Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 7826–7830, August 1992
Medical Sciences

NF-κB-dependent induction of the NF-κB p50 subunit gene promoter underlies self-perpetuation of human immunodeficiency virus transcription in monocytic cells

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Communicated by François Jacob, May 20, 1992

ABSTRACT **The molecular mechanisms underlying the sustained nuclear translocation of NF-κB observed in U937 monocytic cells chronically infected with human immunodeficiency virus (HIV) were studied. The activity of the promoter regulating the synthesis of the p105 precursor of the NF-κB p50 subunit was enhanced in these cells. Deletions in this promoter indicated that this upregulation was mediated through the NF-κB- but not the AP-1-binding motif, by bona fide p50/p65 heterodimers. Analysis of cytosolic extracts indicated that NF-κB levels were increased in HIV-infected cells. In contrast to the transient NF-κB activation induced by phorbol ester, the permanent NF-κB translocation induced by HIV infection was not dependent on PKC isoenzymes α and β as shown by the use of a specific inhibitor (GF 109203X). These observations indicate that during chronic HIV infection of U937 cells, continuous NF-κB (p50/p65) translocation results in p105 promoter upregulation with subsequent cytosolic NF-κB accumulation, ready for further translocation. This HIV-mediated mechanism results in a self-perpetuating loop of NF-κB production.**

A major mechanism of human immunodeficiency virus (HIV) long terminal repeat (LTR) transactivation resulting in viral transcription is mediated by nuclear factor κB (NF-κB), which binds to the two κB motifs constituting the LTR enhancer region (1). NF-κB is a heterodimer composed of 50-kDa (p50) and 65-kDa (p65) subunits (2–4), for which cDNAs have been cloned (5–9). In the majority of cells studied, NF-κB is present in an inactive form in the cytoplasm, bound through the p65 subunit to the inhibitory protein IκB (3, 4, 8, 10–12) and its phosphorylation by kinases prevents this inhibitory binding, allowing NF-κB to translocate to the nucleus (13, 14). The deduced amino acid sequences of the p50 and p65 subunits show that they belong to the same multigene family as the c-*rel* protooncogene product (5, 6, 8, 9). Members of this family associate into multiple homo- or heterodimeric forms that can bind specific decameric DNA sequences (κB motifs) in the regulatory region of multiple genes. The functional activity of a κB motif may depend on both the exact sequence of the motif and the protein subunits involved (2–4, 15–19).

Chronic HIV infection of the monocytic cell line U937 induces nuclear translocation of an HIV enhancer–binding protein complex with functional properties compatible with that of NF-κB (20), thus adding HIV infection itself to the list of events that can induce HIV LTR transactivation, implying that HIV genome transcription may be self-perpetuated in monocytes. The present investigation was aimed at determining, at the molecular level, how U937 cells sustain such an intense and persistent NF-κB translocation as a result of chronic HIV replication. For this to occur, some mechanism of permanent refeeding of the translocable cytoplasmic pool of NF-κB complex must be postulated. Maintenance of NF-κB activity is dependent on the continuous presence of stimuli such as tumor necrosis factor (TNF) or phorbol 12-myristate 13-acetate (PMA) (21). Further, these stimuli can increase the level of mRNA encoding the p105 precursor of the p50 subunit, suggesting that regulation of the p105 gene is NF-κB-dependent (7, 22). Cloning and functional analysis of the cellular promoter regulating the p105 gene showed that it contained binding motifs for the transcription factors AP-1 and NF-κB (23). We have now tested the hypothesis that during chronic HIV infection, continuous nuclear translocation of NF-κB leads to permanent upregulation of p105 gene transcription, which in turn results in cytoplasmic accumulation of the p50 protein in association with p65 and IκB, as a complex ready for further nuclear translocation. Predictions from this hypothesis would be that (*i*) bona fide NF-κB (p50/p65 heterodimer) is translocated into the nucleus of HIV-infected cells from a permanently increased pool of cytoplasmic NF-κB; (*ii*) activity of the p105 promoter is constitutively enhanced in HIV-infected cells, through the NF-κB- and not the AP-1-binding DNA motif, and that it is not mediated by the HIV Tat protein; and (*iii*) intracellular signaling transduction pathways leading to persistent NF-κB translocation are different from those used by protein kinase C (PKC)-activating stimuli such as PMA, which are known to induce a brisk, but not sustained, NF-κB translocation (21). Our observations in chronically HIV-infected U937 cells show that these requirements are met.

MATERIALS AND METHODS

Reagents, Antibodies, and Cells. PKC inhibitor GF 109203X (24) was provided by J. Kirilovsky (Glaxo). Antisera to p50 (5) and against the C terminus of c-Rel (18) were kindly donated by A. Israel (Institut Pasteur, Paris) and I. Verma (Salk Institute), respectively. U937 cells routinely used in our laboratory have been described (20). K562 human erythroleukemia cells were obtained from the ATCC. Cells were periodically tested for mycoplasma and maintained in RPMI 1640 with glutamine, antibiotics, and 5% fetal bovine serum. They were passed twice a week and seeded at 2.5×10^5 cells per ml, at $<3 \times 10^7$ cells per 150-cm^2 flask.

Abbreviations: HIV, human immunodeficiency virus; LTR, long terminal repeat; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; TNF, tumor necrosis factor; rlu, relative light units.

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Expression and Purification of p50 and IκB. DNA coding for the DNA-binding domain of NF-κB p105 (amino acids 38–381) in the sequence of KBF1 (5) was isolated by PCR and inserted into the pGEX-2T expression plasmid (25). Bacterially expressed fusion protein was isolated through double-stranded DNA affinity chromatography on glutathione-agarose and cleaved with thrombin, and the DNA-binding domain of p50 was isolated by affinity chromatography. To isolate DNA coding for IκB, primers based on the sequence of Haskill *et al.* (26) were used for PCR amplification of sequences present in reverse-transcribed HeLa cell mRNA, which were then inserted into pGEX-2T as described above. Expressed fusion protein was isolated by affinity chromatography on glutathione-agarose and IκB was released by thrombin treatment (J. R. Mathews and R.T.H., unpublished work).

HIV Infection of U937 Cells. Infection with mycoplasma-negative LAV-1 Bru strain of HIV was performed as described (20). Reverse transcriptase activity in the supernatant of uninfected and infected U937 cells was tested twice a week at the time of cell passage, and p24 antigen in culture supernatant was measured every other cell passage. Over 80% of the infected cells have been shown to produce HIV *gag* transcripts as determined by *in situ* hybridization (20). Cells were used between days 21 and 120 postinfection. The degree of viral replication, as measured by reverse transcriptase activity and p24 antigen, was essentially constant during this time period. Three different chronic HIV infections of U937 cells were used for these experiments, and cells were used at different times after the onset of infections in each chronically infected culture. TNF and interleukin 1 were not detected in the supernatant of HIV-infected U937 cells as measured by bioassay and enzyme immunoassay.

Gel Mobility-Shift Assays. Nuclear extracts were obtained as described (20). Cells (5×10^6) were seeded at 2.5×10^6 per ml on the day of cell passage and harvested 16 hr later in the presence or absence of stimuli. Cytosolic extracts were prepared by lysing the cells for 6 min at 4°C with 10 mM Hepes, pH 8/10 mM KCl/0.5 M sucrose/1 mM EDTA/0.5 mM spermidine/0.15 mM spermine/0.3% Nonidet P-40/7 mM 2-mercaptoethanol containing phenylmethanesulfonyl fluoride, leupeptin, pepstatin, and aprotinin. Cell lysates were microcentrifuged at 6500 rpm at 4°C and the supernatant was kept at −80°C until tested. Nuclear extract was prepared from the pellet. For mobility-shift assays, 5–7 μg of protein extract was incubated with [γ-^{32}P]ATP-labeled double-stranded synthetic oligodeoxynucleotide probe at 22°C for 15 min in 10 μl of buffer as described (20). The binding reaction was analyzed by electrophoresis in nondenaturing 6% polyacrylamide gels. DNA-binding competition was assessed by preincubating the extract with a 40-fold excess of unlabeled oligonucleotide. In cytosolic extract, sodium deoxycholate was added (0.8%, wt/vol) and incubated at room temperature for 10 min. When indicated, 0.5–3 μl of rabbit antiserum or 15 ng of rIκB was added to the standard reaction mixture 10 min before addition of the radiolabeled probe. The oligonucleotide used in the binding reactions corresponded to the wild-type NF-κB-binding sequence, which constitutes the enhancer of the HIV LTR (5′-ACAAGGGACTTTCCGC-TGGGGACTTTCCAGGGA-3′). The double-stranded probe was labeled with [α-^{32}P]dCTP by Klenow DNA polymerase.

Plasmid Constructs. Plasmid p105-luc consisted of a 1.2-kilobase *Hin*dIII–*Nco* I fragment of the p105 genomic clone, cloned into the *Hin*dIII site of the pc-luc plasmid, upstream of the luciferase reporter gene (23, 27). The p105dkB-luc and p105dAP1-luc vectors represent the p105-luc plasmid with the κB or AP-1 motif deleted, respectively. The HIV LTR wild-type plasmid contained nucleotides −644 (*Xho* I) to +78 (*Hin*dIII) of the HIV LTR (LAV1 Bru strain), cloned into the *Hin*dIII site of the pc-luc plasmid, and was a gift from A. Rabson (National Institutes of Health, Bethesda, MD). The expression vector for the Tax protein of human T-cell lymphotropic virus type I was a gift of M. Brahic (Institut Pasteur, Paris) and contained the Tax cDNA cloned downstream of the cytomegalovirus promoter in the Rc-CMV vector. All plasmids were purified using Qiagen columns (Diagen, Dusseldorf, F.R.G.). Purity, size, and restriction maps were verified by agarose gel electrophoresis.

Transfections. Cells were seeded at 5×10^5 per ml the same day as the routine biweekly cell passage and were transfected 16 hr later by a modified DEAE-dextran technique (28, 29). In brief, 5×10^6 cells were transfected with 2.5 μg of plasmid. In experiments where two plasmids were transfected [the target reporter gene and the effector or control (pc-luc) gene], 2.5 μg of each plasmid was used. Cells were then suspended in 5 ml of RPMI-1640/5% fetal bovine serum in 25-cm^2 flasks, or further aliquoted into 24-well plates at 2 ml per well, and incubated at 37°C for 4 hr. At that time, the various reagents were added. Cells were harvested 36 hr later (29) and luciferase activity was measured in a luminometer (Berthold). The background obtained from the lysis buffer was subtracted from each experimental value. Total protein in an aliquot of each sample was measured by the Bradford technique (Bio-Rad) and the results were calculated as [relative light units (rlu) − background]/mg of protein. Comparison between results of transfection of the same reporter gene into uninfected or HIV-infected cells and the Hirt technique for DNA precipitation were done as described (20). The basal activity of p105dkB-luc and p105dAP1-luc vectors was above background in all experiments.

RESULTS

Activity of the p105 Promoter Is Enhanced in HIV-Infected U937 Cells, Through the NF-κB-Binding Motif of the Promoter, and Is Unresponsive to HIV Tat. To determine whether NF-κB activation by HIV was due to an induction of p105 gene transcription, we performed Northern blot analysis and transient expression assays with various p105 promoter constructs in uninfected and HIV-infected U937 monocytes. Northern blot analysis demonstrated a 2- to 3.5-fold increase in steady-state levels of p105 mRNA in HIV-infected U937 cells as compared to uninfected cells. Hybridization was performed in parallel with glyceraldehyde-3-phosphate dehydrogenase cDNA to control for RNA quantity (data not shown). HIV infection resulted in a 4-fold amplification of the basal activity of the p105 promoter (p105-luc) (Fig. 1). As a positive control, the enhancer region of HIV LTR cloned upstream of the thymidine kinase promoter (3ENH-TKluc) was used, resulting in a similar upregulation in HIV-infected cells, as described (20).

Induction of the p105 promoter activity by HIV infection was dependent on the NF-κB-binding motif present in this promoter, since deletion of this sequence from the transfected p105 promoter plasmid (p105dkB-luc) completely abolished induction in HIV-infected cells. This observation was confirmed when a mutation (CTC instead of GGG) that specifically abolishes NF-κB binding (1) was introduced in an HIV enhancer/thymidine kinase promoter/luciferase vector (data not shown). By contrast, HIV-mediated induction of the p105 promoter was independent of the AP-1-like motif present in this promoter, since deletion of this sequence (p105dAP1-luc) did not change the level of induction.

Since the HIV-encoded Tat protein is known to transactivate the HIV promoter, we tested whether it could mediate the increased activity of the p105 promoter in chronically HIV-infected U937 cells, previously reported to show intense Tat activity (20). Cotransfection experiments were performed in uninfected K562 cells, with the intact p105 promoter and an expression plasmid encoding Tat. K562 cells

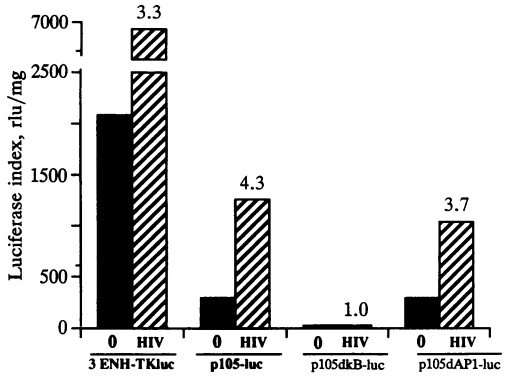

FIG. 1. Activation of p105 promoter activity by HIV. Uninfected (0) or HIV-infected (HIV) U937 cells were transfected with the indicated reporter plasmid. Numbers above the bars indicate fold-stimulation in HIV-infected cells relative to the activity of the same plasmid transfected in uninfected cells.

were used in this experiment because they display a higher basal p105 promoter activity higher than U937 cells. Tat was unable to induce activation of the p105 promoter, whereas it was capable to transactivate the HIV LTR (Fig. 2). Similar data were obtained in uninfected U937 cells. As a positive control we used an expression plasmid encoding the Tax protein of human T-cell lymphotropic virus type 1, which greatly increased the activity of the p105 promoter, again through the κB motif.

NF-κB Protein Complex Translocated into the Nucleus of Chronically Infected U937 Cells Is Made of p50/p65 Heterodimers. Because the p50/p65 heterodimer can transactivate the p105 promoter through the κB motif (23), we wanted to determine the exact molecular composition of the HIV-induced NF-κB complex. Cells infected with HIV showed induction of NF-κB (Fig. 3, lane 2). Complex formation was inhibited by competition with a 40-fold excess of unlabeled oligonucleotide (lane 3). Preincubation of nuclear extracts with an antiserum raised against the p50 subunit, which also recognizes NF-κB (anti-NF-κB; ref. 5), decreased specific DNA-binding activity (lane 4). By contrast, antibodies raised against the C-terminal region of c-Rel, which have been previously shown to upshift the c-Rel complex (18), did not modify the migration of the NF-κB complex induced by HIV (lane 5). This lack of effect was also observed when preimmune serum was used (data not shown). The binding activity induced by HIV infection was blocked when the nuclear

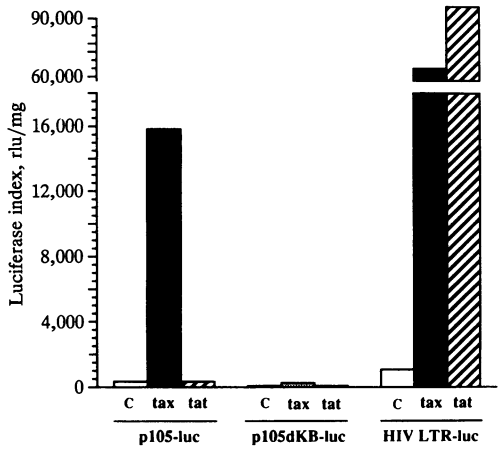

FIG. 2. Effect of Tat on the activation of the p105 promoter. Uninfected U937 cells were transfected with the indicated reporter plasmid alone (control, C) or cotransfected with the reporter plasmid and an expression vector encoding Tat or Tax.

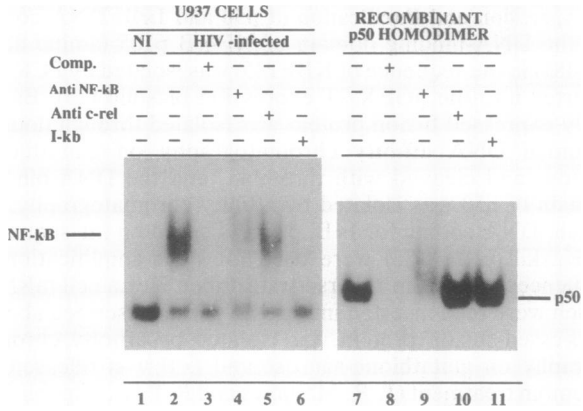

FIG. 3. Gel shift assay using nuclear extracts from uninfected (NI) or HIV-infected (HIV) U937 cells (lanes 1–6) or recombinant p50 (lanes 7–11). Positions of the NF-κB complex and the p50 homodimer are indicated. Comp., competition with 40-fold excess of unlabeled oligonucleotide.

extract was incubated with recombinant IκB (lane 6). No difference in DNA-binding activity was found when nuclear extracts of uninfected or HIV-infected cells were incubated with an oligonucleotide encompassing the AP-1 motif (data not shown). Purified recombinant p50 homodimers were electrophoresed in parallel to confirm the molecular forms defined by the use of antiserum and IκB (lanes 7–11). The recombinant p50 is a truncated form (amino acids 38–381) and thus has an increased electrophoretic mobility. Competition with a 40-fold excess of unlabeled oligonucleotide (lane 8) eliminated DNA binding, and anti-p50 (lane 9) but not anti-c-Rel (lane 10) antiserum upshifted the p50/DNA complex. As expected, addition of recombinant IκB to the reaction mixture did not modify the binding of recombinant p50 (lane 11).

Accumulation of NF-κB in the Cytosol of HIV-1-Infected U937 Cells. Gel shift assays of cytosolic extracts treated with deoxycholate, which dissociates the NF-κB complex from IκB (13), showed that NF-κB was present in both the nuclear and the cytoplasmic compartment of HIV-infected cells (Fig. 4, lanes 4 and 13), as compared to uninfected cells in which NF-κB was only detected in the cytosol (lanes 1 and 7). Three-hour PMA stimulation of uninfected cells resulted in nuclear translocation of NF-κB but was associated with a decreased amount of cytoplasmic NF-κB (lanes 2 and 10). These results were confirmed in four different experiments using the same quantity of cytosolic proteins. These findings indicate that in HIV-infected U937 cells, NF-κB is increased in both the cytoplasmic and the nuclear compartment.

Increased Transcription of the p105 Promoter and Nuclear Translocation of NF-κB in HIV-Infected Cells Are Not Mediated Through PKC-Dependent Cellular Transduction Pathways. Pretreatment with the highly selective PKC inhibitor GF 109203X blocked nuclear translocation of NF-κB 16 hr after PMA stimulation of uninfected cells (Fig. 5, lanes 2 and 4) but did not block the translocation induced by HIV infection during 16 hr (lanes 5 and 7), or even 72 hr. These observations indicate that HIV infection induces NF-κB activity through an α, β, and γ PKC isoenzymes-independent pathway and that PMA induces translocation of NF-κB through these PKC isoenzymes.

Uninfected and HIV-infected cells previously transfected with the p105 promoter construct were treated with the PKC inhibitor or left untreated and then were stimulated with PMA or left unstimulated. PMA-induced activation of the p105 promoter was greatly reduced by the PKC inhibitor, independently of HIV infection (Fig. 6). However, HIV-induced activity of the p105 promoter was not affected by the PKC

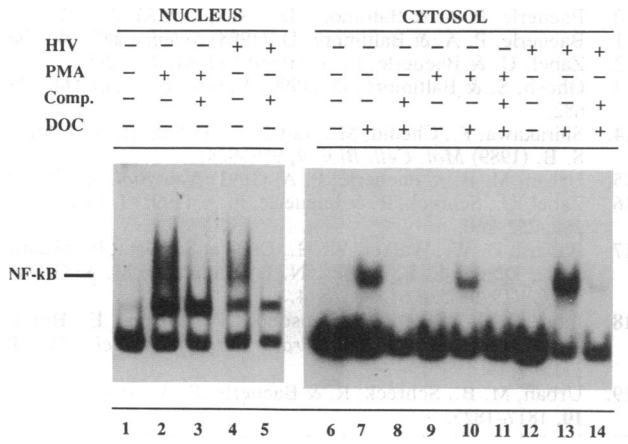

FIG. 4. Gel shift assay with nuclear (lanes 1–5) or cytoplasmic (lanes 6–14) extract of U937 cells. The radiolabeled oligonucleotide and the competition analysis were the same as in Fig. 3. Migration of the NF-κB complex is indicated at left. DOC, deoxycholate.

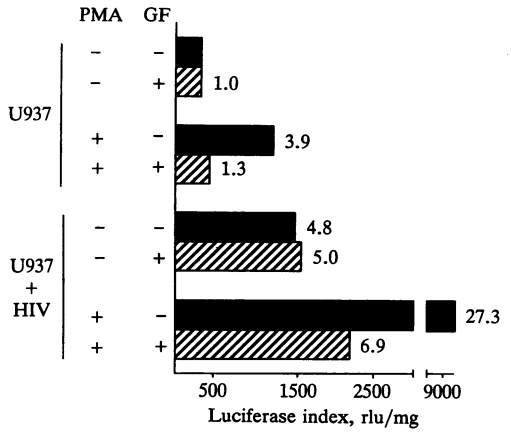

FIG. 6. Effect of PKC inhibitor (GF) on the activation of the p105 promoter by PMA and HIV. Uninfected or HIV-infected U937 cells were transfected with the p105-luc construct. Numbers to the right of the bars indicate fold-stimulation with respect to the promoter activity obtained in uninfected, untreated cells. Concentrations of PMA and PKC inhibitor are the same as in Fig. 5.

inhibitor. The effect of PMA and HIV infection on the activation of the p105 promoter was more than additive. These results indicate that HIV induction of NF-κB activity, at the level of both p105 gene transcription and NF-κB nuclear translocation, is achieved through cellular transduction pathway(s) different from that used by PMA.

DISCUSSION

Study of the mechanism(s) by which HIV infection activates NF-κB in the monocytic cell line U937 may help us to understand the chronic infection with persistent viral replication observed in human brain macrophages of HIV-infected patients (30). Molecular analysis of this model shows that HIV infection induces nuclear DNA-binding activity of p50/p65 heterodimers. The c-Rel protein is known to bind to the p50 subunit of NF-κB and to have DNA-binding activity (18, 31). However, we could not detect c-Rel in the nuclei of infected U937 cells. Antibodies to c-rel did not react with any of the induced complexes, whereas antibodies to p50 did. Addition of recombinant IκB eliminated the binding of the induced NF-κB complex but not that of recombinant p50 homodimers. IκB α does not inhibit DNA binding of c-Rel (26, 32, 33). Therecombinant human IκB that we have used is derived from the MAD-3 sequence (26), which is similar to IκB α (12). Our results indicate that the molecular form of the protein translocated into the nuclei of U937 cells during chronic HIV infection is a heterodimeric complex containing p65 but not c-Rel. The NF-κB complex was found consistently to be increased in cytosolic extracts from HIV-infected cells as compared with extracts of control cells. In some experiments NF-κB could even be detected in infected, but not control, cells in the absence of deoxycholate treatment (data not shown). These observations contrast with results obtained following PMA stimulation of control U937 cells, where a decrease in cytosolic NF-κB and a parallel increase of the complex in the nucleus were seen, as previously reported (32). This suggests that HIV infection of U937 cells continuously induces formation of the NF-κB complex, resulting in its accumulation in both nuclear and cytoplasmic compartments.

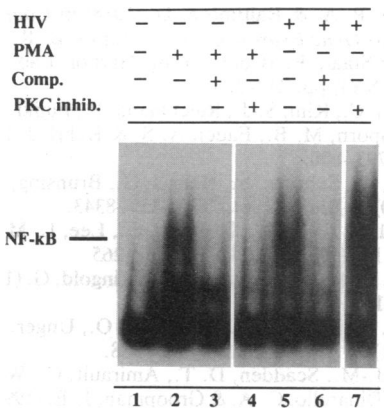

FIG. 5. Gel shift assay of nuclear extracts from uninfected (lanes 1–4) or HIV-infected (lanes 5–7) U937 cells. Cells were treated with 2.5 mM PKC inhibitor (lanes 4 and 7) or left untreated (lanes 1–3, 5, and 6). PMA (20 ng/ml) was added 30 min later (lanes 2–4). Comp., competition with a 40-fold excess of unlabeled oligonucleotide.

As shown by transient transfection experiments, the activity of the p105 promoter was increased in chronically infected cells. This enhancement was dependent on the κB, but not the AP-1, motif of the promoter. This indicated that the κB motif was necessary for p105 promoter induction by HIV, supporting the concept that NF-κB autoregulatory mechanisms are used by HIV to induce and perpetuate NF-κB functions in chronically infected monocytes.

Phosphorylation of IκB through the activation of PKC, as seen following PMA stimulation, is thought to be responsible for translocation of the NF-κB complex to the nucleus (9, 12, 13, 26). Our data show that HIV infection does not use the α, β, or γ PKC pathway utilized by PMA to increase NF-κB nuclear translocation. Using a highly specific inhibitor of these PKC isoenzymes (24, 28), we have found that NF-κB translocation and subsequent stimulation of the p105 promoter by PMA are dependent on PKC isotypes, since these processes were abolished in the presence of this inhibitor. In contrast, the activation of NF-κB induced by HIV infection was not blocked but was, if anything, somewhat increased by incubation of infected cells with this specific inhibitor. Further, the synergistic effect of PMA and HIV infection on the enhancement of p105 gene transcription indicates that these two stimuli use different, complementary transduction pathways. PKC-independent intracellular transduction pathways leading to NF-κB translocation have been described (14, 35–37). TNF upregulates two PKC-independent pathways, those dependent on phospholipase A_2 hydrolysis and phosphatidylcholine-specific phospholipase C activity (38, 39). Chronic HIV infection in U937 cells does not induce TNF or interleukin 1 (ref. 20; data not shown); however, we cannot

formally exclude that TNF is produced, but not exported, and thus acting endogenously. Nevertheless, other authors have shown that chronic HIV infection of monocytic cells does not increase TNF or interleukin 1 mRNA (40), making it unlikely that HIV acts through these cytokines. Another possible mechanism independent from HIV regulation of cellular transduction pathways would be that viral products themselves lead to NF-κB translocation. The HIV protease *in vitro* can process the p105 precursor to give rise to a p50-like molecule (5, 41). However, we have been unable to find nuclear translocation of NF-κB in two lymphoblastoid T-cell lines (CEM and J.Jhan) chronically infected with the LAV-1 Bru strain of HIV under conditions strictly comparable to those used in the present work (data not shown). This argues against a direct role for HIV proteins, presumably produced equally well in infected T cells and monocytes, in the NF-κB activation observed in U937 cells.

Chronic HIV infection of monocytic cells continuously increases HIV transcription itself, in a loop of amplification that may participate in the perpetuation of infection in cells of the myelomonocytic lineage (20). We now show that such a loop may be further amplified by increased synthesis of p105 protein as a result of the continuous HIV-induced translocation of NF-κB. The p105 precursor is evenly distributed in the cytosol, has no DNA-binding activity, and is processed into an active N-terminal p50 subunit (5, 42). Accumulated p50 in the cytosol is likely to form a complex with p65. Preliminary data (not shown) indicate that p65 gene transcription is constitutively high in U937 cells and is not modulated by PMA (in contrast to p105 gene transcription), suggesting that production of p65 is not a limiting factor in the accumulation of p50/p65 heterodimers observed in chronically infected U937 cells. Therefore, upregulation of p105 gene transcription may be critical for replenishment of p50/p65 heterodimers in the cytoplasmic compartment, avoiding the exhaustion of the cytoplasmic pool of NF-κB that would be expected as a consequence of continuous translocation from the cytoplasm to the nucleus of chronically infected cells and thus permitting the sustained levels of HIV transcription observed in cells of the monocytic lineage chronically infected with HIV.

We are grateful to I. Verma for c-Rel antiserum, to A. Israel for p50 antiserum and helpful discussion of the manuscript, to F. Arenzana-Seisdedos for encouragement and discussions, to S. Michelson for useful discussion and review of the manuscript, and to M. Paya and V. del Rocio for continuous encouragement. This work was supported by Association National pour la Recherche du SIDA and Ligue Nationale contre le Cancer. C.V.P. was supported by a Mayo Foundation scholarship and a fellowship from the European Economic Community. R.T.H. was supported by the Medical Research Council AIDS Directed Programme.

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