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Permanent Occupancy of the Human Immunodeficiency Virus Type 1 Enhancer by NF-κB Is Needed for Persistent Viral Replication in Monocytes

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This work aimed to ascertain the role of κB-responsive elements of the human immunodeficiency virus type 1 (HIV-1) enhancer not only in early initiation but also in long-term maintenance of proviral transcription in cells of the monocytic lineage. For this purpose, we used three main approaches. The first was to abruptly terminate tumor necrosis factor-induced NF-κB binding to the enhancer sequences in U1 monocytic cells, using a short pulse of exogenous tumor necrosis factor. This resulted in concomitant decrease in nuclear NF-κB DNA-binding activity and endogenous long terminal repeat transcriptional activity. The second was to suppress the permanent NF-κB translocation induced by HIV-1 replication itself in chronically infected U937 cells, using a specific proteasome inhibitor (Z-LLL-H). As early as 2 h after addition of the inhibitor to the culture medium, there was an inhibition of both constitutive activation of NF-κB and HIV-1 genome expression. The third approach was to monitor the replication competence in U937 cells of an infectious HIV-1 provirus carrying point mutations in the κB-responsive elements of both long terminal repeats. Compared with its wild-type counterpart, this mutated provirus showed a profoundly decreased, Z-LLL-H-insensitive transcriptional and replicative activity in U937 monocytes. Together, our results indicate that occupancy of the viral enhancer by NF-κB (p50/p65) heterodimers is required for ongoing transcription of integrated HIV provirus in monocytes, even in cells chronically infected and permanently producing functional HIV Tat protein. Thus, the ability of HIV-1 replication to activate NF-κB is crucial to the intense self-perpetuated viral transcription observed in cells of the monocytic lineage.

Transcription of the human immunodeficiency type 1 (HIV-1) genome depends on the intracellular environment into which the virus integrates and is regulated by a complex interplay between viral regulatory proteins and cellular transcription factors interacting with the viral long terminal repeat (LTR) region. In 1987, a direct correlation was established between the up-regulation of LTR transcriptional activity during T-cell activation and the induction of the nuclear factor NF-κB, which binds to the two repeated motifs in the core enhancer element of the LTR (45). Since this early study, the notion of the critical dependence of the HIV-1 LTR function on NF-κB DNA-binding activity has received considerable experimental support (reviewed in reference 25).

The human family of Rel/NF-κB proteins is composed of p50, p52, p65, c-Rel, and RelB (reviewed in reference 25). While almost all combinations of homo- and heterodimers may exist, NF-κB, which is rapidly activated in response to extracellular signals, is typically composed of p50 and p65 subunits. While p50 does not appear to possess a domain for transcriptional activation, the p65 subunit does, which accounts for the transcriptional activity of the NF-κB heterodimer (20, 58). The p50 subunit is derived from a p105 precursor by ubiquitin-mediated proteolysis of the C-terminal region of p105 (46). When expressed from an alternatively spliced p105 mRNA lacking the p50 coding sequences, this C-terminal region (IκBγ) preferentially inhibits the DNA-binding activity of p50 homodimers (33, 35). The activity of NF-κB is controlled by the inhibitor protein IκBα (or MAD3) (6, 29), which, in common with IκBγ, Bcl-3, and IκBβ (62, 66), contains multiple ankyrin repeats. Exposure of cells to signals which activate NF-κB results in the rapid phosphorylation and degradation of IκBα (7, 10, 30, 40, 60). Degradation of IκBα is rapidly followed by induction of IκBα mRNA through a mechanism regulated by the interaction of NF-κB with DNA recognition sites located in the promoter of the IκBα gene (14, 34, 60). Newly synthesized IκBα protein appears transiently in the nucleus, where it negatively regulates NF-κB-dependent transcription (3).

The multicatalytic 26S proteasome complex, shown to catalyze the generation of antigenic peptides for presentation of major histocompatibility complex class I antigen molecules (54), is responsible for the ATP-dependent proteolytic degradation of IκBα, as shown by the use of peptide aldehydes which specifically inhibit the chymotrypsin activity of the proteasome (46). Furthermore, it has been proven that covalent attachment of polyubiquitin chains to IκBα is required for proteasome-activated degradation of the protein both in vitro (13) and in vivo following cell activation (55).

Interesting evidence has also emerged that the enhancer-independent inducibility of the HIV-1 LTR may involve the participation of other cellular factors (reviewed in reference 21) and the p53 protein (26). Transcriptional synergism between distinct, virus-inducible enhancer elements on the activity of the human beta interferon gene promoter has been reported (16). Indeed, in the HIV-1 LTR context, NF-κB may act in synergy with the transcription factor Sp1, either through the establishment of a direct interaction (48, 49) or after Sp1

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phosphorylation (64). A complex picture of HIV-1 LTR regulation has emerged from these accumulated data. However, analyses performed in normal T lymphocytes and primary macrophages emphasized the essential role of NF-κB DNA-binding activity for the initiation of LTR transcriptional activity and HIV-1 Tat-dependent LTR transactivation (2, 43). We have recently shown an absolute requirement of enhancer sequences for the establishment of HIV-1 infection in peripheral blood CD4 lymphocytes (2). This study led to the notion that NF-κB p65/p50 heterodimer binding to the enhancer domain plays a driving role in HIV-1 LTR transcriptional activity by both initiating this activity and cooperating with the Tat protein, thus constituting a prerequisite for the establishment of HIV-1 replication in normal T lymphocytes.

In monocytes/macrophages, NF-κB DNA-binding activity and HIV-1 LTR reporter gene expression are also coordinately inducible (5, 24, 43, 47, 56). In this cell type, the contribution of enhancer occupancy by NF-κB to HIV-1 replication has been analyzed primarily in myelomonocytic U1 cells. This cell line, originally cloned from the chronically HIV-1_{LAI}-infected promonocytic cell line U937, was found to have very low basal expression of two integrated HIV genome copies but to retain the ability to produce large quantities of infectious virus when stimulated (19). The inducing effect of tumor necrosis factor (TNF) on endogenous HIV-1 replication in U1 cells has been correlated with the activation of NF-κB binding to the viral enhancer (18, 24, 51) and the stimulation of newly transcribed HIV-1 RNAs (50, 51). In monocytes/macrophages and T lymphocytes, the two main cellular targets of HIV, it appears that of all of the multiple cellular factors which interact with the HIV-1 LTR and modulate its function, the Rel/κB family is the principal inducer of HIV-1 transcription. However, a remarkable feature of the HIV-monocyte/macrophage relationship is that HIV-1 replication itself activates NF-κB transcription factors in this cell lineage (5, 56, 57).

In addition, increased transcription of the p105 precursor replenishes the p50 pool and allows the observed permanent activation of NF-κB to be sustained (47). Increased IκBα turnover appears to account for the constitutive NF-κB DNA-binding activity in HIV-1-infected monocytes (38). However, expression of the viral transactivator Tat in HIV-1-infected U937 cells also participates in maintaining this high-level, permanent HIV-1 genome transcription, since deletion of the Tat-responsive element sequence suppressed most, but not all, transcriptional activity of an HIV-1 LTR luciferase expression vector transiently transfected into chronically infected U937 cells (5). Thus, the role of HIV enhancer occupancy in permanently increased HIV-1 LTR transcriptional activity is still unclear.

Hence, many questions concerning the importance of NF-κB DNA-binding activity in HIV-1 replication in cells of the myelomonocytic lineage remain to be answered. First, is NF-κB LTR-dependent transcription absolutely required for the establishment of HIV-1 replication? Second, is HIV Tat expression, induced as soon as LTR function is ongoing, sufficient to perpetuate proviral transcription in an enhancer-independent manner? Third, the κB binding site in the simian immunodeficiency virus LTR has been demonstrated to be absolutely essential for efficient viral replication in primary macaque macrophages (8). In light of the close relationship between HIV and simian immunodeficiency virus and the high degree of conservation of NF-κB sites, can these results be extrapolated to HIV-1 infection in human monocytic cells? Such questions are particularly relevant with respect to macrophages, in which HIV-1 transcription and replication occur in a persistent manner, in the absence of viral cytopathogenic effects (reviewed in references 22, 32, and 41). They are critical questions, because monocytes/macrophages are likely to be the main cellular sites of the very intense viral replication observed in infected patients (31, 65).

Using the U937 monocytic cell model, we have now addressed these questions by suppressing either the NF-κB-binding elements by specific mutation or the permanent nuclear translocation of NF-κB induced by HIV-1 replication. We monitored the consequences of such manipulations on the transcription of chronic HIV-1 genome expression. In latently infected U1 cells, we have used a recently described (3) method allowing a controlled, transient NF-κB activation to show that early disappearance of nuclear NF-κB-binding activity results in an abortive induction of HIV provirus transcription after a pulse of TNF. Our results provide support for the conclusion that both initiation and perpetuation of integrated HIV provirus transcription are strictly dependent on the actual occupancy of the enhancer sequence by NF-κB.

MATERIALS AND METHODS

Cell cultures and infections. The monocytic cell line U937, latently HIV-infected U1 cells (19) (obtained from the NIH AIDS Research and Reference Reagent Program), and the lymphoblastoid T-cell line CEM were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and glutamine. They were passaged twice a week at a concentration of 2.5×10^5 cells per ml. Infection of U937 cells with mycoplasma-negative HIV-1_{LAI} was performed as described previously (5). The production of infectious supernatants from either wild-type (wt) or κB-mutated (κB-Mut) HIV (see below) was performed in CEM, U937, or Cos-7 cells. Cos-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and glutamine and passaged every 3 days. Ten micrograms of each complementary *Bgl*I-*Nco*I-digested proviral construct was cotransfected for 12 to 16 h into subconfluent Cos-7 cells seeded 1 day before transfection at 4×10^6 cells per 75-cm^2 flask, using Lipofectamine reagent (GibcoBRL). After transfection, cells were trypsinized and cultured for an additional 48 h before harvesting of the viral supernatants. U937 and CEM cells were resuspended in RPMI supplemented with 10% fetal bovine serum and electroporated at 250 V and 960 μF with 15 μg of DNA per 10^7 cells. Cell-free supernatants were assayed for the HIV-1 $p24^{gag}$ protein by enzyme-linked immunosorbent assay (Dupont), and all infections were normalized to the $p24^{gag}$ levels (5×10^6 to 15×10^6 cells infected with a total 20 to 150 ng of $p24^{gag}$ antigen). The threshold of $p24^{gag}$ production detectability was 2.5 pg/ml.

Proviral constructs. The HIV-1 wt and κB-Mut constructs were described previously (28). Plasmid puc5′HIV-arm contains a *Bam*HI-*Sph*I fragment derived from the molecular clone HIV-1SF2 linked to an *Sph*I-*Nco*I fragment originating from the human T-cell leukemia virus IIIB pBH10 clone. puc3′HIV-arm contains the *Nco*I-*Nar*I sequence of HIV-1SF2. The wt and its κB-Mut versions differ only in a 5-bp substitution (AAGGG and TTTAA, respectively) in the enhancer regions of the 5′ and 3′ LTRs. These noninfectious 5′ and 3′ plasmids produce infectious virus upon *Bgl*I-*Nco*I digestion and cotransfection.

Preparation of cytoplasmic and nuclear extracts. U1 cells were treated with 5 ng of TNF (provided by the Medical Research Council AIDS Research and Reference Reagent Program) per ml and cultivated for 2 h at 37°C. After this pulse-chase, experiments were performed as previously described (3). Cells were washed twice with 50 ml of prewarmed phosphate-buffered saline (PBS). Half of the culture was resuspended in prewarmed culture medium in the absence of TNF (chase), and the other half was resuspended in the presence of 5 ng of TNF per ml for an additional 6 h. Unless indicated otherwise, in experiments using the peptide aldehyde Z-LLL-H (46) (provided by F. Baleux, Unité de Chimie Organique, Pasteur Institute), uninfected U937 cells were pretreated for 110 min with this reagent (at concentrations indicated in the figure legends) before the addition of TNF to culture media for an additional 10 min. HIV-1-infected U937 cells were incubated with the inhibitor for the same total time (120 min). In all cases, cells were collected by centrifugation and washed in cold PBS before extraction. Sedimented cells were resuspended in lysis containing protease inhibitors (5) and phosphatase inhibitors (sodium vanadate [1 mM], *P*-nitrophenyl phosphate [10 mM], β-glycerophosphate [10 mM], sodium fluoride [5 mM], and okadaic acid [200 nM]). After microcentrifugation, cytoplasmic fractions were collected and nuclear proteins were extracted in hypertonic buffer as previously described (5).

EMSA. The electrophoretic mobility shift assay (EMSA) was performed with 4 μg of nuclear extract incubated for 15 min at room temperature with a [γ-^{32}P] ATP-labeled, double-stranded oligonucleotide containing either the HIV-1 LTR binding site for NF-κB (5′-ACAAGGGACTTTCCGCTGGGACTTTCCAGG GA-3′) or the consensus binding site for Sp1 (5′-GGGAGGCGTGGCCTGGG CGGGACTGGGGAGTGGC-3′). Samples were analyzed in nondenaturing 6% polyacrylamide gels. Competition experiments were performed by adding a 40-

fold molar excess of homologous, unlabeled oligonucleotide to each sample prior to addition of the [γ-^{32}P]ATP-labeled probe. Components of TNF-induced DNA-binding complexes in U1 nuclear extracts were identified by incubation with specific polyclonal antibodies directed against p50 or p65 recombinant protein. p50 antibodies were affinity-purified immunoglobulins obtained from an immune polyclonal rabbit serum generated by immunization with recombinant p50. p65 antibodies were affinity-purified immunoglobulins obtained from an immune polyclonal rabbit raised again a peptide corresponding to amino acids 531 to 550 mapping within the carboxy-terminal region of human p65 (antibody C20; Santa Cruz Biotechnology).

Nuclear run-on assays. Isolation of nuclei and run-on transcription assays were performed by using a modification (39) of the original procedure (23). To prepare nuclei, cells were quickly disrupted with 1 ml of lysis buffer (10 mM Tris [pH 7.4], 10 mM NaCl, 5 mM $MgCl_2$) containing 0.5% Nonidet P-40 and layered onto 30% sucrose in 50 mM Tris-HCl [pH 8.2]–5 mM $MgCl_2$–0.1 mM EDTA. Pelleted nuclei were resuspended and stored under liquid nitrogen in 40% glycerol–50 mM Tris-HCl (pH 8.2)–5 mM $MgCl_2$–0.1 mM EDTA. Run-on assays were performed on 10^7 nuclei incubated with [α-^{32}P]UTP (3,000 Ci/mmol; Amersham) for 30 min at 37°C. Labeled RNAs were purified by using Qiagen columns as specified by the manufacturer. Labeled RNAs (5×10^6 to 10^7 cpm) were hybridized for 3 days at 50°C with linearized, denatured plasmid DNA immobilized on nitrocellulose membranes (Schleicher & Schuell). The filters were washed and treated with RNase A (100 μg/ml) prior to autoradiography. Plasmids probes were as follows: pLTRXluc (59) linearized by *Hin*dIII (fragment size, 80 bp), pLgagSN containing an *Eco*RI-*Bgl*II PCR-amplified HIV-1_{LAI} fragment cloned into the *Eco*RI-*Bam*HI-digested pLXSN vector and linearized by *Eco*RI (fragment size, 1,500 bp), proviral construct pBru-2 (44) linearized by *Bam*HI (localized in the *env* sequence) (fragment size, 8,063 bp), pβ-actin (provided by A. Hovanessian) containing the β-actin cDNA (53) cloned into the *Bam*HI site of pGEM and linearized outside the β-actin cDNA by *Xba*I (fragment size, 1,170 bp), and control plasmid pUcBM20 (Boehringer) linearized by *Eco*RI (2,300 bp). Hybridization background was estimated by probing in vitro [α-^{32}P]UTP-labeled RNAs with *Eco*RI-linearized pUcBM20. Levels of in vitro-transcribed RNAs was estimated after 2 to 3 days of exposure by scanning the membranes with a digital imaging system, and computer-generated images were analyzed to obtain densitometric values (PhosphorImager model 250 and Molecular Dynamics program).

Nucleic acid purification and PCR analysis. As described previously (4, 67), DNA used for PCR amplification was extracted by lysis of cells in urea lysis buffer (4.7 M urea, 1.3% [wt/vol] sodium dodecyl sulfate, 0.23 M NaCl, 0.67 mM EDTA [pH 8], 6.7 mM Tris-HCl [pH 8]), extracted with phenol-chloroform, and precipitated in ethanol. Total RNA was extracted from cell cultures by using TRIzol reagent as specified by the manufacturer (GibcoBRL). To remove all traces of DNA, RNA preparations were subjected to digestion with RNase-free DNase I (Boehringer) for 1 h at 37°C. RNA ($\leq$3 μg) was subjected to hybridization with 100 ng of the 3′ primer (antisense), and first-strand cDNA synthesis was performed with Moloney murine leukemia virus reverse transcriptase (Superscript; GibcoBRL) in a volume of 20 μl for 1 h at 37°C; 5 to 10 μl of these cDNA products was subsequently subjected to analysis by PCR. PCR amplifications were performed as previously described (67), using 50 ng of the 3′ primer and 25 ng of the 5′ primer (sense) end labeled with ^{32}P (5×10^5 to 10^6 cpm) in a buffer containing 0.25 mM each of the four deoxynucleoside triphosphates, 50 mM NaCl, 25 mM Tris-HCl (pH 8), 5 mM $MgCl_2$, 100 μg of bovine serum albumin per ml, and 1.5 U of *Taq* DNA polymerase (Amersham). Unless indicated otherwise, amplification conditions were as follows: 5 min of incubation at 94°C followed by 1 min at 94°C and 2 min at 65°C for 30 cycles. PCR products were separated by gel electrophoresis on 6% nondenaturing polyacrylamide gels and visualized by direct autoradiography of the dried gels. For quantification analysis, gels were recorded with a digital imaging system, and computer-generated images were analyzed to obtain densitometric values (PhosphorImager model 250 and Molecular Dynamics program). The amount of RNA species was estimated by using a modification of the original method (27). Aliquots of the PCR amplification products were removed during the exponential phase of the PCR. Specific RNA signals were plotted on a semilog scale against the number of cycles, and quantitation of a given RNA species was obtained by extrapolation to the zero cycle.

The PCR primers of HIV-1 used were described previously (1, 4, 67). For HIV-1 DNA detection, primers M667 (5′-GGCTAACTAGGGAACCCACTG-3′) and AA55 (5′-CTGCTAGAGATTTTCCACACTGAC-3′) were used. This pair amplifies a fragment of 140 bp. Quantitation of HIV-1 DNA was performed by analyzing a standard curve of dilutions of cloned HIV-1_{LAI} DNA linearized outside viral sequences. Use of a pair of oligonucleotides complementary to the β-actin cDNA sequence (53), sense (5′-GTGGGGCGCCCCAGGCACCA-3′) and antisense (5′-CGGTTGGCCTTGGGGTTCAGGGGGG-3′), yields a 244-bp amplified product. The oligonucleotide primers used for detection of total HIV-1 RNAs were antisense oligonucleotide M668 (4) (5′-CGCGTCCCT GTTCGGGCGCC-3′) combined with M667 for PCR amplification of a 161-bp product. Oligonucleotide primers LA45 (sense; 5′-GGCTTAGGCATCTC CTATGGC-3′) and LA41 (antisense; 5′-TGTCGGGTCCCCTCGTTGCTGG-3′) (4) allowed the detection of a 123-bp PCR product specific for HIV-1 *tat/rev* spliced RNA. Nested PCR was performed for the detection of HIV-1 RNAs prepared from U937 cells infected with wt and κB-Mut constructs. The outer primers were M661 (67) (antisense; (5′-CCTGCGAGAGAGCTCCTCTGG-3′) and ST1 (1) (sense; 5′-GGGTCTCTCTGGTTAGA-3′), while the inner primer pair was M667-M668. Three microliters of the first-round nonlabeled products was used as the template for the second amplification reaction.

Low-molecular-weight DNA extraction was performed as previously described (5). DNA extracted from cell nuclei isolated 30 min after transfection was analyzed quantitatively after transfer of serial dilutions onto a nylon membrane (Amersham) and hybridization with a γ-^{32}P-labeled HIV-1 *gag* probe.

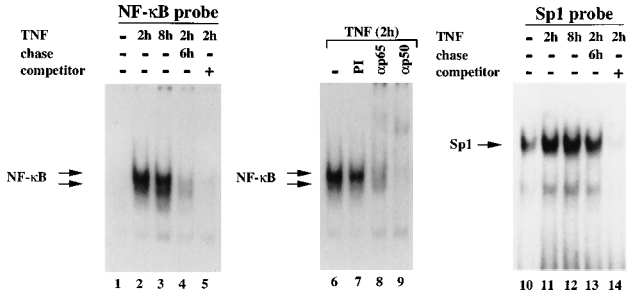

FIG. 1. Inhibition of TNF-induced NF-κB DNA-binding activity in U1 cells. U1 cells were exposed for 2 h to TNF and then extensively washed in prewarmed PBS. Aliquots were processed to obtain nuclear extracts, nuclei (Fig. 2A), and total RNA (Fig. 2B). The remaining cells were reseeded in fresh culture medium in either the absence (chase) or the presence of TNF for an additional 6 h. EMSA was done with 4 μg of nuclear extracts and [γ-^{32}P]ATP-labeled oligonucleotides representing κB (lanes 1 to 9) and Sp1 (lanes 10 to 14) motifs. Competition with a 40-fold molar excess of unlabeled oligonucleotide was used to confirm the specificity of the DNA-binding activity detected (lanes 5 and 14). Anti-p65 and -p50 (αp65 and αp50) polyclonal rabbit antisera or rabbit preimmune serum (PI; 1 μl) was added with the sample 15 min prior to addition of the radiolabeled κB probe (lanes 7 to 9).

RESULTS

U1 cells pulsed by TNF and then maintained in its absence exhibit transient expression of nuclear NF-κB DNA-binding activity. To assess the contribution of activated NF-κB factor to the ongoing transcriptional activity of the endogenous HIV-1 LTR in TNF-treated U1 cells, we used a recently described (3) experimental system in which induction of early NF-κB DNA-binding activity results in a transient expression of this transcription factor, followed by its early disappearance from the nuclear compartment. U1 cells were treated for 2 h with TNF (pulse) and cultured for 6 h in its absence (chase). As shown in Fig. 1, these experimental conditions resulted in transient TNF-induced NF-κB binding to the κB motifs of the HIV-1 enhancer, since this complex is clearly reduced (lane 4) compared with its binding activity in nuclear extracts from cells continuously treated with TNF (lanes 2 and 3). The specificity of the NF-κB/DNA complexes detected in TNF-treated U1 cells was proven by competition with unlabeled DNA oligonucleotides (lane 5). From results of assays using p65 and p50 rabbit polyclonal antibodies (lanes 8 and 9), we concluded that most of the NF-κB DNA-binding activity was due to bona fide heterodimers composed by p50 and p65 proteins. To determine whether primarily NF-κB was affected in these experimental conditions, we analyzed the binding of the transcription factor Sp1, which is essential for HIV-1 LTR transcriptional activity (21). In contrast to NF-κB, Sp1 DNA-binding activity was constitutively detectable in uninduced cells and, depending on the experiment, remained unchanged or increased slightly upon TNF stimulation (lanes 10 and 11). While NF-κB DNA-binding activity is clearly reduced following transient exposure to TNF (lane 4), Sp1 DNA-binding activity is not significantly affected (compare lane 13 with lanes 11 and 12).

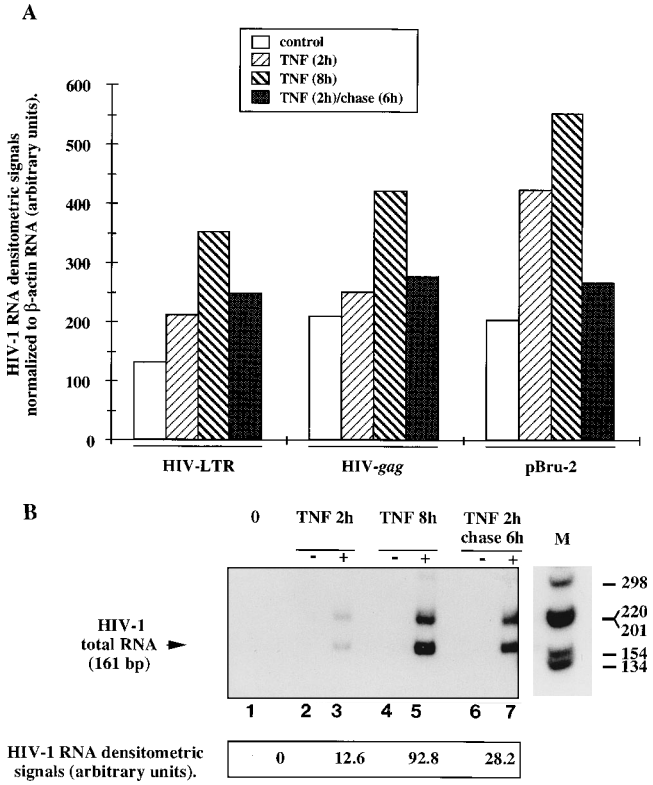

FIG. 2. The decrease of HIV-1-LTR transcriptional activity paralleled the diminution of TNF-induced-NF-κB DNA-binding activity. (A) Nuclear run-on analysis was performed on nuclei obtained from U1 cells treated as described in the legend to Fig. 1. In vitro-labeled nuclear RNAs were hybridized to the double-stranded DNA probes indicated below the columns. The hybridization signals obtained were recorded with a digital imaging system. The densitometric values were corrected relatively to the β-actin values and are expressed in arbitrary units for each series of HIV-1 fragments. The data show a representative study of four similar independent studies. (B) RT-PCR analysis of HIV-1 total RNA extracted concomitantly with the nuclear extracts analyzed in Fig. 1. Reactions without the addition of reverse transcriptase (−) were used to control contamination of RNA samples by genomic DNA. The molecular weight marker (M) is a 1-kb ^{32}P-labeled ladder; positions are indicated in base pairs. The densitometric values obtained from the digital record of the dried gel are given below the gel.

Down-regulation of HIV-1 LTR transcriptional activity parallels the decrease of NF-κB DNA-binding activity in TNF-pulsed U1 cells. We tested whether the TNF-induced HIV-1 LTR transcriptional activity paralleled the transient expression of NF-κB DNA-binding activity. The rate of endogenous HIV-1 proviral transcription was assessed by nuclear run-on analysis in TNF-pulsed U1 cells (Fig. 2A). In vitro [α-^{32}P]UTP-labeled nuclear RNAs were hybridized to immobilized HIV-1 DNA fragments (*Xho*I-*Hin*dIII fragment of the LTR, *gag* sequence, and a pBru-2 subgenomic fragment linearized in the *env* sequence). In vitro-labeled nuclear RNAs were probed with β-actin DNA to ensure that transcriptional activity of this cellular gene was insensitive to the experimental conditions used. The hybridization signals obtained with the three different HIV-1 DNA probes were recorded by using a digital imaging system (Molecular Dynamics program) and normalized relative to the β-actin standard. Corrected densitometric values for the three sets of in vitro-transcribed HIV-1 RNA are shown in Fig. 2A. The expression of newly synthesized HIV-1 RNA in cells continuously exposed to TNF for 2 or 8 h paralleled the induction of NF-κB DNA-binding activity analyzed above (Fig. 1, lanes 2 and 3). As shown above, the EMSA experiments revealed a clear diminution of NF-κB DNA-binding activity when TNF was removed after a 2-h pulse followed by a 6-h chase (Fig. 1; compare lane 4 with lane 2). Nuclear run-on assays were performed to analyze the transcriptional status of HIV-1 genome expression during this transient TNF-induced NF-κB binding. The rates of de novo LTR and pBru-2 subgenomic fragment transcription in TNF-pulsed U1 cells were reduced by 32 and 52%, respectively, compared with those observed in controls continuously stimulated with TNF for 8 h. Figure 2A shows that ongoing HIV-1 transcription induced by a 2-h TNF exposure decreased during the chase period [TNF (2 h/chase (6 h)] in contrast to its further increase when TNF was maintained in the culture medium [TNF (8 h)]. The ongoing HIV-1 transcription observed during the chase period was significantly higher than after a 2-h exposure to TNF, probably as a consequence of residual LTR transcriptional activity. The apparent differences between RNA levels in pulse-chase experiments and in cells treated for 2 h alone for pBru-2 likely reflect experimental variability. These experiments demonstrate the same trend as for the NF-κB DNA-binding activity observed in the nuclei (Fig. 1) and were confirmed by the analysis of steady-state levels of total HIV-1 RNA by reverse transcription-PCR (RT-PCR). To maximize detection of total HIV-1 RNA, a modified PCR method using γ-^{32}P-labeled specific oligonucleotide primers for amplification was used; this procedure was followed by direct autoradiography of the gel-resolved products. RT-PCR products of β-actin were used to normalize the amount of HIV-1 cDNA. As expected from the induction of the HIV-1 LTR transcriptional activity, increased amounts of total HIV-1 RNA were observed in cells continuously treated with TNF (Fig. 2B; compare lanes 3 and 5 with lane 1). Total HIV-1 RNA amounts observed during the chase period were clearly lower than those seen in 8-h TNF-treated control cells. They were significantly higher than HIV-1 RNA levels in cells exposed for 2 h to TNF, as expected from the ongoing accumulation of viral RNAs from the residual LTR transcriptional activity.

Inhibition of constitutive NF-κB DNA-binding activity in chronically infected U937 cells is sufficient to reduce levels of steady-state HIV-1 RNA. We established previously that chronic HIV-1 replication in the monocytic cell line U937 induces permanent nuclear translocation of NF-κB heterodimers (p50/p65) (5). Here, we determined whether inhibition of NF-κB DNA-binding activity could have functional effects on endogenous HIV-1 transcriptional activity, despite the production of the HIV-1 Tat protein, the transactivating function of which is clearly detected in these infected cultures (5). Signal-induced IκBα degradation, which leads to NF-κB activation, has been shown to be mediated by the proteolytic activity of the proteasome (13, 46, 55, 63). To assess whether this HIV-induced activation of NF-κB was ultimately mediated by the proteasome through degradation or processing of NF-κB inhibitors, we treated HIV-1-infected U937 cells continuously with the peptide aldehyde Z-LLL-H, which specifically blocks the proteolytic activity of the proteasome (46). Control, uninfected U937 cells were treated for 2 h with increasing amounts of the peptide aldehyde and then stimulated with TNF for 10 min prior to cell extraction (Fig. 3A, lanes 8 to 10). Treatment of uninfected U937 cells with either 1 or 10 μM Z-LLL-H reduced TNF-induced nuclear NF-κB complexes since the residual activity observed was 57 or 62.2%, respectively (lanes 9 and 10), of that detected in untreated cells (lane 8). In agreement with previous reports (7, 10, 30, 40, 60), we observed that TNF-induced NF-κB DNA-binding activity paralleled the rapid degradation of IκBα and that pretreatment with compound Z-LLL-H blocked the TNF-induced deg-

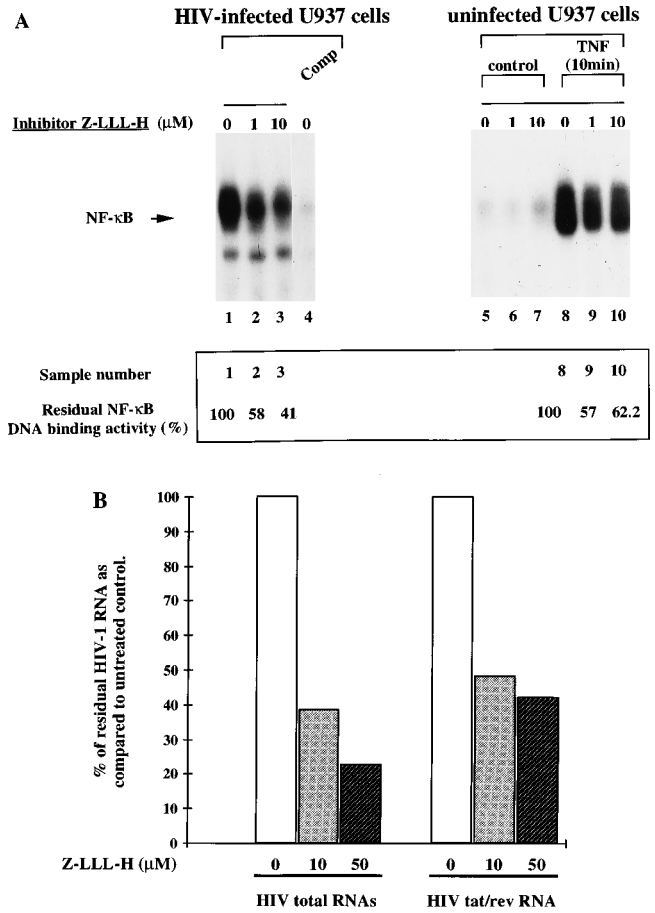

FIG. 3. Inhibition of permanent NF-κB activation by the peptide aldehyde Z-LLL-H paralleled the diminution of endogenous HIV-1 proviral transcription. Infected U937 cells and uninfected controls were treated for 2 h with increasing amounts of Z-LLL-H, and the uninfected cells were exposed to TNF for the last 10 min. Cells were then processed to obtain nuclear extracts and total RNA samples. (A) EMSA using extracts from infected cells (>30 days after infection) (lanes 1 to 4) and uninfected control cells (lanes 5 to 10) with a [γ-^{32}P]ATP-labeled κB oligonucleotide. Identically prepared nuclear extracts from unstimulated cells treated with Z-LLL-H (lanes 5 to 7) were used to control the TNF-induced NF-κB DNA-binding activity (lanes 8 to 10). Competition (Comp) was performed by adding a 40-fold molar excess of the unlabeled oligonucleotide to each sample prior to addition of the labeled probe (lane 4). The residual NF-κB DNA-binding activity was determined by densitometric analysis of the autoradiogram obtained following overnight exposure, and values are given at the bottom. (B) Analysis of the amounts of total and *tat/rev* HIV-1 RNA species by RT-PCR from infected cells treated for 2 h with various concentrations of Z-LLL-H. Equivalent amounts of RNA were subjected to primer extension and subsequent PCR analysis with HIV-1- and β-actin-specific primers. Total and *tat/rev* HIV-1 RNA hybridization signals were recorded with a digital imaging system. The densitometric values were corrected relative to the β-actin standards and expressed as a percentage of the residual HIV-1 transcription level in untreated cells. These percentages of residual activity were plotted versus the various concentrations of Z-LLL-H. The data shown are representative of five separate similar experiments.

radation of IκBα and stabilized phosphorylated forms of the protein (data not shown).

The same treatment was applied to HIV-1-infected cells. Following a 2-h exposure of cells to Z-LLL-H, an inhibition of NF-κB DNA-binding activity was observed. Indeed, infected cells treated with either 1 or 10 μM Z-LLL-H showed 58 or 41%, respectively (Fig. 3A, lanes 3 and 4), of the amount of NF-κB DNA-binding activity detected in untreated cells (lane 1). We next analyzed HIV-1 RNA steady-state levels in Z-LLL-H-treated U937-infected cells by the RT-PCR method described above (Fig. 3B). Figure 3B shows residual HIV-1 total and *tat/rev* transcription in Z-LLL-H-treated infected cells as a percentage of the level in untreated controls cells. Levels of both HIV-1 total RNA and HIV-1 *tat/rev* RNA were reduced by the proteasome inhibitor, since levels of residual viral transcription were, respectively, 38.6 and 48% (10 μM Z-LLL-H) and 23 and 42% (50 μM Z-LLL-H) of the control levels.

These results show that the extent of NF-κB DNA-binding inhibition in Z-LLL-H-treated HIV-1-infected cells paralleled the decrease of integrated provirus transcription, which suggests that maintenance of high-level HIV-1 proviral transcription in U937 monocytic cells depends on the nuclear expression of NF-κB. Furthermore, the down-regulation of NF-κB DNA-binding activity in a Tat-expressing cell environment is sufficient to cause a drastic diminution of transcriptional activity of the integrated HIV-1 provirus.

The NF-κB response element in the viral enhancer is critical for the establishment of efficient HIV-1 replication in U937 cells. To analyze directly the role of viral enhancer κB sequences in the establishment of HIV-1 replication, we monitored the fate of κB-Mut virus in U937 cells (Fig. 4A). wt and κB-Mut HIV-1 constructs have been described previously (28) and differ only in a 5-bp substitution (AAGGG to TTTAA) of the NF-κB response elements in both the 5′ and 3′ LTRs. Kinetics of viral replication in U937 cells were monitored by detection of p24^{gag} production. After the onset of wt virus infection, levels of p24^{gag} production were similar to those observed in CEM cells infected with either wt or κB-Mut virus (data not shown). In clear contrast, κB-Mut viral replication was impaired in U937 cells, with 10^4-fold less virus production than in U937 cells infected with wt virus. To rule out the possibility that different infectivity potentials between the two viral preparations could account for their different replicative patterns, the amount of HIV-1 proviral DNA synthesized 2 h after virus-cell contact was examined by PCR using a primer pair specific for the R/U5 region, which should detect virtually all HIV-1 DNA synthesized (Fig. 4B). An oligonucleotide pair specific for the human β-actin gene was used to estimate the amount of cellular DNA in each sample. Amounts of HIV-1 and β-actin DNAs in experimental samples were estimated by comparison with standards generated with known amounts of either cloned HIV-1_{LAI} DNA or U937 cellular DNA. DNA levels were recorded with a digital imaging system, and values were estimated to be 30 and 11 proviral copies for cells infected with the wt and κB-Mut viruses, respectively. The difference between DNA levels is less than the difference in the intensity of viral replication shown in Fig. 4A.

We tested whether the basal transcriptional capacity of both proviral constructs could have accounted for the replicative defect seen with the κB-Mut virus (Fig. 5). This was assessed by transient transfection of U937 cells with the wt or κB-Mut proviral construct in order to analyze basal LTR transcriptional activity independently of viral entry and proviral integration. Total HIV-1 RNA was extracted 20 h after transfection and analyzed by RT-PCR. Total HIV RNA signals, visualized by direct autoradiography of the dried gel, were recorded, and densitometric values (in arbitrary units) were normalized to values for the β-actin standard and plasmid DNA entry (estimated 30 min after transfection by a modified Hirt technique). The κB-Mut provirus was not less transcriptionally active in the U937 cell environment than its wt counterpart (26,383 and 8,943 arbitrary units of HIV-1 RNA synthesized, respectively). Up-regulation of HIV-1 RNA synthesis was observed when wt- but not κB-Mut-transfected cells were exposed to TNF for 4 h, as expected for a provirus lacking the

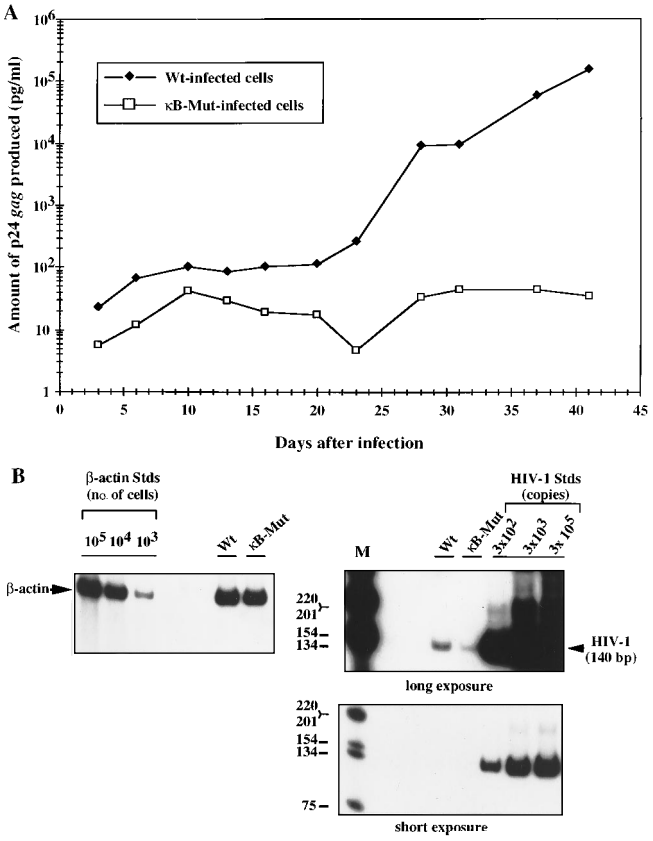

FIG. 4. Establishment of HIV-1 replication in U937 cells is dependent on κB-responsive elements. U937 cells were infected with viral supernatants produced after 50 days of culture of either wt or κB-Mut proviral DNA-electroporated CEM cells. The data shown are representative of three similar studies. (A) Viral replication was determined at the indicated time intervals by the detection of $p24^{gag}$ antigen in the culture medium, and values were plotted on a semilogarithmic scale versus time after infection. (B) Analysis of viral and cellular DNA by PCR performed on DNA samples corresponding to 10^5 cell equivalents. Samples were taken from a 2-h-infected U937 cell culture. Standard amounts of HIV-1_{LAI} proviral DNA (HIV-1 Stds [number of copies]) and U937 cell DNA (β-actin Stds [number of cells]) were amplified in parallel. Assessment of HIV-1 standards after a shorter exposure of the dried gel is shown in the lower panel. Lane M, size marker (positions are indicated in base pairs).

TNF-responsive κB elements. In conclusion, the failure of κB-Mut provirus to achieve productive replication in U937 cells seems not to be a consequence of impaired transcriptional activity or a lower input of infectious particles. Together, these results underlined the critical requirement of the enhancer κB motifs for full replication in monocytic U937 cells.

The proteasome-mediated regulation of NF-κB affects specifically κB-dependent HIV-1 transcription in U937 cells. To examine the specificity of the proteasome inhibitor on NF-κB activation in U937 cells, we measured its effects on the fate of steady-state HIV-1 RNA levels in wt- and κB-Mut-infected cells (Fig. 6). Both cell populations were treated for 2 h with various amounts of the compound Z-LLL-H before harvesting of RNA. When we analyzed HIV-1 RNAs by a classic RT-PCR experimental approach in κB-Mut-infected U937 cells, a very weak amplification was observed. This finding was in agreement with the undetectable viral replication observed in these infected cells as measured by $p24^{gag}$ antigen production. To overcome this problem, we used a more sensitive nested PCR technique on the reverse-transcribed HIV-1 RNA. RT-PCR products of β-actin were used to normalize the amount of HIV-1 cDNA used as the template for the PCR. As shown in Fig. 6B, a dose-dependent inhibition of total HIV-1 RNA levels was observed in extracts from wt-infected-cells treated with Z-LLL-H. The total HIV-1 RNA signals visualized by direct autoradiography of dried gels (Fig. 6A and B) were quantified. Densitometric values were expressed as percentages of the value for HIV-1 residual transcription in corresponding untreated controls (Fig. 6C and D). In Z-LLL-H-treated cells, residual HIV-1 transcription represented 30% of the level in untreated cells (Fig. 6D). At this concentration of the inhibitor (50 μM), we observed no effect on the amount of total HIV-1 RNA accumulated in the κB-Mut-infected U937 population (Fig. 6A and C). The absence of effects of the proteasome inhibitor Z-LLL-H on the steady-state levels of total HIV-1 RNA produced by the κB-Mut virus suggests that enhancer-dependent transcriptional activity of the HIV-1 LTR was mainly, if not solely, affected by this inhibitor. Furthermore, Z-LLL-H treatment had no effect on the transcriptional regulation of a housekeeping gene, encoding β-actin, used as an internal control in our experiments. This compound did not affect the levels of the Sp1 protein as measured by Western blot analysis; the levels of p53 protein were also unaffected (data not shown). In conclusion, these observations and the fact that the proteasome inhibitor Z-LLL-H impaired specifically enhancer-dependent transcription of HIV-1 provirus provides further support for our hypothesis that permanent enhancer occupancy is needed for persistent HIV-1 replication in monocytic cells.

DISCUSSION

We originally reported that NF-κB activation occurs upon chronic active HIV-1 replication in the U937 cell line, a monocytic cell system which expresses very little, if any, nuclear p50/p65 heterodimers (5). This intriguing phenomenon has since been confirmed studies of both U937 cells (47) and monocyte-derived normal blood macrophages (38). Since the

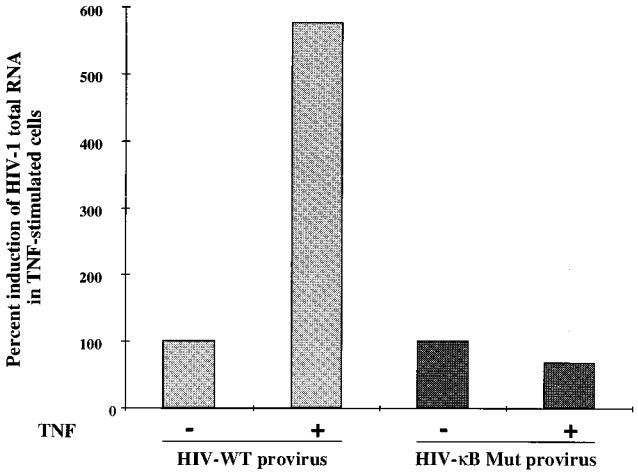

FIG. 5. Analysis of wt or κB-Mut proviral transcription in transiently transfected U937 cells. Twenty hours after transfection, half of the cultures were treated with TNF for the last 4 h prior to harvesting of RNA. Total RNA from 10^6 electroporated cells was subjected to RT-PCR analysis using primers specific for HIV-1 and β-actin. For each proviral construct, corrected densitometric values of HIV-1 RNA signals in TNF-stimulated cells were expressed as percentages of the induction of total HIV-1 RNA into unstimulated control cells. Corrected densitometric values were 8,943, 51,632, 26,383, and 17,848 for HIV wt, HIV wt plus TNF, HIV κB-Mut, and HIV κB-Mut plus TNF, respectively. The data shown are the means of duplicate samples in a single experiment and are representative of three independent studies.

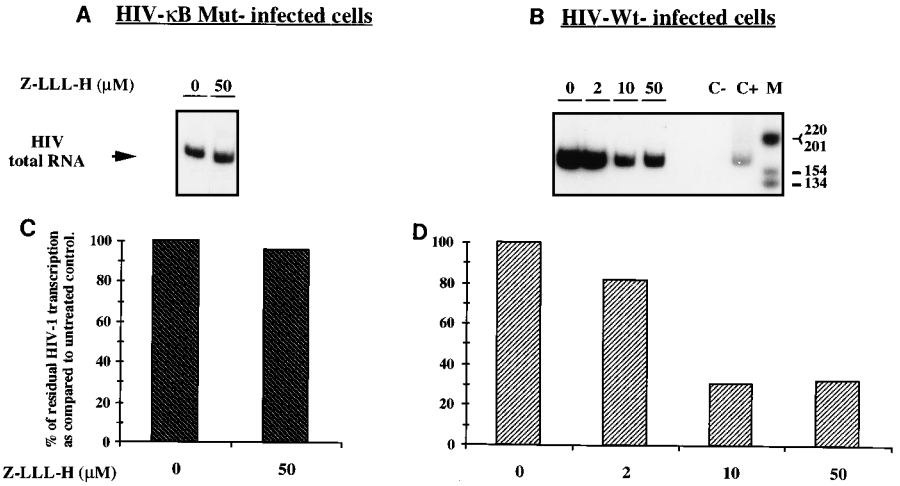

FIG. 6. The peptide aldehyde Z-LLL-H inhibits specifically NF-κB-dependent HIV-1-transcription. U937 cells were infected with virus derived from proviral DNA-transfected Cos-7 cells. κB-Mut- and wt-infected U937 cells (26 days after infection) were treated for 2 h with increasing amounts of the inhibitor Z-LLL-H. (A) RNA (10^5 cell equivalents) extracted from these cells was subjected to primer extension and subsequent PCR with β-actin-specific primers as described above. RT-PCR products of β-actin were used to normalized the amount of HIV-1 cDNA. Nested PCR was performed for the detection of HIV-1 RNAs. PCRs with uninfected cells and HIV-1_{LAI}-infected cells (5×10^2 cell equivalents) served as negative (C−) and positive (C+) controls, respectively. (B) For each set of infected cells, total HIV-1 RNA densitometric values were expressed as percentages of the residual HIV-1 transcription in untreated cells. These percentages were plotted against the various concentrations of the inhibitor Z-LLL-H.

activities of HIV-1 enhancer-driven expression vectors, transiently transfected into chronically infected U937 cells, were found to be constitutively increased as a result of chronic infection, it was possible to postulate a role for NF-κB activation in the indefinite viral transcription and replication observed in this cell type. Alternatively, however, it could be reasoned that HIV Tat protein, produced in a functional way in chronically HIV-1_{LAI}-infected U937 cells, is sufficient to perpetuate transcription of the integrated provirus, which would be merely initiated by NF-κB. Indeed, addition of exogenous HIV Tat protein to U1 cells, a latently infected subclone of U937 cells, induces HIV-1 replication (1, 11, 17). In transient transfection assays, HIV Tat expression transactivates LTR-driven vectors independently of NF-κB DNA-binding elements. However, numerous reports suggested that Tat, in addition to its capacity to elongate viral RNA through an interaction with the Tat-responsive element sequence, must somehow interact with multiprotein complexes associated with the HIV enhancer region and the primary initiation domain to exert its full transactivating effects on the viral LTR (9, 28, 36, 37, 48, 61). Moreover, recent work from our laboratory shows that NF-κB-independent HIV Tat transactivation occurs in transformed lymphoblastoid T-cell lines but not in resting CD4 T lymphocytes purified from the peripheral blood (2). These notions prompted us to revisit the role of NF-κB activation in the long-term control of HIV-1 provirus transcription in cells of the monocytic lineage. In the U1 cell system, it is well known that TNF stimulation activates NF-κB and increases the very low transcription levels of the two integrated provirus copies observed in unstimulated cells (18, 24, 50, 51). Phorbol myristate acetate stimulation of U1 cells, acting through an autocrine secretion of TNF, results in a clear increase in dimethylsulfate sensitivity of a guanine in the downstream enhancer repeat in LTR footprinting experiments that likely is due to the occupancy of the enhancer region (15). Indeed, a correlation between nuclear NF-κB-binding activity and increased transcription was reported (15, 51). From these experiments, however, it cannot be excluded that TNF or phorbol myristate acetate induces, in addition to NF-κB, other molecular events which can participate in the control of HIV-1 genome transcription. We have therefore used a method which allows specific termination of nuclear NF-κB function. We recently reported (3) that TNF stimulation, when used as a transient pulse followed by washing of cells, first induces a nuclear translocation of p50/p65 NF-κB heterodimers and, through the latter, the rapid de novo NF-κB-dependent activation of IκBα gene transcription. Newly transcribed IκBα protein translocates into nucleus, where it binds to NF-κB heterodimers and terminates their function in a highly specific manner (3). This does not happens if TNF is maintained in the culture medium, since permanent degradation of IκBα, including the newly synthesized molecules, occurs. Using this approach, we have now compared the effects on NF-κB translocation and endogenous HIV-1 provirus transcription of prolonged (8-h) or pulsed (for 2 h, followed by 6 h in normal culture medium) stimulation of U1 cells with recombinant TNF. Whereas continuous TNF stimulation induced, as expected, a steady increase in nuclear NF-κB expression and HIV-1 transcription, a 2-h TNF pulse resulted in early activation of both NF-κB DNA binding and viral transcription, followed after a 6-h chase by the disappearance of nuclear NF-κB DNA-binding activity. This phenomenon was accompanied by a brisk decrease of viral transcription to levels similar to those observed in 2-h TNF-stimulated U1 cells. This result indicated that TNF-induced early NF-κB DNA binding to the HIV-1 enhancer is sufficient to launch HIV genome transcription but is, in addition, needed for persistent transcription. This finding provided a first indication that ongoing transcription of integrated HIV-1 provirus depends strictly on actual occupancy of enhancer motifs by NF-κB heterodimers. This interpretation is in agreement with the results of superinfection experiments (12) and in keeping with the notion that the lack of HIV-induced NF-κB activity may be one mechanism which restricts viral replication in U1 cells (12, 52).

Whereas the U1 cell system allowed us to test the role of p50/p65 heterodimers in early steps of HIV-1 reactivation, the chronically HIV-infected U937 cell model offered the opportunity to test the role of enhancer occupancy by NF-κB het-

erodimers in the intense transcriptional activity of integrated HIV-1 provirus in a fully permissive cell environment producing Tat protein on a permanent basis. Partial blockade of this permanent NF-κB translocation induced by viral replication itself in this model system was obtained by inhibition of the proteolytic activity of the proteasome. As early as 2 h after addition of the proteasome inhibitor Z-LLL-H, a dose-dependent inhibition of both nuclear binding activity and endogenous HIV transcription was observed. This finding is a clear indication that viral replication activates NF-κB in U937 cells through a proteasome-dependent pathway and that the HIV-induced, constitutive NF-κB activation observed in the U937 cell line plays a major role in perpetuating HIV-1 genome transcription. It should be stressed that no significant cell toxicity was induced by a 2-h Z-LLL-H treatment of U937 cells, whether infected or not, and that the levels of Sp1 and p53 proteins were not modified by such treatment (results not shown). Eliminating a role for p53 modifications in Z-LLL-H-treated cells was indeed important, since this molecule is also degraded in a ubiquitin proteasome-dependent manner in the presence of the human papillomavirus type 16 (42).

The respective roles of NF-κB and HIV-Tat in HIV-1 transcription in chronically infected U937 cells are not entirely understood. TNF stimulation of U937 cells was shown to induce the replication of Tat-defective virus to near wt levels in transient transfection assays and also to overcome the block of wt HIV replication induced by an inhibitor of Tat (37). We reported earlier that the activities of LTR-driven expression vectors deleted of NF-κB sequences and transiently transfected into chronically infected U937 cells are diminished 20- to 30-fold but are not abolished. It is thus likely that NF-κB and Tat, bound to their respective DNA response elements on the LTR and the nascent transcripts of the integrated HIV provirus, collaborate as part of a large transcriptional protein complex to maintain the high-level viral expression observed in U937 cells. Our present work suggests that NF-κB heterodimers represent a keystone in the building of such a multiprotein complex.

Our final evidence for the critical role of NF-κB in maintaining HIV-1 transcriptional activity was sought by infecting U937 cells with an HIV provirus carrying specific mutations in the 3′ and 5′ LTRs which abolish response to TNF and NF-κB DNA-binding activity. The profound transcriptional defect shown by this construct confirmed that the HIV-1 enhancer must bind NF-κB heterodimers for active viral replication to occur in U937 cells. Inhibition of proteasome activity suppressed the transcription of the wt provirus but not that of its mutated counterpart, providing further evidence for the specificity of the inhibitor used. Our results are consistent with a report showing that a κB-defective simian immunodeficiency virus construct is unable to replicate in primary macrophages (8). Thus, a strategy of self-perpetuation of proviral genome transcription through NF-κB activation appears to be used by HIV-1 in the monocytic lineage. The ability of HIV-1 to induce permissiveness to its own replication in cells of the monocytic lineage may underlie the indefinite and intense production of viral particles observed in infected patients.

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