T-cell Activation Leads to Poor Activation of the HIV-1 Clade E Long Terminal Repeat and Weak Association of Nuclear Factor-κB and NFAT with Its Enhancer Region

Received for publication, August 27, 2004, and in revised form, September 29, 2004
Published, JBC Papers in Press, October 1, 2004, DOI 10.1074/jbc.M409896200

Anne-Marie Lemieux‡, Marie-Ève Paré‡, Brigitte Audet‡, Éric Legault‡, Sylvain Lefort‡, Nancy Boucher†, Sébastien Landry†, Tim van Opjik‡, Ben Berkhour†, Mojgan H. Naghavi¶, Michel J. Tremblay†¶**, and Benoit Barbeau†¶‡‡

From the ‡Centre de Recherche en Infectiologie, Centre Hospitalier Universitaire de Québec, Pavillon CHUL, and the Département de Biologie Moléculaire, Faculté de Médecine, Université Laval, Sainte-Foy, Quebec G1V 4G2, Canada, the §Department of Human Retrovirology, University of Amsterdam, 1105 AZ Amsterdam, The Netherlands, and the ¶Howard Hughes Medical Institute, Columbia University, College of Physicians and Surgeons, New York, New York 10027

The enhancer region in the human immunodeficiency virus type 1 (HIV-1) 5′-long terminal repeat (LTR) is very important for viral transcription. This promoter sequence binds both nuclear factor-κB and NFAT, two important modulators of HIV-1 gene expression. Previous studies have indicated that the enhancer regions of the different HIV-1 clade LTRs differ in their number of NF-κB-binding sites. In this study, we have compared the activation potential of the different HIV-1 clade and HIV-2 LTRs and assessed their interaction with NFAT and NF-κB. In T-cell lines and primary CD4+ T-cells, the results showed that the HIV-1 clade E LTR (with a single NF-κB-binding site) was the weakest LTR regardless of the tested activators, whereas the HIV-2 LTR was the most responsive LTR. The clade E enhancer region was also demonstrated to be the weakest enhancer region in transfection experiments with luciferase reporter-based vectors. Electrophoretic mobility shift assays with extracts from activated CD4+ T-cells indicated that, although NF-κB and NFAT bound all enhancers, HIV-1 clade E and HIV-2 LTR enhancers were poor binding targets for these two factors. Weak NFAT binding to clade E enhancers was also confirmed using NFAT1-expressing 293T cells in competition experiments. We have also shown the absence of interaction of NF-κB or NFAT with the third NF-κB repeat present in clade C. However, the clade C enhancer bound NFAT more efficiently than all other enhancer regions tested. Our results hence demonstrate for the first time that differences in the binding of NF-κB and NFAT to the enhancer regions could be responsible for some of the observed variation in HIV-1 clade LTR activation, whereas HIV-2 LTR activation seems mostly independent of these interactions.

Human immunodeficiency virus type 1 (HIV-1), the causal agent of AIDS, has an important tropism for CD4+ T lymphocytes and macrophages. In T-cells, the replicative cycle of this virus is greatly dependent on the activation state of the infected cells. Hence, in a high activation state, higher replication can be detected, which is mainly consequent to a higher level of transcription. HIV-1 transcription is dependent on the 5′- long terminal repeat (LTR) region, which harbors the important enhancer sequence. Nuclear factor-κB (NF-κB) is a cellular transcription factor that has been demonstrated to be an essential regulator in the activation of the HIV-1 LTR through its interaction with the enhancer region (1). However, a series of studies have suggested that NFAT (nuclear factor of activated T-cells) could also bind to the HIV-1 enhancer region (10 to 81) of the LTR and modulate overall LTR-mediated gene expression (10, 13, 15, 22, 23, 28, 38).

The NF-κB transcription factor is composed of two subunits that are part of the Rel family and is expressed in virtually all cell types. The predominant form of NF-κB implicated in the process of HIV-1 LTR activation is a heterodimer composed of the 50-kDa (p50) and 65-kDa (p65 or RelA) subunits (2–4). Recent findings have suggested that the effect of NF-κB on HIV-1 expression might depend on an increase in both initiation and elongation of transcription (5). The NF-κB complex is usually retained in the cytoplasm as an inactive factor associated with a repressor termed IκBα that masks the nuclear localization signal (6–9). The sudden activation of NF-κB by a range of activators induces the release and degradation of IκBα subsequent to its phosphorylation, thereby allowing NF-κB to move freely from the cytoplasm to the nucleus (10–14). Nuclear NF-κB can subsequently act on the targeted genes.

The NFAT members are part of a family of Rel-related transcription factors that are activated early after T-cell activation. Several NFAT family members are present in human T-cells, such as NFAT1 (NFATp), NFAT2 (NFATc), NFAT3, and NFAT4 (NFATx) (15) and NFAT5 (16). Like NF-κB, NFAT factors are sequestered in the cytoplasm and translocated to the nucleus following an increase in the intracellular calcium content (17, 18). Modulation of intracellular calcium triggers conformational changes in calmodulin and increases its bind-

* This work was supported in part by Have a Heart Grant 507-0154 from the Canadian Foundation for AIDS Research and Grant 003318 from the Fonds de la Recherche en Santé du Québec. To whom correspondence should be addressed: Centre de Recherche en Infectiologie, Centre Hospitalier Universitaire de Québec, Pavillon CHUL, 2705 Blvd. Laurier, Sainte-Foy, Quebec G1V 4G2, Canada. Tel.: 418-654-2705; Fax: 418-654-2196; E-mail: benoit.barbeau@crchul.ulaval.ca.

** Recipient of a Senior Canada Research Chair in Human Immunoretrovirology.

†† To whom correspondence should be addressed: Centre de Recherche en Infectiologie, BC709, Centre Hospitalier Universitaire de Québec, Pavillon CHUL, 2705 Blvd. Laurier, Sainte-Foy, Quebec G1V 4G2, Canada. Tel.: 418-654-2705; Fax: 418-654-2196; E-mail: benoit.barbeau@crchul.ulaval.ca.

1 The abbreviations used are: HIV-1, human immunodeficiency virus type 1; LTR, long terminal repeat; NF-κB, nuclear factor-κB; TNF, tumor necrosis factor-α; PHA, phytohemagglutinin; PMA, phorbol 12-myristate 13-acetate; IκBα, ionomicyn; DTT, dithiothreitol; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; EMSA, electrophoretic mobility shift assay.
ing to the calcineurin serine/threonine phosphatase, leading to its activation. The ensuing NFAT dephosphorylation by calcineurin renders the nuclear localization sequence accessible, allowing nuclear translocation (19). The consensus binding sequence for NFAT is 5′-(T/A)GGAAA(A/N)(A/T/C)-3′ (20). This sequence has similarity to the antisense strand in the NF-κB-binding sites located in the enhancer region of the HIV-1 LTR, which have thus been suggested to represent potential NFAT-binding sites (21, 22). A recent study has provided evidence that NFAT1 interacts as a dimer on the HIV-1 enhancer region through a unique conformation (23).

Although the mechanism of action of these factors on HIV-1 LTR activation is now well documented, their implication in the transcription process following activation has not been clearly assessed in a comparative fashion in the various existing HIV-1 isolates. The known heterogeneity in HIV-1 sequences has led to the classification of the isolates in different clades. Presently, at least 10 distinct genetic subtypes (clades A–J) of HIV-1 have been identified based on phylogenetic analyses of gag and env genes (24). The different subtypes are not distributed evenly throughout the world. For example, subtype B predominates in North America and Europe, subtype C in South Africa and India, and subtype E in Northern Thailand (25). When representative isolates of the different clades are compared within the LTR sequence, a substantial degree of variability is apparent and is preserved in general within each clade (26). Importantly, the number of NF-κB repeats has been observed to vary from one to three between the different clades: two NF-κB-binding sites are present in the enhancer regions of clades A, B, D, F, and G; three in clade C; and only one in clade E (24, 26). Interestingly, the closely related HIV-2 virus has also been demonstrated to vary in terms of its LTR structure compared with the HIV-1 LTR (27, 28). First, the typical enhancer sequence harbors only one NF-κB repeat, but in addition, the LTR region bears DNA-binding sites upstream of this latter enhancer region, which are presumed to play a substantial role in LTR activation (29–31).

In this study, we were interested in determining whether the variable configuration of the different HIV-1 clade LTRs and the HIV-2 LTR could lead to an altered response to various T-cell-activating agents paralleled by a difference in the implication of the NF-κB and NFAT transcription factors. In this study, we report for the first time a comparison of the LTR activation of the different clades with various activators and in relation to the binding of both NFAT and NF-κB. We show that the HIV-1 clade E LTR was the weakest induced promoter in response to stimulation by different tested T-cell-activating agents. Surprisingly, HIV-2 was the most potently activated response to stimulation by different tested T-cell-activating agents. Surprisingly, HIV-2 was the most potently activated response to stimulation by different tested T-cell-activating agents. Surprisingly, HIV-2 was the most potently activated response to stimulation by different tested T-cell-activating agents. Surprisingly, HIV-2 was the most potently activated response to stimulation by different tested T-cell-activating agents.
with Me₂SO (1.25%), and the cells were incubated at 37 °C for 24 h. Transient transfection of 293T cells was conducted with the calcium phosphate transfection protocol as described previously (40). Primary CD4⁺ T lymphocytes were transfected with the Nucleofector device (Amaxa Biosystems, Gaithersburg, MD) according to the manufacturer’s instructions. Briefly, unstimulated primary CD4⁺ T-cells (5 × 10⁶ cells) were washed once with phosphate-buffered saline and then suspended in the human T-cell Nucleofector solution with the indicated plasmids. The cell/DNA mixture was added to a cuvette, inserted in the Nucleofector, and transfected. CD4⁺ T-cells were then activated.

For stimulation, transiently transfected T-cells were seeded at a density of 10⁶ cells/well (100 μL) in 96-well plates. In the form of triplicates, cells were either left untreated or treated with phytohemagglutinin (PHA; 3 μg/mL), ionomycin (1 μM; Sigma), or 2M (10 μM; Alexis Corp., San Diego, CA), or TNFα (20 ng/mL; R&D Systems, Minneapolis, MN) in a final volume of 200 μL. Antibody OKT3 (40 μg/mL) was incubated with paraformaldehyde-fixed DT30 cells (2 × 10⁶ cells), which were used to stimulate Jurkat cells at a ratio of one DT30 cell/10 Jurkat cells. Cells were then incubated at 37 °C for 8 or 16 h for cell lines and primary CD4⁺ T-cells, respectively, and lysed with 1× lysis buffer (25 mM Tris pH 7.8, 2 mM dithiothreitol (DTT), 1% Triton X-100, and 10% glycerol). Luciferase activity was determined as follows. After a freeze/thaw cycle, 20 μL of cell extract was transferred to a 96-well luminescence plate, and luciferase activity was measured on a Dynex MLX microplate luminometer following the addition of 100 μL of luciferase buffer (20 mM Tricine, 1.07 mM (MgCO₃)₂·Mg(OH)₂, 5.67 mM MgSO₄, 0.1 mM EDTA, 220 μM CoA, 4.7 μM β-luciferin potassium salt, 530 μM ATP, and 33.3 mM DTT). To assess transfection efficiency in the different transfected samples, the β-galactosidase activity was monitored in the form of triplicates on the luminometer with a Galacto-Light™ commercial kit (Applied Biosystems, Bedford, MA) according to the manufacturer’s protocol. Values from the luminometer are expressed as relative light units. Fold induction was calculated as the ratio of the mean values of the luciferase activity of treated and untreated samples.

Preparation of Nuclear Extracts, Probe Labeling, and Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts were prepared according to a previously described microscale preparation protocol (41). Jurkat or transfected 293T cells were first washed with ice-cold phosphate-buffered saline. Cells were then resuspended in 400 μL of hypotonic buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5 mM phenylmethylsulfonyl fluoride), kept for 15 min on ice, and lysed with 25 μL of 10% Nonidet P-40. After brief vortexing and centrifugation, the supernatant was discarded, and the pellet was resuspended in hypotonic buffer (20 mM HEPES (pH 7.9), 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride), followed by gentle agitation for 15 min. The solution was then centrifuged, and protein concentration was measured in the supernatant by BCA assay (Pierce) before storage at −85 °C. Radioactive labeling of the oligonucleotides was performed by adding 50 ng of one of the DNA strands in T4 polynucleotide kinase buffer containing 30 μCi of [γ-³²P]ATP and T4 polynucleotide kinase and incubating the mixture for 30 min at 37 °C. The reaction was stopped by the addition of EDTA. After phenol/chloroform extraction, the aqueous phase was spun through a Sephadex G-50 column for further purification. The oligonucleotide was then allowed to hybridize overnight with 200 ng of the complementary strand in annealing buffer (100 mM NaCl, 5 mM Tris (pH 7.5), 10 mM MgCl₂, 20 μM EDTA, and 1 mM DTT). The sequence of each labeled complete enhancer probe is presented in Table 1. Nuclear extracts (10 μg) were mixed with poly(dI-dC) (1 μg/mL), bovine serum albumin (1 μg/mL), and the labeled oligonucleotide in binding buffer (100 mM HEPES (pH 7.9), 40% glycerol, 10% Ficoll, 250 mM KCl, 10 mM EDTA, and 250 mM NaCl). This mixture was incubated for 20 min at room temperature and then run through a 4% or 6% (w/v) polyacrylamide gel for 2 h at 150 V. Competition experiments were performed with a 100-fold excess of unlabeled oligonucleotides containing the NFAT-binding site of the murine interleukin-2 promoter (5'-TCAGCCCCAAGAAGATTTGGTCTACACG-3'). In some experiments, each NF-kB repeat of the clade C enhancer was used as either a probe or unlabeled competition oligonucleotide and identified as follows: repeat I, 5’-CCAGTGGGCGGTCTAGGAGG-3; repeat II, 5’-TCGGCTGGGAGATTTGGGGA-3; and repeat III, 5’-ACAGAAAGGGCTTTGCCGTTACGGG-3’. An additional typical sequence from the extra repeat of clade C was also used in EMSAs: 5’-CCACTGGGGGCTTCAGGGGAGG-3’. Finally, the distal and proximal NF-κB repeats from the NL4-3 clade B enhancer (5’-CCAGCTGAGGATTTGGGGA-3’, i.e. 5’-CCACTGGGGGCTTCAGGGGAGG-3’, and repeat III, 5’-ACAGAAAGGGCTTTGCCGTTACGGG-3’). Certain competition experiments were also performed to evaluate band intensity of the specific complexes. Dried gels were thus placed for 2 h at 85 °C. Certain competition experiments were also performed to evaluate band intensity of the specific complexes. Dried gels were thus placed for 2 h at 85 °C.

RESULTS

HIV-1 Clade E LTRs Are Less Potently Stimulated by Different T-cell-activating Agents—An important divergence in the nucleotide sequence has been demonstrated in the LTR regions of the different clades of HIV-1 (24, 26). The heterogeneity in the enhancer region is of particular interest in the context of T-cell activation known to involve transcription factors binding to this LTR segment. In particular, most clades bear two NF-kB-binding sites in their enhancer region, whereas clades E

| Enhancer | bp | Sequence* |
|----------|----|-----------|
| HIV-1 clade A (UG5218) | 40 | 5’-GAGCTTCTGCTGGGAGGACTTTCCGAGG-3’ |
| HIV-1 clade B (ES6548) | 40 | 5’-GAGCTTCTGCTGGGAGGACTTTCCGAGG-3’ |
| HIV-1 clade C (GM6439) | 42 | 5’-GAGCTTCTTCCGAGGACTTTCCGAGG-3’ |
| HIV-1 clade D (UG6357) | 40 | 5’-GAGCTTCTGCTGGGAGGACTTTCCGAGG-3’ |
| HIV-1 clade E (GM6452) | 40 | 5’-GAGCTTCTGCTGGGAGGACTTTCCGAGG-3’ |
| HIV-2 ROD10 | 40 | 5’-GAGCTTCTGCTGGGAGGACTTTCCGAGG-3’ |
| NL4-3 (B) | 31 | 5’-GAGCTTCTGCTGGGAGGACTTTCCGAGG-3’ |
| HIV-1 clade E (GM6452:2) | 40 | 5’-GAGCTTCTGCTGGGAGGACTTTCCGAGG-3’ |
| HIV-1 clade E (15litr) | 40 | 5’-GAGCTTCTGCTGGGAGGACTTTCCGAGG-3’ |
| HIV-1 clade E (CM240ltr) | 40 | 5’-GAGCTTCTGCTGGGAGGACTTTCCGAGG-3’ |

* NF-κB-binding sites for each Enhancer sequence are indicated in boldface.
and C contain one and three binding sites, respectively. Although early reports had compared activation of the different clade LTRs under certain conditions, we were interested to look at the response of the different HIV-1 clade LTRs to a variety of T-cell activators. We have thus used the standard Jurkat cell line, which is a good T-cell line model for signal transduction analyses.

Different T-cell-activating agents were tested, including T-cell receptor-cross-linking agents as well as more downstream activating agents such as the protein kinase C-activating agent PMA, the calcium ionophore Iono, and the protein-tyrosine phosphatase inhibitor bpV(pic). We have demonstrated previously that this latter activator is a strong inducer of HIV-1 LTR activity, a transcription up-regulation that depends on both NF-κB and NFAT translocation and binding to the HIV-1 enhancer region (38, 42). In addition, in this list of activators, we have also included the DT30 murine cell line, which expresses human B7.1 and the Fc receptor, which, along with antibody OKT3, mimic typical antigen-dependent T-cell activation. Jurkat cells were first transfected with constructs containing HIV-1 clade (A–E) LTRs upstream of the luciferase reporter gene and then stimulated for 8 h (or 16 h for primary cells) with the following agents: PHA (3 μg/ml), PMA (20 ng/ml), PHA (3 μg/ml)/PMA (20 ng/ml), PMA (20 ng/ml)/Iono (1 μM), bpV(pic) (PIC; 10 μM), TNFα (20 ng/ml), and OKT3 (40 μg/ml)/DT30 (1:10 Jurkat). Cells were then lysed and evaluated for both luciferase and β-galactosidase activities as described under “Experimental Procedures.” Values are the means ± S.D. of three different measured samples. Fold inductions are presented for each activating agent. The results are representative of three independent experiments.

**Fig. 1. Differences in LTR activation among the different HIV-1 clades.** Jurkat (A and C) and SupT1 (B) cells were transiently transfected with 5 μg of the pCMVβ vector and 15 μg of the following series of plasmids: pGL3-LTR-LUC(A), pGL3-LTR-LUC(B), pGL3-LTR-LUC(C), pGL3-LTR-LUC(D), pGL3-LTR-LUC(E), and pHIV-2-LTR-LUC. Jurkat cells (D) were transfected with 5 μg of pCMVβ and 15 μg of pBluescript-based cladespecific LTR-driven luciferase constructs. Primary CD4+ T lymphocytes (E) were transfected with 0.5 μg of pCMVβ and 3 μg of either pGL3-LTR-LUC(B) or pGL3-LTR-LUC(E). Following a 24-h incubation period, cells were either left untreated (not treated (NT)) or stimulated for 8 h (or 16 h for primary cells) with the following agents: PHA (3 μg/ml), PMA (20 ng/ml), PHA (3 μg/ml)/PMA (20 ng/ml), PMA (20 ng/ml)/Iono (1 μM), bpV(pic) (PIC; 10 μM), TNFα (20 ng/ml), and OKT3 (40 μg/ml)/DT30 (1:10 Jurkat). Cells were then lysed and evaluated for both luciferase and β-galactosidase activities as described under “Experimental Procedures.” Values are the means ± S.D. of three different measured samples. Fold inductions are presented for each activating agent. The results are representative of three independent experiments.
the different cell extracts prepared from unstimulated trans-
frogated Jurkat cells (data not shown). Our first observations
were that LTRs from the different HIV-1 clades demonstrated
different degrees of response to the tested activators. Most
notably, a significant weaker induction of the LTR segment of
HIV-1 clade E was constantly measured under all stimulating
conditions. The stimulation with the protein-tyrosine phospha-
tase inhibitor bpV(pic) was not found to modulate the LTR of
clade E differently from the LTR of clade A in this experiment,
but other experiments indicated that the clade A LTR under
this activated condition was also stronger than the clade E LTR
data not shown). In general, when the other HIV-1 clade (A–D)
LTRs were compared, no significant differences in terms of
strength of LTR activation were apparent regardless of the
tested activators. Normalized basal activity was also observed
to be weaker for the clade B LTR (2.58 ± 0.04 RLU) in compar-
ison with the clade A (15.5 ± 1.34 RLU), clade C (5.07 ± 1.03
RLU), clade D (8.16 ± 1.81 RLU), and clade E (6.35 ± 0.77
RLU) LTRs, which is consistent with previous data (25).

The response of the various LTRs was also examined in the
T-cell receptor-negative cell line SupT1 (Fig. 1B). Upon testing
the most distinct LTR configuration (i.e., clade B (two NF-κB-
binding sites), clade C (three NF-κB-binding sites), and clade E
(one NF-κB-binding site)), activation of the HIV-1 clade E LTR
resulted again in the weakest response compared with the

Luciferase activity obtained in cells transfected with HIV-1 LTR
subtypes B and C. Again, the basal activity of the clade B LTR
showed the weakest activity compared with the other clade
LTRs (3.77 ± 0.04 RLU (clade B) vs. 7.29 ± 0.2 RLU (clade
C) and 4.23 ± 0.12 RLU (clade E)). Comparison of the induction
of the HIV-1 LTR (containing a single NF-κB-binding site) and
the various HIV-1 LTRs from clades was also performed (Fig.1C).
Upon transfection of constructs bearing LTRs from HIV-1
clades B, C, and E and from the HIV-2 ROD10 strain, the
results demonstrated that, in comparison with the various
HIV-1 LTRs, the HIV-2 LTR was more extensively induced by
all activators except for TNFα, whereas the clade E LTR again
showed the weakest response.

To further corroborate the weak responsiveness of the clade
E LTR to activation, LTRs from various HIV-1 clade E isolates
were compared with two other variants of clade B and C LTRs.
As depicted in Fig. 1D, after activation, clade E LTR constructs
demonstrated weakened induction of luciferase reporter gene activ-
ity in comparison with clade B and C LTRs. To further
assess the weak clade E LTR response in a more physiologically
relevant manner, primary CD4+ T lymphocytes were trans-
fected with clade B and E LTR-driven luciferase gene expres-
sion constructs and then activated. Luciferase readouts after
normalization again concurred toward a general weaker re-
sponse of the clade E LTR upon stimulation compared with the

**Fig. 1—continued**
activation of the clade B LTR (Fig. 1E). Indeed, although the extent of LTR activation was lower in primary CD4+ T lymphocytes compared with our tested CD4+ T-cell lines, LTR activation was generally 20–50% lower in cells transfected with pGL3-LTR-LUC(E) in comparison with pGL3-LTR-LUC(B)-transfected CD4+ T lymphocytes. PHA-mediated LTR activation was not significantly different between both tested clade LTRs, which is consistent with the generally weak differences observed in the response of the different clade LTRs to PHA activation in the T-cell lines. These results demonstrate that LTR activation varies in strength according to the clade of origin, with clade E containing the weakest responsive LTR regardless of the added T-cell activator.

The Enhancer Region of HIV-1 Clade E Is Weakly Induced—Because T-cell activation leads to the up-regulation of HIV LTR-mediated gene transcription and generally involves the enhancer segment, a better assessment of enhancer-dependent modulation of the clade LTRs was next undertaken. To make a clear analysis of the strength of each clade enhancer region, we used pGL2tata-enh-LUC(A–E) plasmids, which contain the isolated enhancer region of each HIV-1 subtype cloned upstream of a minimal TATA box and the luciferase reporter gene. These constructs were cotransfected with the pCMVβ vector in Jurkat cells, and following stimulation, luciferase and β-galactosidase activities were both measured. As demonstrated above, the enhancer region from HIV-1 clade E showed the weakest activation for most of the tested activators (Fig. 2). Although the activation state of the other enhancer regions was higher than for clade E, differences were noted in comparison with the observed differences in full-length LTRs. In fact, PHA-activated T-cells were equally inefficient in activating the enhancers from clades B–E in comparison with the clade A enhancer construct. This overall tendency was also observed when SupT1 cells were transfected with these vectors and stimulated, again demonstrating a weakly induced enhancer region from the clade E LTR (data not shown).

Weak Binding of NF-κB to the HIV-1 Clade E Enhancer—Because the above results suggested that the enhancer might in part contain the important element for the differences in responsiveness of the different LTRs, we set out to focus on the factors that bind to the HIV-1 clade and HIV-2 enhancer regions. Shift assays were thus performed with the same enhancer probes (clades A–E, UG5218, SE5648, GM6439, UG6357, and GM6452, respectively) and the HIV-2 ROD10 enhancer probe. In B, nuclear extracts were preincubated with (lanes 7–12) or without (lanes 1–6) anti-panNFAT antibodies before being incubated with the different probes. Samples were subsequently resolved on a native polyacrylamide gel, which was dried and exposed to Kodak X-Omat film. Free probe and specific complexes are indicated.

\[ \text{FIG. 2. The HIV-1 clade E enhancer is weakly activated following T-cell activation.} \]

Jurkat cells were transiently transfected by the DEAE-dextran protocol with 5 μg of pGL2tata-enh-LUC(A), pGL2tata-enh-LUC(B), pGL2tata-enh-LUC(C), pGL2tata-enh-LUC(D), or pGL2tata-enh-LUC(E). Following a 24-h incubation period, cells were either left untreated or stimulated for 8 h with the following agents: PHA (3 μg/ml), PMA (20 ng/ml), PHA (3 μg/ml/PMA (20 ng/ml), PMA (20 ng/ml)/Iono (1 μμ), bpV(pic) (PIC; 10 μμ), TNFα (20 ng/ml), and OKT3 (40 μg/ml/DT30 (1:10 Jurkat). Cells were then lysed and evaluated for both luciferase and β-galactosidase activities as described under “Experimental Procedures.” Values are the mean ± S.D. of three different measured samples. -Fold inductions are presented for each activating agent. The results are representative of three independent experiments.

\[ \text{FIG. 3. NF-κB binds weakly to the enhancer regions of both HIV-1 LTR clade E and HIV-2.} \]

Jurkat cells were either left untreated or stimulated with TNFα (20 ng/ml) (A) or PMA (20 ng/ml)/Iono (1 μμ) (B) for 1 h. Nuclear extracts were incubated with the different HIV-1 enhancer probes (clades A–E, UG5218, SE5648, GM6439, UG6357, and GM6452, respectively) and the HIV-2 ROD10 enhancer probe. In B, nuclear extracts were preincubated with (lanes 7–12) or without (lanes 1–6) anti-panNFAT antibodies before being incubated with the different probes. Samples were subsequently resolved on a native polyacrylamide gel, which was dried and exposed to Kodak X-Omat film. Free probe and specific complexes are indicated.

The Enhancer Region of HIV-1 Clade E Is Weakly Induced—Because T-cell activation leads to the up-regulation of HIV LTR-mediated gene transcription and generally involves the enhancer segment, a better assessment of enhancer-dependent modulation of the clade LTRs was next undertaken. To make a clear analysis of the strength of each clade enhancer region, we used pGL2tata-enh-LUC(A–E) plasmids, which contain the isolated enhancer region of each HIV-1 subtype cloned upstream of a minimal TATA box and the luciferase reporter gene. These constructs were cotransfected with the pCMVβ vector in Jurkat cells, and following stimulation, luciferase and β-galactosidase activities were both measured. As demonstrated above, the enhancer region from HIV-1 clade E showed the weakest activation for most of the tested activators (Fig. 2). Although the activation state of the other enhancer regions was higher than for clade E, differences were noted in comparison with the observed differences in full-length LTRs. In fact, PHA-activated T-cells were equally inefficient in activating the enhancers from clades B–E in comparison with the clade A enhancer construct. This overall tendency was also observed when SupT1 cells were transfected with these vectors and stimulated, again demonstrating a weakly induced enhancer region from the clade E LTR (data not shown).

Weak Binding of NF-κB to the HIV-1 Clade E Enhancer—Because the above results suggested that the enhancer might in part contain the important element for the differences in responsiveness of the different LTRs, we set out to focus on the factors that bind to the HIV-1 clade and HIV-2 enhancer regions. Shift assays were thus performed with the same enhancer probes (clades A–E, UG5218, SE5648, GM6439, UG6357, and GM6452, respectively) and the HIV-2 ROD10 enhancer probe. In B, nuclear extracts were preincubated with (lanes 7–12) or without (lanes 1–6) anti-panNFAT antibodies before being incubated with the different probes. Samples were subsequently resolved on a native polyacrylamide gel, which was dried and exposed to Kodak X-Omat film. Free probe and specific complexes are indicated.

The Enhancer Region of HIV-1 Clade E Is Weakly Induced—Because T-cell activation leads to the up-regulation of HIV LTR-mediated gene transcription and generally involves the enhancer segment, a better assessment of enhancer-dependent modulation of the clade LTRs was next undertaken. To make a clear analysis of the strength of each clade enhancer region, we used pGL2tata-enh-LUC(A–E) plasmids, which contain the isolated enhancer region of each HIV-1 subtype cloned upstream of a minimal TATA box and the luciferase reporter gene. These constructs were cotransfected with the pCMVβ vector in Jurkat cells, and following stimulation, luciferase and β-galactosidase activities were both measured. As demonstrated above, the enhancer region from HIV-1 clade E showed the weakest activation for most of the tested activators (Fig. 2). Although the activation state of the other enhancer regions was higher than for clade E, differences were noted in comparison with the observed differences in full-length LTRs. In fact, PHA-activated T-cells were equally inefficient in activating the enhancers from clades B–E in comparison with the clade A enhancer construct. This overall tendency was also observed when SupT1 cells were transfected with these vectors and stimulated, again demonstrating a weakly induced enhancer region from the clade E LTR (data not shown).

Weak Binding of NF-κB to the HIV-1 Clade E Enhancer—Because the above results suggested that the enhancer might in part contain the important element for the differences in responsiveness of the different LTRs, we set out to focus on the factors that bind to the HIV-1 clade and HIV-2 enhancer regions. Shift assays were thus performed with the same enhancer probes (clades A–E, UG5218, SE5648, GM6439, UG6357, and GM6452, respectively) and the HIV-2 ROD10 enhancer probe. In B, nuclear extracts were preincubated with (lanes 7–12) or without (lanes 1–6) anti-panNFAT antibodies before being incubated with the different probes. Samples were subsequently resolved on a native polyacrylamide gel, which was dried and exposed to Kodak X-Omat film. Free probe and specific complexes are indicated.

The Enhancer Region of HIV-1 Clade E Is Weakly Induced—Because T-cell activation leads to the up-regulation of HIV LTR-mediated gene transcription and generally involves the enhancer segment, a better assessment of enhancer-dependent modulation of the clade LTRs was next undertaken. To make a clear analysis of the strength of each clade enhancer region, we used pGL2tata-enh-LUC(A–E) plasmids, which contain the isolated enhancer region of each HIV-1 subtype cloned upstream of a minimal TATA box and the luciferase reporter gene. These constructs were cotransfected with the pCMVβ vector in Jurkat cells, and following stimulation, luciferase and β-galactosidase activities were both measured. As demonstrated above, the enhancer region from HIV-1 clade E showed the weakest activation for most of the tested activators (Fig. 2). Although the activation state of the other enhancer regions was higher than for clade E, differences were noted in comparison with the observed differences in full-length LTRs. In fact, PHA-activated T-cells were equally inefficient in activating the enhancers from clades B–E in comparison with the clade A enhancer construct. This overall tendency was also observed when SupT1 cells were transfected with these vectors and stimulated, again demonstrating a weakly induced enhancer region from the clade E LTR (data not shown).

Weak Binding of NF-κB to the HIV-1 Clade E Enhancer—Because the above results suggested that the enhancer might in part contain the important element for the differences in responsiveness of the different LTRs, we set out to focus on the factors that bind to the HIV-1 clade and HIV-2 enhancer regions. Shift assays were thus performed with the same enhancer probes (clades A–E, UG5218, SE5648, GM6439, UG6357, and GM6452, respectively) and the HIV-2 ROD10 enhancer probe. In B, nuclear extracts were preincubated with (lanes 7–12) or without (lanes 1–6) anti-panNFAT antibodies before being incubated with the different probes. Samples were subsequently resolved on a native polyacrylamide gel, which was dried and exposed to Kodak X-Omat film. Free probe and specific complexes are indicated.
enhancer regions of clades A, B, and D (lanes 2, 4, and 8). In contrast, enhancers containing a single NF-κB binding site (HIV-1 clade E and HIV-2) led to a weaker signal of the NF-κB-specific complex in comparison with the other tested enhancer probes (lanes 10 and 12). Interestingly, a very faint band was apparent in unstimulated extracts specifically upon incubation with the clade E enhancer probe (lane 9), which might be reminiscent of the previously identified GA binding protein factor (43, 44). Although the clade C enhancer generated an important band, no clear stronger signal was noted in comparison with the clade A, B, and D enhancer probes, although the signal seemed more diffuse (lane 6).

To further confirm the binding of the NF-κB transcription factor to the different enhancers, nuclear extracts from Jurkat cells stimulated with PMA/Iono were incubated with each enhancer probe (Fig. 3B). As we have demonstrated previously (42, 45), the complexes that bind to the enhancer sequences in PMA/Iono-stimulated Jurkat cells include both overlapping NFAT and NF-κB signals (Fig. 3B, lanes 1–6). To specifically assess the NF-κB signal, nuclear extracts were incubated with a rabbit antibody recognizing all NFAT members (anti-pan-NFAT antibody) (lanes 7–12). The interaction of the antibody with the NFAT-enhancer probe complex resulted in the dissociation of the protein-DNA complex, as no supershift-related signals were identified. However, this antibody interaction allowed the isolation of the NF-κB signal. By comparing the different enhancer probes, similar observations were obtained in experiments using nuclear extracts from PMA/Iono- or TNF-α-treated cells. Indeed, a weaker NF-κB-specific signal was observed when anti-pan-NFAT antibody-treated nuclear extracts were incubated with either the HIV-1 clade E or HIV-2 enhancer probe (lanes 11 and 12). These results thus indicate that the ability of the different enhancers to bind NF-κB is in overall agreement with the results from the above transfection experiments, especially with respect to the binding to the HIV-1 clade E enhancer.

NFAT Binds Weakly to the HIV-1 Clade E Enhancer—Because of the ability of the NFAT factor to stimulate HIV-1 LTR transcription and to act via the HIV-1 enhancer region (21), we directly looked at the association of NFAT with the enhancer regions of the different HIV-1 clades and HIV-2 by performing supershift analysis with an antiseraum directed against the regions of the different HIV-1 clades and HIV-2 by performing transcription and to act via the HIV-1 enhancer region (21), we cause of the ability of the NFAT factor to stimulate HIV-1 LTR (HIV-1 clade E and HIV-2) led to a weaker signal of the NF-AT factor to the different enhancers, nuclear extracts from Jurkat cells stimulated with PMA/Iono or TNF-α-treated cells. Indeed, a weaker NF-κB-specific signal was observed when anti-pan-NFAT antibody-treated nuclear extracts were incubated with either the HIV-1 clade E or HIV-2 enhancer probe (lanes 11 and 12). These results thus indicate that the ability of the different enhancers to bind NF-κB is in overall agreement with the results from the above transfection experiments, especially with respect to the binding to the HIV-1 clade E enhancer.

The interaction of NFAT with clade B and E enhancer regions from different HIV-1 isolates was also compared. As presented in Fig. 4C and as demonstrated above, upon incubation of OKT3/DT30-treated Jurkat nuclear extracts, the NFAT signal isolated upon the addition of anti-p50 antibody was again weaker for all of the tested clade E enhancer representatives compared with the two tested clade B enhancer probes (compare lanes 3–6 with lanes 1 and 2). In addition, a fast migrating signal likely attributable to GA binding protein was observed for all clade E enhancer probes. Binding of NFAT to the clade enhancer was also evaluated using nuclear extracts from primary CD4 + T-cells (Fig. 4D). Nuclear extracts from PMA/Iono-stimulated CD4 + T-cells were hence incubated with clade B and E enhancer probes, and upon anti-p50 antibody incubation, the remaining NFAT-specific signal was again found to be stronger for the clade B enhancer, confirming the above results obtained in Jurkat cells (lane 3 versus lane 4). These results thus indicate that, in the context of activation, for both T-cell lines and primary CD4 + T-cells, HIV enhancer regions containing a single NF-κB repeat are poor binding sites for NFAT.

Weaker Binding Capacity of NFAT1 for Clade E Enhancers—To confirm that NFAT indeed had a weaker association with the clade E enhancer, a different source of NFAT was used. We thus transfected 293T cells with an expression vector for NFAT1 and derived nuclear extracts from these transfected cells. An NFAT1 expression vector was chosen because this isoform has been found to be the major NFAT member interacting with the HIV-1 enhancer from stimulated Jurkat extracts (42, 45). Extracts from transfected 293T cells were then incubated with the probes from HIV-1 clade A–E enhancers (Fig. 5A). A strong signal was observed for most of the enhancer probes incubated with cell extracts from NFAT-1-expressing 293T cells. Again, in comparison with the other tested probes, the clade C enhancer probe produced a more intense signal upon incubation with the transfected 293T cells (lane 8). However, signals were weaker with the enhancer probes from HIV-1 clade E (lanes 14) and HIV-2 (data not shown). Extracts from mock-transfected 293T cells were incubated with these similar probes and gave no specific bands (lanes 1, 4, 7, 10, and 13). Specificity of the signal was demonstrated either through competition experiments with a 100-fold excess of unlabeled enhancer oligonucleotide (lanes 3, 6, 9, 12, and 15) or through supershift assays with anti-NFAT1 antibody (data not shown).

To confirm these results, we conducted competition experiments with increasing concentrations of clade B and E enhancer-derived unlabeled oligonucleotide in nuclear extracts derived from NFAT1-expressing 293T cells and incubated with a clade B enhancer probe. As depicted in Fig. 5B, at a 10-fold excess of oligonucleotides, the signal was greatly diminished with the clade B oligonucleotide, whereas limited competition was observed with the clade E oligonucleotide (lane 2 versus lane 6). In fact, competition was weaker with the clade E oligonucleotide in subsequent competition experiments using higher concentrations of the unlabeled oligonucleotides (lanes 3 and 4 versus lanes 7 and 8). The intensities of the bands from this experiment were subsequently estimated using a Typhoon 9200 imager device and indeed confirmed that the clade E enhancer region was less efficient in competing for the binding of NFAT in comparison with the tested clade B enhancer region (Fig. 5C). Competition experiments using oligonucleotides derived from other clade B and E enhancer regions were similarly
performed and confirmed the above results in that clade E oligonucleotides were poorer competitors than the clade B oligonucleotides (data not shown). As our results also pointed toward a stronger NFAT signal with the clade C enhancer probe, we also compared competition strength of clade B versus clade C enhancer regions, this time at lower concentrations of unlabeled oligonucleotide. As presented in Fig. 5D, Typhoon analysis of band intensities indeed indicated that the clade C enhancer allowed better binding of NFAT than the oligonucleotide corresponding to the clade B enhancer region. These results thus provide evidence that, like NF-κB, NFAT1 might also contribute to reducing LTR activation through the single NF-κB repeat-containing LTRs (clade E). In addition, our results suggest that the clade C enhancer region contains an enhancer sequence that allows better interaction with NFAT1.

**Fig. 4.** NFAT has a weaker binding capacity for the HIV-1 clade E and HIV-2 enhancer regions. A, nuclear extracts from PMA (20 ng/ml)/Iono (1 μM)-treated Jurkat cells were either left untreated (lanes 1, 3, 5, 7, 9, and 11) or preincubated with anti-p50 antibody (lanes 2, 4, 6, 8, 10, and 12). Samples were then incubated with the enhancer regions of HIV-1 clade A (UG5218; lanes 1 and 2), clade B (SE5648; lanes 3 and 4), clade C (GM6439; lanes 5 and 6), clade D (UG6357; lanes 7 and 8), clade E (GM6452; lanes 9 and 10), and HIV-2 ROD10 (lanes 11 and 12). B, nuclear extracts from PMA (20 ng/ml)/Iono (1 μM), bpV(pic) (10 μM), and OKT3 (40 μg/ml/DT30 (1:10 Jurkat)-treated Jurkat cells were either left untreated (lanes 1, 3, 5, 7, 9, and 11) or preincubated with anti-p50 antibody (lanes 2, 4, 6, 8, 10, and 12). Samples were then incubated with the enhancer regions of HIV-1 clade B (SE5648; lanes 1–6) and clade E (GM6452; lanes 7–12). C, nuclear extracts from OKT3 (40 μg/ml/DT30 (1:10 Jurkat)-treated Jurkat cells were preincubated with anti-p50 antibody and incubated with the enhancer probes from clade B (SE5648; lane 1), NL4-3 (B; lane 2), and clade E (GM6452, GM6452.2, 15ltr, and CM240ltr; lanes 3–6). D, nuclear extracts prepared from primary CD4+ T lymphocytes treated with PMA (20 ng/ml)/Iono (1 μM) were incubated with the clade B (SE5648; lanes 1 and 3) and clade E (GM6452; lanes 2 and 4) enhancer probes and incubated with or without anti-p50 antibody. Protein-DNA complexes were subsequently resolved on a native polyacrylamide gel, which was dried and exposed to Kodak X-Omat film. Free probe, specific complexes, and supershift complexes (SS) are indicated.
The Third NF-κB-binding Repeat of the Clade C Enhancer Does Not Bind NFAT or NF-κB—Because it has been postulated that the clade C LTR might support higher transcription activity and that the enhancer allows better NF-κB activation (24–26), the results that we obtained in both transfection and EMSA experiments hence contradicted these previous results. To better assess the involvement of the three repeats in transcription regulation of the clade C LTR, oligonucleotides containing each isolated repeat (from the enhancer region used in the EMSAs) were used in competition experiments with the clade C enhancer-bound complexes (Fig. 6A). The NF-κB repeats were labeled from I to III, starting from the repeat closest to the transcription initiation site. After incubation of the nuclear extract from TNFα-stimulated Jurkat cells with NF-κB repeat I–III probes, signals were detected for each probe, except for the NF-κB repeat I probe. In addition, competition experiments demonstrated that the signals observed for NF-κB repeat II and III probes were out-competed by each other, but...
not by unlabeled NF-κB repeat I oligonucleotides. We similarly analyzed the binding capacity of NF-κB for NF-κB repeat I with a more representative sequence among the different clade C enhancer regions (i.e., 5'-GGGGCGTTCC-3') (24–26). Again, no binding of NF-κB was detected when this oligonucleotide was used as the probe incubated with TNFα-activated Jurkat nuclear extracts (data not shown).

Analysis of the binding of the NFAT1 factor to the three different repeats was also undertaken (Fig. 6B). As expected, NF-κB repeat I (which lacks an NFAT consensus sequence) did not lead to the formation of a complex when the labeled probe was incubated with nuclear extracts from NFAT1-expressing transfected 293T cells. Conversely, excess unlabeled oligonucleotide of this repeat was not efficient in competing for the signals obtained with the labeled probes from the two other repeats (data not shown). Interestingly, the NFAT-specific signal was stronger upon incubation of the extracts with the NF-κB repeat II probe compared with the NF-κB repeat III probe. This higher binding capacity of NFAT for NF-κB repeat II was not paralleled by a stronger NF-κB signal when TNFα-activated Jurkat extracts were incubated with these probes. Because NF-κB repeat II permitted stronger NFAT binding, we also compared the binding ability of both NFAT and NF-κB for each NF-κB repeat from the NL4-3 clade B enhancer. However, no major differences were observed between each repeat of the NL4-3 clade B enhancer in their binding of either NFAT or NF-κB (data not shown). These results thus indicate that the extra binding site of clade C bears no detectable affinity for NF-κB or NFAT. The tested middle repeat from the clade C enhancer bound to NFAT in a stronger fashion than the other repeat from the clade C enhancer.

**DISCUSSION**

Previous studies have determined thatLTRs of various HIV-1 clades show particular differences in their sequences (24, 26). Some of these dissimilarities are included in the enhancer region and relate to the number of NF-κB repeats (from one to three). When HIV-1 LTRs are compared with the HIV-2 LTR, important differences are also noted in the enhancer sequence in that a single NF-κB repeat is present in the LTR sequence of HIV-2 isolates. Because of the role played by NF-κB and NFAT in modulating the LTR activation through the enhancer sequence (1, 21, 22, 46), our objective was to assess the implication of both of these factors in the activation of LTRs from the different clades. Through the use of various T-cell-activating agents, we have demonstrated that the number of NF-κB repeats can affect the binding of both factors to the enhancer regions of the different HIV-1 clades and that this likely reduces the strength of the activation of the clade E LTR, which contains a single NF-κB repeat. However, the HIV-2 LTR, which bears only one NF-κB-binding site, was found to be the strongest activated LTR with most activating agents. Importantly, these results thereby indicate for the first time that, although binding of NF-κB to the different clade enhancers is different, NFAT binding to the enhancer region equally varies significantly among the various tested clade enhancers.

In this study, we have analyzed the LTR response to T-cell activation through the use of LTRs from different clade-specific HIV-1 isolates and in different T-cell settings. These analyses were important to ascertain that the effects were neither cell type-specific nor HIV-1 LTR strain-specific. We have observed in fact that, under all conditions, the tested clade E LTRs were less responsive than any other of the compared clade B LTRs independently of the added activating agent, which also included antigenic-like OKT3/D3T3 stimulation. The weak re-

![FIG. 6. The third repeat in the clade C enhancer does not bind to NFAT or NF-κB. A, nuclear extracts from non-activated (lanes 1 and 6) or TNFα (20 ng/ml)-treated Jurkat cells were incubated with clade C NF-κB repeat I (lanes 1–5), repeat II (lanes 6–10), and repeat III (lanes 11–14). Competition experiments were conducted with excess unlabeled oligonucleotide of repeat I (lanes 2, 3, and 8), repeat II (lanes 4, 13), or repeat III (lanes 5, 10, and 14). B, nuclear extracts from untreated (not treated (NT), lanes 1, 4, and 7) or TNFα (20 ng/ml)-treated (lanes 2, 5, and 8) Jurkat cells or from NFAT1-expressing 293T cells (lanes 3, 6, and 9) were incubated with the following probes: NF-κB repeat I (lanes 1–3), repeat II (lanes 4–6), and repeat III (lanes 7–9). Protein-DNA complexes were subsequently resolved on a native polyacrylamide gel, which was dried and exposed to Kodak X-Omat film. Free probe and specific complexes are indicated. (Top left) A. (Bottom left) B.](image-url)
DEK (49) and Elf-1 (30, 50) likely account for this strong HIV-2 LTR response to T-cell receptor-dependent activators.

The above results obtained from comparison of the responses of the different clade LTRs are in complete agreement with previously published data (25, 26, 51). In fact, these studies had shown earlier that TNFα-stimulated Jurkat cells poorly activate the HIV-1 LTR from clade E in comparison with other clade LTRs. For all other clade LTRs, no significant differences in terms of activation were noticed for all tested activating agents. However, our results contradict previous studies suggesting that the activation of the clade C LTR is higher because of its extra NF-κB-binding site. In fact, Montano et al. (26) demonstrated, using a p65 expression vector, that activation is more pronounced for clade C than clade B in 293T and Jurkat cells. Because our conditions did not involve p65 overexpression, our results are thus more relevant to the actual regulation of the HIV-1 clade C LTR following T-cell activation. In addition, all results partly agree with those obtained by Jeeninga et al. (25), who showed little difference among clades A–D in terms of activation following TNFα stimulation in SupT1 cells.

In our study, we also compared the basal activity of the various clade LTRs. Only clade B LTR basal activity was shown to be different and lower compared with other clade LTR basal activities. Previous studies indicated that LTR basal activity and HIV-1 replication are higher for clade E in unstimulated SupT1 cells (25, 35). We have not confirmed these data through our transfection experiments. Different experimental conditions might account for these discrepancies, although we have confirmed our data in various T-cell lines and using different strains for each clade LTR.

To gain a better and more accurate assessment of the role played by the different enhancer sequences in the modulation of each clade LTR, we have specifically investigated the role of the enhancer sequence in LTR activation following stimulation in Jurkat cells. Again, the clade E enhancer was the weakest activating sequence under all activating conditions. Remarkably, the enhancer sequence from clade C did not appear to be stronger than clades A, B, and D enhancer sequences. Rather, this enhancer was often found to be weaker than the enhancer regions from clades A and B. These results thus further suggest that the clade C LTR does not show a significantly greater level of transcription through more pronounced activation of its enhancer region. It should also be pointed out that, following PHA activation, the clade A enhancer seemed to act in a stronger fashion than the other clades, which is different from the results obtained with the full-length LTR. Other differences are apparent when comparing these data with those from the full-length LTR experiments. Presently, no clear explanations can account for these differences, although the context of the enhancer and the absence of the other LTR sequences are likely to play a major role.

We have analyzed the type of protein/DNA interactions occurring in the various enhancer regions. Using a previously described approach, we have in fact determined that most of the enhancer regions permitted the binding of NF-κB, HIV-1 clade E and HIV-2 enhancers were, however, poor binding sites for NF-κB when analyzed in the presence of TNFα- and PMA/Iono-stimulated Jurkat cells, whereas the other clade enhancers (including the clade C enhancer) showed an NF-κB signal of similar strength. These results are thus a priori in correlation with the data from the transfection experiments in that a similar level of NF-κB binding to the enhancer is paralleled by an equal level of LTR and enhancer activation in clades A–D. A weaker binding of NF-κB to HIV-1 clade E LTRs would thus be consistent with a weaker level of initiation and elongation of transcription, as suggested by West et al. (5). Our analyses have been focused on the p50/p65 heterodimer, the most potent activator of HIV-1 LTR transcription. Other bands are also present in these EMSAs, and we have previously demonstrated that these bands are mainly p50 homodimers (38) and unaltered by T-cell-activating agents. In our study, we have also detected a signal that was specific for the clade E probe and that might represent the previously described interaction with the Ets-related GA binding protein transcription factor (43, 44).

A detailed analysis of the binding of the NFAT factor to the different HIV-1 clade enhancers was also undertaken. Nuclear extracts of stimulated Jurkat cells demonstrated a weaker attachment of NFAT to the HIV-1 clade E and HIV-2 enhancer probes. Differences in binding between clade B and E enhancers were further confirmed using enhancer probes from different isolates. Furthermore, nuclear extracts from stimulated CD4+ T-cells generated a weaker NFAT-specific signal upon incubation with a clade E enhancer probe. This weaker binding to the HIV-1 clade E was also demonstrated by experiments conducted with nuclear extracts from 293T cells transfected with the NFAT1 expression vector and by competitions experiments. These results hence indicate that NFAT could also be an important element in the observed weak activation of the clade E LTR. In addition, a competing event might occur between both NF-κB and NFAT for the only available NF-κB-binding site. Such detrimental action between the activation potential of NF-κB and NFAT in the context of a single NF-κB-binding site has previously been illustrated by Macián and Rao (52). These results also agree with those of Jeeninga et al. (25), who showed that transfection of an NFATc (NFAT2) expression vector leads to a more important activation of a clade B LTR than a clade E LTR. Because of the diluted nature of the NFAT proteins in the tested nuclear extracts, the observed signals detected in our EMSAs are likely representative of bound NFAT monomers. The recent demonstration that NFAT binds as a dimer through the HIV-1 enhancer region (23) suggests that the strength of the interaction of this dimer could also be affected by nucleotide variation in the different clade LTRs. We are hence presently testing the binding of the NFAT dimer to the various clade enhancer regions using purified recombinant NFAT1 proteins.

An interesting observation from these EMSAs is the stronger attachment of NFAT to the clade C enhancer compared with other NF-κB repeats. We have analyzed the third repeat for NFAT binding and found no affinity of this site for NFAT. This was expected given the lack of the NFAT consensus core in this NF-κB repeat (i.e. an antisense 5’-GGAAA-3’ sequence). A more refined analysis of the binding ability of NFAT allowed us to show that repeat II strongly bound NFAT, which might explain the stronger NFAT signal observed with the clade C enhancer probe in our EMSA analysis. No such difference in NFAT binding was apparent when the two NF-κB repeats from the enhancer region of the NL4-3 (clade B) LTR were compared. However, the sequence present between repeats I and II in the clade C LTR (i.e. 5’-ACTG-3’) resembles the previously described AP-2-like binding sequence, which has been shown to be important for the action of NFAT in HIV-1 LTR regulation (21, 24, 26). This new sequence could impart a stronger NFAT affinity for the consensus sequence present in repeat II, as no other differences seem to account for the difference in NFAT binding between repeats II and III. Although we have previously demonstrated that higher activation of NFAT1 suggestively increases LTR transcription via the enhancer (42, 45, 53), the results in the present study indicate that the stronger binding of NFAT1 to the enhancer of clade C does not improve the activation of this enhancer in comparison with the other...
clade enhancer. The preferential positioning of NFAT on repeat II might not be optimal in the context of an activated synergistic response involving both NFAT and NF-κB.

One concern that arises from this study therefore relates to the role that could be played by the third binding site of clade C. Our results demonstrate that neither NF-κB nor NFAT can interact with this repeat and confirm the previous data of Roof et al. (43). This sequence might permit the binding of another factor (for example, Sp1), hence leading to higher basal promoter activity or Tat-induced LTR transcription. Based on our results, such an interaction should not affect the observed induction of transcription in activated T-cells. However, in our EMSAs, we were unable to detect a signal specific for this repeat; a more detailed analysis will be needed to determine whether this repeat allows the binding of a transcription factor.

These studies have thereby demonstrated that the activation of the various LTR regions of the existing clades is affected in HIV-1 only in the context of clade E. On the basis of our analyses, it is likely that this low response from clade E LTRs would be consequential to the single NF-κB repeat-containing enhancer region. On the other hand, the potential of HIV-1 clade C LTRs to be more active upon activation has not been observed in this study and thereby relates to the lack of additional NF-κB binding to the enhancer sequence. A lower level of activated transcription might bear important consequences as to the level of transmission as well as the pathogenicity of particular HIV-1 isolates. However, gag and env sequence as well as the interaction between Tat and TAR RNAs are known to differ between clades and are thus likely crucial determinants of the variations in transmission levels and the degree of pathogenicity of the different HIV-1 clades.

In conclusion, we have evaluated the response of the various HIV-1 clade LTRs to different T-cell-activating agents. HIV-1 clade E LTRs were the weakest responding LTRs, which paralleled weaker binding of NF-κB and NFAT to their enhancer regions. However, clade C LTRs did not lead to a major shift in response compared with the other clade LTRs. Therefore, the number of NF-κB repeats defines definitively act on the strength of promoter activation, but not in all cases. The stronger interaction of NF-κB and NFAT with clade enhancer regions containing two active NF-κB repeats is likely an effect of avidity or cooperative interaction, whereas the affinity per se for the various NF-κB and NFAT-binding sites for both of these factors is not necessarily increased. It is possible, however, that other regions in the LTR, which show divergence between clades, further affect LTR activation and binding of both factors to the enhancer region. Future studies aimed at an understanding of the role of these sequences should shed light on how LTR regulation is orchestrated in response to T-cell activation in the context of the existing LTR sequence heterogeneity among the different HIV-1 clades.