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Negative regulation of human immunodeficiency virus type 1 expression in monocytes: Role of the 65-kDa plus 50-kDa NF-κB dimer

(retroviral transcription/monocyte activation)

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ABSTRACT **Although monocytic cells can provide a reservoir for viral production *in vivo*, their regulation of human immunodeficiency virus type 1 (HIV-1) transcription can be either latent, restricted, or productive. These differences in gene expression have not been molecularly defined. In THP-1 cells with restricted HIV expression, there is an absence of DNA–protein binding complex formation with the HIV-1 promoter-enhancer associated with markedly less viral RNA production. This absence of binding was localized to the NF-κB region of the HIV-1 enhancer; the 65-kDa plus 50-kDa NF-κB heterodimer was preferentially lost. Adding purified NF-κB protein to nuclear extracts from cells with restricted expression overcomes this lack of binding. In addition, treatment of these nuclear extracts with sodium deoxycholate restored their ability to form the heterodimer, suggesting the presence of an inhibitor of NF-κB activity. Furthermore, treatment of nuclear extracts from these cells that had restricted expression with lipopolysaccharide increased viral production and NF-κB activity. Antiserum specific for NF-κB binding proteins, but not c-*rel*-specific antiserum, disrupted heterodimer complex formation. Thus, both NF-κB-binding complexes are needed for optimal viral transcription. Binding of the 65-kDa plus 50-kDa heterodimer to the HIV-1 enhancer can be negatively regulated in monocytes, providing one mechanism restricting HIV-1 gene expression.**

Infection by human immunodeficiency virus type 1 (HIV-1) is clinically characterized by a long latent period preceding the development of overt symptoms of AIDS (1). Several studies have indicated that a persistent state of either latent or chronic low-level (restricted) infection can be found in both fresh and cultured cells (2–4). Thus, elucidating the mechanisms that control viral replication may have important clinical implications in understanding AIDS disease progression. Of particular interest are factors that could play a role in suppressing HIV expression in cells containing integrated proviruses.

Because animal lentiviruses show a tropism for the monocyte cell lineage during viral persistence (5) and HIV-1 infects human monocytes (6, 7), these cells probably serve as circulating or tissue reservoirs for HIV-1. To study the regulatory mechanisms controlling HIV-1 gene expression in monocytes, our laboratory has recently established chronically infected cultures of a monocytic cell line, THP-1, with various levels of HIV-1 expression (2). These cells express either no (latent), low (restricted), or high (productive) HIV-1 levels. In cells with restricted expression, many inductive signals, such as mitogens (8) and cytokines (9, 10), increase viral production; however, the mechanisms by which viral expression is negatively regulated in these cells are poorly understood.

One transcription factor responsive to immune activation is nuclear factor NF-κB, which stimulates the HIV-1 enhancer in T cells and monocytes treated with various agents, including phorbol 12-myristate 13-acetate (11, 12), lipopolysaccharide (LPS) (12), and tumor necrosis factor α (13, 14). NF-κB recognizes an 11-base pair (bp) DNA sequence present in the immunoglobulin light chain (15). Similar sequences are found in the genes encoding class I major histocompatibility antigens (16), interleukin 2 receptor α chain (17), and several cytokines (18, 19). Mutations in the κB-binding sites that abolish binding of NF-κB prevent inducibility of the HIV-1 enhancer. Two NF-κB binding proteins—a 50-kDa protein (p50) (20, 21) and a 65-kDa protein (p65) (22)—have recently been cloned and show considerable homology to c-*rel*. The p50 can form a homodimer and a heterodimer with p65, which bind to DNA (23, 24). Here we show that this heterodimer can be negatively regulated in HIV-1-infected monocytes.

MATERIALS AND METHODS

Cells. THP-1 cells were infected with HIV-1, as described (2). All cell lines were grown in suspension cultures at 37°C in RPMI 1640 medium/10% fetal calf serum, 1% penicillin–streptomycin/1% L-glutamine in a humidified 5% CO_2 atmosphere. Cells were subcultured 1:5 twice weekly. LPS (Difco) was used at 10 μg/ml. HIV p24 antigen Elisa was done by using a Retro-Tek kit (Cellular Products). For transfections, cells at 20×10^6 per 10 μg of plasmid were subjected to a single pulse of 600 μF, 200 V from a Biotechnologies and Experimental Research (San Diego, CA) Electro cell manipulator model 600 apparatus. After 10 min at 4°C, cells were incubated at 37°C in 10 ml of growth medium 36–60 hr. Cells were harvested for nuclear extracts, HIV p24, and chloramphenicol acetyltransferase assay (12–14). NF-κB p50 expression vectors driven by cytomegalovirus promoters were provided by A. Israel (Institut Pasteur, Paris) (21).

Preparation of Nuclear Extracts. Nuclear extracts were made by the procedure of Parker and Topol (25). Briefly, the cell pellet was washed with ten times the pellet volume of hypotonic buffer and resuspended in 5 ml of hypotonic buffer

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Abbreviations: HIV-1, human immunodeficiency virus type 1; NP-40, Nonidet P-40; LPS, lipopolysaccharide; p50, a protein that binds NF-κB; p65, a protein that complexes with p50; LTR, long terminal repeat; IκB, protein that can inhibit the DNA binding of NF-κB; NRE, negative regulatory region.

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with 1 mmol of dithiothreitol per liter of cells. The cells were broken by using a Dounce homogenizer. After restoring the buffer to isotonicity, nuclei were isolated by centrifugation at 10,000 rpm for 10 min with a JA-21 rotor (Beckman). The nuclei were examined under light microscopy, and ≈85% of them were intact with few whole cells seen. The nuclei were lysed by adding 4 M NH_4SO_4 to a final concentration of 0.36 M. DNA was removed from the lysate by centrifugation at 35,000 rpm for 1 hr. Protein was precipitated from the clarified lysate by adding 0.25 g of NH_4SO_4 per ml of lysate. The proteins were pelleted by centrifugation at 35,000 rpm, resuspended, and dialyzed against a buffer containing 10% (vol/vol) glycerol and stored at −70°C. Some nuclear extracts were treated with sodium deoxycholate at 0.8% and then with Nonidet P-40 (NP-40) at 1.2%, as described (26). NF-κB was purified from the cytosol of rabbit lungs with a final step of affinity chromatography, as described (27).

Antiserum. Peptides derived from the C terminus of c-*rel* (28) coupled to hemocyanin as well as a peptide specific for NF-κB (21) were injected in rabbits, as described (28). Polyclonal antisera specific for either c-*rel* or NF-κB were from N. Rice (Advanced Biosciences Laboratories–Basic Science Program, Frederick, MD). Specific peptides at 20 ng/ml were used for unlabeled competition.

Gel-Mobility-Shift Assay. For DNA-binding assays, the pL3CAT plasmid containing HIV-1 long terminal repeat (LTR) was digested with *Eco*RII, dephosphorylated with calf intestinal alkaline phosphate, 5'-end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The labeled fragment was digested with *Eco*RV to give a probe containing only the HIV-1 NF-κB-binding sequences from −117 (CGAGCTTG CTACAAGGGA CTTTCCGCTG GGGACTTTCC AG) to −78. The mutant probe has the GGG motifs replaced by TCT, as described (11–14), which was purified on a 6% polyacrylamide gel and recovered after ethanol precipitation. Assays slightly modified from Kadonaga *et al.* (29) were done in a 20-μl vol containing 25 mM Hepes (pH 7.6); 50 mM KCl; 10% glycerol; 1.0 mM EDTA; 1.0 mM dithiothreitol; 2 μg of poly(dI-dC); 0.05% NP-40; 15,000 cpm of the probe; and nuclear extracts at 10 μg of protein. For competition studies, 50-fold molar excess of unlabeled probe was added. In mixing experiments, 2 μg of protein from the extract to inhibit was mixed with 8 μg of protein from the extract of productively infected cells. The reaction was incubated for 1 hr at 30°C and then electrophoresed at 10 V per cm through a 4% nondenaturing polyacrylamide gel in Tris/EDTA/borate buffer.

DNA–Protein ("Southwestern") Blot Analysis. Nuclear proteins (100 μg) were separated by SDS/PAGE (10% separating gel) and electrotransferred on nitrocellulose, and the membrane was washed, as described (30). The membrane was then placed in a heat-sealable pouch in binding buffer [10 mM Tris·HCl, pH 7.5/40 mM NaCl/1 mM dithiothreitol/1 mM EDTA/8% (vol/vol) glycerol/0.125% nonfat dry milk/60 μg of poly(dI-dC)/5 mM $MgCl_2$/5 × 10^6 cpm of ^{32}P-labeled HIV-1 oligonucleotide from −216 to −156 (negative regulatory region, or NRE, of HIV-1 LTR)]. After 14 hr of constant agitation, the membrane was washed with 10 mM Tris·HCl/50 mM NaCl for 2 hr at 25°C with several changes and exposed on x-ray film.

RESULTS

Chronically infected THP-1 cells, the HIV-1 expression of which is restricted, initially released high reverse transcriptase and p24 levels, but over several weeks, a progressive decrease in these levels was seen until HIV-1 production stabilized (ref. 2, Table 1). The consequences of this restricted expression in THP-1 cells were (*i*) intracellular sequestration of the virions, (*ii*) markedly lower accumulation of viral DNA, and (*iii*) little extracellular virus (2). Terminal dilution analysis of viral DNA by PCR of the THP-1 cultures with restricted expression used in these studies showed equivalent amounts of DNA compared with an 8E5 cell line containing a single HIV-1 copy (ref. 31, data not shown). Nuclear run-on assays indicated a 3- to 4-fold decrease in HIV-1 transcriptional activity in cells with restricted expression compared with the productively infected cells (2). Furthermore, in an *in vivo* transcription assay using a template DNA derived from a 199-bp region of the HIV-1 LTR containing the enhancer and RNA start site, fewer transcripts were seen with extracts from cells with restricted HIV expression compared with transcripts stimulated by extracts from productively infected cells or LPS-treated cells with restricted expression (data not shown).

Because many cellular transcription factors play a role in regulating HIV transcription by binding to the enhancer region of HIV-1 LTR (32), their DNA-binding capability in restrictedly infected cells could be examined. The HIV-1 enhancer contains two tandem repeats of the 11-bp sequence that binds NF-κB, and gel-mobility-shift experiments were done by using this DNA probe (Fig. 1). Affinity-purified NF-κB binding proteins show three DNA-binding complexes—the highest (slowest migration) is a 65-kDa plus 50-kDa heterodimer followed by a lower 50-kDa homodimer and a proteolytic cleavage (p42) of the homodimer (22–24). As shown (12), uninfected THP-1 cells, as well as freshly isolated monocytes and macrophages, constitutively produce active NF-κB leading to all binding complexes (Fig. 1*A*). These complexes were seen in the nuclear extract from the productively infected cells at much higher levels, but the uppermost band (the heterodimer) was absent in extracts from cells with restricted expression (Fig. 1). To verify that specific NF-κB DNA-binding complexes were being measured, several controls were done. NF-κB purified from rabbit lung, as de-

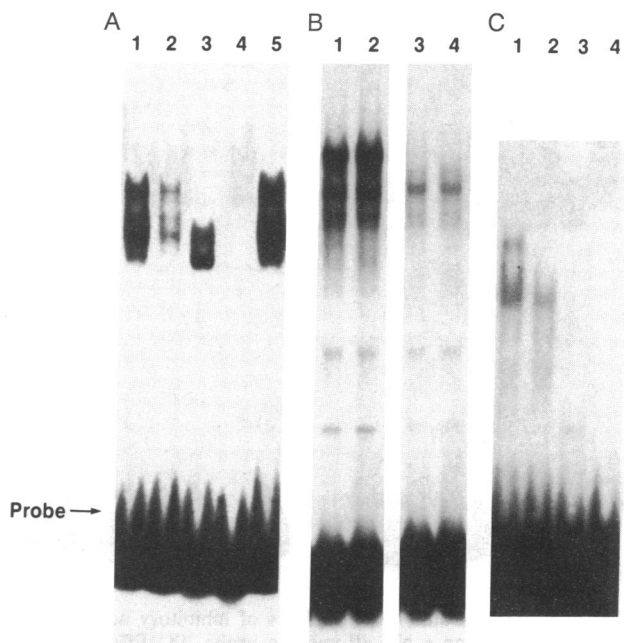

FIG. 1. Gel-mobility-shift analysis of binding to NF-κB sequences. (*A*) Binding to NF-κB sequences. Lanes: 1, 0.4 ηg of purified NF-κB; 2, nuclear extracts (10 μg) from THP-1 cells; 3, nuclear extracts (10 μg) from cells with restricted expression. 4, ^{32}P-labeled probe alone; 5, nuclear extract (10 μg) from productively infected THP-1 cells. (*B*) Effect of GTP on DNA binding. Lanes: 2 and 4, 3 mM GTP in 20 mM Tris·HCl was added to DNA-binding reaction mixture, as described; 1 and 2, THP-1 with productive expression; 3 and 4, THP-1 with restricted expression. (*C*) Lack of binding to mutated NF-κB sequences. Lanes: 1, cells with productive infection; 2, cells with restricted expression; 3, same as lane 2, except mutant probe was used; 4, same as lane 2 except mutant probe was used.

scribed (27), formed the same complexes with the DNA probe (Fig. 1*A*). Because millimolar amounts of GTP can stimulate the DNA-binding activity of NF-κB (33), it was important to show that absence of binding was not due to lower GTP levels. With GTP, the heterodimer complexes were still absent in the cells with restricted HIV expression (Fig. 1*B*). When a DNA probe with mutations in the NF-κB binding sequences was used (11–14), these specific binding complexes were no longer present (Fig. 1*C*).

Because previous results with the whole enhancer region as a DNA probe had shown that nuclear extracts from cells with restricted infection could inhibit complex binding by using extracts from productively infected cells (2), we asked whether the same was true for NF-κB binding to specific sequences (Fig. 2*A*). Nuclear extracts from restricted cells inhibited complex formation to the NF-κB sequences by nuclear extracts from the productively infected cells, whereas extracts from uninfected cells had no effect, suggesting that specific inhibitors of NF-κB binding were present in the extract from cells with restricted expression. Addition of nuclear extracts from restrictedly infected cells to purified NF-κB also reduced binding. In additional specificity controls, competitions with unlabeled oligonucleotides containing normal and mutated NF-κB sequences, as well as transactivation response element (TAR) sequences, were done (Fig. 2*B*). With extracts from productively-infected THP-1 cells, both the p50 homodimer and p50–p65 heterodimer bindings were abolished by unlabeled competition with an NF-κB oligonucleotide, whereas no effect was seen in competitions with a mutated NF-κB or a trans-activation response element-region oligonucleotide. As previously shown, extracts from THP-1 cells with restricted HIV expression bound only p50; this binding was also abolished only by an unlabeled NF-κB probe (Fig. 2*B*).

To determine whether the inhibitory activity in the nuclear extract of THP-1 cells with restricted expression was specific to NF-κB binding and not a general proteolytic degradation of the extract, we determined the ability of this extract to bind to or inhibit specific binding to a different region of the LTR (Fig. 3 *A* and *B*). Southwestern (DNA–protein) analysis of binding to the NRE region of the HIV-1 LTR shows the same distinct bands for all extracts (Fig. 3*A*). In addition, nuclear extracts from HeLa cells can bind to a 30-mer in the NRE region of HIV-1 LTR. Extracts from THP-1 cells with restricted expression could not inhibit this binding (Fig. 3*B*) or binding of the Oct-1 factor (data not shown). These data indicate that protein degradation did not contribute to the inhibitory activity of the extracts. Furthermore, addition of purified NF-κB proteins to the extracts from cells with restricted infection resulted in a recovery of the higher DNA-binding complex (Fig. 3*C*).

Because exogenous addition of NF-κB overcame the inhibitory activity, we asked whether an endogenous treatment could overcome this inhibition. NF-κB had previously been shown to be present in the cytosol of cells bound to an inhibitory protein, IκB, which can inhibit the DNA binding of NF-κB in a reversible and specific manner (26, 34). Sodium

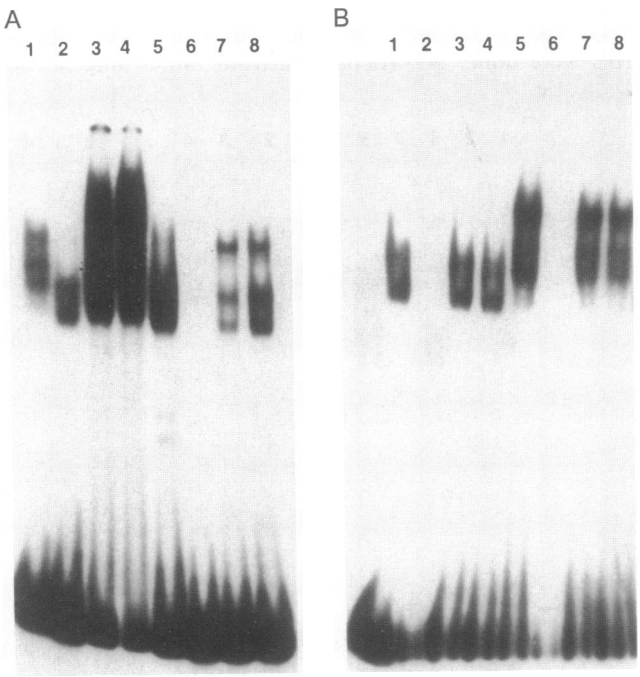

FIG. 2. Gel-mobility-shift analysis of inhibitory activity in nuclear extracts using a NF-κB-specific probe. (*A*) Effect of mixing nuclear extracts on DNA binding. Lanes: 1, THP-1; 2, THP-1 with restricted infection; 3, THP-1 with productive infection; 4, THP-1 with productive infection plus THP-1 (4:1 ratio); 5, THP-1 with productive infection plus THP-1 with restricted infection (4:1 ratio); 6, probe alone; 7, purified NF-κB (0.1 ng) plus THP-1 with restricted expression; 8, purified NF-κB (0.1 ng). (*B*) Oligonucleotide competitions of binding to NF-κB sequences with and without 50-fold excess of unlabeled competitor oligonucleotides. Lanes 1–4, extracts from cells with restricted expression; lanes 5–8, extracts from cells with productive expression. Lanes 1 and 5 contained no competitor; lanes 2 and 6 contained NF-κB sequences; lanes 3 and 7 contained mutated NF-κB sequences; and lanes 4 and 8 contained transactivation response element sequences (−17 to +82).

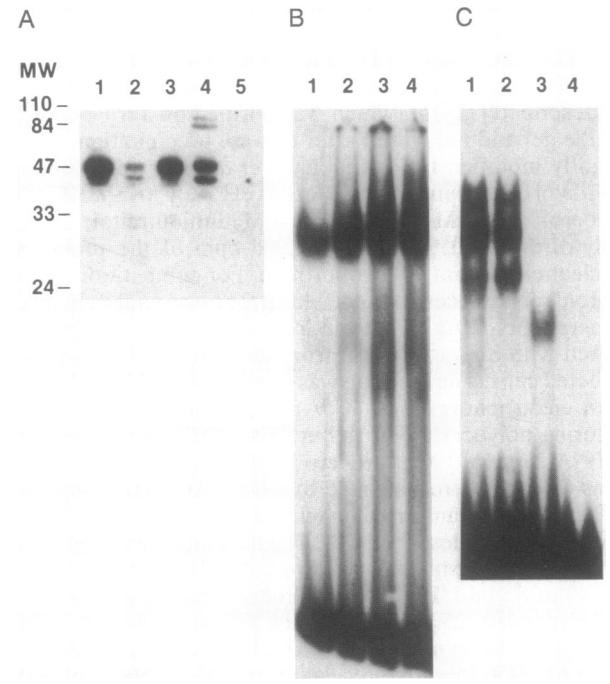

FIG. 3. (*A*) Southwestern (DNA–protein) analysis of binding to NRE region of HIV-1 LTR. A ^{32}P-labeled probe containing the HIV-1 NRE binding sequences from −340 to −216 was used. Lanes: 1, restricted THP-1; 2, productive THP-1; 3, THP-1; 4, HeLa cells; 5, Control. (*B*) Effect of extracts from restricted cells on binding to HIV-LTR NRE region. A ^{32}P-labeled probe containing the HIV-1 NRE binding sequences from −156 to −126 was used. Lanes 1–4 contained extract from HeLa cells (2.5 μg). Lanes: 1, no competitor extract; 2, extract from THP-1 (5 μg); 3, extract from THP-1 with restricted expression (5 μg); 4, extract from THP-1 cells with productive expression (5 μg). (*C*) Effects of mutant NF-κB probe on DNA binding. Lanes: 1, THP-1 with productive infection plus purified NF-κB (0.2 ηg); 2, THP-1 with restricted infection plus NF-κB (0.2 ηg); 3, same as lane 1, except mutant probe was used; 4, same as lane 2, except mutant probe was used. At left are M_r ($\times 10^{-3}$) markers.

deoxycholate can release IκB and thus activate NF-κB (34, 35). Nuclear extracts from uninfected and infected cells were treated with sodium deoxycholate, incubated with NP-40, and then used in gel-mobility assays, as described (34). As has been shown, sodium deoxycholate at 0.8% inhibited NF-κB binding (Fig. 4). However, incubation with NP-40, a nonionic detergent, can sequester sodium deoxycholate out of solution. Under these conditions, nuclear extracts from cells with restricted infection could now form the heterodimer (Fig. 4, lane 3). Extracts from productively infected cells also modestly increased heterodimer-binding activity after treatment with sodium deoxycholate and NP-40 (Fig. 4, lane 6); thus, this increase in NF-κB activity was not limited to cells with restricted expression. This DNA-binding seen after sodium deoxycholate and NP-40 treatment was also specific, as shown by the lack of binding to a probe containing a mutant NF-κB-binding site (Fig. 4, lanes 4 and 8). Several groups have shown that many stimuli can enhance viral expression in chronically infected cells with restricted expression (8–12). In THP-1 cells, LPS at 10 μg/ml for 48 hr stimulates a 10-fold increase in viral production (Table 1). In addition, LPS treatment of THP-1 cells with restricted HIV-1 expression increases binding activity of 65-kDa plus 50-kDa heterodimer, which approaches that of productively infected cells (Fig. 4, lane 10). Transfection of an NF-κB expression vector in these cells increased NF-κB binding activity and viral production (data not shown). All data suggest that lack of optimal NF-κB binding provides at least one mechanism for restricted HIV-1 expression in these cells.

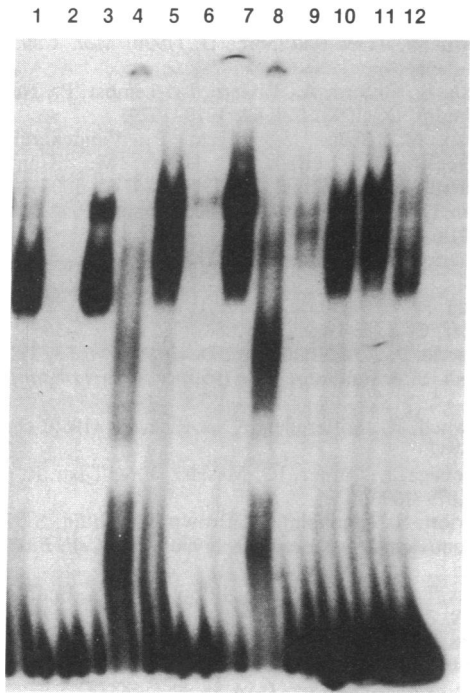

FIG. 4. Effect of dissociating agents on NF-κB activity in nuclear extracts of THP-1 with restricted HIV-1 expression. Nuclear extracts were treated with 0.8% sodium deoxycholate followed by incubation with and without Nonidet P-40 at 1.2% final concentration, as described (24, 32). Lanes: 1, THP-1 with restricted expression; 2, same as lane 1 treated with 0.8% sodium deoxycholate; 3, same as lane 2 followed by NP-40 incubation; 4, same as lane 3, except mutant probe was used; 5, THP-1 with productive HIV-1 expression; 6, same as lane 5 treated with 0.8% sodium deoxycholate; 7, same as lane 6 treated with NP-40; 8, same as lane 7 with mutant probe; 9, extract from THP-1 cells with restricted expression with a second HIV-1 isolate, RF; 10, extract from same cells as lane 9 treated with LPS for 48 hr; 11, extract from cells productively infected with isolate RF; 12, extract from THP-1 cells.

Table 1. Analysis of virally infected THP-1 cells

Viral expression	P24 antigen, ng/ml	CAT activity, % conversion	NF-κB binding (heterodimer)
None	0	12	++
Producer	200–500	78	+++
Restricted	0.05–20	25	+/−
Restricted + LPS*	100–200	75	++

THP-1 cells were grown and infected with the RF strain of HIV-1 as previously described (2). Values are for at least three separate experiments.
*Cells were treated with LPS at 10 μg/ml for 48 hr, and the viral assays were then done. CAT, chloramphenicol acetyltransferase.

Because molecular cloning of c-*rel* and NF-κB binding proteins have shown that they represent a family of cellular transcription factors that can bind to the NF-κB sequences (36, 37), it became important to determine which proteins were being prevented from forming the heterodimer. Addition of an antibody specific to NF-κB profoundly decreased both heterodimer and homodimer binding by extracts from productively infected cells as well as sodium deoxycholate-treated extracts from restrictedly infected cells (Fig. 5). In contrast, two antisera specific for c-*rel* have no effect on complex formation, indicating that the inhibitory activity was predominantly directed against NF-κB.

DISCUSSION

Many cellular proteins contribute to HIV-1 production, including transcription factors such as NF-κB, which bind to sequences in the LTR and increase viral transcription. During productive

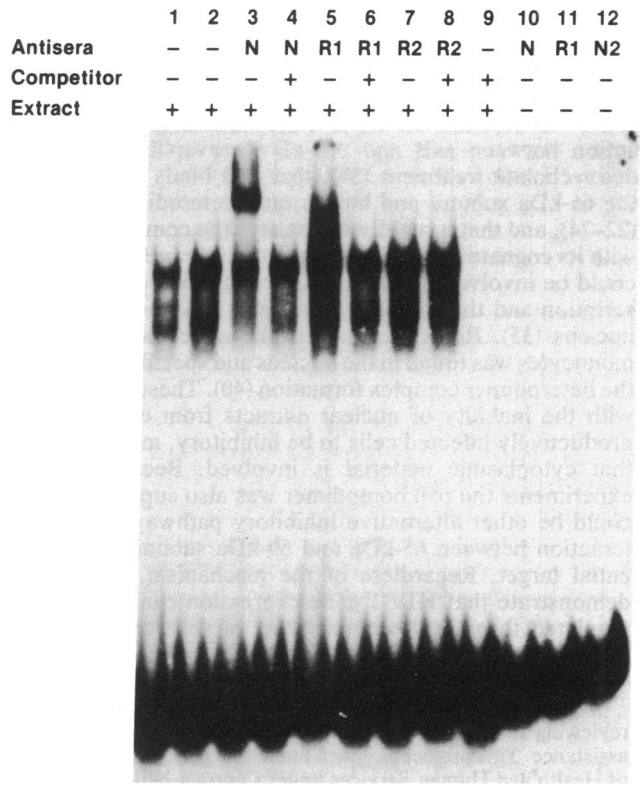

FIG. 5. Reactivity of nuclear extracts with antiserum. Sodium deoxycholate- and NP-40-treated extracts were from restrictedly infected cells. Lane 1 was treated with 0.6% sodium deoxycholate, and all others were at 1.2%. N, NF-κB-specific antiserum; R1 and R2, specific c-*rel* antisera; competitor is peptide used to derive antisera. Lane 8, no probe; lanes 9–12, no extracts added.

infection of U937 cells, NF-κB is specifically induced (38), and it can be regulated by the HIV-1 protease (39). At least in monocytic cells, the formation of both the NF-κB 50-kDa homodimer and the 65-kDa plus 50-kDa heterodimer is required for optimal HIV-1 transcription, producing higher virus levels (Fig. 4 and Table 1). In these chronically infected cells, viral transcription can be negatively regulated by preferentially blocking the formation of the 65-kDa plus 50-kDa heterodimer DNA-binding complex (Figs. 1–3). Many inducible signals such as LPS, phorbol 12-myristate 13-acetate, and tumor necrosis factor α, which increase viral production in these cells (8–12), accomplish this on at least one level by increasing NF-κB-binding activity (11–14). In addition, the production of many cytokines, including tumor necrosis factor α, interleukin 6, and granulocyte/macrophage colony-stimulating factor, which augment HIV-1 production, is stimulated by NF-κB binding (13–19). Thus, positive and negative regulation of NF-κB regulates the level of HIV-1 production. However, other transcription factors like Sp1 may also contribute to this process similarly.

In studying the mechanism leading to a preferential block in formation of the 65-kDa plus 50-kDa heterodimer DNA-binding complex, several results suggest the presence of a specific inhibitor of NF-κB. Mixing nuclear extracts from the cells with restricted expression can block complex formation by nuclear extracts from productively infected cells (Fig. 2). The fact that the extracts from restrictedly infected cells do not inhibit binding to other regions of the LTR and bind normally to other regions indicates that nonspecific degradation is unlikely (Fig. 3 *A* and *B*). Purified NF-κB can restore complex formation when added to nuclear extracts from the cells with restricted expression. Treatment of these nuclear extracts with sodium deoxycholate restores specific heterodimer complex formation. Antisera specific for NF-κB markedly block heterodimer, whereas c-*rel*-specific antisera do not, suggesting that NF-κB binding is specifically inhibited. Finally, an NF-κB expression vector increases LTR activity and viral production in restrictedly infected cell lines.

The protein IκB has been found to be a cytosolic inhibitor of NF-κB-binding activity (34, 35). The facts that the interaction between IκB and NF-κB is reversible by sodium deoxycholate treatment (34), that IκB binds specifically to the 65-kDa subunit and blocks only heterodimer formation (22–24), and that it rapidly dissociates the complex of NF-κB with its cognate DNA (35) suggest that the IκB-like molecule could be involved in this negative regulation of HIV-1 transcription and that IκB-like molecules can be present in the nucleus (35). Recently, an IκB-like molecule cloned from monocytes was found in the nucleus and specifically inhibited the heterodimer complex formation (40). These results, along with the inability of nuclear extracts from uninfected and productively infected cells to be inhibitory, make it unlikely that cytoplasmic material is involved. Because in some experiments the p50 homodimer was also suppressed, there could be other alternative inhibitory pathways, but the interaction between 65-kDa and 50-kDa subunits is a preferential target. Regardless of the mechanism, these results demonstrate that HIV-1 gene expression can be negatively regulated through the interactions of cellular transcription factors such as NF-κB.

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