations are listed with a summary of the results in Table I, and the gels are shown in Fig. 8). We noted that the nucleotides bordering the core 5′-GGAAA-3′ sequence differed by an AA immediately following the core sequence in NFATγ and NFATvδ, in contrast to a GC in NFATvδ, suggesting that these
nucleotides could potentially play a role in the binding of NFATc1 and NF-xB p50. Alternatively, a T rather than an A preceding the core sequence is thought to facilitate stronger binding of NFAT family proteins (56), and this nucleotide was C, T, or A in the NFAT枷, NFATd, and NFATz sequences, respectively. Our rationale was that if these three nucleotides do play an important role in binding, then successively mutating the NFAT枷 sequence to look like the NFATd sequence and vice versa should alter binding accordingly.

Mutation of the first A following the core sequence in NFAT枷 to a G (NFAT枷mut1; Fig. 8A, lane 2) completely abrogated binding of NFATc1 and NF-xB p50 (band A), significantly decreased the binding of NFATc2 (bands B and C) compared with the wild type sequence (NFAT枷wt, lane 1) and provided a pattern similar to that of wild type NFATd (NFATdwt, lane 6). Additionally mutating the second A to a C in NFAT枷 (NFAT枷mut2, lane 3) changed the 3’ sequence to that of NFATd and reduced NFATc2 binding even further. Mutation of the outside A only in the AA pair of NFAT枷 (NFAT枷mut3, lane 4) had a less dramatic effect on the quantity of NFATc2 bound compared with the inside A (lane 2) and did not abrogate binding of NFATc1 and NF-xB p50, although quantitatively all of the complexes were significantly reduced.

Mutation of the C preceding the core sequence in NFAT枷 (NFAT枷mut4, lane 5) to a T, creating the sequence 5’-TG-GAAAAA-3’ (NFAT枷T, lane 10), greatly enhanced the amount of NFATc1, NFATc2, and NF-xB p50 bound to this probe, providing better binding than that observed with any of the wild type sequences.

Alternatively, the reverse mutations in NFATd converted the binding profile of this probe to one similar to NFAT枷 with increased binding of NFATc2 (bands B and C) and de novo binding of NFATc1 and NF-xB p50 (band A) achieved by simply changing the 3’ G (NFATdmut1, lane 6) to an A (NFATdmut1, lane 7). Adding a second A 3’ of the core sequence in NFATd (NFATdmut2, lane 8) further increased the binding of all three complexes (A, B, and C). However, substituting the C for an A in the outside 3’ position did not confer binding of NFATc1 and NF-xB p50, although it did increase the binding of NFATc2 (NFATdmut3, lane 9). Finally, mutation of the T preceding the core sequence to a C, creating the sequence 5’-TG-GAAAAGC-3’, completely abrogated all binding (NFATdmut4, lane 10). Confirmation that the specific binding of NFATc1 and NF-xB p50 was conferred by adding a fourth A to the NFAT core sequence (5’-TG-GAAAAA-3’) was demonstrated by a supershift assay using the NFAT枷mut1 probe (Fig. 8D). This experiment clearly shows that a simple G → A substitution 3’ of the core sequence in NFAT枷 is sufficient to confer binding of NFATc1 and NF-xB p50. Finally, binding to the wild type NFAT枷 sequence is normally weak, and mutation of the A following the core sequence to G completely abrogated binding (NFAT枷mut1, Fig. 8C, lane 2) compared with the wild type (NFAT枷wt, lane 1).

Taken altogether, these mutation experiments demonstrate that a fourth A added to the NFAT core sequence (5’-TG-GAAAAA-3’) is vital for NFATc1 and NF-xB p50 binding and important for the quantity of NFATc2 that binds. They further illustrate the important role that the T preceding the NFAT core sequence (5’-TG-GAAAAA-3’) plays in the quantity or stability of the bound complexes, including both those containing NFATc2 and those containing NFATc1 alone or in association with NF-xB p50.

**DISCUSSION**

We have previously demonstrated that T cell receptor down-modulation, due to a defect in CD3γ gene transcription (15, 20), occurs in a two-phase progression after HIV-1 or HIV-2 infection and can be summarized by the formula TCR-CD3γh ↔ TCR-CD3γl ↔ TCR-CD3γ− in which the forward progression is markedly favored (16). The TCR-CD3γh to TCR-CD3γl phase is characterized by a steady decrease in receptor density on all cells from 100% to 50% of control values, prior to the subsequent conversion of individual cells to the TCR-CD3γ− phenotype (16). The RT-PCR data presented in this study provide further insight into the molecular events generating this progression by showing that the initial conversion from TCR-CD3γh to TCR-CD3γl involves a substantial (80–90%) decrease in the number of CD3γ gene transcripts.

These data answer a fundamental question of why the progression, viewed from the cell surface, appears to be very slow by showing that transcriptional down-modulation is actually initiated very early (or most likely immediately) after infection with a considerable and rapid erosion of transcripts until a threshold is reached where the normal number of complete TCR-CD3 complexes can no longer be assembled and exported to the cell surface (78). The individual TCR-CD3 proteins have been shown to be synthesized in great excess, followed by rapid degradation if they are not stabilized through incorporation into partial or complete complexes (79). The CD3γ protein forms a stable complex with CD3δ (80) and thus can persist both in complete TCR-CD3 complexes, which are continuously recycled to the cell surface in the absence of antigen stimulation, as well as in partially formed complexes in the endoplasmic reticulum. Thus, recycling and partial complex formation precludes an immediate and deleterious effect on surface receptor expression during the initial stages of CD3γ transcript loss.

Our earlier studies examining TCR-CD3 expression over time post-infection found a minor modulation of receptor density immediately following the acute phase of infection (first 4–6 weeks) (15, 16, 20). These studies also revealed that an initial 4- to 5-fold drop in p24 antigen levels in the culture supernatant occurred coincident with down-modulation from TCR-CD3γh to TCR-CD3γl, with a further 4- to 5-fold reduction accompanying the transition from TCR-CD3γh to TCR-CD3γ− (16). However, a subsequent extensive examination of productively infected cells did not reveal a direct relationship between intracellular p24 antigen levels and TCR-CD3 surface density (26). Furthermore, non-productively infected cells expressing the multiply spliced, virally encoded tat, nef, and rev regulatory gene transcripts also demonstrated the same progressive loss of surface TCR-CD3 complexes (26). Treatment of productively infected cells with antisense oligonucleotides targeted to tat, nef, and rev revealed that the relative level of tat and nef gene transcripts could be directly correlated with a loss of CD3γ transcripts (26). Antisense oligonucleotides directed to the splice acceptor of the tat gene were particularly efficient in provoking a coordinate down-regulation of virus expression in concert with an up-regulation of surface TCR-CD3 complexes (26). One interpr-
tion of our previous data in light of the RT-PCR results presented here is that Tat-dependent viral gene expression and the availability of Tat and/or Tat-dependent cellular transcription factors (81) play a crucial role in initiating and maintaining the escalating CD3γ transcription defect.

HIV-1 is known to activate its CD4+ T cell host and trigger the expression of a variety of antigen-induced immune response genes as a means of facilitating virus integration, replication, and expression (82, 83). CD3γ plays an important role in both tyrosine- and PKC-mediated TCR-CD3 down-modulation, and it seems likely that HIV-1 could exert its effect on receptor expression via these normal immune pathways. We asked whether it was possible to restore CD3γ transcription in HIV-1-infected cells by activating or inhibiting steps in the TCR-CD3-directed activation pathway and found that the immunosuppressive drug cyclosporin A could partially restore TCR-CD3 surface expression on infected cells. CsA inhibits the calcium-regulated phosphatase calcineurin, which dephosphorylates NFAT family proteins in response to antigen activation. Dephosphorylation of NFAT proteins is a prerequisite for their translocation to the nucleus, where they function as major players in the transcriptional activation of a wide array of cytokine genes possessing NFAT binding motifs (5'-GGAAAA-3') (56). Four NFAT sites are located in the HIV-1 LTR, one within each of the two NF-κB consensus sequences, and an additional two in the negative regulatory element (52). The HIV-1 κB sequences have been shown to play an important role in the transcriptional regulation of viral gene expression (84, 85) and to competitively bind NF-κB and NFAT family proteins (55, 58, 83). The up-regulation of TCR-CD3 surface expression observed on CsA-treated HIV-1-infected cells suggested that NFAT might also be directly or indirectly involved in the elusive transcriptional control mechanisms that regulate expression of the CD3γ gene.

A search of the 5′-upstream region and exon 1 of the human CD3γ gene revealed three potential binding motifs for NFAT family proteins (NFATc1, NFATc2, and NFATc3). The NFATc1 motif is located in a DNase I-hypersensitive site that has been designated as the putative promoter for CD3γ (71, 86, 87), whereas the NFATc2 motif is nested in a region with sequence homology to the HIV-1 κB elements. Three different molecular weight complexes (A, B, and C) could be induced by PMA+Iono or HIV-1 infection to specifically but differentially bind to these motifs in the CD3γ gene. NFATc2 was shown to be present in both the B and C complexes, as well as in the low abundance D complex found in unstimulated cells. The different electrophoretic mobilities of the three complexes could be correlated with the binding of NFATc2 as a monomer or dimer (56, 88) and/or the presence of other currently unidentified factors, potentially including an additional CsA-sensitive protein in the B complex. The B complex might be the active complex, with the C complex an intermediate stage in assembly and the D complex representing the low level of NFATc2 known to be present in the nucleus of resting T cells (89). Alternatively, the C complex could be a positive transcription complex and the additional protein(s) bound in the B complex could provide a negative signal.

The highest molecular weight A complex was found to contain NFATc1 and NF-κB p50 (but not NFATc2). To determine whether NFATc1 and NF-κB p50 were present in the same protein-DNA complex, we designed a modified supershift assay whose purpose was to reduce the molecular mobility of one or more complexes containing both proteins by the sequential addition of the two different antibodies (referred to as a supershift assay). This experiment demonstrated that some of the A complexes contain both NFATc1 and NF-κB p50, whereas others contain either NFATc1 alone or an inaccessible NF-κB p50. The relatively small impact on the molecular mobility afforded by the additional binding of the anti-NFATc1 antibody in the super-supershift over the band in the anti-p50 antibody simple supershift can be explained by the nature of these antibodies. The anti-NFATc1 used was a mouse monoclonal antibody, whereas the anti-p50 employed was a goat polyclonal antibody. Therefore, the single isotype of the anti-NFATc1 antibody directed to only one epitope of this protein in combination with the repertoire of anti-p50 antibody molecules po-

![Image](http://www.jbc.org/Downloaded_from http://www.jbc.org by guest on July 25, 2018)
potentially bound to NF-κB p50 contributed relatively little additional weight to this already extremely high molecular mass protein-DNA complex, thereby slightly but consistently decreasing its electrophoretic mobility.

The super-supershift approach was designed to demonstrate the dual binding of NFATc1 and NF-κB p50 in a single complex, because both NFAT and NF-κB family proteins are translocated to the nucleus after PMA+Iono stimulation or HIV-1 infection where the preferential and most abundant binding partner for p50 would be another NF-κB family member such as p65 (super-shifts using a NF-κB consensus sequence probe detected abundant amounts of NF-κB p50 and p65 in these nuclear extracts, data not shown). In light of the relatively low levels of the NFATc1:NF-κB p50 complex present, we thought it was important to provide the NFATc1 DNA binding site in the reaction mixture to favor their coordinate binding. Further evidence in support of the dual binding of NFATc1 and NF-κB p50 to NFATc1 and NFATc1 but not NFATc1, was provided by the EMSA experiments using mutant oligonucleotides. Changing the fourth A in the NFATc1 and NFATc1 motifs (5′-GGAAAG-3′ to 5′-GGAAAG-3′) completely abrogated binding of the NFATc1- and NF-κB p50-containing complex, whereas adding a fourth A to the NFATc1 motif (5′-GGAAAG-3′ to 5′-GGAAAG-3′) conferred binding to this sequence. It seems unlikely that simply altering a single nucleotide would have such a dramatic effect on the concurrent binding of NFATc1 and NF-κB p50 binding unless they were present in the same complex.

These data are the first demonstration of a NFAT family member and a NF-κB family member binding together in the same protein-DNA complex. NFAT and NF-κB normally compete for binding to the κB site, and this has been demonstrated to be true for the HIV-1 LTR κB sites (58). The NF-κB/Rel family of transcription factors are defined by a ~300-amino acid region called the Rel homology domain, which contains the residues involved in nuclear translocation, DNA binding, and protein-protein interactions (53, 90). NF-κB p50 and p65 preferentially form a heterodimer, although they are also capable of forming p50/p50 or p65/p65 homodimers. The formation of homo- and heterodimers leading to dimerization is known to be required for binding of the NF-κB family proteins to DNA (91). Crystal structures have shown that NF-κB p50 optimally binds to the 5′-GGAAAG-3′ half site and p65 the 5′-GGAAAG-3′ half site, which are separated by a non-contact base in the palindromic κB sequence (92). Although not all of the known physiological targets have this 10-bp κB consensus sequence, NF-κB proteins are still capable of binding to these non-ideal sequences with similar affinities (56).

A Rel homology domain, with about 20% sequence homology to the NF-κB Rel domain, is also found in all of the NFAT proteins (93, 94). Structural studies have shown that the minimal DNA binding domain of NFATc1 is essentially identical to the N-terminal specificity domain of NF-κB p50, the region involved in the majority of its base specific contacts with DNA (93, 95, 96). NFAT proteins normally bind as monomers in cooperation with other transcription factors such as AP-1. However, they have also been shown to bind as dimers to certain NF-κB/Rel sites (56), and the HIV-1 LTR κB sites are an example of NFATc2 forming both monomeric and dimeric complexes (55, 58, 97). Other common features between the NFAT and NF-κB proteins include their responsiveness to immune activation and their regulation by cytoplasmic to nuclear translocation.

The NFATc1 and NFATc1 probes do not contain a palindromic purine-rich sequence similar to those found in the HIV-1 κB elements, which if present could potentially explain the dual binding of NFATc1 and NF-κB p50. Furthermore, the supershift assay performed on the CsA-treated cells revealed that NF-κB p50 does not bind to the NFATc1 motif in the absence of NFATc1, suggesting that NF-κB p50 binding is completely dependent upon the presence of NFATc1. It was quite intriguing to discover that proteins from these two different transcription factor families bind together to DNA sequences whose only common component is the presence of an extended NFAT binding motif where the fourth adenosine (5′-GGAAAG-3′) was found to be crucial for their binding. This core motif is also the only component common between the NFATc1 and NFATc1 but not the NFATc1 probes and thus emerges as the requisite sequence for binding of the NFATc1:NF-κB p50 complex. Sites in which the 5′-GGAAA-3′ core sequence is preceded by a T rather than an A bind NFAT proteins more strongly (56), and although this was found to be true for NFATc1 by replacing the preceding C with a T, the low level and lack of NFATc1 and NF-κB p50 binding to NFATc1 (5′-TGAAGAG-3′) suggests that the fourth A plays the greatest role in qualitative binding.

The dimerization relationships between the different NF-κB proteins and the combinatorial binding associated with the NFAT family proteins allows a relatively small number of transcription factors to establish an extraordinarily complex and extensive regulatory network with different biological consequences dependent upon selective binding controlled by the flanking sequences. This may be just one more example of how the NFAT family proteins gain specificity and regulatory function through their coordinate binding with other transcription factors. NF-κB p50 could potentially partner with NFATc1 to provide the binding stability it needs and normally acquires through coordinate binding with other transcription factors such as AP-1. The flexibility of binding with different partner proteins may be fundamental to the ability of NFAT proteins to integrate distinct signals through cooperative binding with specific nuclear partners on divergent consensus sequences in diverse genes and different chromatin structures.

In this study, we have shown that a loss of CD3γ gene transcripts is initiated early after HIV-1 infection and rapidly accumulates to a defect of >90% of normal transcript numbers, leading to a down-modulation of surface TCR-CD3 expression and function. We identified three NFAT binding motifs (NFATc1, NFATc1, and NFATc1) in the upstream region of the CD3γ gene and have shown that they differentially bind complexes containing NFATc2, NFATc1, and NF-κB p50. Furthermore, we found that a significant and progressive increase in these protein-DNA complexes could be negatively correlated with CD3γ gene transcript numbers. The NFATc1 site binds the greatest abundance of these transcription factors, which, together with its location in a DNase I-hypersensitive site (86), suggests it may play an active role in CD3γ gene transcription. Normal activation via the TCR-CD3 complex initiates a cascade of molecular events leading to multiple signaling pathways that are integrated to induce the expression of specific cytokine genes. A sustained signal also changes the normal balance in receptor expression, favoring TCR-CD3 internalization and degradation rather than recycling and de novo synthesis (5). Although the accumulation of NFAT family proteins in the nucleus has a positive influence on cytokine gene transcription, it also potentially negatively regulates CD3γ gene transcription as a means of controlling continued TCR-CD3 directed signaling. Thus, HIV-1 may have acquired the ability to intercede in both the positive and negative downstream pathways triggered by the TCR-CD3 as a means of controlling viral gene expression and latency.

Acknowledgments—We are indebted to Dr. F. Barré-Sinoussi for the HIV-1 LAI isolate and Dr. M. J. Crampton for the pBRT7-2 clone containing human CD3γ. The reagent HXB2 was obtained through the National Institutes of Health (NII) AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (from Drs. B. Hahn and G. M. Shaw).
