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Identification of the Human CD3γ Gene Promoter, Characterization of Specific Promoter Elements and Analysis of their Activity in Normal and HIV-infected CD4⁺ T Cells
Identification of the Human CD3γ Gene Promoter, Characterization of Specific Promoter Elements and Analysis of their Activity in Normal and HIV-infected CD4⁺ T Cells
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# Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CBP</td>
<td>CREB Binding Protein</td>
</tr>
<tr>
<td>CREB</td>
<td>Camp-Responsive Element Binding Protein</td>
</tr>
<tr>
<td>CsA</td>
<td>Cyclosporin A</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DBL</td>
<td>DNA-binding loop</td>
</tr>
<tr>
<td>EMSA</td>
<td>Electrophoretic Mobility Shift Assay</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>IkB</td>
<td>Inhibitor of NF-κB</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>Inr</td>
<td>Initiator element</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motifs</td>
</tr>
<tr>
<td>Kda</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LTR</td>
<td>Long Terminal Repeat</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear Factor of Activated T cells</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor Kappa B</td>
</tr>
<tr>
<td>NHR</td>
<td>NFAT-homology region</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear Localization Signal</td>
</tr>
<tr>
<td>NP-40</td>
<td>Nonidet P-40</td>
</tr>
<tr>
<td>NRE</td>
<td>Negative regulatory Element</td>
</tr>
<tr>
<td>P/CAF</td>
<td>p300/CBP-Associated Factor</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositol 3 kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PLCγ1</td>
<td>Phospholipase Cγ</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol-12-Myristate-13-Acetate</td>
</tr>
<tr>
<td>RHD</td>
<td>Rel Homology Domain</td>
</tr>
<tr>
<td>Sp1</td>
<td>Specific Protein 1</td>
</tr>
<tr>
<td>TAD</td>
<td>Transcriptional-activation domains</td>
</tr>
<tr>
<td>TAR</td>
<td>Trans-Activation Responsive Element</td>
</tr>
<tr>
<td>Tat</td>
<td>Trans-activator of Transcription</td>
</tr>
<tr>
<td>ZAP-70</td>
<td>ζ-associated protein</td>
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Summary

Dynamic regulation of T cell receptor (TCR)-CD3 complexes on the cell surface plays a critical role in controlling antigen-dependent T cell mediated immune responses. Our laboratory has clearly demonstrated that the progressive loss of TCR-CD3 receptor expression and function after HIV-1 or HIV-2 infection results from a specific loss of CD3γ gene transcripts, while expression of the other CD3 genes remain unchanged. Northern and dot blot hybridization analyses suggested that the loss of CD3γ does not parallel the downregulation of TCR-CD3 complexes from the surface at a ratio of 1:1. We have used quantitative RT-PCR to gain further insight into the CD3γ gene defect and found that the loss of CD3γ gene transcription begins early after HIV-1 infection and accumulates to a deficiency of more than 90% of transcripts before a significant effect on surface TCR-CD3 density is apparent.

Treatment of infected cells with antisense phosphorothiolate oligodeoxynucleotides demonstrated that HIV-1 tat and/or nef gene products play an important role in the loss of CD3γ transcripts after infection. Therefore, we initiated an investigation designed to identify and characterize the elements responsible for controlling transcription of the human CD3γ gene. We cloned the sequence located between -782 and +286 of the hCD3γ gene and demonstrated that it is transcribed from a weak, non-tissue specific TATA-less promoter. We have located both positive and negative regulatory elements in the promoter including, three NFAT consensus sequences (5'-GGAAA-3') at: -124 to -120 (NFATγ1), -384 to -380 (NFATγ2), and +450 to +454 (NFATγ3) from the first transcription initiation site. EMSA experiments were used to demonstrate that NFATc2 alone binds to the NFATγ2 motif while complexes containing either NFATc2 or the unusual combination of NFATc plus NF-κB p50 bind to the NFATγ1 and NFATγ3 sites. We further demonstrated that an increase in the binding of nuclear NFATc2, in particular, to these motifs is correlated with a progressive loss of hCD3γ transcripts after HIV-1 infection. Mutation and deletion analysis revealed that the NFATγ1 site positively or negatively influences promoter activity depending upon whether NFATc1 plus NF-κB p50 or NFATc2 containing complexes are bound, respectively. Alternatively, the NFATγ2 site, which binds NFATc2 only, negatively regulates promoter activity.

We have located a GC box at -22 to -13 in the hCD3γ promoter, demonstrated that Sp1 specifically binds to this region, and shown that this motif plays a positive role in expression of the hCD3γ gene. We have explored the functional activity of the three transcription initiation sites, located at +1, +6 and +21, and found that a critical element surrounds the +1 and +6 nucleotides. Our preliminary data suggests that this sequence functions as an initiator element, and that an additional critical positive element is present at +12 to +50 of the downstream hCD3γ sequence. The experimental data presented in this thesis adds important information to our understanding of transcriptional regulation of the CD3γ gene and a number of interesting parallels with the HIV-1 LTR, which we shall exploit in future experiments.
Chapter I

Introduction
Figure 1. The T cell receptor TCR-CD3 complex. This figure shows the most common form of the TCR-CD3 receptor, containing the antigen recognition subunits TCRα and TCRβ and the signal transducing subunits CD3ε, CD3γ, CD3δ, and CD3ζ. The interaction of the MHC-antigen complex with the TCR CD3 and CD4 coreceptors is shown.
Introduction

1. The T cell receptor

1.1 General overview

The specific recognition of antigen, presented to T cells by the major histocompatibility complex (MHC), is mediated through a structure known as the T cell receptor (TCR)-CD3 complex (Figure 1) (1). The TCR-CD3 is composed of the antigen recognition subunits TCRα and TCRβ (or TCRγ and TCRδ in 5 to 10% of T cells) and the signal transducing subunits CD3ε, CD3γ, CD3δ and CD3ζ (2, 3, 4). Interactions between the TCR-CD3 and antigen-MHC complex are unusual among known protein-protein interactions because upon engagement a large spectrum of biological and clinical processes are directly affected. The immunological and pathological consequences of these interactions have been extensively studied, but the molecular details of the steps involved are only beginning to be defined. The level of TCR-CD3 expression on the cell surface is the result of a balance between the synthesis of polypeptides leading to the assembly of new complexes and the internalization of surface receptors, which are subsequently recycled or degraded (5, 6, 7). TCR-CD3 binding to antigen-MHC complexes enhances the activation of kinases that phosphorylate components of distinct downstream signaling pathways (8, 9, 10), leading to the activation of Ras and mitogen-activated protein (MAP) kinases among others. TCR-CD3 engagement also activates phospholipase Cγ (PLCγ) provoking a subsequent increase in intracellular free calcium and the activation of protein kinase C (PKC). These two pathways converge at the level of cytokine activation, inducing production of Interleukin 2 (IL-2) and other lymphokines considered to be critical for T cell activation (11, 12, 13, 14).
Figure 2. The human CD3γ, CD3δ and CD3ε gene locations on chromosome 11q23, with CD3γ and CD3δ oriented in a head-to-head arrangement separated by 1.6 kb and CD3ε in the center position of the cluster.
1.2 Molecular structure of the TCR-CD3 complex

The human TCRα and TCRδ genes are located on chromosome 14 while the human TCRβ and TCRγ genes are located on chromosome 7 (the following discussion pertains specifically to the TCRα/β receptor). TCRα and TCRβ exist in their mature forms within the TCR-CD3 complex as a disulfide-linked heterodimer (linked by a single pair of cysteines). The TCRα/β heterodimer confers ligand specificity to the receptor complex. These chains are core polypeptides of 28-30 kDa with several N-linked glycosylation sites resulting in an approximate molecular weight of 40-44 kDa for the mature TCRα or TCRβ chains (as determined by SDS-PAGE). TCRα and TCRβ are notable structurally for their large immunoglobulin (Ig)-like extracellular domains, short cytoplasmic tails, and net positively charged transmembrane regions (15, 16, 17). The positively charged transmembrane region is involved in assembly with the CD3 chains (18) and also acts as a retention signal leading to their rapid degradation in the endoplasmic reticulum (ER) in the absence of assembly (19).

The CD3γ, CD3δ, and CD3ε genes lie together in a 50 kb cluster on human chromosome 11 (Figure 2) (chromosome 9 in mouse) with CD3γ and CD3δ oriented in a head-to-head arrangement separated by 1.6 kb (20, 21, 22, 23). CD3δ is located in the central position of the cluster and flanked on either side by the oppositely transcribed CD3γ and CD3ε genes. CD3γ is encoded by seven exons, CD3δ by five exons and CD3ε by seven or eight exons depending on the presence of two or three miniexons in humans and mice, respectively. Calculations based on sequence divergence demonstrated that these three genes likely arose from an ancestral gene by a process of gene duplication that occurred about 230 million years ago (24). CD3γ, CD3δ, CD3ε, and CD3ζ are all non-covalently associated with the TCRα/β heterodimer (25, 26). In contrast to the TCRα and TCRβ chains, the CD3 chains all possess a net negative charge in their transmembrane domains (27, 28, 29), which allows them to form a salt bridge with the basic amino acids in the transmembrane region of TCRα and TCRβ. Like the TCR chains, the CD3 subunits can also be considered members of the immunoglobulin supergene family because of the Ig domains in the extracellular portion of each polypeptide (30). These CD3 genes are highly
homologous to one another, both in structure and sequence. CD3γ and CD3δ share 57% identity in their cytoplasmic tails, and the CD3γ transmembrane domain can be replaced by that of CD3δ. While CD3γ and CD3δ cytoplasmic domains are not essential for surface expression (31, 32), domain swapping of the CD3γ and CD3δ extracellular domains causes loss of TCR-CD3 surface expression (33). CD3γ and CD3δ are N-glycosylated proteins with a core molecular weight of approximately 16 kDa. The apparent molecular weight (determined by SDS-PAGE) of mature CD3γ is 25 kDa in humans and 20 kDa in mice while mature human CD3δ migrates at 20 kDa with the murine form migrating at 26 kDa (21, 25, 26, 34, 35, 36). CD3ε is not glycosylated and migrates at approximately 20 kDa in humans and 25 kDa in mice on SDS-PAGE gels (25, 26, 34, 35).

The CD3ζ chain is encoded by a gene located on chromosome 1 in both humans and mice (37, 38). In the majority of receptors, CD3ζ exists as a disulfide-linked homodimer CD3ζ-ζ or a heterodimer formed with a related protein called CD3η [the CD3ζ-η heterodimer is present in only a minority of receptors (39)]. The CD3ζ (16 kDa) and CD3η (22 kDa) chains are nonglycosylated proteins that have no sequence or structural homology with any of the other TCR-CD3 chains. CD3ζ and CD3η are formed by alternative splicing of the last exon of the CD3ζ gene and thus are identical through amino acid 122 of the mature protein, but have different carboxy-terminal sequences. The CD3ζ protein has a short extracellular domain of only nine residues, a negatively charged transmembrane region, and a relatively long cytoplasmic tail (37, 40). A single cysteine residue, the site of the disulfide linkage, is located on the outer interface of the membrane. The interaction and assembly of CD3ζ with the other TCR-CD3 chains is thought to be mediated largely through the transmembrane regions. In addition to being a component of the TCR-CD3, the CD3ζ chain has been found grouped with the γ subunit of FcγRIIIA and FceRI where it functions as a signaling chain (41, 42, 43). Finally, CD3ζ chains can be expressed at the surface independently of the hexameric TCR-CD3 complex (44).
Figure 3. **Models for assembly and stoichiometry of the TCR-CD3 complex.** In model A, CD3ζ-TCRα and CD3ζγ-TCRβ associate with the ζ₂ chains to make up the receptor. In model B, CD3ζζ-TCRαβ and CD3ζγ-TCRαβ associate with the ζ₂ to build the complex.
1.3 TCR-CD3 receptor assembly

Assembly of the TCR-CD3 subunits into complete surface complexes has been intensively studied over the past decade. It is initiated in the ER, through a series of tightly regulated, pairwise protein interactions before translocation to the Golgi apparatus (GA) for final complex assembly. Several studies have shown that assembly starts with the formation of heterodimers between CD3ε and CD3γ or CD3δ (45). The CD3γε heterodimers then associate non-covalently with the TCRβ chains to form relatively stable trimers, while the TCRα chains form stable complexes with the CD3δε heterodimers (4, 46). The TCRαCD3δε association appears to take place via the transmembrane region, whereas the TCRβCD3γε trimer seems to be linked through extracellular regions of these proteins (31, 48, 49). Next, disulfide links are formed between the TCRα and TCRβ chains, which is catalyzed by the chaperone CD3ω (50, 51, 52), to produce the hexamer TCRαβCD3γεδ complex, the minimal receptor necessary for translocation to the GA.

The CD3ζ homodimer or CD3ζη heterodimer is the last subunit to be assembled in the complex. Despite its lack of association with the hexameric complex (TCRαβCD3γεδ) in the ER, CD3ζ is transported to the GA (53) where it is assembled with the complex. Complete complexes only are targeted to the cell surface (29, 52) due to interaction of the TCRαβCD3γεδ complex with the ζ-ζ homodimer (or ζ-η heterodimer), which masks a lysosomal targeting motif (di-leucine motif) in the CD3γ chain protecting the complex from degradation and permitting its translocation to the cell surface (4, 45, 46, 53). Only complete receptor complexes, thought to be composed of either TCRαβCD3γεζζ (54) or TCRααββCD3γεζζζ (55) (Figure 3), are efficiently expressed on the mature T cell surface (4, 45, 46, 53). Critical mutations or the lack of any one chain is sufficient to prevent surface expression by retaining partially assembled or unassembled TCR-CD3 components in the ER or GA, targeting them for degradation (56, 57, 58, 59).
Figure 4. Mechanisms of signal transduction and activation. This figure identifies molecule mediators involved in signal transduction after T cell activation, including the phosphorylation of LAT, SLP-76 and other adaptator proteins leading to the activation of NFAT, NF-κB and Ap1 proteins.
1.4 Mechanisms of signal transduction and activation.

The earliest detectable event following engagement of the TCR-CD3 complex with antigen-MHC complexes is phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs), present in the cytoplasmic domains of the CD3 subunits (3, 39, 56, 60, 61, 62). The CD3ε, CD3δ, and CD3γ chains contain one ITAM while three ITAMs are present in each CD3ζ chain for a total of ten ITAMs (63). Tyrosine phosphorylation of the ITAMs is critically dependent on the activity of the Src-family kinases, Lck or Fyn and their action is translated into the recruitment of a 70 kD Syk-family kinase, known as the ζ-associated protein (ZAP-70) (Figure 4). Activation of ZAP-70 by Lck (61) is required for the transmission of signals to the downstream pathway (64, 65, 66), likely through tyrosine phosphorylation of a 36 kD linker molecule known as linker for the activation of T cells (LAT) (67). Tyrosine-phosphorylated LAT then serves to recruit adaptor molecules (like Grb2) that bring with them several signaling molecules like SLP-76, Vav, and Sos. The result is the formation of multimeric complexes based on protein-protein interactions, which ultimately leads to the activation of at least four different signal transduction pathways, including: the PLCγ1 pathway, the ras-mitogen activated protein kinase (MAPK) pathway, the phosphoinositol 3 kinase (PI3K) pathway, and the Itk pathway. Activation of the PLCγ1 pathway is most relevant to our studies.

Activation of the PLCγ1 pathway leads to the generation of inositol 1,4,5 triphosphates (IP3) and diacylglycerol (DAG), which induce a calcium influx and activate PKC, respectively (Figure 4). This pathway converges on the activation of Ca^{2+}-dependent calcineurin phosphatase activity, which in turn, when complexed with calmodulin and cyclophilin, acts on the cytoplasmic component of the NFAT (Nuclear Factor of Activated T cells) family of transcription factors (68, 69, 70). Activation is required for the nuclear translocation of NFAT, which is necessary for optimal IL-2 gene transcription. Activation through this pathway can be blocked by Cyclosporin A (CsA) (71), a drug that inhibits the calcium-dependent translocation of the cytoplasmic component of NFAT.
Figure 5. **TCR-CD3 Internalization and Recycling.** TCR-CD3 complexes are continuously recycled to the cell surface. PKC phosphorylation of CD3γ controls the rate of internalization.
1.5 Degradation of the TCR-CD3 complex

The T cell receptor is constitutively internalized and rapidly recycled back to the surface of normal, unstimulated T cells (6, 7, 72, 73), maintaining a steady state level of surface complexes (72) (Figure 5). The main role of this recycling is to regulate the ability of the T cell to be rapidly activated in response to small changes in the rate constants for internalization and surface expression (74, 75, 76). During constitutive internalization, 70 to 85 % of the cycling TCR-CD3 receptors are on the cell surface and 15 to 30 % are intracellular at any given time (6, 7, 72, 73). Constitutive internalization in unstimulated T cells is dependent on a di-leucine-based (LL) motif (77) in the cytoplasmic tail of CD3γ, which is not fully exposed in the completely assembled TCR-CD3 complex. The CD3γ LL-based motif (Leu 131 and Leu 132) consists of a DxxxLL sequence that binds the adaptor protein AP-2 (an important component of clathrin-coated vesicules) at the plasma membrane (78, 79). AP-2, in turn, links the TCR-CD3 to the clathrin-dependent internalization machinery providing a major signal for receptor internalization. In the absence of antigenic stimulation, CD3γ is dephosphorylated by a serine/threonine phosphatase that allows recycling of the TCR-CD3 complex to the cell surface (80, 81).

Antigen stimulation activates PKC inducing rapid downmodulation of TCR-CD3 complexes from the cell surface (82, 83, 84, 85) by increasing the receptor internalization rate constant more than 10-fold without affecting the exocytic rate constant (73, 82, 86, 87). PKC-mediated phosphorylation of serine 126 on CD3γ, which precedes the LL-based motif, causes a conformational change that fully exposes the LL-based motif and induces its association with the AP-2 complex, thereby increasing its rate of TCR-CD3 internalization. A recent study has shown that ligand-induced TCR-CD3 downregulation does not increase the rate of TCR-CD3 internalization but rather a lack of recycling to the cell surface (Figure 6) (72). Based on this data, it was proposed that the ligand-induced internalization pathway is identical to the constitutive TCR-CD3 internalization pathway and therefore absolutely dependent on a functional constitutive cycling of the TCR-CD3.
Figure 6. **Ligand induced pathway for TCR-CD3 downmodulation.** Antigen interaction with the receptor complex induces tyrosine phosphorylation of the ITAMs, downmodulation, and degradation.
1.6 The role of CD3γ in TCR-CD3 internalization

The key role CD3γ plays in TCR-CD3 internalization is demonstrated by two lines of evidence: first, CD3γ contains functional di-leucine and tyrosine-based motifs in its cytoplasmic tail (88), and second, PKC induced TCR-CD3 downregulation depends upon the presence of the CD3γ di-leucine motif together with the phosphoserine residue at position 126 (89). Phorbol esters, TCR ligands (including antigen-MHC complexes), bacterial toxin superantigens or anti-TCR mAbs all induce TCR-CD3 downregulation (90, 91) via serine phosphorylation of the CD3γ cytoplasmic tail (92, 93). Together these observations demonstrate that TCR-CD3 downregulation, induced by phorbol esters or TCR ligands, occurs through a mechanism involving PKC activation and phosphorylation of CD3γ.

1.7 Control of TCR-CD3 gene expression

In the late 1980's and early 1990's, a number of laboratories were intensely studying transcriptional regulation of the TCR-CD3 subunits, in order to better define the mechanisms that control TCR-CD3 assembly, surface expression, downregulation, and signaling. Activation of T cells, after antigen recognition, induces an alteration of CD3 gene expression (94, 95), which may control TCR-CD3 downregulation and its later re-expression on the cell surface. Studies of TCR-CD3 transcriptional control lead to the identification of promoter and enhancer regions for the TCRα (96, 97), TCRβ (98, 99), TCRγ (100), and TCRδ (101) genes and the CD3δ (102), CD3ε (103), and CD3ζ (95, 104) genes. Despite its highly homology with the CD3δ gene (105, 106) and experimental efforts by investigators in the late 1980's and early 1990's, functional promoter and enhancer regions for the CD3γ gene still have not been identified. A DNase I hypersensitive site was located approximately 100 bp upstream from CD3γ exon 1 (107, 108) and designated as the putative CD3γ promoter, although no functional analysis was performed. Therefore, due to the high sequence homology between the CD3γ and CD3δ genes and the small intergenic region between their transcription initiation sites, it was concluded that they are both controlled by a common regulatory element.
Figure 7. Mechanisms of transcriptional initiation. A, Activation of a prototype gene and assembly of the pol II preinitiation complex. B, sequence elements in a typical promoter. C, the sequence element in a TATA-less promoter.
The CD3γ, CD3δ, and CD3ε genes have several common features including the lack of a TATA or CAAT box immediately upstream from their transcription initiation sites, their location on human chromosome 11q23, and their high homology resulting from a common ancestral gene. The CD3γ and CD3δ genes both have multiple transcription initiation sites [three in CD3γ (23) and two in CD3δ (109)]. A recent study (110) has shown that the mouse CD3δ gene has a T cell specific promoter located from −401 to +48 and contains two “initiator-like” elements, one which has been found to play an important role in its transcriptional regulation (110). The CD3ε promoter is located from −228 to +100 but it is not specific to T cells. Earlier studies located T cell specific enhancers at 0.6 kb 3' from the polyA site for the CD3δ gene and 12 kb from the promoter in the CD3ε gene.

1.8 Eukaryotic promoter and initiator elements

Gene expression in eukaryotes is generally controlled by the binding of a transcription factor complex to the TATA or CAAT box leading to the initiation of transcription. Less frequently, the initiation of transcription by RNA polymerase II can be mediated by an initiator element (Inr) in the promoter (Figure 7). TATA-dependent transcriptional mechanisms are relatively well characterized: the first step involves the binding of transcription factor IID (TFIID) to an A/T rich sequence located 25-30 bp upstream from the transcription start site followed by the formation of a pre-initiation complex containing RNA polymerase II (111). In contrast, the mechanism of transcriptional initiation through initiator regions is less well understood. Initiator elements are found in both TATA-containing and TATA-less promoters, and in general encompass the transcription start site (112). The initiator site alone is sufficient to direct transcription, but transcription is enhanced if an upstream TATA box or Sp1 binding site is present (113, 114, 115). Although the sequence requirements of initiator sites vary, they loosely adhere to a 5'-Py Py A+1 N T/A Py Py-3' consensus, where Py represents a pyrimidine (116). The relative strength of the initiator site appears to be enhanced by the presence of further pyrimidine residues surrounding the core consensus site (116, 117). A wide range of proteins have been shown to bind to initiator-like sites and include TFIID (118, 119, 120), RNA polymerase II (113), Ying Yang A
(YY1) (121, 122), Upstream Stimulating Factor (USF) (123, 124, 125), Sp1 (121), TFII-I (124, 126), E2F-1 (127), GA-binding proteins (GABP) (128), c-myc (129, 130), and various members of the TAFII family (131, 132). It is still not clear, however, which factors influence the binding of these different factors to initiator sites. For example, the mouse CD3ζ promoter lacks a classical TATA or CAAT box and has three highly conserved regions referred to as CR1, CR2, and CR3. The CD3ζ CR3 region contains two “initiator-like” sites (110), and the second of these motifs is adjacent to the first transcription initiation site and has recently been shown to bind TFII-I (110). Potential initiator sequences were located in the murine CD3ζ gene (95) but no functional studies have been performed.

2. Transcription factors implicated in the regulation of HIV-1 and CD3y gene expression

2.1 Transcriptional regulation of HIV-1

T cell activation leads to the induction of many genes that are known to play an important role in the immune response. The activation of specific transcription factors is responsible for the initiation of immune response genes. After HIV-1 infection, the integrated provirus can stay in a latent state until the infected T cell is activated (133, 134). HIV transcription has been linked to T cell activation based on an overlap between signals that activate lymphokine gene expression with those that transactivate the viral LTR (long terminal repeat) (135, 136). Thus, both the cellular response to antigen activation and virus production can be induced by similarities between the architecture of the viral LTR and the immune response genes (Figure 8).

The HIV-1 LTR contains binding sites for several mammalian transcription factors, including NFAT (137), NF-κB (138, 139, 140) and Sp1 (141, 142, 143). The activity of NF-κB can be enhanced through synergism with Sp1, which binds to three sites adjacent to the NF-κB binding sites in the LTR. Several groups have investigated the potential role of NFAT in HIV-1 replication and the interaction between Tat and NFAT, and concluded that different members of the NFAT family of transcription factors may have distinct effects on HIV-1
Figure 8. Transcription factors implicated in HIV expression. The HIV-1 core promoter is composed of the initiator and TATA box flanked by three Sp1, two NF-κB binding sites and the downstream TAR RNA structure which recruits the positive transcription elongation factor b [cyclin T1 (CycT1) plus cyclin-dependent kinase 9 (CDK9)] and the viral transactivator Tat (370).
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 binding sites (144). Recent studies suggest that virally induced immune suppression could be due, in part, to the interaction of Tat with several cellular transcription factors including Oct, Sp1 and NFAT (145, 146), (146) as well as through indirect effects on the transcriptional activity of NF-κB and AP-1 (147). The following section will discuss these specific transcription factors in greater detail.

2.2 The NFAT family

Stimulation of the TCR-CD3 complex with antigen/MHC leads to activation of the tyrosine kinases (Lck, ZAP-70, etc...), necessary for mobilizing calcium and inducing the translocation of NFAT. The activity of NFAT transcription factors is controlled by shuttling the protein between the cytoplasm and the nucleus. NFAT in the nucleus plays a critical role in the coordinate induction of cytokines, including Interleukin 2 (IL-2), IL-3, IL-4, IL-5, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon gamma (IFN-γ), and tumor necrosis factor (TNF)-α as well as cell-surface receptors, such as CD40L, CTLA-4, and FasL (148, 149, 150, 151, 152, 153, 154, 155, 156). Five genes encoding NFAT family proteins have been identified thus far and they are designated NFATc1 (NFATc or NFAT2), NFATc2 (NFATp or NFAT1), NFATc3 (NFAT4 or NFATx), NFATc4 (NFAT3), and NFAT5 (TonEBP) (Figure 9). These proteins have high homology in their DNA binding domains, which in turn are related to the DNA binding domains of Rel/NF-κB family proteins (11, 157, 158, 159). NFATc1 and NFATc2 can be alternatively spliced to produce different isoforms (151, 160, 161) (Figure 8). NFAT proteins are expressed in many cell types, both within and outside the immune system, where they regulate a wide variety of putative target genes (11, 162, 163). Cyclosporin A and FK506 are drugs whose mode of action prevents the calcium-dependent nuclear translocation of NFAT transcription factors (164). Only NFAT5 is not constitutively nuclear or subject to regulation by calcineurin (11, 68, 70, 151, 161, 165, 166, 167, 168, 169, 170, 171).
Figure 9. **NFAT family and their isoforms.** Schematic representations showing different isoforms for each member. The number of amino acid residues is indicated for each protein as well as the RSD, NLS, and TAD (371).
2.2.1 NFATc1 (NFAT2)

NFATc1 was first purified from bovine thymus and subsequently cloned from a Jurkat T cell cDNA library (70). A second isoform, referred to as NFATc.β (160) can be produced by alternative splicing of the gene. NFATc.β differs from NFATc1 in both its amino and carboxy terminal sequences (160) (Figure 9). NFATc1 is expressed at particularly high levels in peripheral lymphocytes, and mice defective for NFATc1 have been shown to be mildly impaired for lymphocyte proliferation and production of IL-4 (156, 172).

2.2.2 NFATc2 (NFAT1)

NFATc2 was first cloned from a murine T cell cDNA library (167), and there are three splice variants in mice and two in humans (151). NFATc2 mRNA is expressed in several different tissues (heart, testis, brain, placenta, muscle, thymus and spleen) (68, 161, 166); however, NFATc2 proteins have a narrow tissue distribution and are found only in the spleen, the thymus and some regions of the murine central nervous system (165, 173). Within the immune system, NFATc2 proteins are present in T cells, B cells, natural killer (NK) cells, mast cells, monocytes, and macrophages but not in neutrophils (173). When over-expressed in the Jurkat T cell line, NFATc2 enhances transcription from the IL-2, IL-4, TNF-α and GM-CSF gene promoters (151).

2.2.3 NFATc3 (NFAT4)

NFATc3 was cloned by screening cDNA libraries at low stringency with DNA fragments from the NFATc2 or NFATc1 genes (161, 166, 174). Analogous to NFATc2, four splice variants (referred to as NFATx1, NFATx2, NFATx3 and NFATx4), have been isolated and display differences in their C terminal regions (91). NFATc3 is highly expressed in double positive thymocytes (154). Mice lacking NFATc3 have defects in positive thymocyte selection (due to increased apoptosis of the double positive thymocytes), T cell hyperactivation and impaired expression of bcl-2 (154).
2.2.4 NFATc4 (NFAT3)

NFATc4 was cloned by hybridization from a Jurkat cDNA library, and is mainly expressed outside the immune system (161). NFATc4 expression has been found to be high in the placenta, lung, kidney, testis, and ovarian tissue, very weak in the spleen and thymus, and undetectable in peripheral blood leukocytes (161). Mice with mutations in NFATc4 are viable and develop normally with no major macroscopic or microscopic abnormalities with a loss of fertility (153).

2.2.5 NFAT5 (TonEBP or NFATz)

NFAT5/TonEBP was originally cloned by a yeast one-hybrid expression cloning screen that used a consensus hypertonicity-responsive DNA-binding site found in the transcriptional regulatory regions of several osmocompensatory genes (therefore this protein was originally designated tonicity enhancer binding protein or TonEBP (169)). NFAT5/TonEBP is a member of the NFAT/Rel family, but it has several specific features that differentiate it from the classical NFATc1-c4 proteins (also called the NFATc family) (168). First, the DNA binding domain of NFAT5/TonEBP does not support cooperative interactions with the AP-1 proteins Fos and Jun (discussed in detail below). Second, the NFAT homology domain responsible for calcium/calcineurin-dependent nuclear localization is not present in NFAT5/TonEBP. NFAT5/TonEBP is the only known mammalian transcription factor that is activated in response to hypertonicity. The following section will focus on the NFATc family excluding NFAT5/TonEBP.

2.3 Cooperative binding with other transcription factors

The specificity of NFAT-dependent gene expression is regulated through its cooperative binding with other transcription factors, including AP-1, Oct1, GATA, Sp1, MEF2, and C/EBP (175, 176, 177, 178, 179, 180). The best-characterized cooperative partner for NFAT is AP-1 (Fos/Jun), and composite NFAT/AP-1 sites have been described in the promoter/enhancer regions of many immune response genes, such as IL-2, IL-3, IL-4 and GM-CSF (142, 149, 181, 182). The IL-2 promoter contains four NFAT binding sites, two of which are composite NFAT/AP-1 binding elements (181, 183). Cooperative binding of NFAT with AP-1
is required for IL-2 gene expression as demonstrated by the use of mutant NFAT proteins that could bind DNA normally but were unable to interact with Fos-Jun or Jun-Jun dimers (184). Other cytokine genes such as those for TNFα and IL-13 apparently have no such requirement.

2.4 Functional domains of NFAT proteins

The NFAT family shares three functional domains: the Rel-similarity domain (RSD), which is responsible for DNA-binding activity as well as the interaction with AP-1; the NFAT-homology region (NHR), which controls intracellular location; and the transcriptional-activation domains (TADs).

2.4.1 The Rel-Similarity Domain

The most well known domain of the NFAT proteins is a region of 290 highly conserved amino acids (~65% identity by pairwise alignment) found in all family members. This region is called the Rel-similarity domain because it shows strong structural similarity with the DNA-binding domains of the Rel/NF-κB transcription factor family (Figure 10) (157, 159, 185). The RSD can be divided into two subdomains: the first containing 190 amino-terminal residues, which are the most conserved (~70% identity), and the second consists of 100 carboxy-terminal residues that are less well conserved (~55% homology) (186). Interestingly, each of these subdomains make a separate protein fold, and the two RSD domains along with the structure of the Rel protein p50 (187, 188, 189, 190) are folded like the Ig superfamily.

The NFAT consensus sequence 5'-GGAAA-3' was derived from the alignment of NFAT binding sites in several different promoters (11). In contrast to the Rel/NF-κB proteins, which bind to the DNA as dimers, the NFAT proteins are able to bind DNA as monomers (161) and have been referred to as monomeric Rel proteins (191). Furthermore, the first Ig-like domain of the NFAT RSD appears to be sufficient for DNA binding specificity and interaction with AP-1, which has been shown both for NFATc2 and NFATc1 (157, 158). The Ig-like domain is marked by the presence of two loops that directly participate in DNA recognition (158). One of these loops, referred to as the DNA-binding loop (DBL),
Figure 10. Alignment of the NFATc2, NFAT5 and NF-κB proteins showing the Rel similarity domain. NFAT and NF-κB proteins show a strong structural similarity in the Rel-homology domain (RHD), which contains a nuclear-localization sequence and a DNA binding domain. In addition, the NF-κB RHD contains a dimerization domain.
contacts the DNA in the major groove of the 5' half site of the NFAT recognition sequence (158). The second loop, referred to as the Rel-insert region (RIR) appears to contact the minor groove of the 3' half site (5'-GGAAA-3') (192).

The function of the second Ig-like domain in the NFAT RSD is not yet clear. The second Ig-like domain in the subunit of NF-κB plays a role in its dimerization (189, 190); however, an analogous role is not obvious for NFAT proteins because there is no evidence that they form dimers in solution. Furthermore, the first NFAT Ig-like domain appears to be sufficient for both DNA binding and cooperation with AP-1 (157, 158). It is thus likely that the second Ig-like domain provides more stability for the NFAT structure and additional surfaces for interaction with other proteins.

In the IL-2 promoter, the distal NFAT binding site, referred to as ARRE-2, is clearly bound with higher affinity by NFATc2 and NFATc1 than by NFATc3 (193). However, the significance of this difference for cytokine expression is still not clear due to conflicting data. First, despite the observed affinity differences for the ARRE-2 site, over-expression of any of the NFAT proteins was found to enhance transcription driven by the IL-2 promoter or multimers of the ARRE-2 site (70, 89, 151, 161). Second, many promoters (IL-2, IL-4, IL-5, TNF-α, and CD40L) and enhancers (GM-CSF and IL-3) with NFAT binding sites tend to have multiple versions of this motif (11, 157, 180, 194). A systematic study to determine which NFAT protein binds to a given NFAT motif has not been performed, and it is quite possible that different members of this family act differentially on the same promoter by binding to alternate sites (180) or by competing for the same site, similar to the differential binding observed in the HIV LTR. Third, gene expression is generally controlled by the concerted action of several transcription factors working on multiple cis-acting elements (195, 196, 197). Thus, other factors likely come into play and directly or indirectly influence the affinity of NFAT proteins for specific sites.

2.4.2 The NFAT-Homology region

Each NFAT protein contains an amino terminal sequence of about 400 amino acids preceded by the RSD (Figure 11). This sequence can be subdivided into an amino-terminal region of 100 residues of low conservation and a serine/proline-
Figure 11. **Functional domain of NFAT proteins.** Schematic representation of the NFAT subdomains. The N- and C-terminal transactivation domains (TAD or AD) are labeled. The eight conserved motifs in the NHR are indicated with small grey boxes. The DNA-binding loop (DBL) and the Rel-insert region (RIR) loops in the RSD domain are shown.
rich region of about 300 residues with even less homology (~35% identity by pairwise alignment), called the NFAT-homology region (NHR). The NHR plays an important role in controlling the intracellular location of NFAT proteins (198, 199, 200, 201). NFAT proteins are translocated from the cytoplasm to the nucleus in response to Ca\(^{2+}\) mobilization and are returned to the cytoplasm when the Ca\(^{2+}\) signal is terminated (11, 69). Deletion of the NHR in NFATc2 or NFATc3 has been shown to produce proteins that remain resident in the nucleus and thus continuously activate transcription in the absence of calcium mobilization (198, 202).

Alignment of the NFAT NHR sequences revealed the presence of eight small motifs composed of conserved residues (151, 198). One of these motifs encodes a highly basic nuclear localization signal (NLS), and mutations of this motif in NFATc1 or NFATc2 reduces nuclear translocation (199, 200). An additional NLS sequence is present in the RSD but it is less efficient than that of the NLS in the NHR (198, 199, 200). Mutating the RSD NLS in NFATc1 has no effect on its nuclear translocation in response to Ca\(^{2+}\) mobilization; however, when both the RSD NLS and the NHR NLS are mutated, NFATc1 remains in the cytoplasm.

In addition to the NLS motifs, the NHR contains a conserved serine-rich region [SRR; also called the calcineurin-regulated-inhibitory (CRI) sequence], three copies of a serine proline (SP) box, and three currently uncharacterized motifs (Figure 11) (151, 166, 198, 199). Mutation of the serines in the NFATc1 SRR motif or deletion of the NFATc3 SRR motif, produce resident nuclear proteins (198, 199). Thus, the SRR motif negatively regulates nuclear translocation by masking the NLS. The majority of serines in the NFATc1 SRR motif have been shown to be constitutively phosphorylated and mediate specific intramolecular interactions with residues in the NLS of the RSD (199). Thus, most likely, the mechanism for NLS masking is that the phosphorylated serines position themselves close to the NLS and neutralize its basic character (11, 198, 199, 201).

2.4.3 Transcriptional-activation domains

Transcriptional activation domains (TAD) have been mapped to both the amino- and carboxy-terminal regions of the different NFAT proteins (202, 203).
For example, two different TADs have been identified in NFATc2 (202). One is located in the first 100 amino acids of the amino terminus (a region rich in acidic and proline residues (11)), and the other in the last 200 amino acids of the carboxy terminus. Both domains are functional when fused to the DNA-binding domain of the yeast GAL4 protein and tested using a reporter plasmid whose activity is dependent on GAL4-binding sites (202). There are several additional mechanisms whereby NFAT proteins enhance transcription. NFAT proteins can recruit the binding of the AP-1 transcription factors Fos and Jun to the NFAT/AP-1-binding site (202). AP-1 then mediates the induction of transcription by recruiting co-activators such as CBP (CREB-binding protein), p300 and JAB1 (Jun activation domain binding protein), through their transcriptional activation domains (204). In addition, it has been shown that NFAT can directly recruit CBP/p300 to its amino and carboxy terminal region where the TADs are located. These co-activators increase transcriptional activity by recruiting the basal transcription machinery through direct protein-protein interactions and by directly acetylating histones, which increases accessibility of nucleosomal DNA to transcription factors (197).

2.5 Cyclosporin A acts on the NFAT pathway

Cyclosporin A is a neutral lipophilic cyclic undecapeptide isolated from the fungus Hypocladium inflatum gams, that has been widely used since 1976 for the treatment of allograft rejection because of its immunosuppressive activity (204). In 1982, studies demonstrated that CsA acts by blocking cytokine gene transcription, including IL-2 and IL-4, and thus leads to an inhibition of T cell activation (204, 205, 206, 207). CsA binds with high affinity to the most abundant cyclophilin (cyclophilin A, 17 kDa) present in the T cell cytoplasm and inhibits its activity (208) (Figure 12)(209). Cyclophilins are cytoplasmic proteins with peptidyl-proline-cis-trans isomerase (PPIase) activity, which play an important role in protein folding (210). However, inhibition of PPIase activity shows that this activity is not implicated in the mechanism of immunosuppression because some CsA analogs can inhibit the PPIase activity without blocking T cell activation (211, 212).
Figure 12. **Mode of action of Cyclosporin A.** Receptor stimulation induces an increase in intracellular calcium leading to the activation of calcineurin which directly dephosphorylates NFAT proteins. This dephosphorylation promotes nuclear translocation of NFAT, where it stimulates the expression of different cytokines including IL-2 (372).
In 1991 Liu, et al. (211) demonstrated that the cyclophilin-CsA complex can associate with another cytoplasmic protein, belonging to the serine/threonine phosphatase family, called calcineurin. Calcineurin (also termed PP2B) is regulated by Ca\(^{2+}\)/calmodulin (213, 214, 215), and it is composed of two subunits, a catalytic subunit called calcineurin A (CnA) and a regulatory subunit called calcineurin B (CnB). Activation of the TCR-CD3 receptor with antigen-MHC complexes induces an increase in the intracellular concentration of calcium, leading to the activation of calmodulin. Activated calmodulin can then interact with CnA and thereby activate its phosphatase activity. The cyclophilin-CsA complex binds directly to the CnA subunit and thus inhibits its phosphatase activity. CsA does not inhibit certain Ca\(^{2+}\) independent T cell activation pathways, including stimulation through CD28 in the presence of PMA (216).

After activation by calmodulin, calcineurin dephosphorylates the NFAT family members, allowing their translocation to the nucleus to activate genes controlled by NFAT (70, 164, 217, 218, 219). Recent studies have shown that activated calcineurin also translocates to the nucleus with NFAT family members, where it may maintain their nuclear localization (201). By preventing calcineurin-mediated dephosphorylation of NFAT proteins, CsA inhibits their nuclear translocation and subsequent activation of gene expression (Figure 12) (11). This inhibition of the calcineurin/NFAT pathway is one important mechanism of CsA-mediated immunosuppression. It has also been shown that the HIV-1 Gag protein can bind cyclophilin in vitro, and that the binding of CsA to cyclophilin A prevents Gag incorporation in the virion, thereby blocking virus assembly. However, recent evidence suggests that CsA may also inhibit HIV-1 gene expression by blocking NFAT nuclear translocation.

2.6 NFAT activation in HIV-1

A variety of cellular transcription factors bind to the HIV-1 LTR and play an important role in regulating HIV-1 gene expression. The NFAT family of transcription factors is among those known to bind, with five NFAT motifs present in the LTR. Several groups have investigated the role of these NFAT binding sites and they found that different NFAT transcription factors have distinct effects on HIV-1 replication. The two NFAT sites present in the NRE were shown to play
Figure 13. Regulation of NF-κB activation by IκB. T cell activation results in the activation of IκB Kinase (IKK) complexes (composed of IKK1, IKK2 and NEMO), which phosphorylate IκB and lead to its degradation. NF-κB free from IκB can translocate to the nucleus where it activates the transcription of genes containing the NF-κB consensus sequence including that of its inhibitor IκBα (373).
a negative role in HIV-1 gene expression; however, the precise NFAT proteins involved were not determined. Two additional NFAT motifs are located within the adjacent NF-κB binding sites and have been shown to bind NFATc2 in the presence of high levels of Tat (146). NFATc2 binding was found to negatively regulate the LTR by competitively binding to the NF-κB for binding sites, whereas has been shown to NFATc1 positively regulate the HIV-1 LTR via the NF-κB binding sites (144). The fifth NFAT binding site is located downstream from the first transcription site and may play a positive role in the expression of the HIV-1 (220).

3. The NF-κB family of transcription factors

The NF-κB/IκB family of transcriptional regulators are known to promote the expression of well over 100 target genes, the majority of which participate in the immune response (221). The NF-κB responsive genes include numerous cytokine and chemokine genes, as well as immune recognition receptors and proteins involved in antigen presentation. NF-κB proteins are vital transcriptional factors for lymphocyte activation and the generation of normal immune responses.

3.1 The biochemistry of NF-κB activation

In response to a variety of stimuli, including viral and bacterial pathogens, cytokines, and stress-inducing agents (222), the quiescent cytoplasmic NF-κB/IκB complex is activated by phosphorylation. IκBα is modified at serines 32 and 36 by the IκB kinase (IκK) complex. Phosphorylation targets IκBα for ubiquitination at lysine residues 21 and 22, as a result of which this inhibitory subunit is degraded by the 26S proteasome. NF-κB free from IκB can translocate to the nucleus where it activates the transcription of genes containing the consensus sequence 5'-GGGRNYYCC-3' (Figure 13). The NF-κB family includes five structurally related proteins (c-Rel, RelA, RelB, p50/p105, and p52/p100) that share a 300 amino acid NH2-terminal Rel homology domain.
containing the sequences essential for dimerization, DNA binding, and nuclear transport. The family members c-Rel, RelA, and RelB possess COOH-terminal transactivation domains. In contrast, p50/p105 and p52/p100 lack this transactivation domain but they can bind to the NF-κB consensus. All of the NF-κB proteins, except RelB, can form homo- or heterodimers with other NF-κB proteins. Depending on which homo- and heterodimers are bound to the NF-κB site, a gene can be positively or negatively regulated. For example, the heterodimer p65/p50 is an activator, whereas the homodimer p50/p50 is a transcriptional repressor (223).

3.2 NF-κB activation in HIV-1 infection

The promoter region in the HIV-1 LTR contains two NF-κB binding sites (located at -109 to -79 from the transcription start site) that play a central role in proviral activation. The HIV motifs are recognized by different combinations of NF-κB proteins, including p65/p50 and p52/p65 heterodimers and p50 and p65 homodimers (224, 225). Mutation of the NF-κB binding sites or IκBα blocks the induction of NF-κB and inhibits de novo infection in T cells by interfering with viral transcription (226, 227). Differences in the molecular organization of HIV-1 subtypes appear to influence the HIV-1 enhancer and thereby viral gene expression and replication. For example, the presence of one NF-κB binding site in the enhancer region of HIV-1 clade E (the predominant subtype in Asia) reduces activation in response to TNF-α (228); whereas, the presence of three NF-κB binding sites in the HIV-1 LTR of clade C (the predominant subtype in Africa) results in higher levels of transactivation than the HIV-1 LTRs from other subtypes. Unexpectedly, clade E, with one NF-κB binding site, has higher transmission efficiency than the North American clade B, which contains two NF-κB binding sites. The explanation for this contradiction is the presence of a single nucleotide deletion in the upstream region of the NF-κB binding site in clade E. This deletion creates a binding site for the GABPα and GABPβ1 transcriptional factors. When the GABP binding site is introduced into the LTR of a clade B virus, the resulting virus replicates better than the wild-type (229).
Figure 14. Structural motifs of Sp factors. Schematic representation of the four Sp family members with the yellow boxes indicating serine/threonine rich regions, red boxes indicating glutamine rich regions, black boxes representing zinc fingers, and the ± indicating highly charged regions.
Binding to the NF-κB motif in the HIV-LTR can be enhanced by the binding of Sp1 to the adjacent sites (224, 230). In addition, several studies have demonstrated that Sp1 and Tat can form a protein-protein interaction in the HIV promoter and influence the expression of HIV. Sp1 belongs to family of transcriptional factors that play an important role in the initiation of transcription.

4. The SP protein family

Proteins with a zinc finger motif constitute a significant proportion of the transcription factor superfamily, and include the Sp and Sp1-like/KLF (specificity protein/Krüppel like) factors (231, 232, 233, 234, 235, 236, 237). The Sp1 and Sp1-like/KLF family bind to various GC-box DNA elements and regulate transcription. The following section will review only the Sp family, which includes Sp1, Sp2, Sp3 and Sp4.

Members of this transcription factor family bind to GC or GT rich motifs commonly found in many gene promoters, including a number of housekeeping genes, which initially led to the notion that Sp1 acts as a basal transcription factor. However, this is not the case and other studies have found that Sp1 can be post-translationally modified by glycosylation, phosphorylation and acetylation. All four Sp proteins contain three zinc fingers at their C-terminal region, which represents the DNA binding domain and is the most conserved part of the protein (Figure 14). Sp1, Sp3, and Sp4 bind with similar affinities to the same consensus DNA site (GC-box) (238, 239, 240, 241), while Sp2 binds to a (GT-box). In addition, it has been shown that Sp3 can function as a competitive repressor of Sp1-induced transcription (239); however, this is not always the case since Sp3 can also act as a transcriptional activator (237). These different effects are still not clearly understood, but a change in the ratio of Sp1 to Sp3 seems to be critical in the differential regulation of gene transcription (237).
4.1. Functional properties of individual Sp-family proteins

4.1.1. Sp1: the family prototype

Sp1 is a 95 to 105 kDa protein that is ubiquitously expressed in mammalian cells. Sp1 contains different functional domains: 1) two major glutamine-rich transactivation domains A and B (Figure 14) that are essential for transcriptional activation (242); 2) located next to the A and B domains are serine/threonine-rich sequences that may be a target for post-translational modification; 3) an inhibitory domain present at the N-terminal region (Figure 14) (243); 4) a highly charged domain, immediately 5' of the zinc fingers, plays a role in stimulating transcription; and 5) a short C-terminal domain (called D) (Figure 14) required for synergistic activation of the promoter by Sp1 through multiple GC-boxes. Sp1 can also form multimeric complexes by interacting with different classes of nuclear proteins. These include factors belonging to the general transcription machinery, such as the TATA-box binding protein (TBP) (244), the TBP-associated factors dTAFII110/hTAFII130 (245, 246), which interact with the glutamine-rich activation domains of Sp1 (A and B), and hTAFII55 that interacts through the C-terminal domain. Sp1 has also been shown to interact with other transcription factors such as YY1 (247, 248), E2F (249, 250) and the cell cycle regulator p107 (retinoblastoma-related protein) (251).

4.1.2. Sp2

The binding site specificity of Sp2 differs from that of the other Sp proteins, explaining its inability to activate promoters containing GC boxes (252, 253, 254). Sp2 also has other characteristics that are different from Sp1, Sp3, and Sp4, including only one glutamine-rich transactivation domain, whereas two domains are required for superactivation and synergistic activation by Sp1 (255). The TCRα gene is thought to be a target for Sp2 since it has been shown to bind to a GT-box element in its promoter in vitro (238).

4.1.3. Sp3

Studies on the transcriptional properties of Sp3 appear to be contradictory. Sp3 has been shown to act as a transcriptional activator similar to Sp1 (256, 257,
258, 259) and activate a variety of promoters from *Drosophila* SL2 cells to mammalian cell lines (256, 258, 260, 261). Furthermore, synergy between Sp3 and Sp1 were observed in co-transfection studies (258, 262, 263, 264). Alternatively, in promoters containing multiple adjacent binding sites, Sp3 can repress transcription driven by Sp1 or other transcription factors (265, 266, 267). Finally, it has been shown that Sp1 can inhibit Sp3-mediated transactivation of the mouse growth hormone L2 promoter (268).

4.1.4. Sp4

Like Sp3 the functional properties of Sp4 are also quite different from those of Sp1, despite their structural similarities. The transactivation function of Sp4 resides in the N-terminal glutamine-rich region (like in Sp1) and it can be activated by Sp1 or repressed by Sp3 (269).

5. Retroviruses

Human immunodeficiency virus, the etiologic agent of acquired immunodeficiency syndrome (AIDS), is a lentivirus that can be considered a prototype for complex retroviruses. Two related but distinct human lentiviruses, HIV-1 and HIV-2, have been identified. HIV-1 has a 9.4 kb RNA genome (its size varies depending upon the viral variant) and encodes 16 distinct proteins (270) (Figure 15). Three of these proteins [Gag, (group-specific antigen), Pol (polymerase), and Env (envelope)] are synthesized as polypeptides, which are subsequently proteolyzed by the viral protease into several individual proteins. The six Gag proteins, matrix (MA), capsid (CA), nucleocapsid (NC), p6, p2, and p1, and the two Env proteins, surface or gp120 (SU) and transmembrane or gp41 (TM), are structural components that make up the core and the outer membrane envelope of the virion, respectively. The three Pol proteins, protease (PR), reverse transcriptase (RT), and integrase (IN) provide essential enzymatic functions and are encapsulated in the virus particle. HIV encodes up to six additional proteins, often referred to as accessory proteins, including the transcriptional transactivator (Tat), the regulator of virion gene expression (Rev), the originally named negative
Figure 15. **Organization of HIV genome.** A, schematic representation of HIV transcripts. Start codons are indicated and the black lines mark unspliced and spliced transcripts. B, schematic representation of HIV proteins showing the precursors Gag and Gag-Pol and the different proteins resulting from its processing by the viral protease. Env is cleaved by cellular proteases into gp120 and gp41. The figure also shows the accessory proteins Tat, Nef, Rev, Vpr, Vpu and Vif (370).
effector (Nef), the viral proteins R (Vpr) and U (Vpu), and the viral infectivity factor (Vif).

5.1 The viral accessory proteins

5.1.1 The Vpr, Vpu and Vif proteins

HIV-1 Vpr is a 14 kDa protein that plays different roles in the virus life cycle. Vpr has been shown to be involved in G2 cell cycle arrest (271, 272, 273), and to play a role in nuclear localization and import of the preintegration complex. A recent study has demonstrated that Vpr can transactivate the HIV-1 LTR through the glucocorticoid response element (274). In addition, Vpr has been shown to induce the transcriptional activity of other heterologous promoters.

Vpu is a 16 kDa protein that plays an important role in CD4 degradation and has also been found to stimulate virion release.

HIV-1 Vif is a 23 kDa protein that has been found to be important for the production of highly infectious virions.

5.1.2 The Nef protein

Nef is a 25 to 34 kDa myristylated protein that has been shown to be a major determinant of virus pathogenicity. Some HIV-1 infected individuals, classified as long-term nonprogressors, have been shown to be infected with nef defective viruses (275, 276). The molecular mechanisms that underlie the pathogenic effect of Nef are still not clear, but Nef has been shown to interact with several cellular signaling proteins (277). A recently described function of Nef is its ability to activate the calcium/calcineurin-signaling pathway and consequently NFAT (278) by interacting with the inositol trisphosphate receptor type-1 (279).

HIV-1, HIV-2, and SIV Nef all have a number of conserved and clearly demonstrated activities including the downmodulation of CD4 (280, 281, 282, 283) and MHC I surface expression (284, 285), the enhancement of virion infectivity (286, 287) and interference with signal transduction and protein trafficking pathways (277, 288). Nef has also been found to induce a state of activation in the infected host cell (289, 290) similar to that observed in uninfected CD3-stimulated T cells (291). Nef's presence in membrane lipid rafts is required
for Nef-mediated hyperinduction of NF-κB, NFAT, IL-2, and the HIV-1 LTR following T cell stimulation via CD3 and CD28 (292). The ability of Nef to downmodulate cell surface receptors is not limited to CD4 and MHC I molecules, since HIV and SIV Nef have been shown to associate with the CD3ζ chain and induce TCR-CD3 downmodulation (293, 294, 295). Many groups have also shown that Nef can downmodulate cell surface levels of the T cell costimulatory receptor CD28 (296, 297).

5.1.3 The Rev protein

HIV-1 Rev contains three distinct domains that are important for its function (298, 299, 300, 301): 1) an arginine-rich region or basic domain found near its N-terminus that mediates specific binding to the Rev-responsive element (RRE) and provides the signal for nuclear localization; 2) sequences flanking the arginine-rich region that are essential for Rev oligomerization; and 3) the C-terminal leucine-rich region, known as the nuclear export signal (NES), that allows Rev to shuttle between the nucleus and the cytoplasm and mediates its interaction with host cell factors.

The Rev protein is responsible for the conversion from early to late HIV gene expression in the newly infected cells. Rev mediates the cytoplasmic delivery of singly spliced and unspliced messages, and thus it coordinates the conversion from predominately nef, tat, and rev regulatory gene transcripts (multiply-spliced) to those involved in virion production (unspliced and singly spliced) (302, 303, 304). This occurs through a physical interaction of Rev with the RRE, which is located within the env gene at the 3' end of the HIV RNA (present in unspliced or singly spliced transcripts). Multiple copies of Rev assemble onto the RRE allowing a different region of Rev to associate with the CRM1 nuclear export protein (305, 306, 307), which mediates transcript transport to the cytoplasm. Finally, the association of RNA-free Rev with importin-β in the cytoplasm shuttles Rev back to the nucleus (308).

5.1.4 The Tat protein

The Tat protein of the HIV virus is a small 16 kDa polypeptide that plays an important role in the stimulation of viral transcription elongation (309). Tat acts by
binding to an RNA structure called TAR (transactivation responsive region) located in the end of all nascent transcripts and interacting with different cellular transcriptional factors, including Sp1, NF-κB, and NFAT. Several reports have demonstrated that Tat can be immunosuppressive both for infected cells and uninfected bystander T cells (310). Tat contains an arginine-rich motif required for binding to the bulge in TAR (311). This region of Tat can interact with the catalytic domain of different histone acetyl transferases (HATs) (312, 313, 314, 315, 316, 317). Interaction between Tat and the transcriptional transactivators P/CAF, p300/CBP and GCN5 increase the transactivator potential of Tat on the HIV1-LTR. GCN5 and p300/CBP acetylate Tat on lysines 50 and 51 and reduce its binding affinity for TAR (318) while P/CAF acetylates Tat on lysine 28, which enhances its interaction with the positive transcription elongation factor complex (P-TEFb) (319).

5.2 Tat-TAR mediated transactivation

HIV-1 and HIV-2 TAR function by recruiting a multi-protein complex, which contains the viral protein Tat and a group of cellular proteins that include a kinase complex known as P-TEFb. P-TEFb is composed of the cellular proteins cyclin T1 (CycT1) and cyclin dependent kinase 9 (CDK9) (320, 321). The association of Tat-P-TEFb with TAR leads to phosphorylation of RNA polymerase II (RNAPII) (322) on its carboxyl-terminal domain, and thereby increases several hundred-fold the steady-state levels of all classes of viral RNAs. Tat has also been shown to interact with CDK7 (a carboxy terminal domain kinase) and increase its activity, with inhibition of CDK7 sufficient to block Tat transactivation and HIV-1 replication (323). Neither CycT1 nor the P-TEFb complex can bind TAR RNA in the absence of Tat (320, 324). Mutagenesis studies revealed that amino acids 1-303 of CycT1 were sufficient to form complexes with Tat-TAR and CDK9 (321, 324, 325, 326, 327, 328, 329).

Impairment of the TCR-CD3 pathway is generally accepted as an early event in the progression from HIV-infection to AIDS, with its functional loss frequently described for T cells from HIV-1-seropositive individuals (330, 331, 332, 333, 334). The molecular or cellular basis for this loss of TCR-CD3-regulated immune function is currently unknown; however, in vitro studies have described
suppression of activation by the virally encoded proteins gp120 (335, 336, 337, 338, 339, 340), Nef (283, 341, 342, 343), and Tat (283, 341, 342, 343).

6. TCR-CD3 downmodulation and its role in human disease

Abnormal expression or functioning of the TCR-CD3 receptor on the cell surface is more and more frequently observed in a wide range of human clinical conditions. Defects in TCR-CD3 expression appear to render patients more prone to infection by provoking a mild to significant immunodeficiency. These defects have been reported in healthy aged humans (344), chronic fatigue syndrome (345), and in rare genetic defects characterized by either the loss of ZAP70 expression (346) or an individual receptor chain [reviewed in (347)]. An increasing number of studies have associated a similar defect with T cells isolated from cancer patients, where interference with receptor expression and function led to defective signal transduction [reviewed in (348)]. Moreover, most T cell malignancies (except Sezary syndrome (349) and T cells infiltrating solid tumors have been found to have TCR/CD3 defects. Patient studies, using anti-CD3ε and anti-CD3ζ antibodies, found that CD3ζ but not CD3ε was decreased (350, 351, 352, 353), while a study in mice found CD3ζ absent and CD3γ reduced (354). Although only one study has analyzed mRNA expression, downregulation of CD3γ, CD3δ and CD3ζ but not CD3ε gene transcripts were detected (355).

Infection of CD4+ T cells has also been found to target the expression and function of the TCR-CD3 pathway. Infection with HTLV-I in vitro using T cell clones (356) and in T cells isolated from patients with ATL (357, 358, 359) has shown that TCR-CD3 expression is specifically targeted. This has also been demonstrated for HIV-1 (360) and HIV-2 (361, 362) infected T cell lines and in HIV-1 infected individuals (363, 364, 365). Finally, HHV-6 (366, 367) and HHV-7 (368) infected mature T cell populations have been reported to have a TCR-CD3 expression defect.
Figure 16. Specific loss of CD3γ after infection by HIV. A, The histogram overlays showing the progressive loss, as a function of time, of TCD-CD3 from the cell surface after HIV-1 infection. B, Analysis of the expression of mRNA for TCRα, TCRβ, CD3δ, CD3ε, and CD3γ and CD3ζ by dot blot in uninfected WE17/10 cells (as a positive control), OVK (ovine kidney cell line, as a negative control), and the infected WE/HIV-1, WE/HIV-2, WE/HIV-1_HXB2, and WE/HIV-1_LAI cell lines (360, 369). C, Dot plots showing the percentage of TCR-CD3 and p24 in uninfected untreated WE17/10 or productively infected WE/HIV-1_HXB2 cells treated with Tat-S or Tat-AS.
An interesting aspect of TCR-CD3 receptor downregulation provoked by lymphotropic viruses is that it is achieved through the absence of a generalized suppression of host protein synthesis and thus appears to be receptor specific. Studies done in our laboratory have demonstrated specific downregulation of surface TCR-CD3 complexes due to a transcription defect in CD3γ in HIV-1 (360) or HIV-2 (362) infected T cells. Expression of the other TCR-CD3 chain genes (TCRα, TCRβ, CD3δ, CD3ε and CD3ζ) was not altered. In studies by Ginaldi, et al. (365), quantitative levels of CD3ζ protein expression were correlated with HIV disease progression, and our laboratory has recent experimental evidence demonstrating that decreases in CD3ζ protein levels are consequent to the progressive CD3γ transcription defect. In addition, it has been shown that HTLV-1 dowregulates CD3γ, CD3δ, CD3ε, and CD3ζ gene expression (369), with CD3γ transcripts lost first (unpublished data from our laboratory, H. Akl, et al.), while a decrease in transcripts encoding the CD3γ, CD3δ and CD3ε chains has been reported in HHV-6 and HHV-7 infected T cells (366) (CD3ζ expression was not examined).

7. Role of the viral transactivating proteins in dowregulating the TCR-CD3 complex

Our laboratory has clearly demonstrated that the progressive loss of TCR-CD3 receptor expression and function after HIV-1, HIV-2 or HTLV-I (Figure 16A) infection results from a specific loss of CD3γ gene transcripts, while expression of the other CD3 components remain unchanged. Northern and dot blot hybridization analyses suggested that the loss of CD3γ does not parallel the downregulation of TCR-CD3 complexes from the surface at a ratio of 1:1 (360) (Figure 16B)(362). Experiments in which tat and/or nef gene expression was specifically blocked by treating HIV-1 infected WE17/10 cells with antisense phosphorothioate oligodeoxynucleotides (P-OdN) to these viral genes [Tat-AS (Figure 16C) or Nef-AS] delayed the loss of receptor expression. However, using antisense to block rev gene expression (Ref-AS) accelerated TCR-CD3 downmodulation. RT-PCR was used to quantify the expression of these multiply
spliced HIV-1 transcripts, and Tat-AS and Nef-AS were found to reduce the level of tat, nef, and rev transcripts, whereas Rev-AS increased the level of tat, nef transcripts in infected cells. Thus, when intracellular conditions favor the expression of tat and/or nef in the absence of rev, CD3γ gene transcripts and TCR-CD3 surface density are downmodulated (370). These results suggested that perhaps a common denominator(s) exists between Tat-mediated transcriptional control of HIV-1 and CD3γ gene transcription, and/or Nef's ability to associate with clathrin adaptors and downregulate surface TCR-CD3 complexes. The significance of our antisense experiments is their clear demonstration that the CD3γ gene transcription defect is results from viral regulatory gene expression, and that through specific interference with tat gene expression it is possible to alter the balance in favor of CD3γ gene expression and restore TCR-CD3 complexes on the cell surface.
Chapter II

Specific Aims
Specific Aims

Successful antigen-specific triggering of the TCR-CD3 complex initiates a cascade of molecular events in multiple signaling pathways, which are integrated to induce cytokine gene expression. Engaged receptors are internalized and counted until a necessary minimum threshold has been reached. A sustained signal therefore changes the normal balance in receptor expression, favoring TCR-CD3 internalization and degradation rather than recycling and de novo synthesis. Abnormal downmodulation of TCR-CD3 receptors in clinical conditions would thus disable the T cell specific immune response and favor disease progression.

Data from our laboratory have demonstrated that HIV-1 and HIV-2 specifically interfere with transcription of the human CD3γ gene and thereby affect the T cells ability to form new TCR-CD3 complexes destined for the cell surface (360, 362, 371). Treatment of infected cells with antisense phosphorothiolate oligodeoxynucleotides demonstrated that HIV-1 tat and/or nef gene products play an important role in the loss of CD3γ transcripts after infection (370). Overall, data from our laboratory suggests that a common denominator exists between transcriptional control of HIV-1 and the human CD3γ gene.

The initial goal of this thesis research was to better define the relationship between the number of CD3γ gene transcripts and the density of TCR-CD3 complexes on the HIV-1 infected cell surface using quantitative competitive RT-PCR. The subsequent and major goal of this thesis research was to identify and characterize the human CD3γ promoter and the specific transcription factors complexes involved in regulating its normal expression as foundation for understanding the mechanisms exploited by HIV in regulating this important immune response gene.
Chapter III

Results
Figure 17. Quantitative competitive RT-PCR for CD3γ. Panel A, Ethidium bromide staining of CD3γ RT-PCR products (647 bp) from total RNA extracted from 100% TCR-CD3⁺ uninfected and 100%, 90%, 64%, 25%, and 5% TCR-CD3⁺ HIV-1 infected WE17/10 cells. The RNA was co-amplified with the same stock series of dilutions of the CD3γ competitor (pUC18yc; 1717 bp). Panel B, Graphic representation of CD3γ transcript numbers as estimated by RT-PCR relative to the percentage of TCR-CD3⁺ cells as determined by flow cytometry. Panel C, CD3γ RT-PCR products amplified from total RNA extracted from 100% TCR-CD3⁺ uninfected cells, TCR-CD3⁺ HIV-1 infected cells and the B cell line Raji (negative control). Panel D, CD3δ RT-PCR products (660 bp; using the same cDNAs as in Panel A) and the B cell line Raji (negative control).
Results

1. Further insight into the CD3γ transcription defect in HIV-1 infected cells.

1.1 Quantitative analysis of CD3γ mRNA from uninfected TCR-CD3⁺ and HIV-1 infected cells with downmodulated TCR-CD3 surface complexes

Our previous work, using dot and Northern blot hybridization showed that while the levels of TCRα, TCRβ, CD3ζ, CD3ζ, and CD3ε transcripts remain unchanged CD3γ transcripts are progressively downmodulated after HIV-1 (360, 371) or HIV-2 infection (362). In these studies, measurement of the amount of radioactivity bound to dot blots using the CD3γ probe and mRNA from infected cells expressing different amounts of TCR-CD3 suggested that the loss of CD3γ transcripts does not parallel the downregulation of TCR-CD3 complexes from the surface at a ratio of 1:1 (360, 362). To better define the relationship between the number of CD3γ gene transcripts and the surface density of TCR-CD3 complexes, we used quantitative competitive RT-PCR to examine transcript levels in uninfected and HIV-1 infected WE17/10 cells. RNA was extracted from cells at different stages in the progression from TCR-CD3ʰⁱ → TCR-CD3ʰ⁰ → TCR-CD3⁻ [previously described in (371); the uninfected cells designated as 100% TCR-CD3⁺ are all TCR-CD3ʰⁱ, whereas, the HIV-1 infected cells described as 90% TCR-CD3⁺ (for example) are 10% TCR-CD3⁻ and 90% TCR-CD3ʰ⁰]. cDNA's, reverse transcribed from the native RNA preparation, were co-amplified with serial dilutions of a competitor specific for the human CD3γ gene (pUC18yc), which had been engineered to produce a larger PCR product (Figure 17A, upper band) than the cellular CD3γ RNA (Figure 17A, 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 Figure 17A. In the uninfected 100% TCR-CD3⁺ cells, the competitor was initially detected when 3.3 x 10⁵ molecules were added.
to the reaction mixture, followed by a corresponding decrease in native CD3\text{y} transcripts until they are no longer detectable in the presence of $>6.6 \times 10^8$ molecules of the competitor. The competitor was detected earlier (at $6.6 \times 10^6$ molecules) in RNA amplified from 100% TCR-CD3\text{+} HIV-1 infected cells [the mean fluorescence revealed that these cells were actually 100% TCR-CD3\text{lo} with a receptor density equal to 85% of the uninfected control cells analyzed in parallel], and indicated that these TCR-CD3\text{lo} cells had already lost ±80% of their CD3\text{y} gene transcripts. Amplification of RNA from 90% TCR-CD3\text{+} HIV-1 infected cells initially detected the competitor at a concentration of $1 \times 10^5$ molecules, revealing a further decline equivalent to a total loss of $>90\%$ of CD3\text{y} gene transcripts. This extensive loss of transcripts prior to significant TCR-CD3 downmodulation 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\text{+} (64% is shown in Figure 17A) were competed at essentially the same concentrations as the 90% TCR-CD3\text{+} 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 CD3\text{y} gene transcripts before substantial numbers of TCR-CD3\text{−} 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 CD3\text{y} transcripts (represented graphically in Figure 17B) continues in 25% and 5% TCR-CD3\text{+} cells (Figure 17A) and were completely undetectable in HIV-1 infected TCR-CD3\text{−} cells and the B cell line Raji (Figure 17C). Under the same standardized RT-PCR conditions, transcript levels for the highly homologous CD3\text{z} gene were unchanged in all of the RNA preparations (Figure 17D). These data demonstrate that the loss of CD3\text{y} 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.
Figure 18. Treatment with activators and inhibitors of the TCR-CD3-directed pathway. Histogram overlays showing the distribution of anti-CD3 antibody labelling on uninfected (Panel A) and HIV-1 infected (50% TCR-CD3*) (Panel B) WE17/10 cells before and after treatment with 2.5 M EGTA, 10 μM BAPTA/AM, 10 ng/ml Staurosporine A, 10 ng/ml PMA and 10 ng/ml PMA+30 ng/ml Iono. Panel C, uninfected and HIV-1 infected cells (34% TCR-CD3*) treated for 3 and 7 days with 0.1-1.0 μg/ml CsA. Panel D, uninfected and HIV-1 infected (42% TCR-CD3*) cells treated for 5 days with 0.1 μg/ml CsA. Panel E, CD3y RT-PCR products co-amplified with the CD3y competitor (as described in Figure 1) from HIV-1 infected (85% TCR-CD3*) cells before (top) and after (bottom) CsA treatment (0.1 μg/ml).
1.2 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 surface receptor expression. Uninfected and HIV-1 infected WE17/10 cells at different stages of receptor downmodulation were treated with the PKC activator, PMA and the calcium ionophores, A23187 and ionomycin (which can induce phosphorylation of CD3γ on Ser126 without activation of PKC), a combination of PMA plus ionophore (PMA+lono), 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.

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 is shown in Figure 18. As expected, activation by PMA, PMA+lono, or anti-CD3 resulted in further downmodulation of receptors on TCR-CD3^hi uninfected or TCR/CD3^lo HIV-1 infected cells but had no effect on the TCR-CD3^- infected cells (Figure 18A & B; histograms for anti-CD3 are not shown but were similar to those shown for PMA or PMA+lono). Staurosporine and Herbimycin A had a deleterious effect on cell viability after 48 hours, but no discernable positive or negative effect on receptor surface density after treatment for 18-24 hours where viability was not affected (the histogram profiles shown in Figure 18A & B for Staurosporine are identical to those for Herbimycin A). Cells treated with BAPTA/AM, but not EGTA, exhibited a slight but consistent downmodulation of TCR-CD3 complexes on both uninfected and HIV-1 infected cells, particularly noticeable as an increased number of cells in the TCR-CD3^lo range (Figure 18A & 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
annotated hCD3γ gene sequence. Partial sequence of the 5'-upstream region of the hCD3γ gene (NCBI accession number X06026) corresponding to the 1068 bp fragment cloned to produce the various hCD3γ constructs. The transcription start sites identified by Tunnacliffe, et al. (23) are indicated as +1, +6, and +21. Binding sites for NFAT and Sp are capitalized and indicated in bold face type, while the underlined nucleotide sequences define the NFATγ, NFATγ2, NFATγ3, Spγ1, and Spγ3 probes. The 5' end of the various cloned lengths are indicated with a green box. Forward and reverse primers used to clone the full length CD3γ vector are indicated in a yellow box.
graphically as an increase in the percentage of TCR-CD3⁺ cells after treatment with 0.1-1.0 μg of CsA for three or seven days (Figure 18C), as well as by histograms that illustrate the evolution of cells from the negative to positive phenotype after five days of treatment with 0.1 μg of CsA (Figure 18D). CsA also provoked a slight downmodulation of CD3 density on the surface of uninfected cells (Figure 18D), which was augmented with increased time and drug concentrations (Figure 18C). No cytotoxicity was observed in any of the CsA-treated cell cultures likely due to the fact that WE17/10 cells are grown in the presence of an excess of exogenously added IL-2 (376).

RNA from CsA-treated HIV-1 infected cells (85% TCR-CD3⁺) was analyzed by quantitative competitive RT-PCR in parallel with RNA from the untreated control (Figure 18E). CD3γ transcripts were initially detected at a competitor concentration of 8.3 x 10⁴ for the CsA-treated cells compared to 4.9 x 10⁴ for the untreated cells, which represents an approximate two-fold increase in transcript numbers. Taken together with the FACS data, these results suggest that CsA treatment has a net positive effect on CD3γ gene transcription in HIV-1 infected cells, resulting in the formation of more complete TCR-CD3 complexes that can then be processed to the cell surface. CsA controls nuclear translocation of the NFAT family of transcription factors, which is essential for immune response-directed cytokine gene expression.

The molecular mechanisms exploited by HIV in altering expression of the human CD3γ gene are currently unknown. Therefore, it became apparent that we first must identify and characterize the elements involved in regulating normal expression of the human CD3γ gene. Thus, the majority of this thesis is focused on transcriptional regulation of the human CD3γ gene.
Figure 20. Activity of the human CD3ε promoter in human cell lines. Jurkat, WE17/10, SupT1, Raji, GM607, HeLa, and 293T cells were transiently transfected with the pLuc-782/+286_{WT} construct. Firefly luciferase activities were measured after 40 h of incubation, and normalized to the Renilla luciferase activity from a co-transfected internal control plasmid, pRL-TK vector. Data is expressed as 100 x the value of the firefly luciferase over renilla luciferase value and the standard deviation (SD) bars are shown. The pGL3-Basic vector was used as a negative control and pGL3-promoter vector (pGL3-PV) containing the SV40 promoter was used as a positive control.
2. Transcriptional regulation of the human \( \text{CD3}_y \) gene

2.1 General characteristics of the 5' flanking sequence of the \( \text{hCD3}_y \) gene

The 5' flanking sequence of the \( \text{hCD3}_y \) gene (Figure 19) lacks a classical TATA or CAAT box or any sequences similar to the "initiator-like" elements recently described for the highly homologous \( \text{CD3}_\delta \) gene (110). Fifteen years ago Tunnacliffe, et al. (23) identified three transcription initiation sites and an Alu-type repeat element, extending from -726 to -415 [NCBI accession #X06026 (23); Figure 19]), but they did not experimentally identify the \( \text{hCD3}_y \) promoter sequences. We searched this region for transcription factor binding sites and located three potential NFAT consensus sequences (5'-GGAAA-3'). Two are located upstream from the first transcription initiation site at -124 to -120 (NFAT\(_{y1}\)) and -384 to -380 (NFAT\(_{y2}\)) while the third is located downstream from the first transcriptional initiation site at +450 to +454 (NFAT\(_{y3}\)). In addition, we have located a GC box as a potential Sp1 binding site upstream from the first transcription start site (-22 to -13).

2.2 Tissue specificity of the \( \text{hCD3}_y \) promoter

We cloned a 1068 bp upstream fragment of the \( \text{hCD3}_y \) gene into a luciferase reporter gene and called this construct pLuc-782/+286\(_{\text{WT}}\). This 1068 bp sequence (forward and reverse primer positions are shown in Figure 19) spans from -782 to +286 relative to the first transcription initiation site (23) and corresponds to approximately one-half of the \( \text{CD3}_y\)-\( \text{CD3}_\delta \) intergenic region plus exon 1 of \( \text{CD3}_y \). Investigation of the potential promoter activity of this region was achieved by transient transfection of the pLuc-782/+286\(_{\text{WT}}\) construct into a variety of human T cell lines, including Jurkat and WE17/10. These experiments revealed that this sequence contains weak promoter activity, with levels varying from 2- to 5-fold that of the empty vector, pGL3-basic vector (pGL3-BV; Figure 20). Interestingly, promoter activity was consistently found to be much lower in SupT1 (a 1- to 2-fold increase over the pGL3-basic), which is an immature \( \text{CD3}^{\beta}\text{CD4}^-\text{CD8}^- \) T cell line and by nature expresses low levels of the pre-
Figure 21. Deletion analysis of the human CD3γ promoter. Promoter activity of constructs containing different fragments of the hCD3γ upstream region cloned into pGL3-Basic. The 3' end of each insert is at position +286 except for the clone pLuc-782/-95 where the 3' end is at -95. Jurkat cells were transiently transfected with these constructs and after 40 h of incubation cells were harvested. Controls and calculations were described in Figure 20. The SD is shown.
TCR-CD3 complex. A plasmid encoding *Renilla* luciferase under control of the thymidine kinase promoter (pRL-TK) was cotransfected as an internal control, and the pGL3-promoter vector (pGL3-PV) under control of the SV40 promoter was used as a positive control.

The T cell specificity of the hCD3γ promoter was investigated by transient transfection of pLuc-782/+286WT in two B cell lines, Raji and GM607, as well as the epithelial cell line HeLa and the fibroblast cell line 293T (Figure 20). In the B cell lines, promoter activity was similar to that of the T cell lines, with Raji cells generally having higher activity (4-fold increase) compared to GM607 (2- to 3-fold increase). The Raji cell line is considered to be a pre-B cell line expressing intracellular IgM whereas the GM607 cell line secretes IgM and is thus a terminally differentiated plasma cell. This difference in differentiation stage may affect promoter activity due to the presence or absence of specific transcription factors in these cells.

In the non-lymphoid cell lines, HeLa and 293T, on average hCD3γ promoter activity was two to three times greater than the empty vector (Figure 20). However, the positive control pGL3-PV was one to two orders of magnitude higher in the adherent HeLa and 293T cell lines compared with all of the suspension T and B cell lines, reflecting the higher efficiency of transient transfection in the adherent cells. Thus, the relatively high ratio of the pGL3-PV control relative to hCD3γ promoter activity in the adherent cells suggests that this result may reflect a higher transfection efficiency rather than true promoter activity in non-lymphoid cells. Despite tremendous effort in our laboratory over a considerable number of years, WE17/10 cells (and other mature T cell lines) have remained refractory to transfection by all currently available techniques. In recent weeks, we have finally succeeded in some initial transfection experiments with WE17/10; however, this thesis research was performed using Jurkat due to these technical constraints.

### 2.3 Activity of the hCD3γ promoter

In order to define the potential positive and negative regulatory sequences within the hCD3γ promoter, increments of the 1068 bp fragment were cloned upstream from a luciferase reporter gene. All of the fragments begin at +286
Figure 22. **EMSA competition experiments using the NFAT^., probe.** Panel A, EMSAs were performed using the ^32^P-labeled NFAT^., probe and nuclear extracts from untreated (lane 1) and PMA+Iono (each 30 ng/ml) stimulated 100% TCR-CD3^° uninfected (lane 2) as well as TCR-CD3^° HIV-1 infected WE17/10 cells (lane 3). Nuclear extracts from TCR-CD3^° infected cells were competed with a 4- or 20-fold molar excess of the homologous oligonucleotide (lanes 4 and 5), an oligonucleotide containing the NFAT consensus sequence from the IL-2 promoter (lanes 6 and 7), an oligonucleotide containing the IL-2 promoter NFAT consensus sequence mutated to abrogate NFAT binding GGAA to CCTT (lanes 8 and 9), an oligonucleotide containing the NFAT^., sequence mutated from GGAA to CCTT (lanes 10 and 11). Panel B, Nuclear extracts from TCR-CD3^° infected cells were competed also with an oligonucleotide containing one NF-κB binding site from the HIV-1 LTR promoter (lanes 12 and 13), and a mutated version of the NF-κB binding site known to abrogate NF-κB binding (GGG to CTC) (lanes 14 and 15). Bands A, B, C and D indicate the four different protein:DNA complexes that specifically bind to the NFAT^., probe. Panel C, Binding of proteins from the same nuclear extracts shown in Panel A to a ^32^P-labeled Oct-1 probe in an EMSA performed as a control (lanes 1-3).
(downstream from the exon 1 splice donor site) and terminate at the various upstream locations highlighted in green in Figure 19 (these constructs are named according to the position of the terminal nucleotide relative to the first transcription initiation site). Transient transfection of these incremental hCD3γ promoter constructs in Jurkat reveals that both positive and negative regulatory regions are contained in the entire pLuc-782/+286WT construct. In the experiment shown in Figure 20, luciferase activity of the full-length pLuc-782/+286WT was at the lower end of the 3- to 5-fold increase; however, the relative activity (increase or decrease) of all of these constructs was reproducible in more than three replicate experiments.

The shortest construct pLuc-52/+286 clearly has lost an important element and displays a significant decrease in promoter activity (Figure 21); whereas the pLuc-80/+286 vector is similar to pLuc-782/+286WT. hCD3γ promoter activity continues to increase with sequence length up to pLuc-192/+286. The interruption in this positive progression of pLuc-142/+286 potentially reflects differential activity of the NFAT1 site, which will be discussed in greater detail in the following section. Interestingly, it appears that there are two regions that have a major negative influence on promoter activity, the first contained in the pLuc-232/+286 construct and the second in the pLuc-412/+286 vector (which contains the NFAT2 site, also discussed further below) with an additional positive sequence found inbetween (pLuc-302/+286). Finally, activity of the pLuc-412/+286 construct was consistently lower than pLuc-782/+286WT, suggesting the presence of a positive regulatory element upstream from nucleotide -412, possibly contained in the Alu repeat. The pLuc-782/-95 construct was deleted at the 3' end to eliminate the transcription initiation sites and any potential RNA polymerase binding sites as a negative control, while the pGL3-PV was used as a positive control in all experiments.

2.4 Characterization of the NFAT motifs in the hCD3γ gene

2.4.1 Nuclear protein complexes bind to the NFAT1 motif

An oligonucleotide probe extending from -132 to -113 (underlined in Figure 19) was used in EMSA experiments to examine the in vitro binding of nuclear
proteins to the NFATγ1 motif. 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 (Figure 22A, A-D), representing DNA-protein complexes with different electrophoretic mobility bind to the NFATγ1 probe. Nuclear extracts from uninfected, unstimulated cells contain only nominal amounts of the lower molecular weight bands C & D (lane 1). Stimulation for 18 hr with PMA+Iono (lane 2) both downregulated TCR-CD3 surface complexes (TCR-CD3⁺; Figure 18) and induced binding of B & C and to a lesser extent A (but not D) to the NFATγ1 probe. A similar binding profile is observed for the TCR-CD3⁻ HIV-1 infected cells (Figure 22A, lane 3). The differential binding observed between nuclear extracts from uninfected/unstimulated TCR-CD3⁺ cells and TCR-CD3⁺ PMA+Iono stimulated cells or TCR-CD3⁻ HIV-1 infected cells is reproducible among different preparations of nuclear extracts and specific because binding of the constitutively expressed Oct-1 transcription factor to its consensus sequence does not vary (Figure 22C, lanes 1-3).

The specificity of the complexes bound to the NFATγ1 probe was further investigated by competition experiments using the homologous oligonucleotide (NFATγ1; lanes 4 & 5), an oligonucleotide containing the NFAT consensus sequence in the human IL-2 promoter (377, 378, 378) (NFAT-IL-2wt; lanes 6 & 7) or versions of NFAT-IL-2wt and NFATγ1 mutated to abrogate binding (157) (GGAA → CCTT; NFAT-IL-2mut, lanes 8 & 9; NFATγ1mut, lanes 10 & 11) (Figure 22A). The homologous and the NFAT IL-2wt probes efficiently compete for binding, while the NFAT IL-2mut and the NFATγ1mut probes are unable to compete. Furthermore, oligonucleotides containing the HIV-1 LTR NF-κB consensus sequence, either wild-type or mutated (GGG → CTC, known to abrogate NF-κB but not NFAT binding) (379), both efficiently compete for binding (Figure 22B, lanes 12, 13, 14 &15). 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.
Figure 23. Supershift and super-supershift analysis of NFAT, NF-κB and AP-1 protein binding to the NFAT^i probe. The 32P-labeled NFAT^i probe was used in a supershift assay with nuclear extracts from TCR-CD3^- 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), anti-c-Jun (lane 12), anti-p65 (lane 13), anti-c-Rel (lane 14), anti-Rel B (lane 15), and anti-p52 (lane 16) 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 is 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).
2.4.2 The nuclear protein complexes bound to NFAT\(_{1}\) contain NFATc1, NFATc2 and NF-\(\kappa\)B p50

Identification of some of the proteins present in the complexes bound to the NFAT\(_{1}\) probe was achieved using antibodies to the NFAT family members, NFATc1 and NFATc2, the NF-\(\kappa\)B 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-CD3\(^-\) HIV-1 infected cells in a supershift assay (Figure 23). Antibodies specific for NFATc1 (lane 2), NFATc2 (lane 3), and NF-\(\kappa\)B p50 (lane 4) all supershift a DNA-protein complex, whereas antibodies to the AP-1 proteins c-Jun (lane 11), c-Fos (lane 12), and NF-\(\kappa\)B proteins p65 (lane 13), c-Rel (lane 14), Rel B (lane 15) and p52 (lane 16) do not. The A complex can be supershifted with either the anti-NFATc1 or the anti-p50 antibody, although the electrophoretic mobility of the anti-p50 supershifted complex (upper A↑) is slower than the anti-NFATc1 supershifted complex (lower A↑). The B and C complexes are both supershifted to a similar electrophoretic mobility with the anti-NFATc2 antibody only (B↑+C↑). This data suggested that there were at least three different nuclear complexes bound to the NFAT\(_{1}\) probe in activated or infected cells, one containing NFATc1 and NF-\(\kappa\)B p50 (band A; present at lower concentrations) and the other two containing NFATc2 (bands B & C; present at higher concentrations; the low molecular weight NFATc2 containing band D was detected only in the unstimulated, uninfected cells).

Confirmation of this observation was achieved by developing a modified supershift assay where combinations of the anti-NFATc1, anti-NFATc2 and anti-p50 antibodies were added sequentially to the binding reaction. These experiments lead to two distinct results: 1) a double-supershift where each antibody binds to a separate complex and individually supershifts the band(s) and 2) a super-supershift where the two antibodies bind to the same complex and their synergy further increases its molecular weight thereby reducing its electrophoretic mobility. Combining the anti-NFATc1 and anti-NFATc2 antibodies (Figure 23, lanes 7 & 10) or the anti-NFATc2 and anti-p50 antibodies (lanes 6 & 9) in either order produced double-supershifts where the A, B and C complexes were all supershifted (A↑ and B↑+C↑), migrating with the same electrophoretic mobility as with the individual antibody alone (lanes 2-4). Alternatively, both
Figure 24. Binding to the $^{32}$P-labeled NFATc1 probe was examined in a supershift assay using nuclear extracts from TCR/CD3+ HIV-1 infected WE17/10 cells. Untreated cells (lane 1) or cells 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-NFATc2 (lane 3), anti-NFATc1 (lane 4), anti-NFATc2 (lane 5) antibodies.

Figure 25. Correlation between TCR-CD3 surface expression and binding of NFATc1, NFATc2 and NF-κB p50 to the NFATc1 probe. Panel A, EMSA experiments were performed with the $^{32}$P-labeled NFATc1 probe and nuclear extracts from 100% TCR/CD3+ uninfected (lane 1) and 98% (lane 2), 87% (lane 3), 39% (lane 4) and 0% TCR/CD3+ (lane 5) HIV-1 infected WE17/10 cells. Panel B, The same nuclear extracts were assessed using a $^{32}$P-labeled Oct-1 probe.
combinations of anti-NFATc1 + anti-p50 (lanes 5 & 8) produced a super-supershift where the electrophoretic mobility of the A complex (A↑↑) was consistently further retarded compared with the anti-p50 antibody alone (upper A↑↑, lane 4). A second A band, migrating with the same electrophoretic mobility as with the anti-NFATc1 antibody alone (lower A↑, lane 2), was also detected when the anti-p50 and anti-NFATc1 antibodies were used together. This suggests that while some of the A complexes contain NFATc1 and NF-κB p50 others contain NFATc1 alone. Alternatively, NF-κB 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 NFATyi probe including: two abundant complexes that contain NFATc2 (bands B & C) but not NFATc1 or NF-κ B p50 and two low concentration complexes (band A) devoid of NFATc2, one which contains NFATc1 and NF-κB p50 and the other either NFATc1 alone or NFATc1 and an inaccessible NF-κB p50.

Uninfected TCR-CD3+ and HIV-1 infected TCR-CD3- cells were treated with CsA and then stimulated with PMA+lono 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 NFATyi probe in EMSA binding studies, which is in agreement with the ability of CsA to block T cell activation via NFAT (380). These extracts were also used in a supershift assay with the NFATyi probe and anti-NFATc1, anti-NFATc2 and anti-NF-κB p50 antibodies (Figure 24). Binding of the NFATc1 and NF-κB 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-NF-κB p50 antibodies).

The NFATc2 containing B and C complexes are both largely inhibited by CsA, and while a faint B complex could be detected in longer exposures, the normally weaker C complex is readily detectable in lower exposures of the gels (Figure 24). 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.
**Figure 26. EMSA analysis of nuclear factors bind to the NF-κB like probe.**

**Panel A,** The NF-κB homology region in the 5 upstream region of the human CD3γ gene identified by alignment with the HIV-1 \textsubscript{HXB2} (NCBI Accession \#K03455) and HIV-2 \textsubscript{BEN} (NCBI Accession \#M30502) LTRs using MegaAlign.

**Panel B,** The CD3γ HIV homology probe was examined, using nuclear extracts from PMA-stimulated WE17/10 cells, in competition experiments with increasing concentrations (4-, 20-, 100-, and 500-fold molar excess) of the homologous oligonucleotide.

**Panel C,** the CD3γ HIV homology probe was incubated with nuclear extracts from different human cell lines [(WE17/10 induced for 18h with 30ng/ml of PMA (lane 6); HeLa (lane 7); Raji (lane 8); SupT1 (lane 9); SupT1 induced for 16h with 30ng/ml PMA (lane 10) and Jurkat (lane 11)]. DNA-protein complexes are labeled from 1 to 10.

**Panel D,** The 20L and 20M oligonucleotide probes were incubated with nuclear extracts from uninfected, PMA-stimulated WE17/10 cells (Lanes 12 and 14) and HIV-1 TCR-CD3$^\gamma$-infected cells. These complexes are labeled from A to E).
2.4.3 The quantity of nuclear NFATc1, NFATc2 and NF-κB p50 is negatively correlated with TCR-CD3 surface expression in HIV-1 infected cells.

The relationship between the presence of NFATc1, NFATc2 and/or NF-κB p50 in the nucleus and the concentration of CD3γ gene transcripts was assessed by examining differential binding to the NFATγ probe of nuclear extracts prepared during the progression of HIV-1 infected cells from TCR-CD3hi → TCR-CD3lo → TCR-CD3− (Figure 25). Characteristically, only low levels of the NFATc2 containing complexes (bands C & D) were detectable in the uninfected and unstimulated 100% TCR-CD3+ cells (lane 1). Alternatively, increased binding of the NFATc1 plus NF-κB p50 containing complex (band A) and NFATc2 containing complexes (bands B & C) to NFATγ occurs in parallel with a decrease in surface TCR-CD3 expression from 98% (lane 2) to 87% (lane 3) to 39% (lane 4) to 0% (lane 5) of normal receptor levels. A non-specific band (indicated as NS) was also detectable in these nuclear extracts. This escalation in binding to the NFATγ probe is specific because similar amounts of the constitutively expressed Oct-1 protein from each extract bound to an Oct-1 sequence specific probe (Figure 25). These results suggest that a correlation exists between the quantity of NFATc2, and to a lesser extent NFATc1 and NF-κB p50, in the nucleus and downmodulation of CD3γ transcripts and TCR-CD3 complexes after HIV-1 infection.

2.4.4 Nuclear factor binding to the HIV homology region surrounding the NFATγ motif

Alignment of the 5' upstream region of CD3γ gene with the 5' LTR's of HIV-1 (Strain HXB2, NCBI Accession # K03455) and HIV-2 (Strain BEN, NCBI Accession # M30502) revealed that the second NFAT motif, NFATγ2 (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 from the Sp1 binding sites and the TATA promoter in both the HIV-1 and HIV-2 LTR's (Figure 26A). However, the first NF-κB consensus sequence in the HIV-1 and HIV-2 LTR's varies from the potential site in CD3γ by two nucleotides (GGGACTTTCC in HIV compared to GIGGCTTTCC in CD3γ) of which the first three G's are thought to
Figure 27. Characterization of factors that bind to the NFATγ2 probe. Panel A, The NFATγ2 32P-labeled probe was tested in competition experiments after incubation with WE17/10 (lane 1), PMA+Iono (each 30 ng/ml) stimulated 100% TCR/CD3+ uninfected (lane 2) and TCR/CD3- HIV-1 infected WE17/10 cells (lane 3) and in the presence of increasing concentrations (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 (TTCC to AAGG lanes 8 and 9), and an oligonucleotide containing the NFATγ2 sequence with the same mutation (lanes 10 and 11). The presence of bands B, C and D indicate the three different protein:DNA complexes that specifically bind to the NFATγ2 probe. Panel B, Binding of proteins from the same nuclear extracts shown in Panel A to a 32P-labeled Oct-1 probe performed as a control (lanes 1-3).
be critical for NF-κB binding (379, 381, 381, 381). Examination of the in vitro binding of nuclear factors isolated from PMA stimulated, uninfected TCR-CD3⁺ cells to a 41 nucleotide (nt) sequence surrounding the NFAT₂ motif (hereafter referred to as the CD3γ HIV homology probe; this sequence is overlined in red in Figure 19) was performed by EMSA experiments. As shown in Figure 26B the CD3γ HIV homology probe is capable of binding up to ten DNA-protein complexes that can be competed with the homologous oligonucleotide (Figure 26B, Lanes 1 to 5). Nuclear extracts from a variety of cell lines, including WE17/10 plus PMA, HeLa, Jurkat, Raji, SupT1, and SupT1 plus PMA+lono (Figure 26C; lanes 6 to 11) demonstrated that most of these complexes are present in varying amounts in lymphoid cells but only one complex (Band 8) is present in epithelial cells (HeLa). Due to the complexity of protein binding to the CD3γ HIV homology probe, we decided to simplify the profile by breaking the sequence into 20 nt probes corresponding to the left (hereafter referred to as 20L), right (hereafter referred to as NFAT₂) and the middle (hereafter referred to as 20M) regions of the original oligonucleotide. At least four bands (Figure 26D; lanes 14 & 15), representing DNA-protein complexes with different electrophoretic mobilities bind to the 20M probe. These complexes are present at lower levels in PMA stimulated WE17/10 cells compared with receptor negative HIV-1 infected WE17/10 cells (TCR-CD3⁻) (Figure 26D, lanes 14 & 15; A, B, C, D), where the bands A and D are significantly more intense. The 20L probe binds three nucleoprotein complexes (Figure 26D, lanes 12 & 13; C, D, E), whose abundance is similar in nuclear extracts from PMA stimulated WE17/10 cells (100% TCR-CD3⁺), and receptor negative HIV-1 infected WE17/10 cells (TCR-CD3⁻). The most abundant protein complexes bound to the 20 nucleotide right probe, NFAT₂, which contains the NFAT motif (Figure 27). These complexes could be induced by PMA+lono or HIV-1 infection and therefore we initially focused our attention on this 20 nt sequence.

2.4.5 Nuclear protein complexes bind to the NFAT₂ and NFAT₃ probes

We asked whether members of the NFAT and/or NF-κB protein families could also bind to the NFAT₂ and/or the NFAT₃ probes. EMSA experiments using the
Figure 28. Characterization of factors that bind to the NFATγ2 probe. Panel A, Binding to the NFATγ2 probe was examined in a supershift assay using nuclear extracts from TCR-CD3- HIV-1 infected WE17/10 cells without antibody (lane 1) or with anti-NFATγ2 (lane 2), anti-NFATγ1 (lane 3), anti-NF-κB p50 (lane 4), anti-c-Jun (lane 5), anti-c-Fos (lane 6), anti-NF-κB p65 (lane 7), anti-NF-κB c-Rel (lane 8) anti-NF-κB Rel B (lane 9), or anti-NF-κB p52 (lane 10) antibodies. Panel B, Binding to the 32P-labeled NFATγ3 probe was examined in a supershift assay using nuclear extracts from TCR-CD3- HIV-1 infected WE17/10 cells without antibody (lane 1) or with anti-NFATγ1 (lane 2), anti-NFATγ2 (lane 3) or anti-NF-κB p50 (lane 4) antibodies. Panel C. The relative quantity of proteins bound to the 32P-labeled NFATγ1, NFATγ2 and NFATγ3 probes was examined using nuclear extracts from uninfected 100% TCR/CD3+ (lanes 1, 3 and 5) and TCR-CD3- HIV-1 infected WE17/10 cells (lanes 2, 4 and 6).
NFATγ2 probe (−392 to −372, underlined in Figure 19) and extracts from unstimulated cells, PMA+Iono stimulated cells and TCR-CD3− HIV-1 infected cells (Figure 27A & B, lanes 1 to 3) showed a similar binding pattern to NFATγ1 (Figure 22A) except that only the NFATc2 containing (bands B, C & D) but not the NFATc1 plus NF-κB p50 containing complex (band A) were detected. Competition with the homologous oligonucleotide (NFATγ2; lanes 4 & 5), an oligonucleotide containing the NFAT consensus sequence in the human IL-2 promoter (378)(NFAT-IL-2wt; lanes 6 & 7) or versions of NFAT-IL-2wt and NFATγ1 mutated to abrogate binding (GGAA → CCTT; NFAT-IL-2mut, lanes 8 & 9; NFATγ2mut, lanes 10 & 11) demonstrate that this complex is specific for the NFAT family. The binding pattern for the NFATγ3 probe (+447 to +466, underlined in Figure 19) was found to be identical to the NFATγ1 probe (Figure 28B and C), except that the complexes were at much lower abundance.

2.4.6 Differential binding of NFATc1, NFATc2 and NF-κB p50 to the NFATγ1, NFATγ2, and NFATγ3 motifs.

The differential binding of NFATc1, NFATc2 and NF-κB p50 to the NFATγ1, NFATγ2, and NFATγ3 sequences was confirmed in a supershift assay using antibodies to the NFAT, AP-1 and NF-κB family members and nuclear extracts from TCR-CD3− HIV-1 infected cells (Figure 28). Only the anti-NFATc2 antibody specifically shifted the complex bound to the NFATγ2 probe (Figure 28A, lane 2, bands B & C), while no band shift was observed with antibodies to NFATc1 (lane 3), to the NF-κB proteins p50 (lane 4), p65 (lane 7), c-Rel (lane 8), Rel B (lane 9) or p52 (lane 10) 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-κB family proteins bind to the NFATγ2 sequence, in spite of its homology with the NF-κB region in the HIV-1 LTR. On the contrary, a supershift assay using the NFATγ3 probe (Figure 28B) was qualitatively similar to the NFATγ1 probe, with supershifted complexes observed for the anti-NFATc1 (band A, lane 2), anti-NFATc2 (bands B & C, lane 3), and anti-NF-κB p50 antibodies (band A, lane 4). We compared the relative binding of the NFATc1 plus NF-κB p50 and NFATc2 containing complexes to the NFATγ1, NFATγ2, and NFATγ3 motifs (Figure 28C) and found
Table I: NFATγ1, NFATγ2, and NFATγ3 sequence mutants

<table>
<thead>
<tr>
<th>Probe Name</th>
<th>Sequence 5' → 3'</th>
<th>Binding of bands A, B &amp; C</th>
</tr>
</thead>
<tbody>
<tr>
<td>NFATγ1 wt</td>
<td>cGGAAAAaa</td>
<td>A, B &amp; C</td>
</tr>
<tr>
<td>NFATγ1 mut1</td>
<td>cGGAAAAga</td>
<td>A lost, B &amp; C ↓</td>
</tr>
<tr>
<td>NFATγ1 mut2</td>
<td>cGGAAAagc</td>
<td>A lost, B &amp; C ↓↓</td>
</tr>
<tr>
<td>NFATγ1 mut3</td>
<td>cGGAAAAgc</td>
<td>A, B, &amp; C ↓</td>
</tr>
<tr>
<td>NFATγ1 mut4</td>
<td>tGGAAAaaa</td>
<td>A, B, &amp; C ↑↑</td>
</tr>
<tr>
<td>NFATγ2 wt</td>
<td>tGGAAAagc</td>
<td>B &amp; C</td>
</tr>
<tr>
<td>NFATγ2 mut1</td>
<td>tGGAAAac</td>
<td>A acquired, B &amp; C ↑</td>
</tr>
<tr>
<td>NFATγ2 mut2</td>
<td>tGGAAAaaa</td>
<td>A acquired, B &amp; C ↑↑</td>
</tr>
<tr>
<td>NFATγ2 mut3</td>
<td>tGGAAAgca</td>
<td>B &amp; C</td>
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<tr>
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</tr>
<tr>
<td>NFATγ3 wt</td>
<td>aGGAAAaas</td>
<td>A, B &amp; C</td>
</tr>
<tr>
<td>NFATγ3 mut1</td>
<td>aGGAAAaga</td>
<td>A, B &amp; C lost</td>
</tr>
</tbody>
</table>

The sequence shown was integrated in the full length 20 nucleotide probes for NFATγ1, NFATγ2, and NFATγ3.

Band A contains NFATc1 alone or in combination with NF-κB p50, Bands B and C contain NFATc2.
that NFATy1 binds significantly more of these protein complexes than NFATy2 and NFATy3, with binding to the NFATy3 probe the weakest among the three motifs. Furthermore, there does not appear to be cooperative recruitment of c-Jun and c-Fos in any of the complexes bound to the NFATy1, NFATy2, and NFATy3 probes.

2.4.7 Sequence variation is responsible for the differential binding of NFATc1, NFATc2 and NF-κB p50 to the NFATy1, NFATy2, and NFATy3 motifs.

In an effort to understand the basis for the qualitative and quantitative differences in binding to the NFATy1, NFATy2, and NFATy3 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 Figure 29). We noted that the nucleotides bordering the core 5'-GGAAA-3' sequence differed by an AA immediately following the core sequence in NFATy1 and NFATy3 in contrast to a GC in NFATy2, suggesting that these nucleotides could potentially play a role in the binding of NFATc1 and NF-κB p50. Alternatively, a T rather than an A preceding the core sequence is thought to facilitate stronger binding of NFAT family proteins (11), and this nucleotide was C, T or A in the NFATy1, NFATy2, and NFATy3 sequences, respectively. Our rationale was that if these three nucleotides do play an important role in binding, then successively mutating the NFATy1 sequence to look like the NFATy2 sequence and visa-versa should alter binding accordingly.

Mutation of the first A following the core sequence in NFATy1 to a G (NFATy1mut1; Figure 29A, lane 2) completely abrogated binding of NFATc1 and NF-κB p50 (band A), significantly decreased the binding of NFATc2 (bands B & C) compared with the wild-type sequence (NFATy1wt; lane 1), and provided a pattern similar to that of wild-type NFATy2 (NFATy2wt; lane 6). Additionally mutating the second A to a C in NFATy1 (NFATy1mut2; lane 3) changed the 3' sequence to that of NFATy2 and reduced NFATc2 binding even further. Mutation of the outside A only in the AA pair of NFATy1 (NFATy1mut3; 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-κB p50, although quantitatively all of the complexes were significantly reduced. Mutation of the C
Figure 29. Mutation analysis of the NFATc1, NFATc2 and NFATc3 probes. Panel A, EMSAs were performed using nuclear extracts from TCR-CD3+ HIV-1 infected WE17/10 cells and the 32P-labeled NFATc1 and NFATc2 probes mutated as shown in Table 1 (lanes 1-10). Panel B, Binding to the 32P-labeled NFATc2mut1 probe was examined in a supershift assay using nuclear extracts from TCR-CD3+ HIV-1 infected WE17/10 cells and anti-NFATc1 (lane 2), anti-NFATc2 (lane 3) and anti-NF-κB p50 (lane 4) antibodies. Panel C, EMSA binding to the 32P-labeled NFATc3wt (lane 1) and NFATc3mut1 (lane 2) probes using nuclear extracts from TCR-CD3+ HIV-1 infected WE17/10 cells.
preceding the core sequence in NFAT\(_{\gamma 1}\) (NFAT\(_{\gamma 1}\)mut4; lane 5) to a T, creating the sequence 5'-TGGAAAAA-3', greatly enhanced the amount of NFATc1, NFATc2 and NF-\(\kappa\)B p50 bound to this probe, providing better binding than that observed with any of the wild-type sequences.

Alternatively, the reverse mutations in NFAT\(_{\gamma 2}\) converted the binding profile of this probe to one similar to NFAT\(_{\gamma 1}\) with increased binding of NFATc2 (bands B & C) and de novo binding of NFATc1 and NF-\(\kappa\)B p50 (band A) achieved by simply changing the 3' G (NFAT\(_{\gamma 2}\)wt; lane 6) to an A (NFAT\(_{\gamma 2}\)mut1; lane 7). Adding a second A 3' of the core sequence in NFAT\(_{\gamma 2}\) (NFAT\(_{\gamma 2}\)mut2; lane 8) further increased the binding of all three complexes (A, B, & C). However, substituting the C for an A in the outside 3' position did not confer binding of NFATc1 and NF-\(\kappa\)B p50 although it did increase the binding of NFATc2 (NFAT\(_{\gamma 2}\)mut3; lane 9). Finally, mutation of the T preceding the core sequence to a C, creating the sequence 5'-CGGAAAGC-3', completely abrogated all binding (NFAT\(_{\gamma 2}\)mut4; lane 10). Confirmation that the specific binding of NFATc1 and NF-\(\kappa\)B p50 was conferred by adding a fourth A to the NFAT core sequence (5'-GGAAAAA-3') was demonstrated by a supershift assay using the NFAT\(_{\gamma 2}\)mut2 probe (Figure 29B). This experiment clearly shows that a simple G \(\rightarrow\) A substitution 3' of the core sequence in NFAT\(_{\gamma 2}\) is sufficient to confer binding of NFATc1 and NF-\(\kappa\)B p50. Finally, binding to the wild-type NFAT\(_{\gamma 3}\) sequence is normally weak, and mutation of the A following the core sequence to G completely abrogated binding (NFAT\(_{\gamma 3}\)mut1; Figure 29 B) compared to the wild-type (NFAT\(_{\gamma 3}\)wt; lane 1).

Taken altogether, these mutation experiments demonstrate that a fourth A added to the NFAT core sequence (5'-GGAAAAA-3') is vital for NFATc1 and NF-\(\kappa\)B 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'-TGGAAAAA-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-\(\kappa\)B p50.
Figure 30. **Mutation of the NFAT motifs.** Jurkat cells were transiently transfected with pLuc-782/+286\(\text{wt}\), pLuc-782/+286\(\text{NFAT1\text{mut}}\), pLuc-782/+286\(\text{NFAT2\text{mut}}\) and pLuc-782/+286\(\text{NFAT1\text{+2\text{mut}}}\). After 40h, the cells were harvested and assayed for firefly and renilla activities. The results shown are each representative of three experiments, each performed in triplicate. Error bars indicate the SD of the mean. pGL3-Basic was used as a negative control and pGL3-PV as a positive control (the latter is not shown). **Panel B.** Co-transfection of the pLuc-782/+286\(\text{wt}\) construct with 85ng of a plasmid expressing NFATc1, NFATc2 or NF-\(\kappa\)B p50 in Jurkat cells.
2.4.8 The rôle of the NFAT$_{1}$ and NFAT$_{2}$ motifs in the hCD3$_{Y}$ gene promoter

Investigation into the functional rôle of the NFAT$_{1}$ and NFAT$_{2}$ motifs in the hCD3$_{Y}$ promoter was carried out by introducing mutations known to abolish NFAT binding (GGAA → CCTT) in the pLuc–782/+286$_{WT}$ construct (Figure 19) and examining their activity by transient transfection in Jurkat. Promoter activity was consistently increased (more than 80%) in cells transfected with the NFAT$_{2}$ mutated construct (pLuc–782/+286$_{NFAT_{2}mut}$) compared with the wild-type vector (Figure 30A), which is in agreement with the differences observed between the pLuc–302/+286 (NFAT$_{2}$ is present) and the pLuc–412/+286 (NFAT$_{2}$ is absent) constructs (Figure 21). In addition, co-transfection of an NFATc2 expression vector with the pLuc–782/+286$_{WT}$ construct results in decreased promoter activity (a more than 35% decrease; Figure 30B).

In contrast, transient transfection of a construct mutated in the NFAT$_{1}$ binding motif (pLuc782/+286$_{NFAT_{1}mut}$) decreases promoter activity by >15% (Figure 30A). Co-transfection of either NFATc1 or NF-κB p50 expression vectors with the pLuc-782/+286$_{WT}$ construct (Figure 30B) increases promoter activity up to 20% over the wild-type vector alone. This result reflects the same differences observed between the pLuc -142/+286 (NFAT$_{1}$ is present) and pLuc-116/+286 (NFAT$_{1}$ is absent) constructs (Figure 21). The effect on promoter activity of co-transfecting an NFATc2 expression vector with a construct containing the NFAT$_{1}$ but not the NFAT$_{2}$ motif is currently in progress. Transient transfection of a construct containing mutations in both the NFAT$_{1}$ and NFAT$_{2}$ motifs appears to neutralize the negative activity of these two sites with luciferase activity generally higher than in the wild-type construct, likely due to the presence of other positive acting elements (Figure 30A). Taken altogether, these data suggest that NFATc2 bound to the NFAT$_{2}$ motif plays a negative rôle in regulating the hCD3$_{Y}$ gene. While the rôle of the NFAT$_{1}$ motif is currently undergoing further investigation, it appears that NFAT$_{1}$ plays a positive rôle if NFATc1 plus NF-κB p50 are bound and an additional negative rôle when NFATc2 is bound.
Figure 31. Mutation analysis of the transcription initiation sites.

Panel A, Transient transfection of the pLuc-782/+286\textsubscript{wt}, pLuc-782/+286\textsubscript{del+1}, pLuc-782/+286\textsubscript{del+5}, and pLuc-782/+286\textsubscript{del+21} constructs in Jurkat cells as described in Figure 20. The results shown are a represent three individual experiments performed in triplicate with the SD shown. The pGL3-Basic was used as a negative control.

Panel B, nuclear extracts from TCR-CD3- HIV-1 infected WE17/10 cells were incubated in the presence of CD3\textsubscript{ylnrwt} (lane 1), CD3\textsubscript{ylnrdel+1} (lane 2), CD3\textsubscript{ylnrdel+6} (lane 3), and CD3\textsubscript{ylrmut+1} (lane 4).

Panel C, Binding to the CD3\textsubscript{ylnrwt} probe was examined in a supershift assay using nuclear extracts from TCR-CD3- HIV-1 infected WE17/10 cells without antibody (lane 1) or with anti-Sp1 (lane 2), anti-Sp2 (lane 3) or anti-Sp3 (lane 4) antibodies.
2.5 Activity of the hCD3γ transcription initiation sites

Multiple transcription initiation sites are commonly observed in promoters lacking a TATA or CAAT box (382, 383, 384, 385, 386, 387). In 1987, Tunnacliffe, et al. (23) used S1 mapping and primer extension analysis to identify three transcription initiation sites for the hCD3γ gene (shown in Figure 19 as +1, +6 and +21) to determine the precise distance between the CD3γ and CD3δ genes (human CD3δ transcription initiation sites had been previously identified (109, 388). Thinking at the time, which has continued into the present, was that CD3γ and CD3δ gene expression is controlled by common sequences located in the 1625 bp region between the 5' ends of these two genes.

We further explored the functional activity of the three hCD3γ transcription initiation sites by individually deleting the +1, +6 and +21 nucleotides in the pLuc-782/+286WT construct. Transient transfection of these 1067 bp constructs (pLuc-782/+286del+1, pLuc-782/+286del+6, and pLuc-782/+286del+21) in Jurkat revealed that deletion of the cytidine at +21 does not significantly alter reporter gene activity in comparison with the wild-type construct (Figure 31 A). However, the single nucleotide deletion of the thymidine at +1 or the cytidine at +6 dramatically decreases hCD3γ promoter activity (Figure 31 A). This result was surprising since one would expect that if there were two critical transcription initiation sites, then deletion of only one would greatly reduce but not abolish transcriptional activity. Furthermore deletion of the +6 cytidine could theoretically be replaced by the cytidine at +7 (Figure 19), which would logically become +6. Therefore, we interpret this data to mean that we have disrupted the binding of a critical element, possibly an initiator, by changing the spatial configuration when the +1 or +6 nucleotide is absent.

This led us to examine protein binding to the region surrounding the +1 and +6 transcription initiation sites. Using an oligonucleotide probe extending from −8 to +14 (CD3γInrwt) in EMSA experiments, we found that two major complexes (Figure 31 A and B, lane 1) bind to this sequence in nuclear extracts from TCR-CD3− HIV-1 infected WE17/10 cells. Interestingly, deletion of the +1 nucleotide in the CD3γInrwt probe (CD3γInrdei+1) leads to the formation of a very large protein:DNA complex that was unable to enter a 6% acrylamide gel in four
Figure 32. **Characterization of factors that bind to the Sp1γ1.** Panel A, EMSAs were performed using the $^{32}$P-labeled Sp1γ1 probe and nuclear extracts from untreated 100% TCR-CD3$^\text{hi}$ WE17/10 cells in the absence of competitor (lane 1) or in the presence of increasing concentrations (4- and 20-fold molar excess) of the homologous oligonucleotide (lanes 2 and 3), an oligonucleotide containing the Sp1 consensus sequence in the IL-12Rβ2 promoter (lanes 4 and 5), an oligonucleotide containing the IL-12Rβ2 promoter Sp1 consensus sequence mutated to abrogate Sp1 binding (lanes 6 and 7), an oligonucleotide containing Sp1γ1 sequence mutated from AGCC to AGAA (lanes 8 and 9) and the unrelated NFAT$_1$ sequence (lanes 10 and 11). Panel B, the Sp1γ1 probe was used in a supershift assay with the same WE17/10 nuclear extracts in the absence of antibody (lane 12) or in the presence of anti-Sp1 (lane 13), anti-Sp2 (lane 14), anti-Sp3 (lane 15), anti-AP-2α (lane 16), anti-AP-2β (lane 17), or anti-AP-2γ (lane 18) antibodies.
separate experiments (lane 2). This complex could potentially be formed from the A and B bands (possibly with additional proteins), since both are completely lost in this binding reaction. Alternatively, deletion of the +6 (CD3γlnrdel+6) or mutation of the +1 from T to G (CD3γlnrmut+1) in the wild-type probe significantly reduces but does not completely abolish binding of the A and B complexes (lanes 3 and 4) without the formation of a large complex in the well. Current experiments are in progress to further elucidate the nature of the nuclear protein complexes that bind to what we now know is a critical control sequence in the hCD3γ gene promoter. This region may also play an important role in the T cell specificity of the hCD3γ promoter since the A and B complexes were not detected in EMSA experiments using nuclear extracts from HeLa. The only protein:DNA complex detected in HeLa nuclear extracts had a different electrophoretic mobility from the A and B complexes and bound with equal abundance to all four probes (wild-type, +1 and +6 deleted and +1 mutated) (data not shown).

Identification of specific proteins present in the complexes bound to the CD3γlnrwt probe was achieved using antibodies to the Sp family members (Sp1, Sp2, and Sp3) and TFIIID and nuclear extracts from TCR-CD3+ HIV-1 infected cells in a supershift assay (Figure 31C). Antibodies specific for Sp1 (lane 2), Sp3 (lane 4), and TFIIID (lane 5) all supershift a protein-DNA complex, while an antibody to Sp2 (lane 3) has no effect. This experiment clearly demonstrates that Sp1, Sp3 and TFIIID all bind to the CD3γlnrwt probe, and their role in regulating the hCD3γ gene promoter is currently under investigation.

2.6 Identification of a functional Sp1 binding motif in the hCD3γ promoter

Computational analysis of the hCD3γ promoter sequences using TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) and TESS (http://www.cbil.upenn.edu/teess/) detected an Sp1 binding site at -22 to -13 (Figure 19). An oligonucleotide probe extending from -30 to -9 (Sp1yi) was used in EMSA experiments to examine the in vitro binding of nuclear proteins from WE17/10 (unstimulated, uninfected 100% TCR-CD3+). At least three bands (Figure 32A), representing protein-DNA complexes with different electrophoretic mobilities, specifically bind to the Sp1yi probe. The specificity of the complexes
Figure 33. Mutational analysis of the Sp1γ1 motif. Jurkat cells were transiently transfected as described in Figure 20 with the wild-type (pLuc-782/+286\textsubscript{wt}) or mutated (pLuc-782/+286\textsubscript{Sp1γ1mut}) or deleted (pLuc-782/+286\textsubscript{Sp1γ1del}) Sp1γ1 binding site.
bound to the Sp1,1 probe was further investigated in competition experiments with the homologous oligonucleotide (Sp1,1; lanes 2 and 3), an oligonucleotide containing the Sp1 consensus sequence in the IL-12Rβ2 (389) (IL-12Rβ2; lanes 4 and 5) or versions of the IL-12Rβ2 (IL-12Rβ2mut, lanes 6 and 7) and Sp1,1 (Sp1,1mut, lanes 8 and 9) probes mutated to abrogate binding (389) [CC → AA (shown in Figure 19)]. An unrelated oligonucleotide containing the NFAT,1 binding site (NFAT,1; lanes 10 and 11) in the hCD3γ promoter was used as an additional control. The homologous and IL-12Rβ2 probes efficiently compete for binding whereas the IL-12Rβ2mut, Sp1,1mut and NFAT,1 probes are unable to compete. This binding to the wild-type but not the Sp1 mutant probes suggests that the nuclear protein complexes bound are specific for the Sp family of transcription factors.

Identification of the specific Sp protein(s) present in the bound complexes was accomplished by performing a supershift assay. Antibodies to the Sp family members Sp1, Sp2 and Sp3 and the AP-2 family members AP-2α, AP-2β, and AP-2γ (which also specifically bind to a GC rich consensus sequence) were incubated with nuclear extracts from unstimulated WE17/10 cells (Figure 32B). Only the antibody specific for Sp1 (lane 13) supershifted the protein-DNA complex in band A accompanied by a moderate decrease in bands B and C. Antibodies to Sp2 (lane 14), Sp3 (lane 15), AP-2α (lane 16), AP-2β (lane 17), and AP-2γ (lane 18) had no effect on the migration pattern of any of the protein-DNA complexes. These results confirm that Sp1 specifically binds to the consensus sequence located from -22 to -13 of the hCD3γ gene promoter.

In an effort to understand whether Sp1 binding to the hCD3γ gene promoter has a functional role, we mutated the Sp1 motif located at -22 to -13 in the pLuc-782/+286WT construct from 5'-CCCCAGGCCCC-3' to 5'-CCCCAGMCC-3'. The mutant construct (pLuc-782/+286Sp1mut) was tested in transient transfection experiments in Jurkat. Mutation of these two nucleotides alone results in a reduction of promoter activity by approximately 50% compared with the wild-type vector (Figure 33). In addition, a second construct with a 23 nucleotide deletion (pLuc-782/+286Sp1,1del, deleted from -31 to -8) that completely removes the sequence covered by the -28 to -9 Sp1,1 EMSA probe, reduced promoter activity
Figure 34. Characterization of the Sp1γ3wt motif. Panel A, The Sp1γ3 sequence was used as probe and incubated with nuclear extracts from unstimulated WE17/10 cells in the absence of competitor (lane 1) or in the presence of increasing concentrations (4-, and 100-fold molar excess) of the homologous Sp1γ3wt oligonucleotide (lanes 2 and 3), an oligonucleotide containing the Sp1 consensus sequence in the IL-12Rp2 promoter (lanes 4 and 5), an oligonucleotide containing the IL-12Rp2 promoter Sp1 consensus sequence mutated to abrogate Sp1 binding (lanes 6 and 7), and an oligonucleotide containing the Sp1γ3 sequence mutated from GCGG to TCTT (lanes 8 and 9). Panel B, Transient transfection of the pLuc-782/+286wt and pLuc-782/+286Sp1γ3mut construct in Jurkat cells as performed described in figure 4.
to 30% of the wild-type construct in transient transfection experiments (Figure 33). These experiments demonstrate that Sp1 plays a significant role in regulating the hCD3γ promoter and suggests that other protein-DNA complexes may be bound to motifs adjacent to the Sp1 consensus sequence (to other deleted nucleotides) and also positively regulate the hCD3γ gene promoter.

2.7 Nuclear protein complexes bind to the Sp1γ3 motif in the hCD3γ promoter

The transient transfection experiments using the different length constructs (Figure 21) drew our attention to a second (in addition to NFATγ2) potential negative regulatory region in the hCD3γ gene promoter (Sp1γ3; Figure 19), located in the region between pLuc-232/+286 and pLuc-192/+286 (Figure 21). pLuc-192/+286 lacks only 40 nt compared with pLuc-232/+286 but has a two-fold increase in luciferase activity (Figure 21). To investigate nuclear protein binding to this region, an oligonucleotide extending from -216 to -195 (hereafter referred to as Sp1γ3) and nuclear extracts from unstimulated WE17/10 cells (100% TCR/CD3+) were used in EMSA experiments. A very abundant protein-DNA complex, which may actually be composed of more than one individual complex, binds to the Sp1γ3 probe (Figure 34A). The specificity of this complex was investigated in competition experiments using the homologous oligonucleotide (Sp1γ3wt; lanes 2 & 3), an oligonucleotide containing the Sp1 consensus sequence from the IL-12Rβ2 promoter (IL-12Sp1wt; lanes 4 & 5), or versions of IL-12Rβ2wt (lanes 6 and 8) and Sp1γ3 (lanes 8 and 9) mutated to abrogate Sp1 binding (GCGG→TCTT). The homologous and the IL-12Rβ2wt probes efficiently compete for binding, whereas the IL-12Rβ2mut and the Sp1γ3mut probes are unable to compete. These results show that a specific complex binds to the Sp1γ3wt probe that can be competed with an Sp consensus sequence. Preliminary supershift assays suggest that one or more members of the Sp family of transcription factors are present in this complex(es).

We then asked whether this complex is responsible for the two-fold increase in activity observed between the pLuc-232/+286 and pLuc-192/+286 constructs. The same mutation (GCGG→TCTT) that abrogated binding in the
Figure 35. Activity of the -8 to +286 region in the hCD3γ gene. Panel A, Jurkat, Raji, and 293T cells were transiently transfected with the pLuc-8/+286 construct as described in Figure 20. Panel B, Jurkat cells were transiently transfected with the pLuc-782/+286, pLuc-782/+286ΔD, and pLuc-782/+286Tid.
EMSA experiments (Figure 34A) was introduced in the pLuc-782/+286_{WT} construct. This construct, pLuc-782/+286_{Sp1γ3mut}, was assessed in transient transfection experiments in Jurkat (Figure 34B), and revealed that this mutation increases activity more than two-fold over the wild-type construct. These data demonstrate that the negative regulatory activity present in the 40 nt sequence between pLuc-232/+286 and pLuc-192/+286 lies in the Sp1γ3 motif and is likely conferred by the nuclear protein complex(es) that we have shown bind to this sequence.

3. Preliminary data on downstream regulatory sequences in the hCD3γ gene promoter

Previous published experiments from our laboratory (370) used antisense oligonucleotides targeted to splice donor and acceptor sites in the HIV-1 genome and found that specifically influencing tat and/or nef gene expression could affect downregulation of surface TCR-CD3 complexes (Figure 16C). This data led us to identify a RNA stem-loop structure with sequence similarity to the Tat-responsive RNA structure TAR in HIV-1 (Figure 8) that could potentially be formed from the first 60 nucleotides of the hCD3γ transcript (K. Willard-Gallo, personal communication). Experiments using AS-P-OdN’s targeted to the CD3γ “TAR-like” structure were efficient at downmodulating TCR-CD3 surface expression, most likely as a result of efficient degradation of the hCD3γ transcripts by RNase H (K. Willard-Gallo, personal communication). Unexpectedly, an antisense P-OdN targeted to the terminal half of this potential stem-loop structure (but not the sense or a random sequence control) also significantly inhibited the number of tat, nef and rev gene transcripts in HIV-1 infected WE17/10 cells (K. Willard-Gallo, personal communication).

Experiments to investigate the potential role of this downstream sequence in regulating the hCD3γ gene promoter are currently in progress; however, we believe that our preliminary results add interesting information concerning the functioning of the hCD3γ promoter. We cloned a 294 bp fragment, extending from -8 to +286 of the hCD3γ gene, upstream from a luciferase reporter gene (pLuc-
Transient transfection of this construct in different cell lines (Jurkat, Raji, and 293T cells) revealed that it has between two to three-fold more activity than the full-length wild-type construct, (pLuc-782/+286WT) (Figure 35A) or the pLuc-116/+286 construct which contains the NFATγ1 site but lacks the negative regulatory sequences (Figure 21). Potentially, the activity of pLuc-8/+286 could be related to the complexes that bind to the -8 to +14 sequence (discussed above, Figure 31). Therefore, generated a pLuc-782/+286WT constructs where the ten nucleotides (+28 to +37) forming the terminal end of a potential stem-loop CD3γ "TAR-like" structure were deleted (pLuc-781/+286DLD). Transient transfection of the pLuc-781/+286DLD construct in Jurkat reduced promoter activity by 50% compared to the wild-type construct (pLuc-782/+286WT) (Figure 35B). We then deleted the nucleotides from +12 to +50 corresponding to the upper half of a potential CD3γ "TAR-like" structure to produce the pLuc-781/+286TLD vector. Transient transfection of the pLuc-781/+286TLD construct in Jurkat revealed that this deletion completely abolishes promoter activity. The pLuc-781/+286TLD no longer contains the +21 transcription initiation site; however, since deletion of this third transcription initiation site (Figure 31) had no effect on promoter activity, this important loss of activity cannot be due to deletion of the +21 start site. These data suggest that control of the hCD3γ gene is also positively influenced by important downstream RNA or DNA sequences, and further characterization of this region is currently in progress.
Chapter IV

Discussion
Discussion

1. Further insight into the CD3y transcription defect in HIV-1 infected cells

Previous results in our laboratory demonstrated that T cell receptor downmodulation, due to a defect in CD3y gene transcription (360, 362, 362), occurs in a two-phase progression after HIV-1 or HIV-2 infection and can be summarized by the formula TCR-CD3^{hi} \rightarrow TCR-CD3^{lo} \rightarrow TCR-CD3^{-} in which the forward progression is markedly favored (371). The TCR-CD3^{hi} to TCR-CD3^{lo} 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-CD3^{-} phenotype (371). The RT-PCR data presented in this thesis provide further insight into the molecular events generating this progression by showing that the initial conversion from TCR-CD3^{hi} to TCR-CD3^{lo} involves a substantial (80-90%) decrease in the number of CD3y 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 downmodulation is actually initiated very early (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 (390). 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 (391). The CD3y protein forms a stable complex with CD3\varepsilon (28) 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 CD3y 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) (360, 362, 362, 371, 371). These studies also revealed that an initial 4- to 5-fold drop in p24 antigen levels in the culture supernatant occurred coincident with downmodulation from TCR-CD3$^{hi}$ → TCR-CD3$^{lo}$, with a further 4- to 5-fold reduction accompanying the transition from TCR-CD3$^{lo}$ → TCR-CD3$^{-}$ (371). 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 (370). 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 (370). 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 CD3$\gamma$ transcripts (370). Antisense oligonucleotides directed to the splice acceptor of the tat gene were particularly efficient in provoking a coordinate downregulation of virus expression in concert with an upregulation of surface TCR-CD3 complexes (370). One interpretation 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 (392) plays an important role in initiating and maintaining the escalating CD3$\gamma$ 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 (134, 393, 393). CD3$\gamma$ plays an important role in both tyrosine and PKC-mediated TCR-CD3 downmodulation, 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 CD3$\gamma$ 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'-GGAAA-3') (11). 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 (394). The HIV-1 κB sequences have been shown to play an important role in the transcriptional regulation of viral gene expression (135, 135, 395) and to competitively bind NF-κB and NFAT family proteins (144, 144, 146, 146, 393). The upregulation 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.

2. Identification and characterization of the hCD3γ promoter

Transcriptional regulation of most eukaryotic genes requires both specific transcription factors that bind proximal to the transcription start site (i.e. to the promoter) and cis-acting elements that modify gene expression at a distance from the gene (i.e. enhancers and silencers). The specificity of gene expression is dependent upon the expression of tissue specific promoter and/or enhancer elements. Understanding the mechanisms that lead to a specific loss of CD3γ transcripts after HIV-1 and HIV-2 infection first requires identification and characterization of the transcriptional control elements that govern CD3γ gene expression. In the late 1980's and early 1990's, a number of different laboratories were intensively studying the control elements for the various TCR-CD3 genes, and succeeded in identifying promoter and enhancer elements for all of them (TCRα (96, 97), TCRβ (98, 99), TCRδ (101), TCRγ (100), CD3ε (103), CD3β (102), and CD3ζ (95, 104)) except CD3γ. hCD3γ is located in a head-to-head orientation with hCD3δ and separated by only 1.6 kb, and thus it was concluded that CD3γ and CD3δ are controlled in a manner similar to other divergently transcribed genes that share common bi-directionally active regulatory elements.
(396, 397, 398, 399, 400). However, data from our laboratory and others on the
differential expression of CD3γ and CD3δ during T cell differentiation and in a
growing number of human pathologies, strongly suggests that these two genes
are controlled independently.

The data presented in this thesis describes, for the first time, specific promoter
sequences involved in regulating transcription of the human CD3γ gene. The
region between −782 and +286 of hCD3γ (corresponding to approximately one-
half of the CD3γ-CD3δ intergenic region and extending 3' through CD3γ exon 1)
was cloned by PCR into a luciferase expression vector. The full-length clone and
increasingly shorter increments of this sequence were functionally characterized
in transient transfection experiments. These experiments show that hCD3γ is
controlled by a weak, non-TATA promoter similar to those previously described
for the highly homologous CD3ε and CD3δ genes. Furthermore, this promoter
activity does not appear to confer T cell specificity on CD3γ gene expression.
Originally, both the CD3δ and CD3ε promoters were found to lack T cell
specificity (102, 401) and to achieve this specificity through T cell specific
enhancers. A recent study (110) has shown, however, that specific upstream
elements in the CD3δ promoter also contribute to T cell specific expression of this
gene. Although the CD3γ promoter was found to also be equally active in B cell
lines, we have not excluded the possibility that unidentified T cell specific
transcription factors could play a critical role in optimal expression from the
promoter.

Constructs containing incrementally decreasing lengths of the CD3γ
downstream sequence (from −782 to −52 at the 5' end, all 3' ends extend to
+286; Figures 19 & 21) demonstrated that the CD3γ half of the intergenic region
contains both positive and negative regulatory elements. Two clearly negative
regions were located from −412 to −302 and −232 to −192, the former one
containing the NFATγ2 motif and the latter one the Sp1γ3 motif. The NFATγ2 motif
binds NFATc2 containing complexes, and the negative role of this complex is
discussed in detail in the following section on NFAT. An oligonucleotide probe
covering the Sp1γ3 motif was used in EMSA experiments and found to bind an
abundant and specific complex whose binding was abolished by mutating three
nucleotides known to affect Sp1 binding (389). When this mutation was
introduced into the full-length pLuc-782/+286 construct, a two-fold increase in luciferase activity was observed signifying a negative role for the complex bound to the Sp1y3 motif. Attempts to determine whether Sp family proteins are present in this complex have not yet met with success, perhaps due to inaccessibility of the epitope or affinity of the antibodies tested; however, experiments using different anti-Sp antibodies are currently underway.

The sequences lying between the negative activities conferred by NFATy2 and Sp1y3 appear to contain positive acting elements. A search for transcription factor binding sites detected potential GATA and Oct binding sites located in the sequence from -302 to -232 (downstream from the NFATy2 element). The GATA family of transcription factors includes GATA3, which is expressed exclusively in T cells(402). And while Oct 1 is ubiquitous, Oct 2 is lymphoid specific and is expressed in CD4⁺ T cells but not CD8⁺ T cells. Potentially, one or both could play a role in regulating CD3γ promoter activity in T cells. The sequence downstream from the Sp1y3 motif (-192 to -142) contains a potential Ikaros/LyF-1 binding site, both of which are lymphoid specific zinc finger transcription factors. Continuing in the 3' direction, the NFATy1 motif is located in the region between -142 to -116, and its positive role in CD3γ promoter activity will also be discussed in the following section on NFAT. Moving further downstream, the sequence located between -80 to -52 clearly contains an important positive regulatory element. This region has a six nucleotide sequence homologous with the CD3δ repeat (23), which is two copies of the sequence 5'-AAGCAGA-3' located exactly 28 bp from each of the two major start sites of the human CD3δ gene. These elements in CD3δ were proposed to play a role like that of the TATA-box (generally located ~30 bp upstream from the start site); however, this function has never been investigated (388). In addition, this sequence also contains an eight nucleotide motif (-58 to -51) identical to the critical sequence surrounding the +1 transcription initiation site (5'-GGGIGGAG-3'). The role of this motif at both the +1 and upstream locations is currently under investigation. Finally, the sequence downstream from -52 contains the Sp1y1 motif, the three transcription initiation sites and an important positive regulatory region located between +12 and +50. An in depth discussion of our data relevant to these regions is discussed in Section 4.
3. The NFAT motifs in the hCD3γ gene promoter

We located three potential binding motifs (5'-GGAAA-3') for NFAT family transcription factors in the 5' flanking sequence of the hCD3γ gene, and named them NFATγ₁, NFATγ₂ and NFATγ₃. The NFATγ₁ motif, located between −124 and −120 and present in the pLuc−142/+286 construct, is a region that apparently possesses both positive and negative regulatory activity. The NFATγ₂ motif, located between −184 to −380, is nested in a region with sequence homology to the HIV-1 kB elements. The NFATγ₂ motif is present in the pLuc−412/+286 construct, which we have shown contains a negative regulatory element. The third NFAT motif, NFATγ₃, is located in the intron between CD3γ exon 1 and exon 2, and its potential function is currently unknown.

We used EMSA experiments to study the binding of nuclear proteins to the CD3γ NFAT motifs. 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 all three NFAT motifs. 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 mobility's of the three complexes could be correlated with the binding of NFATc2 as a monomer or dimer (11, 152, 152) 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 (403). 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). In order 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 a complex(es) containing both proteins by the sequential addition of the two different antibodies (referred to as a super-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 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 in order 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 (supershifts 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 plus NF-κB p50 complex present, we thought it was important to provide the NFATy1 DNA binding site in the reaction mixture in order to favor their coordinate binding. Further evidence in support of the dual binding of NFATc1 and NF-κB p50 to NFATy1 and NFATy3 but not NFATy2 was provided by the EMSA experiments using mutant oligonucleotides. Changing the fourth A in the NFATy1 and NFATy3 motifs (5'-GGAAAA-3' to 5'-GGAAAG-3') completely abrogated binding of the NFATc1 and NF-κB p50 containing complex whereas adding a fourth A to the NFATy2 motif (5'-GGAAAG-3' to 5'-GGAAAA-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.

This data is 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 (146). The NF-κB/Rel family of transcription factors are defined by a ~300 aa region called the Rel homology domain (Figure 10), which contains the residues involved in nuclear translocation, DNA binding, and protein-protein interactions (191, 404) (404). 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 (405). Crystal structures have shown that NF-κB p50 optimally binds to the 5'-GGAAA-3' half site and p65 the 5'-GGAA-3' half site which are separated by a non-contact base in the palindromic κB sequence (406). 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 (11).

A Rel homology domain, with about 20% sequence homology to the NF-κB Rel domain, is also found in all of the NFAT proteins (Figure 10) (70, 70, 158). 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 (158, 158, 189, 190, 190). 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 (11), and the HIV-1 LTR κB sites are an example of NFATc2 forming both monomeric and dimeric complexes (144, 146, 146, 407, 407). 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 NFATγ1 and NFATγ3 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 NFATγ1 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'-GGAAA^-3') was found to be crucial for their binding. This core motif is also the only component common between the NFAT\textsubscript{1} and NFAT\textsubscript{3} but not the NFAT\textsubscript{2} probes, and thus emerges as the requisite sequence for binding of the NFAT\textsubscript{c1} plus 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 (11), and while this was found to be true for NFAT\textsubscript{1} by replacing the preceding C with a T, the low level and lack of NFAT\textsubscript{c1} and NF-κB p50 binding to NFAT\textsubscript{2} (5'-TGGAAAG-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 NFAT\textsubscript{c1} 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.

Investigation of the functional role that binding of the NFAT\textsubscript{c2} and/or NFAT\textsubscript{c1} plus NF-κB p50 containing nuclear complexes plays in regulating the hCD3\gamma promoter was accomplished by mutating NFAT\textsubscript{1} and/or NFAT\textsubscript{2} in the pLuc-782/+286\textsubscript{WT} construct. Mutation of the NFAT\textsubscript{2} motif substantially increased luciferase activity and together with the negative activity observed in the −412 to −302 sequence, strongly suggests that NFAT\textsubscript{c2} negatively regulates transcription of the hCD3\gamma gene. Alternatively, mutation of the NFAT\textsubscript{1} motif slightly decreases luciferase activity. Together with our data demonstrating that NFAT\textsubscript{1} can bind both NFAT\textsubscript{c1} plus NF-κB p50 and NFAT\textsubscript{c2} containing complexes, suggests that
the balance between its positive and negative activity may depend upon the nuclear abundance and binding of these two different NFAT containing complexes. Mutation of both NFATγ1 and NFATγ2 in the same construct neutralizes the effect of the single motif mutation, adding weight to the argument that the role of NFATγ1 is more positive and NFATγ2 more negative for promoter activity. Experiments where NFATc1, NF-κB p50, and NFATc2 were co-transfected with the pLuc-782/+286WT construct demonstrated that the NFATc1 plus NF-κB p50 complex has a positive influence on the hCD3γ promoter whereas the NFATc2 complex has a negative effect. Taken altogether, these data strongly suggest that binding of NFATc2 to the NFATγ2 motif plays a negative role in hCD3γ gene expression. Alternatively, binding of the NFATc1 plus NF-κB p50 complex to the NFATγ1 motif has a positive effect that can be counteracted through successful competition by NFATc2 for binding. Our data on the hCD3γ promoter thus provides another example of a gene where members of the NFAT family can differentially regulate the same promoter.

4. A beginning to our understanding of transcription initiation from the hCD3γ promoter

The majority of characterized eukaryotic gene promoters have been found to initiate transcription through binding of a pre-initiation complex to a TATA or CAAT box sequence. In the absence of a classical TATA or CAAT box, a GC box (where Sp family transcription factors bind) has been frequently shown to be involved in the initiation of transcription (408, 409, 410). hCD3γ is one of the minority of genes that do not contain a TATA or CAAT box; however, we located an atypical GC box sequence at -22 to -13 and demonstrated that Sp1 specifically binds to this element. Introduction of a mutation known to abolish Sp1 binding or deletion of the entire GC box revealed that this element has a significant positive effect on hCD3γ promoter activity. The other CD3 genes (CD3δ, CD3ε, and CD3ζ) have not been found to possess a classical GC box consensus sequence (5'-CCGCCC-3' or its complement) (95, 411, 412, 413).
The mechanism of Sp1-mediated activation is not yet fully understood, but Sp1 has been shown to interact with components of the TFIID complex and stabilize their binding to initiator elements. An "initiator-like" element that binds TFII-I was recently shown to play a role in initiating transcription of the mouse CD3δ gene (110), and our ongoing experiments have been designed to determine whether hCD3γ also possesses an initiator sequence. The positive role of Sp1 in transcription initiation has been shown to be repressed by Sp3 in several genes (265, 266, 267) through its ability to block Sp1 binding to the promoter. Sp1 can also interact with other transcriptional factors such as YY1 (247, 248) and E2F (249, 250), and a recent study has shown that YY1 binding to the CD3δ promoter plays a negative role in core promoter activity (110).

Three transcription start sites (Figure 19) have been previously identified for the hCD3γ gene at +1, +6 and +21. Our experiments demonstrated that deletion of a single nucleotide at position +21 (in the 1068 bp wild-type construct) has no effect on promoter activity. Alternatively, deletion of a single nucleotide at +1 or +6 abolishes or severely diminishes promoter activity, respectively. This extensive loss of activity was unexpected because if both the +1 and +6 are functional then loss of a single initiation site should allow some transcription to continue initiating from the other. Furthermore, there are cytidines both at +6 and +7, so logically deletion of the C at +6 would be replaced by the C at +7. We interpret our results to indicate that deletion or mutation of a single nucleotide in the region surrounding the +1 and +6 nucleotides has a deleterious effect on the binding of an essential transcription factor complex, perhaps the pre-initiation complex.

Binding to the region surrounding the +1 and +6 nucleotides was investigated in EMSA experiments using wild-type and mutated oligonucleotides that span from -8 to +14. These experiments revealed that two specific nuclear protein complexes bind to this region. Deletion of the +1 nucleotide from the oligonucleotide leads to the formation of a very high molecular weight complex that is unable to enter the gel, whereas mutation of the +1 substantially reduces but does not abolish binding of the two complexes. These data suggest that changing the spatial arrangement of the nucleotides surrounding the +1 by removing the thymidine alters the binding of the two complexes in a manner that
results in the formation of one large complex. The total lack of luciferase activity in the pLuc-782/+286\textsubscript{del+1} construct suggests that this large protein:DNA complex is non-functional. Elimination of the +6 nucleotide reduces binding in a manner similar to the mutation of +1, suggesting that while these alterations dramatically affect binding they do not alter the nature of the complexes that bind. The low level of binding observed with the +6 deleted oligonucleotide is compatible with the low level of luciferase activity we detected after transfection of the pLuc-782/+286\textsubscript{del+6} construct. Overall, these data suggest that the sequence surrounding the +1 initiation site is a critical element and may function as an initiator sequence.

5. A downstream positive element is present in the hCD3\textsubscript{y} promoter

Deletion of ten nucleotides in the hCD3\textsubscript{y} promoter (from +28 to +37) reduces activity by approximately one-half while deletion of the +12 to +50 sequence completely abolishes promoter activity. This preliminary experimental data suggests that these downstream sequences play an important role in expression of the hCD3\textsubscript{y} gene. Structural analysis from our laboratory found that the +1 to +60 sequence in hCD3\textsubscript{y} potentially forms an RNA stem loop structure similar to TAR of HIV-1 (+1 to +60 of the viral transcript, Figure 8). Our ongoing experiments are designed to investigate whether the positive activity of these downstream sequences is contained in a DNA or RNA element and to further characterize this activity.

6. A proposed model for transcriptional regulation of the human CD3\textsubscript{y} gene promoter

In our search for the molecular mechanisms regulating expression of the hCD3\textsubscript{y} gene, we have shown that this gene is transcribed from a weak, non-tissue specific TATA-less promoter. In the absence of a TATA or CAAT box the initiation of transcription can be mediated by an initiator element. Our results
Figure 36. Model for transcriptional regulation of the hCD3γ gene
show that a critical element is present in the area surrounding the +1 and +6 transcription initiation sites and that two initiator elements are potentially located in this region. The first element from -2 to +5 (5'-GGTGGAC-3', where T is +1) is complementary to a classic initiator, with a sequence of 5'-Pu Pu T(+1) N G/C Pu Pu-3' instead of the typical 5'-Py Py A (+1) N T/A Py Py-3'. The second sequence located from +6 to +12 (5'-CCAGTCG-3', where the first C is +6) follows the classic configuration of 5'-Py Py A (+1) N T/A Py Py-3'. However, the +8 nucleotide (A) has not been shown to be a major transcription initiation site (23). This does not exclude the possibility that this element functions as an "initiator-like" sequence similar to those found for CD3δ (110), although our recent experimental evidence does suggest that the sequences surrounding the +1 site are the most critical.

Although the mechanism of transcription initiation through initiators is not well defined, it is generally accepted that the presence of one or more Sp1 binding sites helps to recruit the pre-initiation complex (113, 114, 115). We have shown that two adjacent Sp1 binding motifs (Sp1γ1 and CD3γlnr) are present in the hCD3γ gene promoter (Figure 36). The Sp1γ1 oligonucleotide (-30 to -9) binds a complex containing Sp1, whereas the CD3γlnr oligonucleotide (-8 to +14) binds two complexes that contain Sp1, Sp3 and/or TFIID. We postulate that the CD3γlnr sequence binds Sp1, which recruits TFIID and the other TATA binding proteins to the promoter, leading to formation of the pre-initiation complex. In this model, the Sp1γ1 sequence also binds an Sp1 containing complex whose role would be to further activate transcription. We also found that Sp3 can bind to the CD3γlnr sequence in nuclear extracts from HIV-1 infected TCR/CD3− cells, and postulate that it may play a negative role (238) by repressing Sp1-mediated activation of the hCD3γ promoter.

We have further identified two negative regulatory elements in the upstream hCD3γ gene sequence. The first is located in the Sp1γ3 sequence and binds an abundant complex whose composition is currently undefined. The second contains the NFATγ2 motif, which we have shown binds increasing amounts of an NFATc2 containing complex in cells with progressively downmodulated TCR-CD3 surface receptors, suggesting that it is involved in negative regulation of the hCD3γ promoter. We have also shown that the NFATγ1 motif can bind either
NFATc1 plus NF-κB p50 containing complexes that have a positive effect on the promoter, and that they can be outcompeted by NFATc2 containing complexes for further suppressive activity at this site. Finally, we have found an important positive regulatory element located in the downstream sequence (between +12 to +50); however, it is still unclear whether this reflects the presence of a DNA or an RNA regulatory element.

T cell activation induces the activation of different transcription factors, including NF-κB, NFAT, and AP-1, which ultimately lead to the induction of cytokine gene expression (e.g. IL-2). At the same time, these factors also transactivate expression from the HIV-1 LTR. Thus, this overlapping use of the same cellular mediators involved in normal immune responses and viral gene expression ultimately results in the downregulation of surface TCR-CD3 complexes due to a defect in CD3γ gene transcription. This transcriptional downmodulation could be due to a direct negative role by elevated levels of cellular transcription factors such as NFATc2 and Sp3, or it could result from abnormal combinations of cellular and viral transacting factors acting preferentially to regulate viral gene expression over that of the CD3γ gene.
Figure 37. CD3γ TAR-like sequence. Possible TAR-like RNA secondary structure with similarity to HIV-1 and HIV-2 TAR of the CD3γ sequence from +1 to +60. Location of the 10 nt (DLD) and 29 nt (TLD) deletions are indicated with arrows.
Chapter V

Future Directions
Future Directions

The experimental data presented in this thesis adds important information to our understanding of transcriptional regulation of the CD3γ gene. We have shown that a loss of CD3γ gene transcripts is initiated early after HIV-1 infection and accumulates to a defect of >90% of normal transcript numbers prior to an effect on surface TCR-CD3 expression. We have identified the human CD3γ promoter sequences and characterized it as a weak, TATA-less promoter without apparent T cell specific expression. In addition, we have located positive and negative regulatory elements involved in controlling the promoter and identified some of the transcription factors present in the complexes bound to these sequences, including NFATc1, NFATc2, NF-κB p50, Sp1, Sp3 and TFIID. While identification and characterization of the other elements involved in transcriptional regulation of the human CD3γ gene is not yet complete, we have located two critical elements, one surrounding the +1 transcription initiation site and the other between +12 to +50 of the downstream sequence.

The arrangement of the hCD3γ promoter has revealed a number of interesting parallels with the HIV-1 LTR. Previous results from our laboratory found that when intracellular conditions favor expression of tat and/or nef in the absence of rev, CD3γ gene transcripts and TCR-CD3 surface density are downmodulated (368). This data led to the identification of an RNA stem-loop structure with sequence similarity to the Tat-responsive RNA structure TAR in HIV-1 (Figure 37) that could potentially be formed from the first 60 nucleotides of the hCD3γ transcript (K. Willard-Gallo, personal communication). The CD3γ "TAR-like" region differs from HIV-1 TAR by the presence of double instead of a single apical loop (Figure 37), a bulge consisting of two oppositely placed uridines and a third loop located between the bulge and the double apical loop. Transfection of the pLuc-782/+286 vector where the double apical loop (nt +28 to +37) was deleted had a 50% decrease in promoter activity, whereas deletion of all three loops plus the bulge (nt +12 to +50) completely eliminated promoter activity. These experiments demonstrate that this downstream region plays a critical positive role in expression of the hCD3γ promoter, but does not reveal whether
this is via a DNA or RNA element. Our data from antisense experiments, showing that P-OdN’s directed to the CD3γ "TAR-like" structure effectively suppress CD3γ gene transcription (but not the sense or random controls) and had a significant negative effect on the level of tat, nef and rev gene transcripts in infected cells, suggesting that this region may function as an RNA transcriptional activator.

Further investigation of this hypothesis shall be accomplished by extending our gel retardation assay to the use of RNA probes to investigate the nature and identity of proteins bound to the CD3γ TAR “like” element in comparison with binding to HIV-1 TAR. We also intend to swap the +1 to +60 nucleotides of HIV-1 (TAR element) into the hCD3γ promoter vector and the +1 to +60 nucleotides of the hCD3γ promoter (TAR “like” element) into an HIV-1 LTR construct. Transient transfection of these swapped TAR elements in uninfected cells should demonstrate whether HIV-1 TAR is able to transactivate hCD3γ gene expression and visa versa. We postulate that if these two elements function as transcriptional activators, then the effect of transfecting uninfected TCR-CD3γ cells with the CD3γ TAR “like” sequence inserted in the HIV-1 LTR in should induce higher activity then the wild-type HIV-1 TAR construct in the absence of Tat. Alternatively, transfection of the CD3γ TAR “like” sequence inserted in the HIV-1 LTR in TCR-CD3γ HIV-1 infected cells producing virus should have a negative effect on HIV-1 LTR function, similar to that observed with the intact hCD3γ gene promoter. Alternatively, insertion of HIV-1 TAR into the hCD3γ promoter vector should reduce CD3γ gene expression in uninfected TCR-CD3γ cells and increase its expression in TCR-CD3γ HIV-1 infected cells producing Tat. Our continued investigations will center on the identification and characterization of other elements involved in normally regulating the hCD3γ gene to further our understanding of the mechanisms whereby HIV-1 has imitated its control.
Experimental Procedures

1. Cell culture conditions and reagents

The WE17/10 cell line is a human interleukin 2 (IL-2) dependent CD4⁺ T cell line (360, 414) that was established and is maintained in RPMI 1640 containing 20% fetal bovine serum (FBS), 1.25 mM L-glutamine, 0.55 mM L-arginine, 0.24 mM L-asparagine, and 100 units of recombinant human IL-2 per ml (Cetus Corp, Emeryville, CA). WE17/10 cells infected with the HIV-1 isolate LAI (415) or the molecular clone HXB2 (416) were used in the experiments described (360, 414). The human B lymphocyte line, Raji was obtained from the American Type Culture Collection, Rockville, MD USA and maintained in RPMI 1640 supplemented with 10% fetal bovine serum.

The human B lymphocyte line, Raji (obtained from the American Type Culture Collection (ATCC), Rockville, MD USA) was maintained in RPMI 1640 supplemented with 10% fetal bovine serum. The human T cell lines, Jurkat and SupT1 (both obtained from the ATCC) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 1.25 mM L-glutamine, 0.55 mM L-arginine, and 0.24 mM L-asparagine. The human epithelial HeLa cell line (ATCC) is derived from a cervical carcinoma and is transformed by human papilloma virus type 18 and was maintained in Dulbecco’s medium containing 10% fetal bovine serum. The 293T cell line (ATCC) a human embryonic kidney epithelial cell line was maintained in cell line Dulbecco’s medium containing 10% fetal bovine serum. And finally, the human B cell line GM-607 (obtained from the Human Genetic Mutant Repository, Rockville, MD, USA) was maintained in Dulbecco’s medium containing 10% fetal bovine serum, 1.25 mM L-glutamine, 0.55 mM L-arginine, and 0.24 mM L-asparagine. Media for all cell lines were supplemented with 100 U/ml penicillin and 50 µg/ml streptomycin prior to transfection but not during routine culture.

WE17/10 cells were treated for 18 hours with the calcium channel blockers EGTA [2.5 M; ethylene-bis-(oxyethylenenitrilo)-tetraacetic acid] and BAPTA/AM [1-10 µM, bis-(o-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid], the PKC
activator PMA [30 ng/ml; phorbol-12-myristate-13-acetate], the calcium ionophores A23187 or Ionomycin (30 ng/ml), and the protein kinase inhibitors Herbimycin A (10^{-8} M) and Staurosporin [1-30 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\textsuperscript{+} cells were pretreated for 1 hour with CsA followed by overnight stimulation with PMA+Iono (in the continuous presence of CsA) to achieve the maximum potential induction of NFAT translocation to the nucleus in the presence of the inhibitor.

2. Flow cytometry

Cells were analyzed for CD3 surface expression by flow cytometry as previously described (370). In brief, cells were labeled with the murine monoclonal antibody OKT.3 (directed against CD3e) in a two-step process (using 1 μg/ml of antibody to ensure saturation binding) followed by the manufacturer's recommended dilution of fluorescein-conjugated goat anti-mouse immunoglobulin (BD Biosciences, Erembodegen, Belgium). The labeled cells were fixed in 2% paraformaldehyde and fluorescence was analyzed on a FACS Calibur (BD Biosciences).

3. Preparation of nuclear extracts

Nuclear extracts were prepared from 2 x 10^7 cells according to a modified version of the method described by Osborn (417). All buffers contained a mixture of protease inhibitors (Complete, Roche Diagnostics, Brussels, Belgium) in order to minimize proteolysis. The cellular pellet was washed with ice-cold phosphate-buffered saline and then resuspended twice with 1 ml of ice-cold buffer A (10 mM HEPES buffer, pH 7.9, 1.5 mM MgCl\textsubscript{2}, 10 mM KCl). Cells were collected by centrifugation (600 x g for 10 min), resuspended and incubated for 10 min with 40 μl of ice-cold lysis buffer A containing 0.2% NP-40 (this step was repeated twice). The pellet (nuclear fraction) was incubated with 30 μl of ice-cold extraction buffer C (20 mM HEPES buffer, pH 7.9, 25% glycerol, 1.5 mM MgCl\textsubscript{2}, 420 mM NaCl, 0.2 mM EDTA) for 20 min at 4°C and then centrifuged at 20,800 x g for 10 min at
The nuclear supernatants were diluted 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 (418).

4. Electrophoretic mobility shift assay (EMSA)

EMSAs were performed as described by Van Lint (419) with some modifications. Single-stranded oligonucleotides were 5' end-labeled with [γ-32P] ATP (>5000 Ci/mmol; Amersham Pharmacia Biotech Benelux, AT Roosendal, Netherlands) using T4-polynucleotide kinase; annealed, isolated on a polyacrylamide gel and extracted from the gel using the QIAEX II kit (Westburg, AE Leiden, The Netherlands) prior to their use in EMSA experiments. Nuclear extracts (10 µg of protein) were pre-incubated for 10 min in a reaction mixture containing 10 µg of bovine serum albumin (Sigma-Aldrich, Bornem, Belgium), 1.5 µg of the non-specific competitor DNA poly(dl-dC) (Amersham Pharmacia Biotech Benelux), 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% (vol/vol) glycerol. 15,000 cpm of the 32P-labeled probe were subsequently added to the mixture (final volume 20 µl) and incubated for a further 20 min at room temperature before being loaded onto a 6% nondenaturing polyacrylamide gel (1 x Tris-Glycine-EDTA buffer; migrated at 50 V overnight). The radiolabeled proteins were detected by autoradiography on Biomax MR film (Amersham Pharmacia Biotech Benelux).
5. Oligonucleotide Probes

Oligonucleotides encoding wild type and mutated NFAT binding motifs in the 5' upstream region of the human CD3γ gene are as follows:

- 5'-TCCTTAACGGAAAAACAAAA-3' (NFATγwt),
- 5'-TCCTTAACCCCTAAACAAAA-3' (NFATγmut),
- 5'-TCCTTAACGGAAAAACAAAA-3' (NFATγmut1),
- 5'-TCCTTAACGGAAAAACAAAA-3' (NFATγmut2),
- 5'-TCCTTAACGGAAAAACAAAA-3' (NFATγmut3),
- 5'-TCCTTAACGGAAAAACAAAA-3' (NFATγmut4),
- 5'-GAGGTGGCCCTTTCCATTTGGA-3' (NFATγwt),
- 5'-GAGGTGGCCCTTTCCATTTGGA-3' (NFATγmut),
- 5'-GAGGTGGCCCTTTCCATTTGGA-3' (NFATγmut1),
- 5'-GAGGTGGCCCTTTCCATTTGGA-3' (NFATγmut2),
- 5'-GAGGTGGCCCTTTCCATTTGGA-3' (NFATγmut3),
- 5'-GAGGTGGCCCTTTCCATTTGGA-3' (NFATγmut4),
- 5'-AAAGGAAAAAGTATATGTTC-3' (NFATγawt),
- 5'-AAAGGAAAAAGTATATGTTC-3' (NFATγsmut).

Oligonucleotides encoding wild type and mutated Sp1γ3 binding sites in the hCD3γ are:

- 5'-CATCCATTGCCGTTCCCTGTCG-3' (Sp1γ3wt),
- 5'-CATCCATTGCCGTTCCCTGTCG-3' (Sp1γ3mut).

Oligonucleotides encoding wild type, deleted and mutated CD3γlnrwt binding sites are:

- 5'-GTGATGGGTGGAGCCAGTCTAG-3' (CD3γlnrwt),
- 5'-GTGATGGGTGGAGCCAGTCTAG-3' (CD3γlnr-del+1),
- 5'-GTGATGGGTGGAGCCAGTCTAG-3' (CD3γlnr-del+6),
- 5'-GTGATGGGTGGAGCCAGTCTAG-3' (CD3γlnr-mut+1).
Oligonucleotides encoding wild type and deleted Sp1yi binding sites are:

5'-CTCAAAGGCCGCCCAGCCCAACA-3' (Sp1yiwt)
5'-CTCAAAGGCCGCCCAGCAACACA-3' (Sp1yimut)

Oligonucleotides encoding wild type and mutated NFAT binding sites in the human IL-2 promoter are:

5'-AGAAAGGAGGAAAAACTGTT-3' (NFAT-IL-2wt),
5'-AGAAAGGACCTTAAACTGTT-3' (NFAT-IL-2mut).

Oligonucleotides encoding the wild type and mutated NF-κB consensus sequence (Santa Cruz, Boechout, Belgium) are:

5'-TTGAGGGGACTTTCCCAGGC-3' (NF-kBwt),
5'-TTGAGCTCACTTTCCCAGGC-3' (NF-kBmut).

The oligonucleotide for the Oct-1 binding site (Santa Cruz) is:

5'-TGTCGAATGCAAATCACTAG-3'.

Oligonucleotides encoding wild type and mutated Sp1 binding sites in the human IL-12R2β promoter are:

5'-CTCCAGTGGGCGGTCTTGTG-3' (IL-12R2βwt)
5'-CTCCAGTGTTCGGTCTTGTG-3' (IL-12R2βmut).

6. Supershift assay

Antibodies directed against the NFAT family proteins NFATc1 (SC-7294X), NFATc2 (SC-7295X), NFATc3 (SC-8321X), NFATc4 (SC-1153X), and NFAT5 (SC-5501X), the NF-κB family proteins p50 (SC-1190X), p65 (SC-109X), c-Rel (SC-6955X), Rel-B (SC-226X) and p52 (SC-7386X), and the AP-1 and Ap2 family proteins c-Jun (SC-1694X) and c-Fos (SC-52X), AP2α (SC-184X), AP2β (SC-6310X), AP2γ (SC-8977), (all from Santa Cruz) were pre-incubated with nuclear extracts for 1 h on ice prior to the addition of the radiolabeled probe for the supershift assay. In the super-supershift and double-supershift assay, the first antibody was pre-incubated with the nuclear extract for 45 min on ice followed by a subsequent incubation with the second antibody for an additional 45 minutes on ice before a final 20 min incubation with the radiolabeled probe at room temperature. Antibodies directed against the Sp family proteins Sp1 (SC-59X),
Sp2 (SC-643X), Sp3 (SC-644X) (Santa Cruz) were added to the EMSA reaction 25 min after the radiolabeled probe and were incubated for 1h.

7. Quantitative Competitive RT-PCR

Total cellular RNA was extracted from 5 x 10⁶ cells using the SV total RNA isolation system (Promega Benelux, AJ Leiden, Netherlands) following the manufacturer's recommendations and using the optimal DNase treatment to remove contaminating genomic DNA. The primers used to specifically amplify the CD3γ and CD3δ genes have been previously described (420, 421). Forward (F) and reverse (R) primer pairs are as follows:

CD3γ F: 5'-CATTGGCTTTGATTCTGGGAACTGAATAGGAGGA-3';
CD3γ R: 5'-GGCTGCTCCACGCTTTTG-CCGGAGACAGAG-3',
which yields a 647 bp product and

CD3δ F: 5'-TTCCGGTACCTGTGAGTCAGC-3';
CD3δ R: 5'-GGTACAGTTGGTAATGGCTGC-3', which yields a 660 bp product.

Five micrograms of total RNA from uninfected and HIV-1 infected WE17/10 cells at various stages of TCR-CD3 downmodulation were reverse-transcribed into cDNA in the presence of Moloney murine leukemia virus reverse transcriptase (2.5 U/μl; Roche Diagnostics, Brussels, Belgium), 0.5 mM of each dNTP, 1 U/μl RNase inhibitor, 30 pmole of the forward primers for CD3-γ or CD3-δ, 0.01 M DTT, 20 μl of 5X first strand buffer (250 mM Tris-HCl, 200 mM KCl, 25 mM MgCl₂, 2.5% Tween 20 (v/v), pH 8.3) in a total volume of 100μl. The RT mix was incubated at 30°C for 10 min and 42°C for 45 min.

An internal standard for use in the competitive RT-PCR assay was constructed from a full-length cDNA sequence of the human CD3-γ gene subcloned from pJ6T3γ-2 (21) into the EcoRI site of pUC18 (Life Technologies, Merelbeke, Belgium), and the resulting plasmid was called pUC18γ. This recombinant plasmid was then used to construct a competitor by cutting a 1071 bp XhoI fragment from pV344 (422) and ligating it into XhoI digested pUC18γ, producing the plasmid pUC18γc. The competitor copy number was calculated using the concentration measured by absorbance at 260 nm and the molecular
weight of pUC18γc [i.e. 1 mole of the full length pUC18γc DNA is equal to 4557 bp x 700 Da (the average molecular weight of a deoxynucleotide base pair) = 3.1899 x 10^5].

Human CD3γ gene expression was measured in a quantitative competitive RT-PCR assay, where the target cDNA was co-amplified with the same stock dilution series of the pUC18γc competitor in all experiments. For each target sequence, 20 sequential dilutions of the pUC18γc competitor DNA (from a minimum of 3.3 x 10^3 to a maximum of 6.6 x 10^6 copies) were co-amplified with 100 ng of cDNA, 1 U of Taq polymerase (Amersham Pharmacia Biotech Benelux), 0.2 mM dNTP, 0.4 μM of each primer, 15 mM of MgCl₂ in a final volume of 50 μl in Taq DNA polymerase buffer (Amersham Pharmacia Biotech Benelux). Amplification of CD3-γ was performed with an initial denaturation step of 5 min at 94°C followed by 35 cycles of amplification: 10 cycles of denaturation at 94°C for 35 s, annealing at 50°C for 20 s, extension at 72°C for 30 s followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 65 °C for 20 s, extension at 72 °C for 45 s with a 1 s/cycle automatic prolongation of the extension period. Amplification of CD3-δ was performed with an initial denaturation step of 5 min at 94°C followed by 40 cycles of denaturation at 94°C for 20 s, annealing at 58 °C for 15 s, and extension at 72 °C for 1 min. After amplification, the samples were incubated at 72°C for 7 min, separated on a 1% agarose gel and stained for 10 min with a freshly prepared ethidium bromide solution (0.5 μg/ml).

8. Plasmid and reporter constructs

A 1068-bp fragment spanning from -782 to +286 relative to the first transcription initiation site (a fragment corresponding to approximately one half of the CD3γ-CD3δ intergenic region plus exon 1 of CD3γ) was amplified from PBMC by PCR using 5'-ATTGTTCACCTATTGCCCTTCC-3' as a forward primer and 5'-GAAGGGCAAAATGGAGGCT-3' as a reverse primer (custom made from Invitrogen, Merelbeke, Belgium). It was then cloned into the Sfi site of the pPCR-Script plasmid (Stratagen). This recombinant plasmid was digested with SacI and
HindIII enzymes and the resulting fragments were cloned into SacI and HindIII digested pGL3-Basic vector.

To obtain various 5' deletion constructs, fragments were generated by PCR using sets of oligonucleotide primers. The latter were specific for the promoter sequence and included a 5'-linked SacI site in the forward primer and a 3'-linked HindIII site in the reverse primer.

The forward primers used were:

- pLuc-8/+286: 5'-GCGCGAGCTCGTGATGGGTGGAGCCAGTC-3'
- pLuc-52/+286: 5'-GCGCGAGCTCAGGCTCTGGGTTCTTGCCT-3'
- pLuc-80/+286: 5'-GCGCGAGCTCTGCTGCTCACACTTGAGCAG-3'
- pLuc-116/+286: 5'-GCGCGAGCTCAAAAGGCATCTCGACCTGC-3'
- pLuc-142/+286: 5'-GCGCGAGCTCCTCACCTCCTCCTTAACGGGCA-3'
- pLuc-192/+286: 5'-GCGCGAGCTCGATGAGTCTCTGAGTGGGAATCC-3'
- pLuc-232/+286: 5'-GCGCGAGCTCACCATCCCTCCACCCAGCA-3'
- pLuc-302/+286: 5'-GCGCGAGCTCAACAACTGGCTACGATCCTAACAA-3'
- pLuc-342/+286: 5'-GCGCGAGCTCCCTGATGGAAGGGTCCTGACT-3'

The reverse primer for all of these constructs was

5'-GCGCAAGCTTAGCCTCCATTTTGCCTTC-3'

All PCR products were digested with SacI and HindIII, and re-cloned into the SacI/HindIII site of pGL3-Basic vector.

9. Site-directed mutagenesis

Mutation in NFATγ1, NFATγ2, Sp1γ1, Sp1γ3 and +1 as well as deletion +1, deletion +6, deletion +21, Sp1γ1 probe (which contains the Sp1 binding site), from +28 to +37 (pLuc-782/+286<sub>DelD</sub>) and from +16 to +29 ((pLuc-782/+286<sub>DelD</sub>) were derived from pLuc-782/+286<sub>wt</sub> and generated by using the QuikChange site-directed mutagenesis kit (Stratagene) with the following primers:

- pLuc-782/+286<sub>NFATγ1mut</sub> 5'-CCTTCACCCTCCTTAAACCAAGGATCATCTGC-3'
- pLuc-782/+286<sub>NFATγ2mut</sub> 5'-CTGGACTAGGATGGGTGATTGGAGGTCCTGAGG-3'
- pLuc-782/+286<sub>Sp1γ1mut</sub> 5'-CTGGACTAGGATGGGTGATTGGAGGTCCTGAGG-3'
5'-CCCACCCAGCATCCATTTCTTTTCCCTGTGCAAGATG-3',
\[\text{pLuc-782/+286}_{\text{Sp1y1del}}\]
5'-GGGTCTTTGCTTTCGTGATGGGTGAGC-3'
\[\text{pLuc-782/+286}_{\text{Sp1y3mut}}\]
5'-CTCTCAAGGCCCCAGAACACAGTGATGGGTG-3'
\[\text{pLuc-782/+286}_{\text{DLD}}\]
5'-GCTGCTGCACAGGCCTGGCTGCTAAGG-3'
\[\text{pLuc-782/+286}_{\text{TLD}}\]
5'-GGGTGGAGCCAGTCGGCTGCTAAGGGC-3'
\[\text{pLuc-782/+286}_{\text{del+1}}\]
5'-CCAAAGGTAGGAGGGGAGCCAGTCTAGC-3'
\[\text{pLuc-782/+286}_{\text{del+6}}\]
5'-GTGATGGGGTGGAGCAGTCTAGCTGC-3'
and \[\text{pLuc-782/+286}_{\text{del+21}}\]
5'-GCCAGCCAGCCTGTCAGCAGCTAGGTGG-3'

10. Transient transfection

Jurkat, Raji, SupT1, HeLa, and 293T cells were all transiently transfected using the DEAE-dextran procedure as described by Van Lint (419) with some modifications. In brief, exponentially growing cells (3 x 10^6 for Jurkat, Raji, SupT1, GM607 cells and 2 x 10^6 for HeLa and 293T cells) were washed once with STBS 1X and resuspended in 410 µl of STBS (25 Mm Tris-HCl, pH 7.5, 1.37 mM NaCl, 5 mM KCl, 500 µM CaCl₂, 500 µM MgCl₂ and 600 µM Na₂HPO₄) containing 450 µg/ml of DEAE-dextran, 0.5 µg of the reporter plasmid (containing the regulatory region being tested), and 0.05 µg of an internal control plasmid containing the Renilla luciferase gene under control of the herpes simplex virus-1 thymidine kinase promoter pRL-TK vector (Promega Benelux). In the co-transfection experiments, an additional 0.085 µg of the NFATc1, NFATc2, or NF-κB p50 expression plasmids were transfected together with the reporter plasmid constructs in Jurkat.
11. Luciferase assay

The luciferase assay was done using a Dual-Luciferase Reporter Assay System (Promega, Benelux) according to the manufacturer's instructions. After incubation in complete medium for 40 h, cells were washed once with PBS and harvested in 75 µl of 1x passive lysis buffer for Jurkat, SupT1, Raji, GM607 and WE17/10 and 100µl of passive lysis buffer for HeLa and 293T cells. Firefly luciferase activity was measured from 20µl of the cell extract and the values for Firefly luciferase activity were normalized to the Renilla luciferase activity. The promoterless luciferase construct pGL3-Basic and pGL3-promoter vector (which encodes the luciferase gene under the control of SV40 promoter) were used as controls (Promega). Each experiment was performed in triplicate. All of the experiments shown are representative of at least three independent experiments to guarantee the reproducibility of the results.
Annex I

Literature Cited
Literature Cited


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Annex III

Publications
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'-GGAAAA-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 NFAT2 alone binds to the NFATγ3 motif; however, complexes containing either NFATc1 or NFATc1 plus NF-κB p50 bind to the NFATγ2 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'-GGAAAA-3') in NFATγ1 and NFATγ2 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 CD3δ (8, 9). A recent study has shown that the di-leucine motif in CD3δ 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 interferring 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 to 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 CD3δ (10, 30–32) and thus conditions favoring Nef expression could potentially enhance the activity of the CD3δ di-leucine motif.

The viral transcripational 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
typical TATA or CAAT box, despite the identification of promoter and enhancer sequences for the other TCR-CD3 genes: TCRI (36, 39), TCRB (40–43), TCRγ (42), TCRδ (40, 46), and the highly homologous CD3ζ (45–47). However, the recurring defect in CD3δ 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 transcriptional response element RNA element present at the 5'-ends of all nascent HIV-1 transcripts and subsequently activate 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 binding sites that have been shown to be a major cis-acting element for viral gene expression (52). The NFκBRel family of transcription factors (p50, p65, RelB, c-Rel, and p52) are induced in response to T cell activation signals to bind to the NFκB consensus sequence (5'-GGGACTTTCC-3'). Members of the NFκB family of transcription factors (NFκB1 (NFκB 1, NFκB2; NFκB2 (NFκB 2, NFκB2); NFκBc2 (NFκB c2, NFκB2); NFκBc2) and NFκBc2 have distinct effects on HIV-1 replication. NFκBc2 is thought to have major potentiation of NFκB translocation to the nucleus in the presence of the inhibitor. Flow Cytometry—Cells were analyzed for CD3 surface expression by flow cytometry as previously described (53). Briefly, cells were labeled with the murine monoclonal antibody OKT3 (directed to CD3) in a twostep process using 1 μg of antibody to CD3 followed by goat antimouse IgG (69) (Amersham, Arlington Heights, Ill.). The labeled cells were fixed in 2% paraformaldehyde, and fluorescence was analyzed on a FACScan (Becton Dickinson). Electrophoretic Mobility Shift Assay—Nuclear extracts were prepared from 2 × 10^6 cells according to a modified version of the method described by Osbom et al. (66). All buffers contained a mixture of protease inhibitors (Complete, Roche Diagnostics, Brussels, Belgium) to minimize proteolysis. The nuclear pellet was washed with ice-cold phosphate-buffered saline and then resuspended twice with 1 ml of ice-cold buffer A (20 mM HEPES buffer, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl). Cells were centrifuged (400 × 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 C (20 mM HEPES buffer, pH 7.9, 25% glycerol, 1.0 mM MgCl₂, 420 mM NaCl, 0.2 mM EDTA) for 20 min at 4 °C and then centrifuged at 20,800 × g for 10 min at 4 °C. The nuclear supernatants were diluted with 150 μl of buffer D (20 mM HEPES buffer, pH 7.9, 25% glycerol, 50 mM NaCl, 0.2 mM EDTA) and a mixture of protease concentration determinations were determined by the Bradford method (64). EMSAs 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, Arlington Heights, Illinois) using T4-polynucleotide kinase, annealed, isolated on a polyacrylamide gel, and extracted from the gel using the QiAEX II kit (Qiagen, Kleefeld, Germany) 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), 60 μM ZnCl₂, 0.25 mM diithothreitol, 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). Experimental Procedures—Cell Culture Conditions and Reagents—The WE1710 cell line is a human interh buffet 2 (IL-2)-dependent CD4⁺ T cell line (15, 60) that was established and is maintained in RPMI 1640 containing 20% fetal bovine serum, 1.25 mM l-glutamine, 0.55 mM l-arginine, 0.24 mM l-lysine, and 160 units of recombinant human IL-2 per ml (Cetus Corp., Emeryville, CA). WE1710 cells infected with the HIV-1 isolate LAI (61) or the molecular clone HXB2 (62) were used in previous experiments (13, 69). The human B lymphocyte line, Raji, was obtained from the American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 supplemented with 10% fetal bovine serum. WE1710 cells were treated for 18 h with the calcium channel blockers EGTA (2.0 μM) and BAPTA/AM (1–10 μM, bis-o-carboxymethylxanthine-4,4'-N,N'-tetraacetic acid), the P38 activator PMA (30 ng/ml, phorbol 12-myristate 13-acetate), the calcium ionsophores A23187 or ionomycin (30 ng/ml), and the protein kinase inhibitors herbimycin A (10 μM) and staurosporine (1–30 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 preseeded for 1 h with CsA followed by overnight stimulation with PMA and lono (in the continuous presence of CsA) to achieve the maximum potential induction of NFAT translocation to the nucleus in the presence of the inhibitor.
RESULTS

Measurement of the Relative Amounts of CD3y 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 CD3y 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 (16, 20). To better define the relationship between the number of CD3y 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 WE17/10 cells. RNA was extracted from cells at different stages in the progression from TCR-CD3⁺—TCR-CD3⁻—TCR-CD3⁻⁻ (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⁻). cDNAs, reverse-transcribed from the native RNA preparation, were co-amplified with serial dilutions of a competitor specific for the human CD3y gene (pUC18y), which had been engineered to produce a larger PCR product (Fig. 1, upper band) than the cellular CD3y 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 x 10⁶ molecules were added to the reaction mixture, followed by a corresponding decrease in native CD3y transcripts until they are no longer detectable in the presence of ~6 x 10⁵ molecules of the competitor. The competitor was detected earlier (at 6.6 x 10⁶ molecules) in RNA amplified from 100% TCR-CD3⁺ HIV-1-infected cells (mean fluorescence of control thymocyte 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 CD3y gene transcripts. Amplification of RNA from 90% TCR-CD3⁺ HIV-1-infected cells initially detected the competitor at a concentration of 1 x 10⁵ molecules, revealing a further decline equivalent to a total loss of >90% of CD3y gene transcripts. This extensive loss of transcripts prior to significant TCR-CD3 down-modulation was consistent for cells infected with a wide variety of viral variants. RNA extracted from HIV-1-infected cell lines expressing 60–80% 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 CD3y 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 CD3y 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 CD3y 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 induce phosphorylation of CD3γ on Ser-126 without activation of PKC), a combination of PMA plus ionophore (PMA-flono), 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 CD3γ, 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-CD3γuninfected or TCR-CD3γ HIV-1-infected cells but had no effect on the TCR-CD3γ-uninfected cells (Fig. 2, A and B). 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-CD3γ 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-CD3γ cells after treatment with 0.1–1.0 μg 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 μg of CsA (Fig. 2D). No cytokotoxicity 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-CD3γ) 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 (85% TCR-CD3<sup>+</sup>) (B) WE17/10 cells before and after treatment with 2.5 μM EGTA, 10 μM BAPTA/AM, 10 ng/ml staurosporine, 10 ng/ml PMA, and 10 ng/ml PMA + 30 ng/ml ionomycin. C, uninfected and HIV-1-infected cells (84% TCR-CD3<sup>+</sup>) treated for 3 and 7 days with 0.1–1.0 μg/ml CsA. D, uninfected and HIV-1-infected (42% TCR-CD3<sup>+</sup>) 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. 3) from HIV-1-infected (85% TCR-CD3<sup>+</sup>) cells before (top) and after (bottom) CsA treatment (0.1 μg/ml).
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We further asked whether the alignment of the 5'-upstream region of the human CD3γ gene is essential for immune response-directed cytokine gene expression, and it is via this pathway that CsA exerts its immunosuppressive activity. Therefore, we identified three NFAT consensus sequences (5'-GGAAAaa-3') at -124 to -120 (NFATγ1), -384 to -380 (NFATγ2), and -1450 to -1454 (NFATγ3) from the transcription initiation site (Fig. 3A), based on the published sequence NCBI accession number X06026 (71)). We further asked whether alignment of the 5'-upstream region of the human CD3γ gene is essential for CsA treatment to have a net positive effect on transcript numbers. Taken together with the fluorescence-extraction competition experiments shown in A to a 32P-labeled Oct-1 probe in an EMSA performed in vitro using the NFATγ1 probe, A, EMSAs were performed using the 32P-labeled NFATγ1 probe and nuclear extracts from unstimulated 100% TCR-CD3γ (lane 1) and PMA+Iono (each 30 ng/ml) stimulated 100% TCR-CD3γ (lane 2) and PMA+Iono-stimulated WE17/10 cells (lane 3). Nuclear extracts from PMA+Iono-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 7 and 8), and an oligonucleotide containing the IL-2 promoter NFAT consensus sequence mutated to abrogate NFAT binding (lanes 6 and 9), and an oligonucleotide containing the NFATγ3 sequence mutated from GGAGA to CCTT (lanes 10 and 11). Bands A-D indicate the four different protein-DNA complexes that specifically bind to the NFATγ3 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 region of the human CD3γ gene. We identified three NFAT consensus sequences (5'-GGAAAaa-3') at -124 to -120 (NFATγ1), -384 to -380 (NFATγ2), and -1450 to -1454 (NFATγ3) from the transcription initiation site (Fig. 3A), based on the published sequence NCBI 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, NCBI accession number K03455) and HIV-2 (Strain BEN, NCBI accession number M30502) would expose regions of sequence homology. This analysis revealed that the first motif, NFATγ3 (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 from the SPI 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 (GGGACTTTCC in HIV compared with CTGCGCTTCC 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 NFATγ 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 NFATγ1 motif by EMSA. Nuclear extracts of unstimulated WE17/10 cells (100% TCR-CD3γ), PMA+Iono-stimulated WE17/10 cells (100% TCR-CD3γ), and receptor-negative HIV-1-infected WE17/10 cells (TCR-CD3γ) were analyzed in parallel. At least four bands (Fig. 4, A-D), representing DNAprotein complexes with different electro-
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Fig. 5. Supershift and super-supershift analysis of NFAT, NF-κB, and AP-1 protein binding to the NFATγ probe. A, the 32P-labeled NFATγ probe was used in a supershift assay with nuclear extracts from TCR-CD3 HIV-infected cells in the absence of antibodies (lane 1) or in the presence of anti-NFATc1 (lane 2), anti-NFATc2 (lane 5), 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 is 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 NFATγ probe was examined in a supershift assay using nuclear extracts from TCR-CD3 HIV-infected WE17/10 cells untreated (lane 1) or treated with CsA (0.1 μg/ml) and PMA-Mono (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.

Phoretic mobility levels bind to the NFATγ probe. Nuclear extracts from uninfected, unstimulated cells contain only nominal amounts of the lower molecular weight bands C and D (lane 1). Stimulation for 18 h with PMA+Iono (lane 2) both down-regulated TCR-CD3 surface complexes (Fig. 2) and induced binding of E and C and to a lesser extent A (but no D) to the NFATγ probe. A similar binding profile was observed for the TCR-CD3 HIV-1-infected cells (Fig. 4, lane 1). The differential binding observed between nuclear extracts from uninfected/unstimulated TCR-CD3γ cells and TCR-CD3γ PMA+Iono-stimulated cells or TCR-CD3 HIV-1-infected cells was reproducible among different preparations of nuclear extracts and specific, because binding of the constitutively expressed Oct-1 transcription factor to its consensus sequence did not vary (Fig. 4f, lanes 1–3).

The specificity of the complexes bound to the NFATγ 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-2mut and NFATγ1 mutated to abrogate binding (76) (GGAAG → CCTT; NFAT-IL-2mut, lanes 8 and 9; NFATγ1mut, lanes 10 and 11). The homologous and the NFAT IL-2mut 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 NFκB but not NFAT binding), both efficiently compete for binding (data not shown). These experiments indicate that the nuclear protein complexes binding to the NFATγ 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 NFATγ Contain NFATγ1, NFATγ2, and NF-κB p50—Identification of some of the proteins present in the complexes bound to the NFATγ probe was achieved using antibodies to the NFAT family members, NFATc1 and NFATc2, the NF-κB family members, p50, p65, c-Rel, RelB, and p52, and the AP-1 family members, c-Fos and c-Jun, with nuclear extracts from TCR-CD3 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, RelB, 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
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anti-NFATc1 or the anti-p50 antibody, although the electrophoretic mobility of the anti-p50-supershifted complex (upper A↓) was slower than the anti-NFATc1-supershifted complex (lower A↓). The B and C complexes were both supershifted to a similar electrophoretic mobility with the anti-NFATc2 antibody only (B↓+C↓). The C and D complexes expressed at low levels in unstimulated, uninfected WE17/10 cells could be supershifted entirely and exclusively with the anti-NFATc2 antibody indicating that these lower molecular weight complexes contain NFATc2 but not NFATc1 or NF-xB p50 (data not shown). These data suggested that there were at least three different nuclear complexes bound to the NFATγ, probe in activated or infected cells, one containing NFATc1 and NF-xB p50 (band A; present at lower concentrations) and the other two containing NFATc2 (bands B and C; present at higher concentrations; the low molecular weight NFATc2 containing band D was detected only in the unstimulated, uninfected cells).

Confirmation of this observation was achieved by developing a modified supershift assay where combinations of the anti-NFATc1, anti-NFATc2, and anti-p50 antibodies were added sequentially to the binding reaction. These experiments lead to two distinct results: 1) a double-supershift where each antibody binds to a separate complex and individually supershifts the band(s) and 2) a super-supershift where the two antibodies bind to the same complex and their synergy further increases its molecular weight thereby reducing its electrophoretic mobility. Combining the anti-NFATc1 and anti-NFATc2 antibodies (Fig. 5A, lanes 7 and 10) or the anti-NFATc2 and anti-p50 antibodies (lanes 8 and 9) in either order produced double-supershifts where the A, B, and C complexes were all supershifted (A↓, B↓+C↓), migrating with the same electrophoretic mobility as with the individual antibody alone (lanes 2–4). Alternatively, both combinations of anti-NFATc1 + anti-p50 (lanes 5 and 8) produced a super-supershift where the electrophoretic mobility of the A complex (A↓↓) was consistently further retarded compared with the anti-p50 antibody alone (upper A↓↓, lane 4). A second A band, migrating with the same electrophoretic mobility as with the anti-NFATc1 antibody alone (lower A↓↓, lane 2), was also detected when the anti-p50 and anti-NFATc1 antibodies were used together. This suggests that although some of the A complexes contain NFATc1 and NF-xB p50 others contain NFATc1 alone. Alternatively, NF-xB 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 NFATγ, probe, including: two abundant complexes that contain NFATc2 (bands B and C) but not NFATc1 or NF-xB p50 and two low concentration complexes (band A) devoid of NFATc2, one which contains NFATc1 and NF-xB p50 and the other either NFATc1 alone or NFATc1 and an inaccessible NF-xB p50.

Uninfected TCR-CD3+ and HIV-1-infected TCR-CD3- cells were treated with CsA and then stimulated with PMA+Ion 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 NFATγ, 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 NFATγ, probe and anti-NFATc1, anti-NFATc2, and anti-NF-xB p50 antibodies (Fig. 5B). Binding of the NFATc1 and NF-xB p50 containing A complex was totally inhibited by CsA treatment (overexpression 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-xB p50 is Negatively Correlated with TCR-CD3 Surface Expression in HIV-1-infected Cells—The relationship between the presence of NFATc1, NFATc2, and/or NF-xB p50 in the nucleus and the concentration of CD3γ gene transcripts was assessed by examining differential binding to the NFATγ, probe of nuclear extracts during the progression of HIV-1-infected cells from TCR-CD3+→TCR-CD3−→TCR-CD3γ− (Fig. 6A). Characteristically, only low levels of the NFATc2-containing complexes (bands C and D) were detectable in the uninfected and unstimulated 100% TCR-CD3+ cells (lane 1). Alternatively, increased binding of the NFATc1/NF-xB p50-containing complex (band A) and NFATc2-containing complexes (bands B and C) to NFATγ, occurs in parallel with a decrease in surface TCR-CD3 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).
Fig. 7. Comparative binding to the NFATy, NFATy, and NFATy probes. A, binding to the ^P-labeled NFATv probe was examined in a supershift assay using nuclear extracts from TCR-CD3 HIV-1-infected WE17/10 cells without antibody (lane 1) or with anti-NFATc (lane 2), anti-NFATc (lane 3), anti-NFATc (lane 4), anti-c-Jun (lane 5), or anti-c-Fos (lane 6) antibodies. B, binding to the ^P-labeled NFATv probe was examined in a supershift assay using nuclear extracts from TCR-CD3 HIV-1-infected WE17/10 cells without antibody (lane 1) or with anti-NFATc (lane 2), anti-NFATc (lane 3), or anti-NF-xB p50 (lane 4) antibodies. C, the relative quantity of proteins bound to the ^P-labeled NFATv, NFATv, and NFATv probes was examined using nuclear extracts from uninfected 100% TCR-CD3 HIV-1-infected WE17/10 cells (lanes 2, 4, and 6).

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escalation in binding to the NFATv 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 NFATc, and to a lesser extent, NFATc and NF-xB p50, in the nucleus and down-modulation of CD3y transcripts and TCR-CD3 complexes after HIV-1 infection.

Differential Binding of NFATc, NFATc, and NF-xB p50 to the NFATy Motif—We next asked whether members of the NFAT and/or NF-xB protein families could also bind to the NFATy and/or the NFATy motifs. EMSA experiments using the NFATv probe (−392 to −372, underlined in Fig. 3) and extracts from unstimulated cells, PMA- and/or-stimulated cells, and TCR-CD3 HIV-1-infected cells bound in a similar pattern to NFATv (Fig. 4) except that only the NFATc-containing complexes (bands B, C, and D) but not the NFATc:NF-xB p50-containing complex (band A) were detected (data not shown). Alternatively, binding to the NFATv probe (+447 to +466, underlined in Fig. 3) was identical to NFATv with all four of the complexes bound (A–D; data not shown). An experiment using the NFATv and NFATv probes in competition with the homologous or the NFATv and NFATv-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 NFATc, NFATc, and NF-xB p50 to the NFATv, NFATv, and NFATv sequences was confirmed in a supershift assay using antibodies to the NFAT, AP-1, and NF-xB 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 NFATv probe (Fig. 7A, lane 2, bands B and C), whereas no band shift was observed with antibodies to NFATc (lane 3), to the NF-xB proteins p50 (lane 4), p65, c-Rel, Rel B, or p52 (data for the latter four antibodies were 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 NFATc but not NFATc, AP-1, or NF-xB family proteins bind to the NFATy probe, despite its homology with the NF-xB region in the HIV-1 LTR. On the contrary, a supershift assay using the NFATv probe (Fig. 7B) was qualitatively similar to the NFATv probe, with supershifted complexes observed for the anti-NFATc (band A, lane 2), anti-NFATc (bands B and C, lane 3), and anti-NF-xB p50 antibodies (band A, lane 4). We compared the relative binding of the NFATc plus NF-xB p50- and NFATc-containing complexes to the NFATv, NFATv, and NFATv motifs (Fig. 7C) and found that NFATv binds significantly more of these protein complexes compared with NFATv and NFATv, with binding to the NFATv 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 NFATv, NFATv, and NFATv.

Sequence Variation Is Responsible for the Differential Binding of NFATc, NFATc, and NF-xB p50 to the NFATy Motif—In an effort to understand the basis for the qualitative and quantitative differences in binding to the NFATv, NFATv, and NFATv 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 5'-GGAAA-3' sequence differed by an AA immediately following the core 5'-GGAAA-3' sequence. In contrast to a GC in NFATy, suggesting that these
nucleotides could potentially play a role in the binding of NFATc1 and NF-kB 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, NFAT, and NFAT sequences, respectively. Our rationale was that if these three nucleotides do play an important role in binding, then successively mutating the NFAT, sequence to look like the NFAT, sequence and vice versa should alter binding accordingly.

Mutation of the first A following the core sequence in NFAT, to a G (NFAT,mut1; Fig. 5A, lane 2) completely abrogated binding of NFATc1 and NF-kB p50 (band A), significantly decreased the binding of NFATc2 (bands B and C) compared with the wild type sequence (NFAT,wt, lane 1) and provided a pattern similar to that of wild type NFAT, (NFAT,wt, lane 6). Additionally mutating the second A to a C in NFAT, (NFAT,mut2, lane 3) changed the 3’ sequence to that of NFAT, and reduced NFATc2 binding even further. Mutation of the outside A only in the AA pair of NFAT, (NFAT,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-kB p50, although quantitatively all of the complexes were significantly reduced. Mutation of the C preceding the core sequence in NFAT, (NFAT,mut4, lane 5) to a T, creating the sequence 5’-GGAAAAGC-3’, greatly enhanced the amount of NFATc1, NFATc2, and NF-kB p50 bound to this probe, providing better binding than that observed with any of the wild type sequences.

Alternatively, the reverse mutations in NFAT, converted the binding profile of this probe to one similar to NFAT, with increased binding of NFATc2 (bands B and C) and de novo binding of NFATc1 and NF-kB p50 (band A) achieved by simply changing the 3’ G (NFAT,mut4, lane 6) to an A (NFAT,mut1, lane 7). Adding a second A 3’ of the core sequence in NFAT, (NFAT,mut2, 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-kB p50, although it did increase the binding of NFATc2 (NFAT,mut3, lane 9). Finally, mutation of the T preceding the core sequence to a C, creating the 5’-CGGAAAGC-3’, completely abrogated all binding (NFAT,mut4, lane 10). Confirmation that the specific binding of NFATc1 and NF-kB p50 was conferred by adding a fourth A to the NFAT core sequence (5’-GGAAAAA-3’) was demonstrated by a supershift assay using the NFAT,mut2 probe (Fig. 5B). This experiment clearly shows that a simple G → A substitution 3’ of the core sequence in NFAT, is sufficient to confer binding of NFATc1 and NF-kB p50. Finally, binding to the wild type NFAT, sequence is normally weak, and mutation of the A following the core sequence to G completely abrogated binding (NFAT,mut3, lane 2) compared with the wild type (NFAT,wt, lane 1).

Taken altogether, these mutation experiments demonstrate that a fourth A added to the NFAT core sequence (5’-GGAAAAA-3’) is vital for NFATc1 and NF-kB 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’-GGAAAA-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-kB p50.

FIELD OF 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-CD3αβ = TCR-CD3αβ*, in which the forward progression is markedly favored (16). The TCR-CD3αβ to TCR-CD3γ 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-CD3αβ to TCR-CD3γ 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 precedes 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 4–5 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-CD3αβ to TCR-CD3αβ*, with a further 4- to 5-fold reduction accompanying the transition from TCR-CD3αβ* to TCR-CD3αβ* (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 interpre-
Differential NFAT Binding in the Human CD3γ Chain Gene

Fig. 8. Mutation analysis of the NFAT\textsubscript{c1}, NFAT\textsubscript{c2} and NFAT\textsubscript{p50} probes. 
A, EMSAs were performed using nuclear extracts from TCR-CD3\textsuperscript{+} HIV-infected WE17/10 cells and the \textsuperscript{32}P-labeled NFAT\textsubscript{c1} probe as shown in Table I (lanes 1–10). B, binding to the \textsuperscript{32}P-labeled NFAT\textsubscript{c1max} probe was examined in a supershift assay using nuclear extracts from TCR-CD3\textsuperscript{+} HIV-1-infected WE17/10 cells and anti-NFATc1 (lane 2), anti-NFATc2 (lane 3), and anti-NF-p50 p50 (lane 4) antibodies. C, EMSA binding to the \textsuperscript{32}P-labeled NFAT\textsubscript{c2} (lane 1) and NFAT\textsubscript{p50} (lane 2) probes using nuclear extracts from TCR-CD3\textsuperscript{+} HIV-1-infected WE17/10 cells.

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A search of the 5′-upstream region and exon 1 of the human CD3γ gene revealed three potential binding motifs for NFAT family proteins (NFAT\textsubscript{c1}, NFAT\textsubscript{c2}, and NFAT\textsubscript{p50}). The NFAT\textsubscript{c1} motif is located in a DNase I-hypersensitive site that has been designated as the putative promoter for CD3γ (71, 86, 87), whereas the NFAT\textsubscript{c2} motif is nested in a region with sequence homology to the HIV-1 \textit{k}B elements. Three different molecular weight complexes (A, B, and C) could be induced by PMA + ion 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 Ca\textsuperscript{2+}-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-\textit{k}B p50 (but not NFATc2). To determine whether NFATc1 and NF-\textit{k}B 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 super-supershift assay). This experiment demonstrated that some of the A complexes contain both NFATc1 and NF-\textit{k}B p50, whereas others contain either NFATc1 alone or an inaccessible NF-\textit{k}B 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 was used as 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-
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The dual binding of NFATc1 and NF-κB p50 contributed relatively little additional weight to the 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 NFATc2, but not NFATc4, was provided by the EMSA experiments using mutant oligonucleotides. Changing the fourth A in the NFATc1 motif (5'-GGAAAAG-3' to 5'-GGAAAAA-3') completely abrogated binding of the NFATc1-NF-κB p50-containing complex, whereas adding a fourth A to the NFATc4 motif (5'-GGAAAGAG-3' to 5'-GGAAAAA-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. NFATc1 and NF-κB normally compete for binding to the p50 site, and this has been demonstrated to be true for the HIV-1 LTR p50 site (61). 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 also can be capable of forming p50/p50 or p65/p65 homo-dimers. The formation of homo- and heterodimers leading to dimerization is known to be required for binding of the NFAT family proteins to DNA (91). Crystal structures have shown that NF-κB p50 optimally binds to the 5'-GGAAA-3' half site and p65 the 5'-GGAAA-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 affinity (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 (35, 58, 97). Other common features between the NFAT and NF-κB proteins include their responsiveness to immune activation and their regulation by cytokispic to nuclear translocation.

The NFATγ and NFATδ 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 Ca2+-treated cells revealed that NF-κB p50 does not bind to the NFATc4 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'-GGAAAAA-3') was found to be crucial for their binding. This core motif is also the only component common between the NFATc1 and NFATc2, but not the NFATγ probes and thus emerges as the requisite sequence for binding of the NFATc1-NF-κB p50 complex. Sites in which the 5'-GGAAAAA-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 NFATγ (5'-TGGAAG-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 functions 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 (NFATγ, NFATδ, and NFATc2) 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 NFATγ 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 (50). Although the accumulation of NFAT family proteins in the nucleus has a positive influence on cytokine gene transcription, it also potentially negatively regulates CD3γ 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.

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Summary

Dynamic regulation of T cell receptor (TCR)-CD3 complexes on the cell surface plays a critical role in controlling antigen-dependent T cell mediated immune responses. Our laboratory has clearly demonstrated that the progressive loss of TCR-CD3 receptor expression and function after HIV-1 or HIV-2 infection results from a specific loss of CD3γ gene transcripts, while expression of the other CD3 genes remain unchanged. Northern and dot blot hybridization analyses suggested that the loss of CD3γ does not parallel the downregulation of TCR-CD3 complexes from the surface at a ratio of 1:1. We have used quantitative RT-PCR to gain further insight into the CD3γ gene defect and found that the loss of CD3γ gene transcription begins early after HIV-1 infection and accumulates to a deficiency of more than 90% of transcripts before a significant effect on surface TCR-CD3 density is apparent.

Treatment of infected cells with antisense phosphorothiolate oligodeoxy-nucleotides demonstrated that HIV-1 tat and/or nef gene products play an important role in the loss of CD3γ transcripts after infection. Therefore, we initiated an investigation designed to identify and characterize the elements responsible for controlling transcription of the human CD3γ gene. We cloned the sequence located between −782 and +286 of the hCD3γ gene and demonstrated that it is transcribed from a weak, non-tissue specific TATA-less promoter. We have located both positive and negative regulatory elements in the promoter including, three NFAT consensus sequences (5'-GGAAA-3') at: −124 to −120 (NFATγ1), −384 to −380 (NFATγ2), and +450 to +454 (NFATγ3) from the first transcription initiation site. EMSA experiments were used to demonstrate that NFATc2 alone binds to the NFATγ2 motif while complexes containing either NFATc2 or the unusual combination of NFATc1 plus NF-κB p50 bind to the NFATγ1 and NFATγ3 sites. We further demonstrated that an increase in the binding of nuclear NFATc2, in particular, to these motifs is correlated with a progressive loss of hCD3γ transcripts after HIV-1 infection. Mutation and deletion analysis revealed that the NFATγ1 site positively or negatively influences promoter activity depending upon whether NFATc1 plus NF-κB p50 or NFATc2 containing complexes are bound, respectively. Alternatively, the NFATγ2 site, which binds NFATc2 only, negatively regulates promoter activity.

We have located a GC box at −22 to −13 in the hCD3γ promoter, demonstrated that Sp1 specifically binds to this region, and shown that this motif plays a positive role in expression of the hCD3γ gene. We have explored the functional activity of the three transcription initiation sites, located at +1, +6 and +21, and found that a critical element surrounds the +1 and +6 nucleotides. Our preliminary data suggests that this sequence functions as an initiator element, and that an additional critical positive element is present at +12 to +50 of the downstream hCD3γ sequence. The experimental data presented in this thesis adds important information to our understanding of transcriptional regulation of the CD3γ gene and a number of interesting parallels with the HIV-1 LTR, which we shall exploit in future experiments.