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The κB Sites in the Human Immunodeficiency Virus Type 1 Long Terminal Repeat Enhance Virus Replication yet Are Not Absolutely Required for Viral Growth

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The dependence of human immunodeficiency virus type 1 (HIV-1) on its NF-κB binding sites (κB sites) for replication in transformed and primary T-cell targets was examined by infecting cells with HIV-1 reporter viruses containing κB site enhancer mutations. Viral transcription was measured either with luciferase-expressing HIV-1 that infects for a single round or by flow cytometric analyses with HIV-1 expressing placental alkaline phosphatase (PLAP) or green-fluorescent protein (GFP). Both PLAP- and GFP-expressing viruses spread from cell to cell and allowed analysis of viral gene expression patterns in single cells. Infection of a panel of T-cell lines with different basal levels of NF-κB demonstrated a direct correlation between the amount of constitutive nuclear NF-κB and the degree to which a wild-type virus outperformed κB site mutants. One T-cell line with a constitutively high level of nuclear NF-κB, PM1, showed a 20-fold decrease in transcription when its κB sites were mutated. In contrast, in a T-cell line with a low basal level of NF-κB, SupT1, mutation of the κB site in the enhancer had no effect on viral transcription or growth rate. Phytohemagglutinin-activated peripheral blood mononuclear cells showed a large dependence on the κB sites for optimal virus growth. Viruses without marker genes corroborated the finding that mutations to the κB sites impair virus production in cells with a high basal level of NF-κB. These data show that in T cells, HIV-1 can use NF-κB to enhance its growth but the virus is clearly able to grow in its absence.

The transcription factor NF-κB is thought to play a major role in the regulation of human immunodeficiency virus type 1 (HIV-1) gene expression (20). Originally characterized as a transcription factor important for immunoglobulin κ light-chain gene transcription, NF-κB has since been more broadly implicated in regulating many other immunomodulatory genes, including those for interleukin 2 (IL-2), the IL-2 receptor, tumor necrosis factor alpha (TNF-α), IL-6, granulocyte-macrophage colony-stimulating factor, and the class II major histocompatibility complex (3, 16). NF-κB is induced upon activation of primary human T cells (13) and is also found at high levels in cultured monocytes; these are the two major targets of HIV-1 infection. Two tandem consensus binding sites for NF-κB are highly conserved on virtually all isolates of HIV-1, and single sites are found on HIV-2 and most isolates of simian immunodeficiency virus. Numerous studies have observed a correlation between activation of NF-κB and the stimulation of transcription of long terminal repeat (LTR) reporter constructs or of integrated proviral sequences in chronically infected cell lines (8, 12, 21).

Earlier work used transient transfection of LTR-driven reporter constructs to dissect the functional *cis* elements important for activation of HIV-1 transcription (11, 20, 23). Although this approach has been fruitful in uncovering important *cis*-acting elements, it fails to recapitulate many aspects of normal viral transcriptional regulation because of several limitations. First, transient transfection methods involve the introduction of much higher copy numbers of LTR-containing DNA to each cell than that seen during infection of cells. Second, because transfected DNAs are not incorporated into chromatin they may not be regulated in the same manner as integrated retroviral promoters. Several recent studies suggest that alterations of chromatin structure correlate with induction of NF-κB and HIV-1 transcription (22, 28). Third, the process of infection with HIV-1 may influence levels of NF-κB (2, 4, 25). Therefore, much of the previous work measuring the importance of NF-κB outside the context of infection has neglected virus-host interactions that may affect NF-κB levels and function. Lastly, measurement of the effects of enhancer mutation must be made during infection to assess the potential effects of HIV-1 regulatory genes such as *tat* or *nef* on NF-κB-directed transcription.

The few studies investigating the role of the *cis* elements in the HIV-1 LTR on viral growth and replication have come to conflicting conclusions regarding the importance of the κB sites during infection of cells. Initial reports suggested that the κB sites are largely dispensable for viral growth in cell culture (15, 24). However, a more recent report suggests that there is an absolute requirement for κB sites for replication in primary cells (1). These disparate observations leave the issue of how the κB elements function during viral growth unresolved.

To clarify the issue of how discrete LTR mutations change viral transcription and growth properties of HIV-1 in human T cells, we examined the question of the relative importance of intact κB binding elements with more efficient virus production and more quantitative virus analytic technology. Our experiments avoid the use of prolonged virus cultivation to produce mutant virus prior to measurement of its growth, as has been done in previous viral growth studies (1, 15, 24). We achieve this by producing high initial titers of HIV-1 by transient transfection of 293 cells. Here we provide analyses of the effects of a discrete enhancer mutation on the levels of viral gene expression during single-round infection and correlate such de-

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fects with gene expression in individual cells and the subsequent growth of HIV-1. We examine the effects of enhancer mutations in primary peripheral blood mononuclear cells (PBMC) and in T-cell lines with various levels of NF-κB binding activity and show that mutation of the enhancer can have a dramatic effect or no effect, depending upon variations in the basal levels of nuclear NF-κB found in a given target cell. We conclude that NF-κB can play a central role in enhancing HIV-1 growth, but we cannot show an absolute requirement for this factor.

MATERIALS AND METHODS

Cells. SupT1 is a $CD4^+$ $CD8^+$ $CD3^-$ T-leukemic line isolated from a pleural effusion of a non-Hodgkin's lymphoma patient (27). Jurkat clone E6 is a $CD4^+$ $CD3^+$ T-leukemic line derived by limit dilution cloning over macrophages (29). PM1, a derivative of HUT78, is a $CD4^+$ T-leukemic line that has been found to support infection of a wide variety of primary and laboratory-adapted isolates of HIV-1 (18). SupT1 from James Hoxie, Jurkat clone E6 from Arthur Weiss, and PM1 from Paolo Lusso and Marvin Reitz were obtained from the AIDS Reagent Repository. PBMC were purified twice by centrifugation over a Ficoll-Hypaque (Pharmacia) gradient and incubated in RPMI medium containing 10% fetal calf serum, either with or without 2 μg of phytohemagglutinin (PHA) per ml for 36 to 40 h prior to infection and maintained in medium containing 20 U of recombinant human IL-2 (Genzyme) per ml.

Electrophoretic mobility shift assays (EMSA). Nuclear extracts for EMSA were prepared with a modification of the protocol of Dignam et al. (7, 17). Briefly, 20×10^6 cells, treated with 20 ng of recombinant human TNF-α (Genzyme) per ml or untreated, were washed once with ice-cold phosphate-buffered saline, resuspended in ice-cold buffer A (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES, pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl), and then lysed by addition of an equal volume of buffer A containing 0.2% Nonidet P-40. Nuclei were pelleted by low-speed centrifugation and resuspended in buffer C (25% glycerol, 20 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 0.6 M KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol) with vigorous pipetting. Extracts were cleared by high-speed ultracentrifugation and stored at −70°C. EMSA was performed as described previously (17), with a hairpin consensus HIV-immunoglobulin κ probe (10).

Viruses. Viruses were produced from molecular clone HXB-2D in proviral vector R7 (9). PCR-mediated, site-directed mutagenesis was performed by amplifying a 772-bp fragment encompassing the 3′ LTR region and subcloning the recombinant fragment into the place of the 3′ LTR via a unique *Xho*I site and a recombinant *Asc*I site engineered into the 3′ end of the proviral sequence. The primers used for PCR amplification were 5′-GATGGGGTGGGAGCAGCATCT-3′ and 5′-ATGCTCTAGAGGCGCGCCAGAGTCACACAACAGACGGGCA-3′.

The resulting provirus contains a deletion of a portion of the sequence flanking the 3′ end of the provirus. LTR sequences were confirmed by dideoxy sequencing (ABI Sequenator; Applied Biosystems). The HXB-ΔκB-alt provirus was made by replacing the *Xho*I-*Sac*I fragment containing the 3′ HIV-1 LTR in proviral clone R7 with a mutant LTR from proviral clone BH8 with site-directed mutations to both κB sites. Construction of HXB-Luc (luciferase) and HXB-PLAP (placental alkaline phosphatase have been described previously (5, 6). HXB-GFP (green-fluorescent protein) was engineered in an analogous fashion to the luciferase- and PLAP-expressing viruses, by using a modified allele of the *Aequorea victoria* GFP from Clontech, Enhanced GFP. Enhanced GFP was PCR amplified and cloned into unique *Not*I and *Xho*I sites in HXB-Luc. HXB virus that has not been engineered to express marker genes does express a functional repaired *nef* open reading frame. Virus was produced by calcium phosphate methods and harvested at 48 h following transfection as previously described (5). Virus was quantitated by p24 enzyme-linked immunosorbent assay (19) to normalize all infections to equivalent antigenic input.

Luciferase assays and flow cytometry. Luciferase assays were performed as described previously (6). Flow cytometry was performed on a FACScan (Becton Dickinson) as previously described (5). A modified allele of GFP which has had its coding sequence changed to allow optimal translation in mammalian cells was obtained from Clontech and detected by FACScan on the FL1 channel, 530/30 nm.

Reverse transcriptase-mediated PCR of cultured HIV-1. Cell-free supernatants precleared of cellular debris by low-speed centrifugation were ultracentrifuged at 28,000 rpm (Beckman SW60.1) to pellet virions, and viral RNAs were prepared with Tri-Reagent (Molecular Research Center) in accordance with the manufacturer's instructions. Random hexamer (for the U3 region) and oligo(dT) (for the U5 region) primers were used to prime cDNA syntheses. The U3 and U5 regions were amplified with 26 cycles of PCR with native *Pfu* polymerase (Stratagene) from the viral cDNAs with the following sets of primers and subcloned into pBluescript: U3, 5′-GAT GGG GTG GGA GCA GCA TCT-3′ and 5′-TTT ATT GAG GCT TAA GCA GTG-3′; U5, 5′-GGA ACC CAC TGC TTA AGC CTC-3′ and 5′-CAT GGC GCG CCT GCT AGA GAT TTT CCA CA-3′. No amplification products were detected in control cDNA synthesis reactions that did not include murine leukemia virus reverse transcriptase. Duplicate bacterial colony lifts were created from bacterial transformations containing U3 region PCR products from samples described in Results. Filters were hybridized with a ^{32}P-radiolabelled oligonucleotide complementary to the wild-type (WT) κB site (5′-AAG GGA CTT TCC GCT GGG GAC-3′) or to the mutated κB site (5′-CAA TCT ACT TTC CGC TGT CTA CT-3′) in accordance with standard methods (26). Sequence analysis was performed with a dideoxy terminator cycle sequencing kit (Applied Biosystems) and an ABI Sequenator.

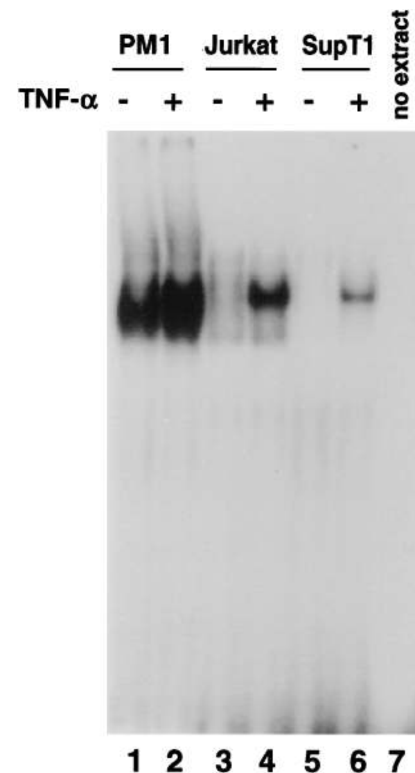

FIG. 1. EMSA on nuclear extracts binding to κB site-containing DNA. Nuclear extracts were prepared from the indicated T-cell lines following a 30-min treatment with TNF-α or no treatment.

RESULTS

Determination of endogenous NF-κB activity in three T-cell lines. We prepared nuclear extracts from three human T-lymphoblastoid cell lines routinely used to propagate HIV-1 in tissue culture, SupT1, Jurkat clone E6, and PM1. EMSA were used to determine both basal and TNF-α-activated levels of NF-κB, and the three cell lines tested differed markedly. No basal NF-κB activity was detected in SupT1, a small amount of basal activity was found in Jurkat cells, and high basal activity was observed in PM1 cells (Fig. 1, lanes 5, 3, and 1, respectively). Constitutive activation of NF-κB in PM1 cells may be due, in part, to a genetic mutation found in the parental cell line HUT78 which created an activated form of the NFKB2 gene product (30). As might be expected, TNF treatment gave the greatest relative increase in specific DNA binding activity in SupT1 cells, an intermediate response in Jurkat cells, and only a small increase in PM1 cells (Fig. 1, lanes 2, 4, and 6, respectively).

Measurement of viral transcriptional activity in a single round of infection. To measure the role of the HIV-1 κB sites in viral growth in these human T cells, we employed an infectious luciferase-expressing HIV-1 vector, HXB-Luc, which infects cells efficiently for only a single round (6). To study the

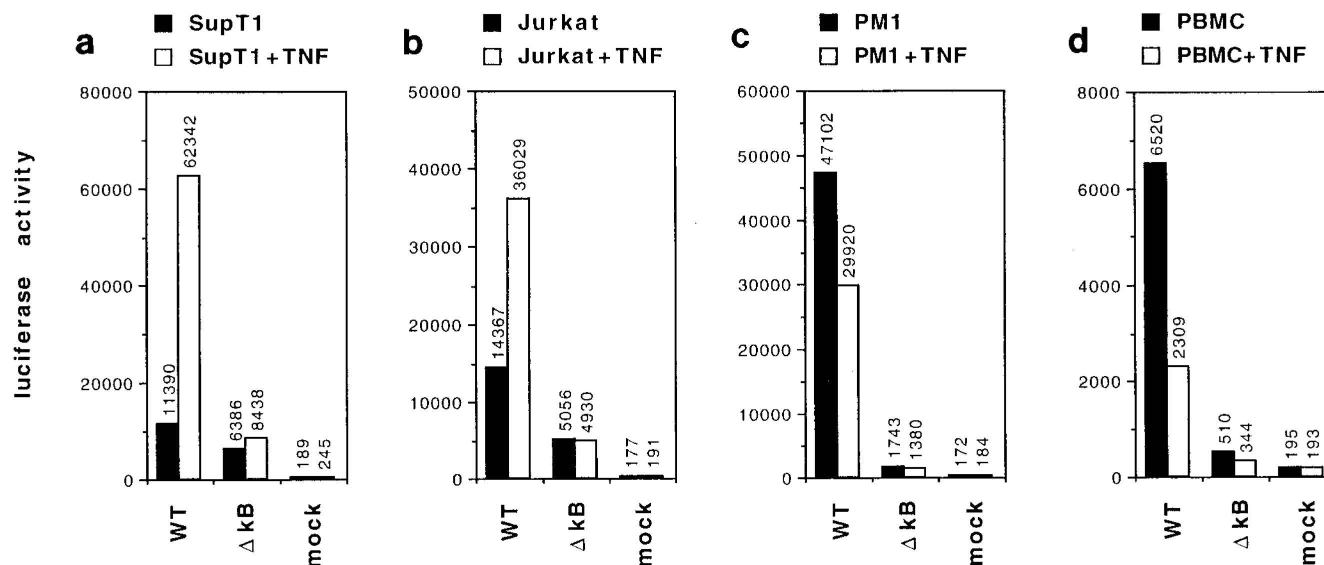

FIG. 2. Luciferase-expressing virus infection of SupT1, Jurkat, and PM1 cells and PBMC. Infected cells were stimulated with TNF-α at 48 h postinfection or left unstimulated, and luciferase activity was measured at 72 h postinfection. Results are representative of three independent experiments with independent virus preparations.

role of the κB sites, we selectively mutated both κB sites in the 3′ U3 region of the proviral DNA, changing the initial GGG of both κB sites to TCT, thus eliminating NF-κB binding to the HIV-1 enhancer (14). While the 5′ U3 region controls transcription, only the 3′ U3 region of the retrovirus is transcribed into genomic viral RNA. This 3′ U3 sequence is copied during reverse transcription, providing the template for the 5′ U3 region in progeny provirus. Therefore, transfection of a 3′ U3 mutant proviral construct should yield predominantly proviral progeny with two mutated LTRs. This strategy was used to allow us to produce high-titer virus by transient transfection with transcription directed by the 5′ LTR so that WT and U3 mutant viruses can be generated with similar efficiencies. In fact, transfection of mutant and WT HXB-2D proviral plasmids into 293 cells yielded similar levels of p24 from both constructs (data not shown). This approach avoids the need to amplify virus in cells prior to experimentation as has been the case in previous studies.

Using the 293 cell supernatants for infection of T-cell lines, we found differences between WT viruses (HXB-Luc-WT) and mutant viruses (HXB-Luc-ΔκB) dependent upon the target cells tested. These differences provide a measure for the dependence of HIV-1 on κB sites in each cell type tested. In SupT1, HXB-Luc-WT was less than twofold more active than HXB-Luc-ΔκB (Fig. 2a). In PM1 cells, a much larger difference was observed: 20- to 30-fold greater activity from the WT than from a ΔκB virus (Fig. 2c). In Jurkat cells, an intermediate three- to fivefold difference was found (Fig. 2b). The magnitude of the difference between WT- and ΔκB-directed luciferase activities correlated well with the basal level of DNA-binding NF-κB in each case.

Treatment of HXB-Luc-infected cells with TNF-α resulted in a large activation of luciferase activity in SupT1 cells, a smaller induction in Jurkat cells, and small inhibition in PM1 cells (Fig. 2a to c). The inhibition of luciferase activity in PM1 cells was less prominent at earlier time points (data not shown) and could be due to antiproliferative or other pleiotropic effects of TNF-α which occur following prolonged exposure to the cytokine. The mutation of the NF-κB site completely abrogated the ability of TNF-α to elicit activation in all T cells tested, showing that in the intact virus, mutation of these sites is sufficient to completely eliminate TNF-α activation of HIV-1. This analysis shows that the relative dependence of the HIV-1 LTR on NF-κB sites correlates well with basal levels of NF-κB activity. A strong, inducible NF-κB activity can be observed in SupT1 cells, yet they do not show dependence upon NF-κB following infection. This provides strong indirect evidence that the infection process itself does not activate NF-κB activity in cell lines with low basal NF-κB activity.

Infection of PHA-stimulated PBMC was also performed (Fig. 2d). Although the levels of luciferase activity from the primary cells was lower than that of the T-cell lines tested, the Luc activity was well over the background levels for both the WT and mutant LTR luciferase-expressing viruses. The lower activity in primary cells may, in part, be reflective of a smaller fraction of total cells that actively replicate under such activation conditions. We found in independent experiments that roughly 10% of the total PBMC actively incorporated bromodeoxyuridine following PHA activation at 24 h (data not shown). The pattern of luciferase expression observed in the activated cells most closely resembled that of PM1 cells. Thus, activated PBMCs have high levels of NF-κB-dependent transcription and also display inhibition by TNF-α treatment. The cellular environment found in PHA-activated PBMC thus appeared to be maximally activated for NF-κB in a manner which functionally resembles that of PM1 cells.

Single-cell analysis of HIV-1 infection. To analyze on a single-cell basis how mutations in the enhancer affect the virus' ability to progress through its life cycle, we engineered the κB mutant LTR and WT HIV-1 to carry genes for the cell surface protein PLAP or for cytoplasmic GFP. In both constructs, the marker gene is put in the place of the HIV-1 *nef* reading frame. Although previous reports have suggested that Nef fusion proteins may activate NF-κB, we have found no evidence for an increase in NF-κB-dependent viral growth in the presence of *nef* (3a). Staining for PLAP or GFP in conjunction with stain-

ing for the viral receptor, CD4, allows discrimination between the early and late phases of viral replication (5).

Shortly following infection with HXB-PLAP virus, cells in the early phase of infection are marker gene positive yet still retain cell surface CD4 ($CD4^+$ $PLAP^+$). When the levels of marker genes increase during the late phase of the viral life cycle, Env protein is expressed, which down-modulates CD4 ($CD4^-$ $PLAP^+$). Due to the absence of functional *nef* and *vpu* in this HXB-2 molecular clone of HIV-1, Env is the only gene product which can affect the levels of CD4 during this infection. Following infection of Jurkat cells with HXB-PLAP-WT and HXB-PLAP-ΔκB, the two viruses infected similar numbers of cells (within twofold) based upon the percentage of total cells that became PLAP positive after 3 days (Fig. 3a). Notably, the cells infected with the mutant virus showed similar levels of PLAP-associated fluorescence and also showed a similar pattern of CD4 down modulation in PLAP-high cells, suggesting that the level of viral transcription in these cells is sufficient for the virus to progress into the late phase of viral gene expression. However, over time we saw that the WT virus spread to infect a greater fraction of the cell culture at a significantly faster rate.

From fluorescence-activated cell sorter (FACS) plots like those in Fig. 3a, growth curves were then plotted (Fig. 3b) which illustrate the ability of the WT and mutant viruses to infect and deplete CD4-positive Jurkat cells. The upper right panel in Fig. 3b shows the rise and fall of cells exhibiting a $CD4^+$ $PLAP^+$ phenotype indicative of the early stages of infection. The lower right panel in Fig. 3b shows the rise and fall of cells exhibiting a $CD4^-$ $PLAP^+$ phenotype indicative of the late stages of infection. In all of the infections analyzed in this manner, the peak in the number of $CD4^+$ $PLAP^+$ cells precedes the peak in $CD4^-$ $PLAP^+$ cells, illustrating a clear progression from early to late infection. In all cases, there is a significant lag in the rise and fall of these infected populations in the κB site-mutated viruses. Similar results were obtained with the two different starting concentrations of virus used (Fig. 3b). In Jurkat cells, a small percentage of what are initially $CD4^-$ cells resist infection and maintain a $CD4^-$ $PLAP^-$ phenotype. Over time, these become the dominant cell type in the culture (Fig. 3b, lower left panel), following the growth inhibition and death of the virus-infected cells. We found that the NF-κB mutant virus exhibited a 6- to 10-day delay in its ability to deplete the entire culture of $CD4^+$ cells (Fig. 3b, upper left panel).

We then tested GFP-expressing viruses in SupT1, Jurkat, and PM1 cells (Fig. 4). Comparing the GFP results with the PLAP results allowed us to determine whether the results of single-cell analyses may be peculiar to a particular reporter gene. Interestingly, with the three cell lines we saw a strong correlation between the basal level of NF-κB and the effect of κB site mutation on the rate of viral spread. In SupT1 cells, which have low basal NF-κB activity, there was no observable delay in the ability of HXB-GFP-ΔκB to spread compared with HXB-GFP-WT (Fig. 4a). In Jurkat cells infected with GFP-expressing viruses, we observed a growth delay caused by the ΔκB construct consistent with the PLAP virus data shown above (Fig. 4b). In PM1 cells, which have high basal NF-κB activity, a more dramatic delay in the spread of HXB-GFP-ΔκB was observed, consistent with the large 20-fold transcriptional defect observed during HXB-Luc-ΔκB infection (Fig. 4c). At 9 days following infection of PM1 cells with HXB-GFP-WT, virtually all of the cells had rapidly progressed to become $CD4^-$ GFP^+. At this same time point, only a small fraction of the cells had been infected by the mutant virus. At day 12, HXB-GFP-ΔκB did spread in PM1 cells to infect a significant fraction of the cells; however, the average intensity of GFP expression in cells with the highest GFP expression was almost 10-fold lower than that seen in cells infected with HXB-GFP-WT. The lower level of GFP found in these $CD4^-$ GFP^+ cells suggests that in PM1 cells, the virus with a mutant enhancer can produce a spreading infection but does so at a lower peak level of viral gene expression. These studies illustrate a range of NF-κB dependence for viral growth which depends upon the basal NF-κB level in the target cell. Interestingly, despite a significant growth lag in some cells, enhancer mutant HIV-1 was still able to exert cytopathic effects in all three $CD4^+$ T-cell lines (data not shown).

Assessment of viral growth by p24 production. To correlate our marker gene studies with virus production, we tested viral constructs which did not contain marker genes to monitor the rate of p24 production during viral infection. Again, growth defects of the mutant virus correlated well with the basal level of NF-κB found in the T-cell lines. No detectable difference in the rate of p24 production was found in SupT1 cells (Fig. 5a). Notably, SupT1 cells supported a highly cytopathic infection which depleted cells rapidly, forming giant ballooning syncytia. These syncytia were found equally in infections with both the WT and mutant viruses (data not shown). In contrast, infection of Jurkat cells with HXB-ΔκB showed a consistent lag in p24 production; the mutant required 3 to 4 more days to reach similar levels of p24 than did the WT (Fig. 5b). Infection of PM1 cells with mutant virus revealed the most prominent delay in p24 production (Fig. 5c). Eight days after the infection of PM1 cells, there was a more-than-100-fold difference in p24 production between the two viruses. At a later time point, when the WT has already largely depleted the $CD4^+$ cells, the mutant reaches a peak virus production roughly fourfold lower than that of the WT.

We next examined the ability of PHA-activated PBMC to support infection of WT or mutant virus. A marked growth lag was observed in PBMC infected with HXB-ΔκB compared to those infected with HXB-WT. Because previous reports have shown a prominent defect in cells that are infected in a quiescent state and subsequently activated, we tested both cells activated before infection (Fig. 5d) and cells activated following infection (Fig. 5e). The delay in viral growth was observed whether the cells were stimulated prior to infection or 24 h following infection of quiescent PBMC. The peak titer of the HXB-ΔκB virus was more than 10-fold lower than that of HXB-WT whether cells were stimulated before infection (Fig. 5d) or 24 h following infection (Fig. 5e). However, in contrast to previous reports (1), the mutant virus is still capable of producing levels of virus 3 orders of magnitude above the threshold of detection (Fig. 5d and 5e).

Analysis of LTR sequences arising during in vitro infections. The slowed growth kinetics of the ΔκB virus raise the issue of whether reversions in the mutant virus may occur to compensate for the low levels of transcription of ΔκB virus. Such mutations would cause us to underestimate the functional defect of the NF-κB mutations which we have observed by growth curve analysis. In addition, because our methods of mutant virus production involve transfection of proviral constructs containing a WT 5′ LTR, we must monitor our experiments for potential recombination between the 5′ and 3′ LTRs. If recombinant virus represented a significant fraction of the input virus, this too could cause us to underestimate the growth defect of the ΔκB virus. To investigate these possibilities, we utilized an alternate variant of the enhancer mutant, designated HXB-ΔκB-alt, a viral construct which contains small differences in its 3′ LTR in comparison with that of HXB-WT (Fig. 6). These sequence differences between the 3′

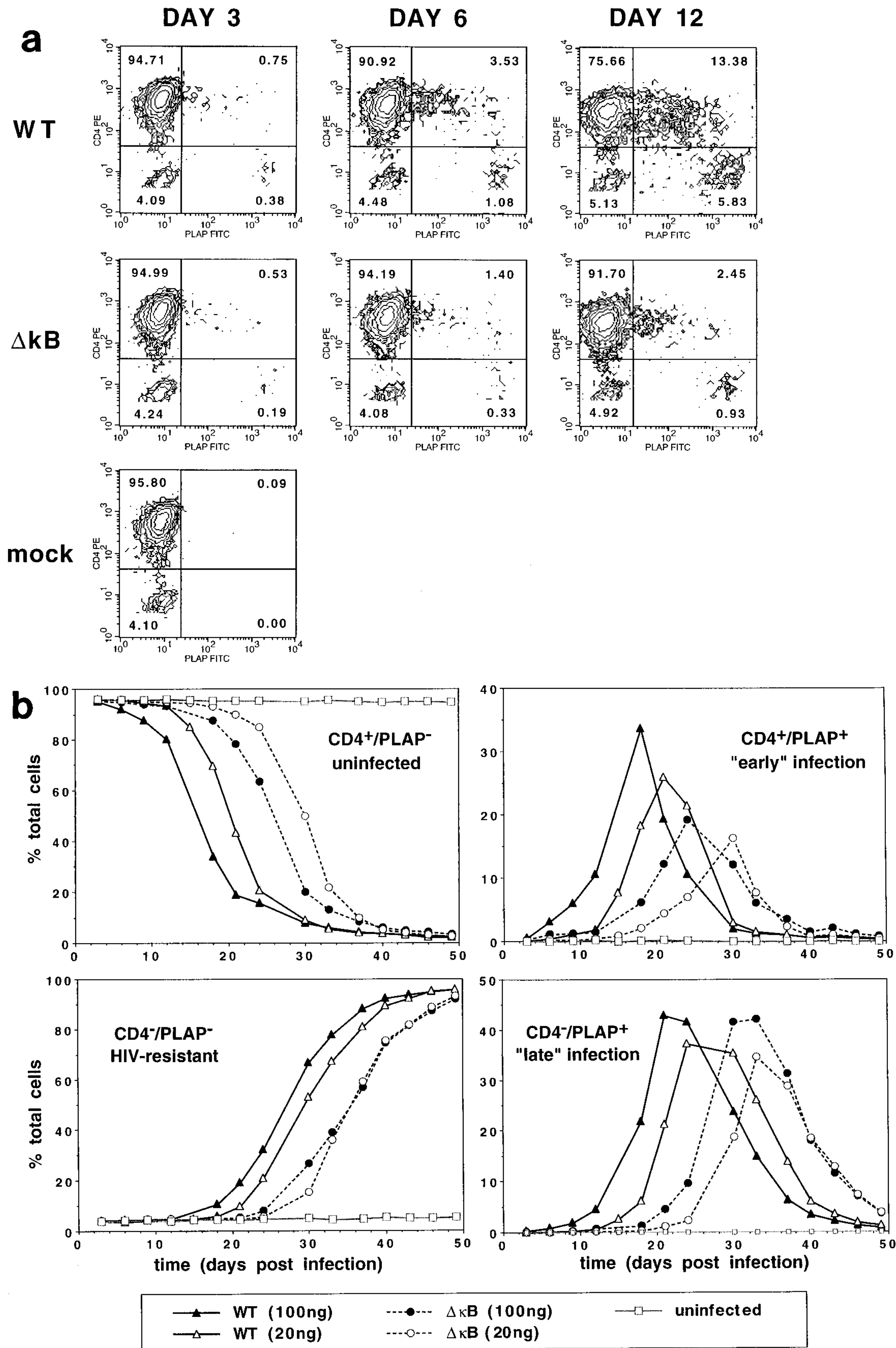

FIG. 3. Time course of infection of Jurkat cells with HXB-PLAP-WT and HXB-PLAP-ΔκB viruses. (a) FACS plots of HXB-PLAP-WT (top row)- and HXB-PLAP-ΔκB (second row)-infected Jurkat cells showing progression of infection at 3, 6, and 12 days following infection with 100 ng of input p24. The single plot at the bottom is representative of samples stained from mock-infected cells for all time points. The *x* axis represents PLAP fluorescence, and the *y* axis represents CD4 fluorescence, both on logarithmic scales. The number in each corner of the plot represents the percentage of total gated cells that fall into that quadrant. FITC, fluorescein isothiocyanate; PE, phycoerythrin. (b) Time course of HXB-PLAP infections graphing the percentage of total cells which fall into each of the quadrants at sequential time points. Changes in uninfected $CD4^+$ $PLAP^-$ cells from the upper left quadrant (upper left graph), $CD4^+$ $PLAP^+$ cells from the upper right quadrant (upper right graph), $CD4^-$ $PLAP^+$ cells (lower right graph), and $CD4^-$ $PLAP^-$ cells (lower left graph) are graphed versus time.

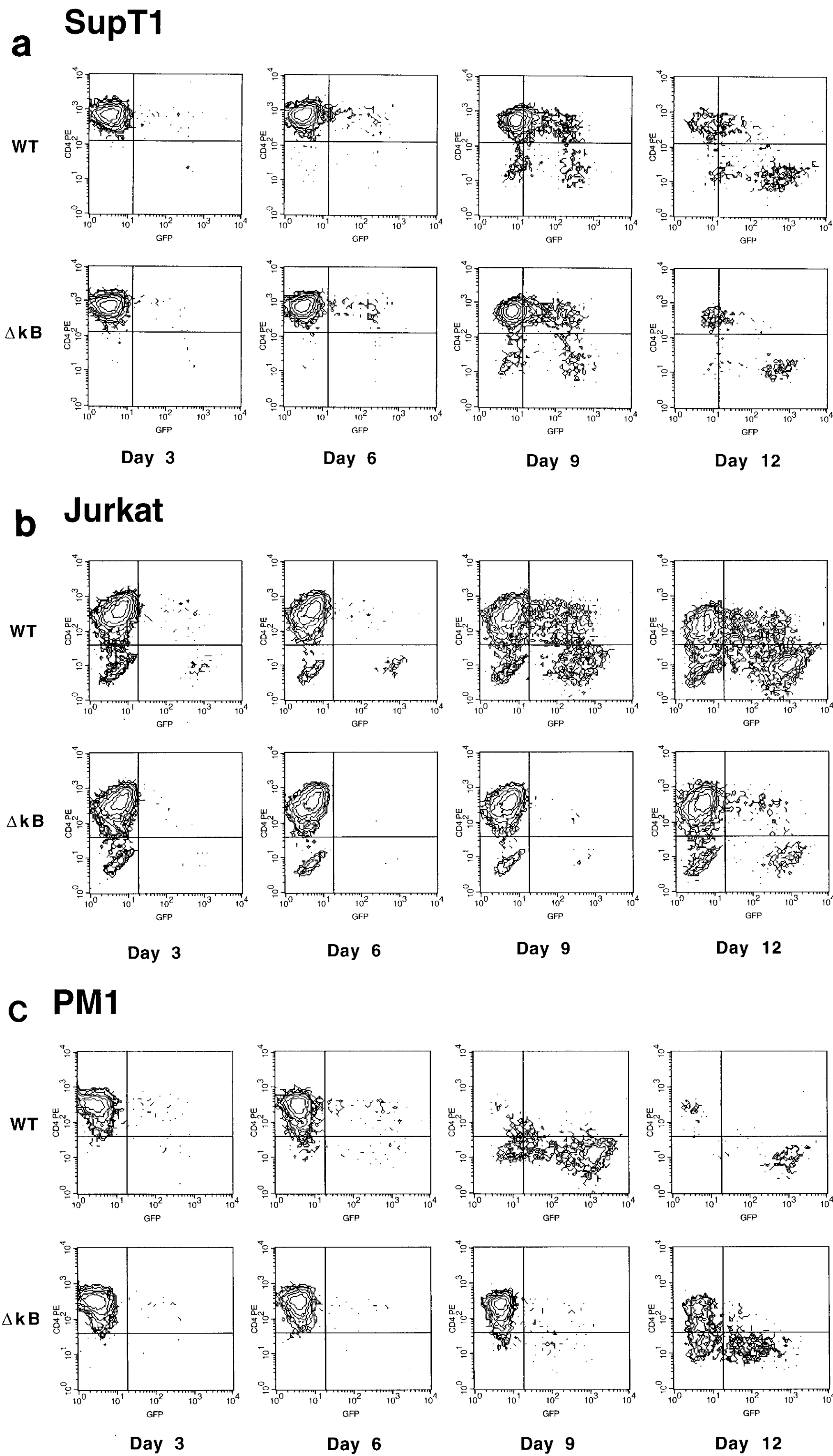

FIG. 4. Infections of different T-cell lines with HXB-GFP-WT and HXB-GFP-ΔκB viruses. FACS plots of HXB-GFP-WT (top row)- and HXB-GFP-ΔκB (bottom row)-infected SupT1 (a), Jurkat (b), and PM1 (c) cells showing progression of infection at 3, 6, 9, and 12 days postinfection. The *x* axis represents GFP fluorescence, and the *y* axis represents CD4-associated fluorescence, both on logarithmic scales.

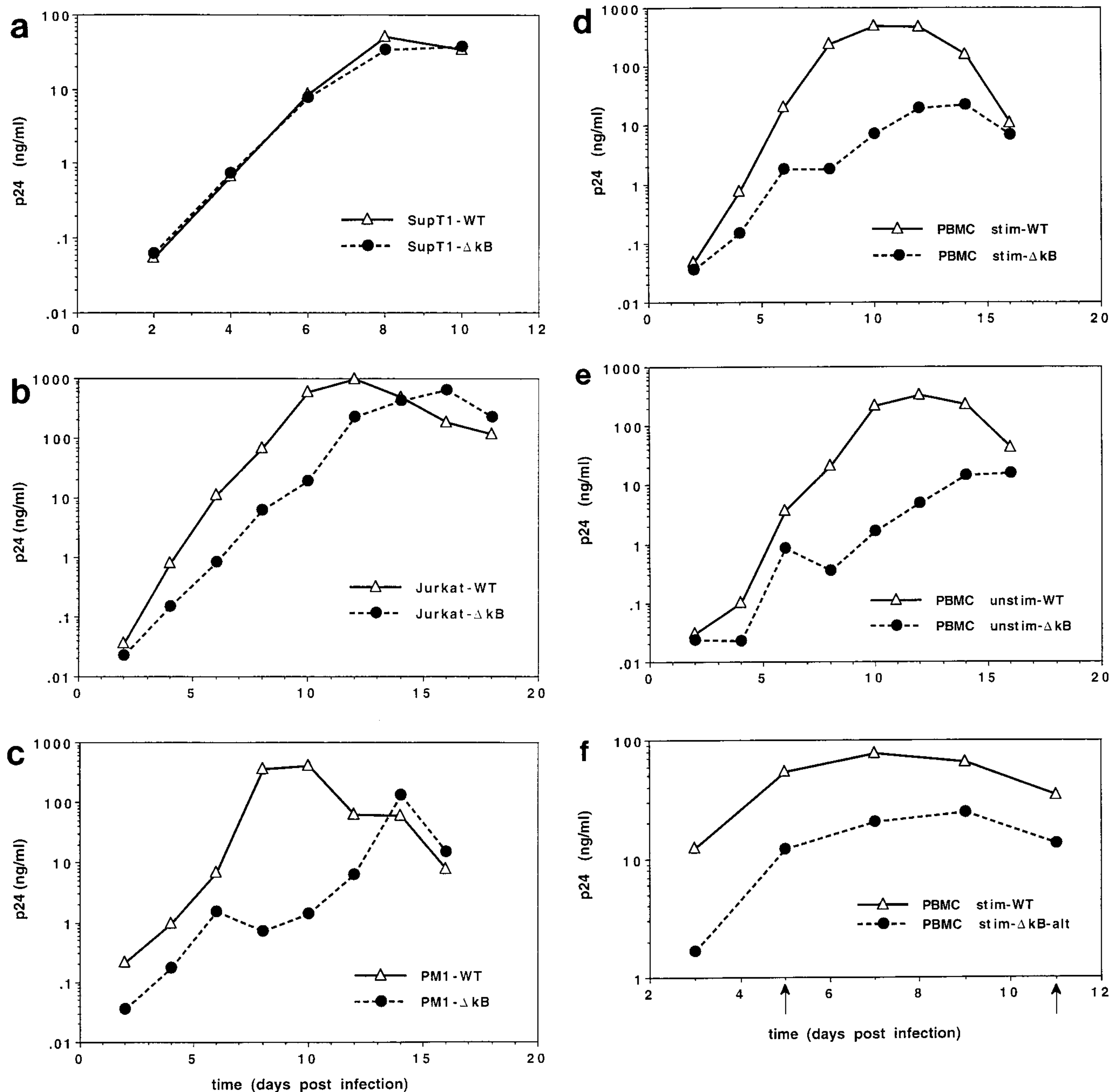

FIG. 5. Analysis of growth of HXB-WT and HXB-ΔκB in SupT1 (a), Jurkat (b), and PM1 (c) cells, PHA-stimulated (stim) PBMC (d), unstimulated (unstim) PBMC stimulated 24 h postinfection with PHA (e), and PBMC infected with HXB-ΔκB-alt or HXB-WT, which contain distinct 3′ LTRs (f). The arrows indicate times when viral sequence analysis was performed. Virus production, measured by p24 enzyme-linked immunosorbent assay, is shown on a logarithmic scale.

LTRs allow us to distinguish between reversion events and potential cross-contamination of virus during experimentation. Additionally, the 5′ and 3′ LTRs of HXB-2D can also be distinguished based upon sequence differences so that recombination between the 5′ and 3′ LTRs can also be readily detected.

HXB-ΔκB-alt produced a growth lag relative to HXB-WT comparable to that observed in other experiments conducted with completely isogenic viruses (compare Fig. 5f with d and e). Viruses from two time points were isolated from the infection for sequence analysis. In addition, virus from transfected supernatants was also tested for the presence of contaminating WT virus in the input mutant virus preparation (see Materials and Methods).

RNAs from the input virus and the virus collected at two time points were subjected to reverse transcriptase-mediated PCR and cloned into pBluescript. Subcloned LTRs were sequenced, and a few subclones from the HXB-ΔκB infections were, in fact, found to contain small numbers of WT LTRs. Examinations of these WT sequences determined that these WT LTRs were largely identical to the 5′ LTR of the proviral HXB-2D molecular clone. Therefore, these are most likely to

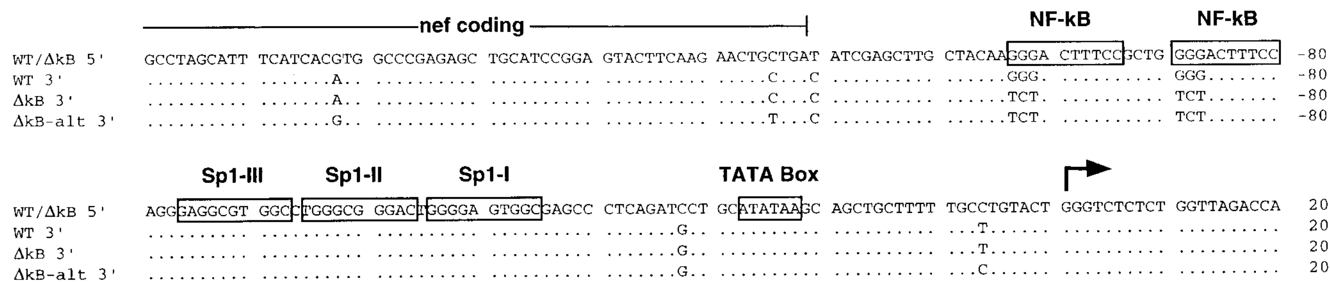

FIG. 6. Sequences of 5′ and 3′ LTRs of proviral constructs used for infections. The 5′ LTRs of all of the constructs were identical. In Fig. 2 to 5e, the 3′ LTRs used are completely isogenic except for the κB sites. The ΔκB-alt LTR used especially for sequence analysis in Fig. 5f is indicated at the bottom. Sequence analyses of the virus allows discrimination between cross-contamination, recombination, and reversion mutations. LTR sequence differences between ΔκB and ΔκB-alt extend beyond the 200-bp region designated here, into the 5′ *nef* sequence.

have arisen from low levels of WT virus generated by recombination during transfection (data not shown). To determine the frequency of WT LTRs found at different time points, oligonucleotide hybridization to WT or mutant κB site probes was performed on the U3 cDNAs to determine the ratios of WT to mutant LTR sequences found in all of the samples (Table 1). Analysis of the input inoculum detected no WT sequence in the input virus from a screen of 107 colonies. In addition, no mutant sequence was found in the WT inoculum. However, 6% of the WT sequence was found in mutant virus infections at day 5, which increased to represent 25% of all LTRs at day 11. A careful inspection of the U5 sequences of 18 day 11 clones and 6 day 5 clones did not reveal any repetitive mutations which could be interpreted as second-site revertants (data not shown). In conclusion, we found that although our system initially produced less than 1% recombinant WT virus during virus production by transfection, we did detect significant levels of recombinant virus at late time points. However, even at a later point, the majority (75%) of the virus still contained mutant sequence points confirming that the mutant virus is able to replicate and produce progeny.

DISCUSSION

This study clearly defines the relative importance of the κB sites in the HIV-1 life cycle in human T cells. We have measured LTR function during infection with three independent assays and made correlations with a biochemical measure of NF-κB activity. When considered together, the single-round luciferase assay, single-cell flow cytometric analyses, and virus production analyses provide a coherent picture of how a *cis*-acting promoter element is important in the HIV-1 life cycle. It is clear that the PM1 cells that showed the largest effect when mutating the κB sites by luciferase assay were also the only cells to show a significant decrease in the peak level of GFP expression on the single-cell level. This also correlates with the largest growth delay and a decrease in peak titer for infections with a mutant virus in these cells. Furthermore, these cells also demonstrated the highest basal level of NF-κB activity.

By both luciferase assay and viral growth analysis, PBMC appeared to behave most like PM1 cells in having the most dramatic effects when mutating the enhancer element. This similarity suggests that NF-κB plays an important role in highly activated T cells, although it is unclear to what extent a PHA-activated cell resembles an activated T cell in vivo. In other cells, however, we found little effect of mutation of the enhancer. We found that in SupT1 cells, a T-cell line with little or no basal NF-κB activity, there is no dependence upon these *cis* elements for optimal viral replication or spread. This shows that the HIV-1 LTR is not absolutely dependent on its κB sites for substantial growth. In all of the assays, Jurkat cells consistently displayed phenotypes intermediate between those of SupT1 and PM1 cells, concordant with their intermediate levels of basal NF-κB activity.

In contrast to previous studies, our analysis of viral growth in different T-cell environments correlates NF-κB DNA binding activity with the phenotypes that we observed and differs technically from previous studies in key ways. Use of a more robust viral production method which employs transfection of 293 cells allowed us to produce mutant virus in large quantities without the need to amplify them in other cells prior to infection. Our marker gene methods enabled us to look at the effects of mutations in a single round of infection, as well as in multiple rounds, and find confirmatory results between the various assays. In one previous case, in which the investigators found mutant virus completely growth deficient (1), it is likely that the methods they used to generate virus were not sufficient for detection of the lower κB mutant virus levels, which can be as much as 100-fold lower than those of a WT virus. In agreement with that study, we did observe a marked effect of enhancer mutation in PBMC; however, in contrast to their study, we clearly showed that an enhancer mutant can grow to 3 orders of magnitude above the limit of detection of our p24 assay. In earlier studies, in which little or no difference was ascribed to the viruses lacking κB sites, it is possible that the smaller differences observed could be due to selection of viral variants during the production of viral stocks prior to experimentation or, perhaps, to differences in the level of κB activation achieved by variations in PHA or IL-2 treatment.

Our sequence analysis did reveal that one shortcoming of our methods was the generation of WT recombinants at low levels during mutant virus production. These contaminants were detectable following multiple infection cycles. However,

TABLE 1. Oligonucleotide hybridization analysis of viral U3 sequences cloned at different times of infection[a]

Virus	ΔkB infection			WT infection		
	Mut[b]	WT[b]	% WT[c]	Mut[b]	WT[b]	% WT[c]
Input	107	0	0	0	160	100
Day 5	108	7	6	0	11	100
Day 11	219	73	25	0	137	100

[a] As shown in Fig. 5f.
[b] Values are numbers of individual bacterial colonies hybridizing to mutant (Mut) or WT oligonucleotides as described in Materials and Methods.
[c] No. of WT colonies ÷ (no. of mutant colonies + no. of WT colonies) × 100%.

the hybridization analysis confirmed that the preponderance of virus was mutant throughout the period of time during which we studied virus growth. This suggests that the mutant virus could, indeed, replicate to a significant level. Because the replication of HXB-ΔκB could be readily detected in primary cells, our data do not support the hypothesis that the activity of Tat in primary cells is fundamentally dependent upon NF-κB sites (1).

Because recombination was detected in the PBMC experiment, experiments involving HXB-PLAP and HXB-GFP infection of Jurkat or PM1 cells should provide a conservative estimate of the differential between the WT and ΔκB viruses. Consequently, the dependence on κB sites is possibly larger than that shown by PLAP staining of cells. However, we corroborated our results with single-cycle infections with luciferase-encoding viruses that should be less affected by recombination. These experiments confirm the disposition of the enhancer mutants to transcribe HIV RNA at decreased levels consistently in proportion to the levels of basal NF-κB. The especially low level of transcription by HXB-Luc-ΔκB in PM1 cells could be suggestive of a global inhibitory effect on non-κB-dependent transcription or a relative absence of other factors, e.g., Sp1, that may act to promote HIV transcription even in the absence of NF-κB. In a similar vein, in SupT1 cells, perhaps levels of these non-NF-κB HIV activators are high, allowing robust replication in the absence of basal NF-κB.

Importantly, in both transformed T-cell lines and primary cells, mutant virus was eventually able to achieve levels of gene expression sufficient to allow virus production and spread to neighboring cells. Single-cell analysis of infection by HXB-PLAP-ΔκB or HXB-GFP-ΔκB suggests that the enhancer mutation does not significantly impede the progression of virus from early to late stages of infection. Especially early in the infections, very similar levels of cells were initially targeted and these contained similar ratios of cells in early (CD4 high) or late (CD4 low) stages of infection. Flow cytometric analyses of SupT1 cells further suggest that there is no absolute requirement for the NF-κB sites in cell lines and that other cellular factors can promote viral transcription even in their absence.

Our report shows that the enhancer plays an important role by increasing the level of virus production from each cell. While mutant virus is capable of infecting cells to completion and depleting CD4-positive cells, the peak p24 titers which arise from WT virus can be 100-fold higher when the virus contains intact NF-κB binding elements. When p24 values were measured as a percentage of infected, $PLAP^+$ Jurkat cells observed by flow cytometric analyses, we found that the WT virus produced severalfold more p24 per infected cell, suggesting that the burst size of WT viruses is larger than that of the enhancer mutant (data not shown). From these results, one might predict that infection of individuals with ΔκB virus might give rise to decreased viral loads and a slower disease course.

Interestingly, a recent report describes several viral isolates from a single patient which are pathogenic despite containing deletions of the NF-κB binding sites (31). While the patient was initially characterized as belonging to a long-term survivor cohort, he later progressed to AIDS. The virus isolated from this patient, described by Zhang et al. (31), was also found to have a duplication of a putative TCF-1 site in the U3 region. We were therefore interested in identifying potential second-site reversions in mutant virus from our own experiments which might complement its slow-growth phenotype in vitro. However, we failed to find any recurring mutations which appeared over time. Nonetheless, it is interesting to speculate that such compensatory mutations could occur in vivo. In the studies of Zhang et al. (31), the LTRs were characterized with luciferase reporter viruses and found to have basal transcriptional rates only a fewfold lower than that found in WT virus, in contrast to the 20-fold difference which we observe with a discrete mutation of κB binding alone, suggesting that the TCF-1 repeat may compensate for the lack of NF-κB sites in this unique in vivo case.

From this study, a rationale for the extraordinary sequence conservation of these sites in vivo becomes evident. During virus growth in activated PBMC, an intact enhancer element appears to contribute to an earlier and higher peak titer of HIV-1. The finding that a tiny contaminating fraction of WT virus in a ΔκB infection can achieve 25% of the total by the end of an infection is a testimony to the competitive advantage of a virus with an intact enhancer. It is clear from our studies that activated, primary $CD4^+$ human lymphocytes provide a transcriptional environment that heavily utilizes and provides significant selective pressure to maintain functional κB sites.

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