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Human Immunodeficiency Virus Type 1 Nef Protein Down-Regulates Transcription Factors NF-κB and AP-1 in Human T Cells In Vitro after T-Cell Receptor Stimulation

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Human immunodeficiency virus type 1 (HIV-1) negative factor (Nef) has been shown to down-regulate the transcription factors NF-κB and AP-1 in vitro. To define the mechanism of action of the Nef protein, the signal transduction pathways which may be affected in T cells by constitutive expression of the *nef* gene were examined. Stimulation of T cells with tumor necrosis factor, interleukin-1, or lipopolysaccharide resulted in the recruitment of transcriptional factors to a similar level whether or not the cells expressed the *nef* gene. On the other hand, stimulation of T cells by mitogens or antibodies to the T-cell receptor (TCR)-CD3 complex resulted in the down-regulation of transcriptional factors NF-κB and AP-1 in cells expressing the *nef* gene compared with cells not expressing the *nef* gene. Because the Nef protein does not affect the surface expression of the CD3-TCR complex, we conclude that the Nef protein down-regulates the transcriptional factors NF-κB and AP-1 in T cells in vitro through an effect on the TCR-dependent signal transduction pathway.

Human immunodeficiency virus type 1 (HIV-1) negative factor (Nef) is dispensable for virus replication in vitro (10, 44) but is essential for the pathogenicity of the closely related simian immunodeficiency virus (6, 22). We have previously reported that HIV-1 and simian immunodeficiency virus Nef proteins affect virus replication in vitro (33, 34) and that this effect is related to down-regulation of transcriptional factors NF-κB and AP-1 (30, 31). These transcriptional factors normally regulate the expression of genes involved in T-cell activation and proliferation (16). The HIV-1 promoter possesses multiple binding sites for both NF-κB and AP-1 which allow the virus to subvert the normal activity of these factors to enhance its own replication (29).

The mechanism by which HIV-1 Nef protein affects signal transduction is unknown. Although HIV-1 Nef protein also down-regulates the cell surface expression of CD4 (13), the mechanism for this effect is also unknown. However, this function requires the cytoplasmic domain of CD4 (12). Because Nef protein localizes preferentially in the cytoplasm, membranes, and cytoskeleton (11, 32), its effects on nuclear factors must be mediated by a physical interaction or enzymatic regulation of one or more factors involved in signal transduction.

Several pathways of signal transduction for activation of T cells have been described. Antigenic stimulation of the T-cell receptor (TCR)-CD3-CD4 complex results in translocation of NF-κB into the nucleus. The mechanism of NF-κB activation in T cells is not completely understood, but it may involve dissociation of NF-κB from I-κB as a result of phosphorylation and/or proteolysis of I-κB (3, 8, 14, 19–21, 28, 40). Alternatively, T cells can be activated by cytokines and other surface receptors (tumor necrosis factor alpha [TNF], interleukin-1 [IL-1], lipopolysaccharide [LPS]), also resulting in activation of transcriptional factors (4, 9, 35, 36, 39).

In this report, we describe the signal transduction pathway(s) affected in T cells by constitutive expression of the *nef* gene. We found that stimulation of these cells through pathways originating from the TNF or IL-1 receptors is not affected by Nef protein, whereas stimulation of these cells through the TCR-CD3-CD4 complex resulted in down-regulation of NF-κB and AP-1 in the presence of Nef protein compared with the parental cell line not expressing the *nef* gene.

MATERIALS AND METHODS

Cell lines and nuclear extract preparation. The cell lines used included Jurkat (J25) human T-cell clone 133, which stably expresses the Nef protein derived from the HIV-1 isolate NL-43, and its parental counterpart, clone 22F6, which does not express Nef protein (26). Confirmation of Nef protein production was performed by immunoprecipitation with rabbit anti-Nef serum and Western blot (immunoblot) analysis as reported previously (30). Cells were maintained in the logarithmic growth phase in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml.

Cells were stimulated with different combinations of 50 to 75 ng of phorbol 12-myristate 13-acetate (PMA) per ml (Sigma, St. Louis, Mo.), 13 μg of phytohemagglutinin (PHA) per ml (Sigma), 1,000 U of TNF per ml (Genentech Inc., San Francisco, Calif.), 15 ng of recombinant human IL-1 per ml (generously provided by D. Chaplin, Washington University, St. Louis, Mo.), or 10 μg of lipopolysaccharide (LPS) per ml (Sigma). Antibody cross-linking was performed as described previously (17). Briefly, sheep anti-mouse immunoglobulin G (IgG) antibody (Becton Dickinson, San Jose, Calif.) diluted 1:100 in 0.1 mM Tris buffer (pH 9.5) was immobilized onto plastic microtiter plates by incubation at room temperature for 1 h and then washed with phosphate-buffered saline three times. One microgram of monoclonal antibody to CD3 per ml (Leu-4, Becton Dickinson) was added to the Jurkat cells, and then they were incubated for 4 h at 37°C in the microtiter plates in the presence or absence of sheep anti-mouse IgG. The protein kinase inhibitor isoquinol methylpiperazine (H-7)

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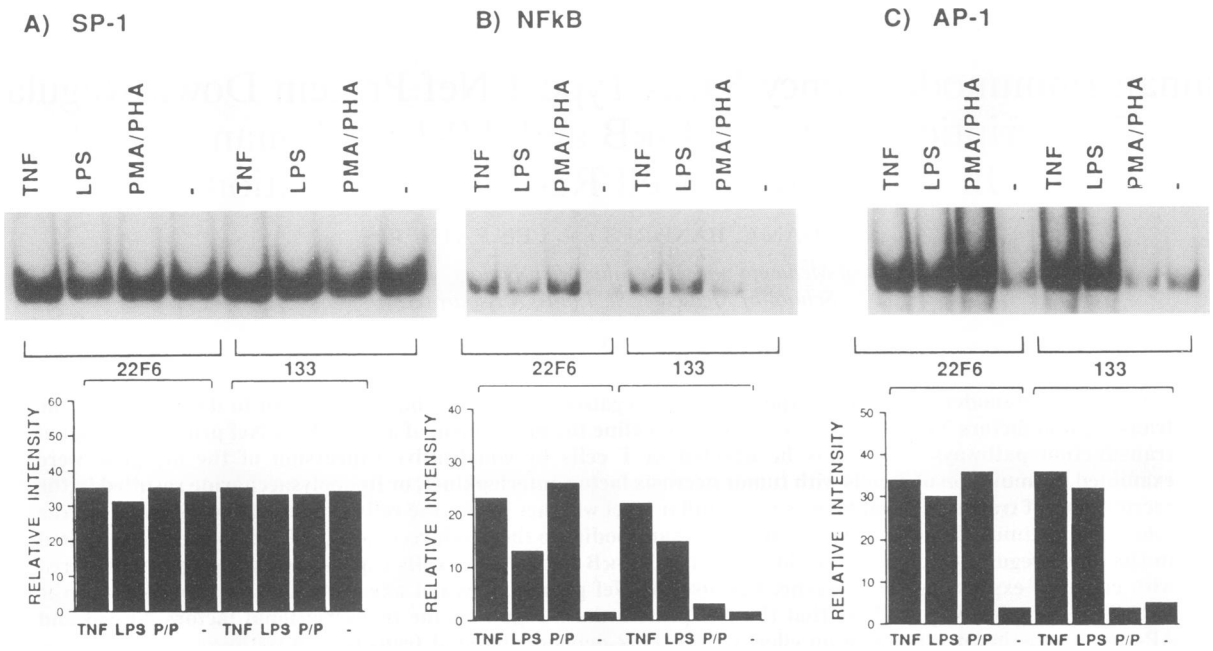

FIG. 1. Effect of Nef protein on induction of transcriptional factors AP-1 and NF-κB in T cells stimulated with TNF, LPS, or PMA and PHA. Gel shift analysis shows induction of transcriptional factors SP-1, NF-κB, and AP-1 in Jurkat T cells (22F6 is the parental cell line, and 133 is the Nef protein-expressing line). Cells were incubated for 4 h with saline (−), TNF, LPS, or PMA and PHA (P/P). Retarded DNA-protein complexes are shown; free DNA complexes are not shown but were equivalent in each lane. The experiments were performed three times with similar results. The relative intensity of the bands was evaluated by laser densitometry.

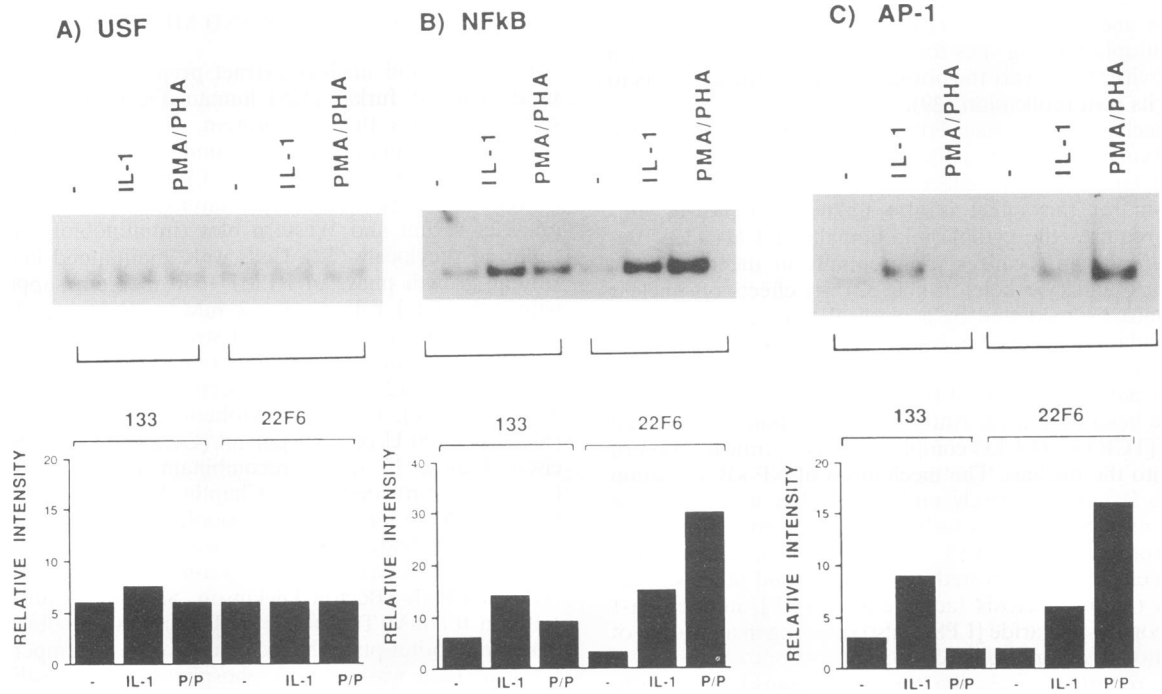

FIG. 2. Effect of Nef protein on induction of transcriptional factors AP-1 and NF-κB in T cells stimulated with recombinant human IL-1 or PMA and PHA. Gel shift analysis shows induction of transcriptional factors USF, NF-κB, and AP-1 in Jurkat T cells (22F6 is the parental cell line, and 133 is the Nef protein-expressing line). Cells were incubated for 4 h with IL-1, PMA and PHA (P/P), or sterile saline (−). Retarded DNA-protein complexes are shown; free DNA complexes are not shown but were equivalent in each lane. The experiments were performed three times with similar results. The relative intensity of the bands was evaluated by laser densitometry.

(Sigma), when used, was added to the cells 30 min prior to the stimulants to a final concentration of 100 μM. Nuclear extracts were prepared from 5×10^7 cells as previously described (30, 31) with the following modifications: after ammonium sulfate precipitation, nuclear proteins were resuspended in 100 μl of 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [pH 7.9])–20 mM KCl–1 mM $MgCl_2$–2 mM dithiothreitol–17% glycerol, with the addition of 10 mM sodium fluoride, 0.1 mM sodium vanadate, and 50 mM β-glycerolphosphate. Binding reaction mixtures contained 2 μg of nuclear extract, 2 μg of poly(dI-dC) (Pharmacia, Milwaukee, Wis.), 20,000 to 40,000 cpm of end-labeled oligonucleotide probe, and the equivalent amount of labeled probe in each assay for a given experiment, with or without a 100-fold molar excess of unlabeled oligonucleotide, in DNA binding buffer at a final volume of 30 to 40 μl. Reactions were performed at 30°C for 25 min.

Gel retardation assays. For the gel retardation assays, the following double-stranded oligonucleotides were used: NF-κB, ACAAGGGACTTTCCGCTGGGACTTTCCAGGGA; SP-1, CAGGGAGGCGTGGCCTGGGCGGGACTGGGGAG TGGCGTCC; AP-1, CAGGGCCAGGAGTCAGATATCCA CTGACCTTTGGATGGTGCT; and USF, GCCGCTAGCA TTTCATCACGTGGCCCGAGAGCTGC.

All DNA probes were gel purified and end labeled with [γ-^{32}P]ATP. Gel retardation assays were performed as previously described (30) by using the radiolabeled probes, with DNA-protein complexes being separated from the free DNA probe by electrophoresis through low-ionic-strength 4.5% polyacrylamide gels and run at 200 V with Tris-borate-EDTA (3.7 g of Na EDTA, 54 g of Tris, and 27.5 g of boric acid per liter). The intensity of indicated bands for DNA-protein complexes was determined by laser densitometry scanning.

CAT assay. Chloramphenicol acetyltransferase (CAT) assays were performed as described previously (15) with samples with the same protein concentration as determined by the method of Bradford (Biorad, Hercules, Calif.). Transfections were performed in parallel with previously described reporter plasmids Rous sarcoma virus-CAT, HIV-1-CAT, and IL-2-CAT (1, 41) in both cell lines, and the results were normalized to the values obtained in parallel from the noninducible Rous sarcoma virus-CAT to control for transfection efficiency. Reaction products were analyzed by thin-layer chromatography followed by autoradiography and liquid scintillation counting. CAT activity was expressed as fold increases in the percentage of radioactivity in the acetylated forms compared with the sum of that of the acetylated and unacetylated forms.

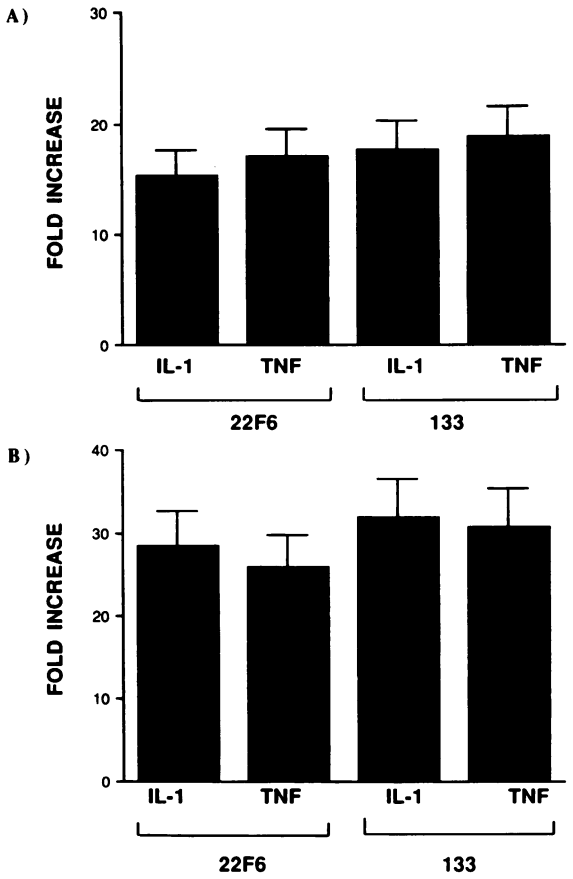

FIG. 3. CAT assay showing the effect of Nef protein on HIV and IL-2 transcription. Jurkat T cells (22F6 is the parental cell line, and 133 is the Nef protein-expressing cell line) were transfected with HIV-1-long terminal repeat-CAT (A) and incubated for 4 h with recombinant human IL-1 or TNF. Similar experiments with IL-2-CAT (B) were performed with 22F6 and 133 cells. Cells were transfected four times with each plasmid, a mean fold induction was calculated, and the standard deviation of the mean was determined. The percentages of acetylation of HIV-1-CAT were 4.6 ± 0.7 (IL-1) and 4.4 ± 0.4 (TNF) for 22F6 cells and 3.9 ± 0.6 (IL-1) and 4.7 ± 0.3 (TNF) for 133 cells. The percentages of acetylation for IL-2-CAT were 14.9 ± 0.9 (IL-1) and 17 ± 0.7 (TNF) for 22F6 cells and 12.7 ± 0.6 (IL-1) and 12.9 ± 0.3 (TNF) for 133 cells.

RESULTS

To evaluate the effects of Nef protein on activation of transcriptional factors through protein kinase C-independent mechanisms, Jurkat cells carrying the *nef* gene (133) and the parental cell line (22F6) were stimulated with either TNF or LPS, and the results were compared with those from stimulation with PMA and PHA (Fig. 1). A noninducible factor, SP-1, was used to control for protein concentration and quality of the extracts (Fig. 1A). TNF or LPS induction of NF-κB (Fig. 1B) and AP-1 (Fig. 1C) was not affected by the presence or absence of Nef protein in 133 and 22F6 cells, respectively. In contrast, the level of induction of both transcription factors was decreased, by 6-fold in the case of NF-κB and by 14-fold in the case of AP-1, when the same cells were stimulated with PMA and PHA.

Similar results were obtained when the comparison was made between the stimulation with IL-1 and that with PMA or PHA (Fig. 2). Noninducible transcription factor USF was used as a control (Fig. 2A). NF-κB (Fig. 2B) and AP-1 (Fig. 2C) activation by IL-1 was not significantly affected by the presence or absence of Nef protein in 133 and 22F6 cells, respectively. Stimulation with PMA or PHA resulted in down-regulation of NF-κB by 5-fold and AP-1 by 15-fold in the presence of Nef protein in this experiment.

To determine if induction of NF-κB and AP-1 correlated with transcriptional activity, cells were transfected with DNA plasmids which use the HIV-1 long terminal repeat to direct expression of a heterologous gene product, CAT. Cells were stimulated with TNF or IL-1 (Fig. 3). No difference in acetylation between the cells expressing the *nef* gene, 133, and the parental cell line, 22F6, was found when both cell lines were stimulated with either IL-1 or TNF (Fig. 3A). Because Nef protein also has been reported to suppress the induction of CAT activity directed by the IL-2-CAT plasmid (26), both 133 cells and 22F6 cells containing this plasmid were stimulated

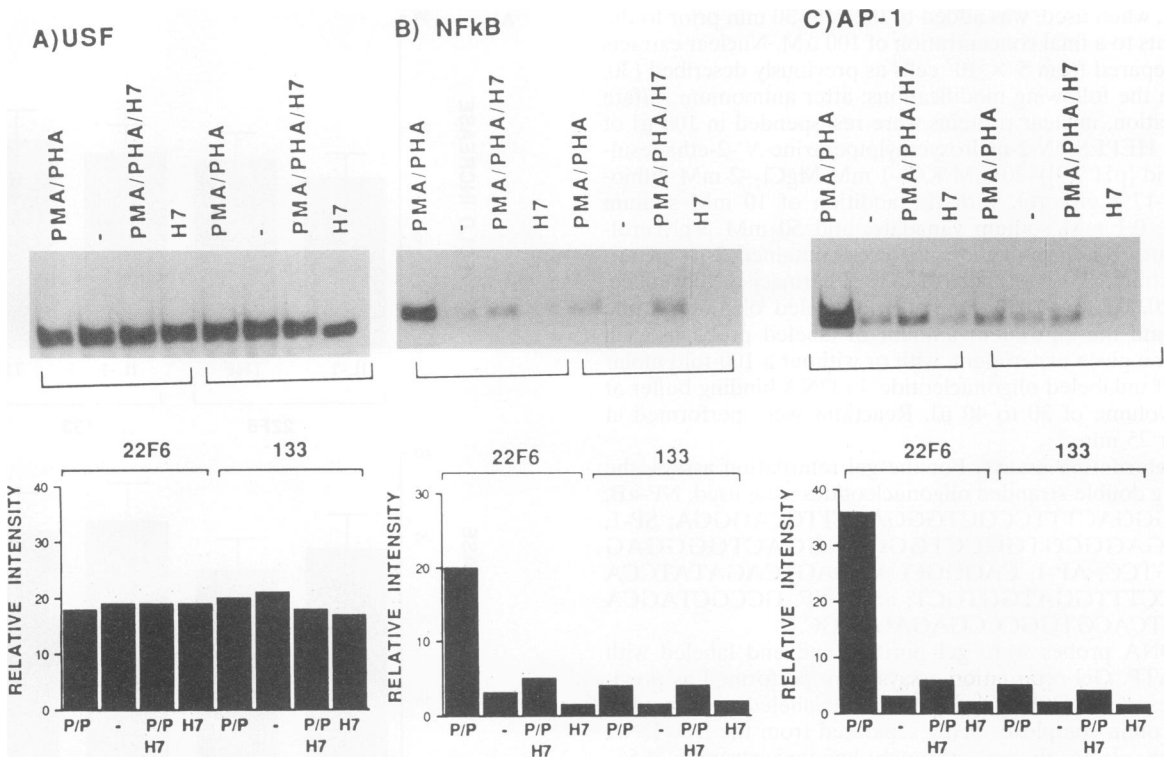

FIG. 4. Effect of Nef protein on induction of transcriptional factors AP-1 and NF-κB in T cells stimulated with PMA and PHA in the presence or absence of H-7. Gel shift analysis shows induction of transcriptional factors USF, NF-κB, and AP-1. Jurkat T cells (22F6 is the parental cell line, and 133 is the Nef protein-expressing cell line) were incubated for 4 h with PHA and PMA (P/P) or sterile water (−). H-7 was added 30 min prior to the incubation. Retarded DNA-protein complexes are shown; free DNA complexes are not shown but were equivalent in each lane. The experiments were performed three times with similar results. The relative intensity of the bands was evaluated by laser densitometry.

with TNF or IL-1 (Fig. 3B). The results paralleled those obtained with HIV-1-CAT. These data indicate that Nef protein does not affect the induction of transcription factors by the pathway(s) of T-cell activation originating from the TNF or IL-1 receptors.

To further evaluate the effects of Nef protein on signal transduction after PMA or PHA stimulation, the effect of addition of a protein kinase inhibitor, H-7, was examined (Fig. 4). The noninducible factor USF was used as a control (Fig. 4A). PMA or PHA induction of NF-κB (Fig. 4B) was 10-fold higher in the 22F6 cells than in the 133 cells. The use of H-7 resulted in a significant decrease in NF-κB induction in the cells that did not express Nef protein. Similarly, PMA and PHA resulted in the induction of AP-1 (Fig. 4C) to a level that was 15 times higher in the cells not expressing Nef protein than in the cells expressing Nef protein. H-7 blocked the induction of AP-1 in the 22F6 cells. In both cases, the addition of H-7 to the Nef protein-expressing cells did not cause any further decrease in the already low level of expression of the respective transcription factors.

To further establish that the effects of Nef protein were related to stimulation through the TCR-CD3 complex, both Jurkat 22F6 and 133 cells were stimulated by anti-CD3 cross-linking in the presence or absence of H-7 (Fig. 5). Noninducible transcription factor USF was used as a control (Fig. 5A). NF-κB induction (Fig. 5B) in 22F6 cells was increased fivefold by the addition of the antibodies to the TCR-CD3 complex. The presence of H-7 attenuated the induction to a level comparable to that achieved in the 133 cells. Again, induction was not further decreased by H-7 in the 133 cells, because levels were already fivefold lower for the cells stimulated with anti-CD3 than for the Nef protein-expressing cells. When the oligonucleotide for AP-1 was used (Fig. 5C), similar results were obtained, with differences of eightfold for the anti-CD3 antibody stimulation of 22F6 cells compared with the 133 cells.

To corroborate that the effects on the regulation of the transcription factors were correlated with transcriptional activity, CAT assays were performed (Fig. 6). The percentage of acetylation was decreased by sixfold with the use of H-7 in 22F6 cells stimulated with anti-CD3 for HIV-1-CAT (Fig. 6A). The difference between the percentage of acetylation in 22F6 and 133 cells (Fig. 6B) in the absence of H-7 was 5.5-fold when cells were stimulated with anti-CD3. The results with the IL-2-CAT plasmid with both 22F6 (Fig. 6C) and 133 (Fig. 6D) cells again paralleled those of the HIV-1-CAT plasmid, except for the greater differences found between the Nef protein-expressing cells and the parental cell lines. Furthermore, the findings with IL-2-CAT reflect previously described Nef protein effects on endogenous IL-2 expression (26). The results presented above indicate that Nef protein down-regulates the induction of NF-κB and AP-1 by interacting with one of the steps involved in T-cell activation from the TCR-CD3-CD4 complex.

DISCUSSION

The function of HIV-1 Nef protein remains unclear. Whether the results of the experiments in vitro correlate with an in vivo situation is, at this point, impossible to ascertain. The only available animal data from the experiments with simian

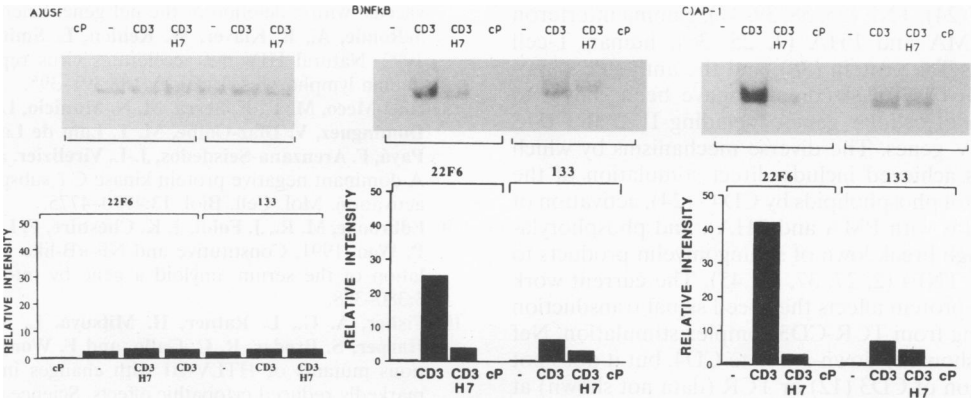

FIG. 5. Effect of Nef protein on induction of transcriptional factors AP-1 and NF-κB in T cells stimulated with cross-linked antibody to CD3 (CD3) or soluble CD3 in the absence of immobilized anti-mouse IgG (−). Gel shift analysis shows induction of transcriptional factors USF, NF-κB, and AP-1. H-7 was added 30 min prior to the incubation. cP, cold probe. Retarded DNA-protein complexes are shown; free DNA complexes are not shown but were equivalent in each lane. The experiments were performed twice with similar results. The relative intensity of the bands was evaluated by laser densitometry.

immunodeficiency virus may indicate the importance of the gene for the pathogenicity of the virus (6, 22) in vivo. Differences in the results of the in vitro experiments among various groups may be due to the use of different *nef* alleles (18, 43, 48), the conditions of the experiment (23), or the use of peripheral blood lymphocytes (7) versus stable cell lines. In our in vitro system, Nef protein has been shown to down-regulate both NF-κB (30) and AP-1 (31) after PMA and PHA stimulation.

The current work aimed at clarifying the mechanism of action of Nef protein by examining the pathways of signal transduction affected by this protein in T cells. Several signalling pathways have been described, although further clarification is still required. Multiple stimuli, including IL-1 (4, 9, 38),

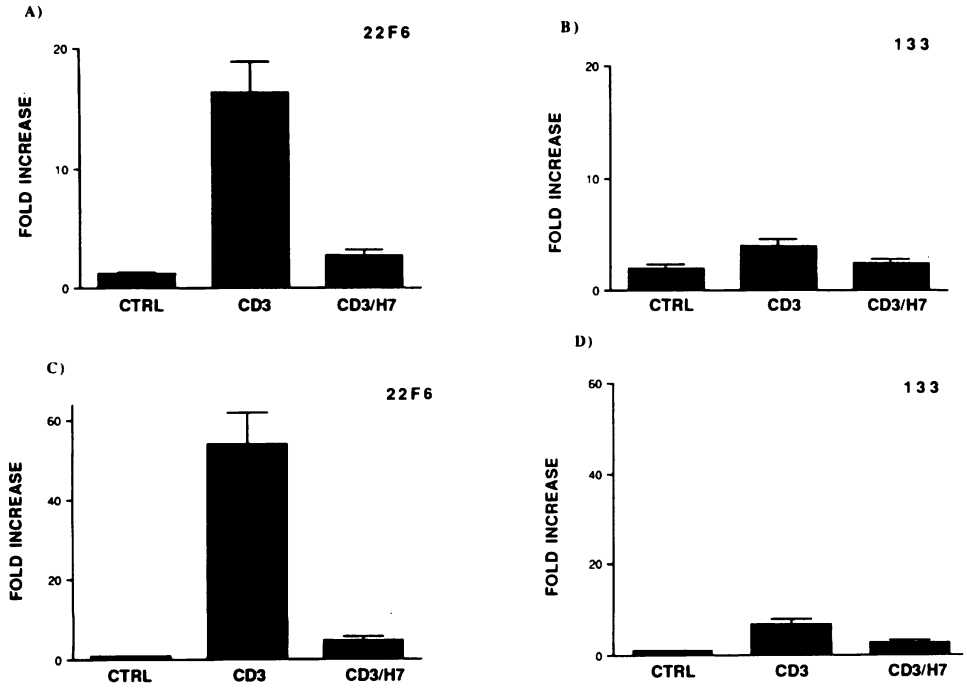

FIG. 6. CAT assay showing the effect of Nef protein on HIV and IL-2 transcription. Jurkat T cells (22F6 [A] is the parental cell line, and 133 [B] is the Nef protein-expressing cell line) were transfected with HIV-1-long terminal repeat-CAT and stimulated with cross-linked antibody to CD3 (CD3) or soluble CD3 in the absence of immobilized anti-mouse IgG (CTRL). H-7 was added 30 min prior to the incubation. Similar experiments with IL-2-CAT were performed with 22F6 cells (C) and 133 cells (D). Cells were transfected four times with each plasmid, a mean fold induction was calculated, and the standard deviation of the mean was determined. The percentages of acetylation for HIV-1-CAT were 0.3 ± 0.02 (CTRL), 4.1 ± 0.45 (CD3), and 0.75 ± 0.2 (CD3/H7) for 22F6 cells and 0.3 ± 0.06 (CTRL), 0.7 ± 0.1 (CD3), and 0.4 ± 0.06 (CD3/H7) for 133 cells. The percentages of acetylation for IL-2-CAT were 0.2 ± 0.03 (CTRL), 16.2 ± 0.8 (CD3), and 1.5 ± 0.25 (CD3/H7) for 22F6 cells and 0.4 ± 0.04 (CTRL), 2.1 ± 0.35 (CD3), and 0.9 ± 0.25 for 133 cells.

CD28 (46), CD45 (24), TNF (25, 38, 39, 47), gamma interferon (47), LPS (4), PMA and PHA (4, 25, 38), human T-cell lymphotropic virus Tax protein (38), and the antigenic stimulation of the TCR-CD3-CD4 complex, have been shown to induce expression of cellular genes, including IL-2 and IL-8 and surrogate HIV genes. The diverse mechanisms by which this stimulation is achieved include direct stimulation of the hydrolysis of inositol phospholipids by CD45 (24), activation of protein kinase C (as with PMA and PHA), and phosphorylation of I-κB through breakdown of sphingomyelin products to ceramide (as with TNF) (2, 27, 37, 42, 45). The current work indicates that Nef protein affects the T-cell signal transduction pathway originating from TCR-CD3 complex stimulation. Nef protein has been shown to down-regulate CD4, but it does not affect the expression of CD3 (12) or TCR (data not shown) at the cell surface. Therefore, Nef protein probably affects one of the downstream steps resulting from stimulation of the TCR-CD3 complex. Whether this is due to physical interaction with a component or components of this pathway, like the ζ subunit of the TCR, or whether it is due to enzymatic regulation of pathway intermediates like tyrosine kinases (*lck*) or tyrosine phosphatases remains to be determined. It is interesting that the effect of Nef protein on CD4 expression requires the cytoplasmic domain of CD4 (12), which includes two sites that bind to $p56^{lck}$ (5). Whether Nef affects CD4 expression and signal transduction by the same molecular interaction or through a different mechanism needs to be clarified.

To determine if the effects in vitro on transcriptional regulation correlate with the in vivo effects of Nef protein requires first an understanding of the molecular mechanisms of its action in vitro. It needs to be kept in mind that our in vitro system does not take into account circumstances that could be important in an in vivo situation and could be affected by Nef protein, such as reinfection of T cells. Therefore, we are unable to ascertain the relative importance of diverse activities of Nef protein. This report indicates that Nef protein interacts with the signal transduction pathway originating from the TCR-CD3 complex and does not affect the stimulation of T cells with the cytokines IL-1 and TNF. The complete definition of this system is required to determine if Nef protein is involved in the cellular latency of HIV and, if so, through which mechanism.

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