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Immunology

Tumor necrosis factor α and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor κB

(cytokines/T-cell activation/human immunodeficiency virus latency)

LAURELEE OSBORN*, STEVEN KUNKEL†, AND GARY J. NABEL*‡

*Howard Hughes Medical Institute, Departments of Internal Medicine and Biological Chemistry; and †Department of Pathology, University of Michigan Medical Center, Ann Arbor, MI 48109

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ABSTRACT **Binding of peptide hormones to surface membrane receptors leads to the transcription of specific genes within relevant target cells. How these signals are transduced to alter gene expression is largely unknown, but this mechanism probably involves a sequence of enzymatic steps that activate factors in the nucleus that modulate transcription. We now demonstrate that two different peptide hormones, or cytokines, stimulate the human immunodeficiency virus enhancer, and this effect is mediated by nuclear factor (NF) κB (nuclear factor that binds the κ immunoglobulin light chain gene enhancer). These cytokines, tumor necrosis factor α and interleukin 1, act on multiple cell types and represent the only naturally occurring activators of this transcription factor among eight cytokines examined. Although NF-κB binding can be stimulated by phorbol 12-myristate 13-acetate, tumor necrosis factor α acts through an independent mechanism, inducing NF-κB binding in HT-2 cells, which did not show increased binding in response to phorbol 12-myristate 13-acetate, and causing superinduction in Jurkat T-lymphoma cells. Tumor necrosis factor α is also a more selective activator of T cells than phorbol 12-myristate 13-acetate, having no effect on lymphokine production in EL-4 cells at the same time it induces NF-κB. These findings suggest that human immunodeficiency virus gene expression can be induced in T cells without activating lymphokine secretion and that the role of these cytokines in the activation of latent human immunodeficiency virus infection deserves further clinical evaluation. Finally, this link between binding at the surface membrane and stimulation of a specific transcription factor should help define intermediates for these cytokine activation pathways.**

Immunologic mediators affect genes that regulate cell proliferation and differentiation. The action of these peptide hormones, or cytokines, at the surface membrane probably leads to the activation of factors in the nucleus that modulate transcription. One transcription factor responsive to cellular activation is nuclear factor κB (NF-κB), which stimulates the human immunodeficiency virus (HIV) enhancer in T cells treated with phorbol esters (1). NF-κB recognizes an 11-base-pair DNA sequence present in the immunoglobulin light chain (2) and several primate virus enhancers (3–6). Sequences resembling κB sites are also associated with cell-surface products, including the class I major histocompatibility antigen (7, 8), β_2-microglobulin (9), and the interleukin 2 (IL-2) receptor α chain (6, 10, 11). Thus, NF-κB-like transcription factors may normally regulate expression of cellular genes associated with cell proliferation and recognition.

Mutations of the κB sites in the HIV enhancer that abolish binding of NF-κB eliminate inducibility of the HIV enhancer. Binding of NF-κB can be activated by phorbol 12-myristate 13-acetate (PMA) (1, 12), protein synthesis inhibitors such as cycloheximide or anisomycin (12), or the transactivator of human T-cell lymphotropic virus type I, tax_1 (6). In 70Z or Hela cells, NF-κB binding is superinduced by PMA in combination with protein synthesis inhibitors (12), indicating multiple mechanisms of activation. Recently, a specific inhibitor has been implicated as one such regulator of NF-κB (13, 14). Although treatment with PMA stimulates NF-κB binding activity, physiologic activators of this transcription factor were unknown. In this report, we have examined whether peptide hormones activate NF-κB binding and stimulate the HIV enhancer and have analyzed the mechanism and specificity of NF-κB activation in T cells.

MATERIALS AND METHODS

Cell Culture. Cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (50 units/ml), and streptomycin (50 μg/ml) with freshly added glutamine in a humidified incubator containing 5% CO_2. The 70Z leukemia cell medium was additionally supplemented with 2-mercaptoethanol (50 μM), BaF3 cell medium was supplemented with recombinant interleukin 3 (IL-3) (100 units/ml), and HT-2 cell medium was supplemented with rat spleen Con A-conditioned medium (Collaborative Research). The 70Z cells were obtained from the laboratory of D. Baltimore, BaF3 cells from R. Palacios, and HT2 from M. Kluger.

Nuclear Extracts and Electrophoretic Mobility-Shift Assay. Electrophoretic mobility-shift assay was performed with a κB probe (6) as described (1) using 20 μg of cell extract in the presence of 1 μg of dIdC. Extracts were prepared for analysis from whole cells or nuclei by a rapid method, modified from Dignam *et al.* (15). Briefly, for cellular extracts, 10^7 cells were washed once with phosphate-buffered saline and twice with buffer A (15). The cell pellet was suspended in buffer C (15)/0.1% Nonidet P-40 (20 μl per 10^7 cells). After incubating for 15 min on ice, the lysed cellular suspension was briefly mixed on a Vortex and microcentrifuged for 10 min at 4°C. The supernatant was diluted with 80 μl per 10^7 cells of buffer D (15) modified to contain 0.05 M KCl instead of 0.1 M and stored at −70°C. Nuclear extracts were prepared by resuspending 10^7 washed cells (see above) in 20 μl of buffer A/0.1% Nonidet P-40, incubating 10 min on ice, mixing

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Abbreviations: CAT, chloramphenicol acetyltransferase; HIV, human immunodeficiency virus; IL-1, -2, -3, and -4, interleukin 1, 2, 3, and 4, respectively; NF-κB, nuclear factor κB (nuclear factor that binds the immunoglobulin κ light chain gene enhancer); PMA, phorbol 12-myristate 13-acetate; TNF-α, tumor necrosis factor α; ICPO, infected cell protein; GM-CSF, granulocyte/macrophage colony-stimulating factor.

‡To whom reprint requests should be addressed.

briefly, and microcentrifuging for 10 min at 4°C. The supernatant was removed, and the nuclear pellet was suspended in 15 μl of buffer C, incubated 15 min at 4°C, mixed briefly, and microcentrifuged for 10 min at 4°C. The supernatant was diluted with 75 μl of modified buffer D (see above) and stored at −70°C. Comparable NF-κB binding activity was seen with ≈20 μg of cellular extract, 8 μg of nuclear extract, or 8 μg of nuclear extract prepared by the method of Dignam *et al.* (15) using stimulated Jurkat or 70Z cells.

Chloramphenicol Acetyltransferase (CAT) Assay. Cell extracts were prepared 44 hr after transfection, protein concentrations were normalized, and conversion of chloramphenicol to its acetylated forms was assayed by standard methods (1, 16). Results are representative of at least three independent transfections, and SD for each CAT determination was ≤ 10%. Percentage conversions of [^{14}C]chloramphenicol to its acetylated forms are indicated.

Cell Proliferation. Supernatants from EL-4 cells incubated with different agents were prepared in RPMI 1640 medium/5% fetal bovine serum/penicillin at 50 units/ml and streptomycin at 50 μg/ml and collected after 24 hr. Measurement of secreted growth factor was accomplished by using the HT-2 cell line and a colorimetric assay to determine proliferation (17).

RESULTS

Stimulation of the HIV Enhancer in Jurkat Cells: Dependence on κB Sites. To determine whether cytokines affect gene expression dependent on the HIV enhancer, we transfected Jurkat cells with a plasmid containing the HIV enhancer linked to the CAT gene, HIV–CAT (1). One day after transfection, cells were incubated for an additional 20 hr with recombinant human interleukin 1 (IL-1) (α plus β forms), IL-2, interleukin 4 (IL-4), interferon γ, tumor necrosis factor α (TNF-α) or granulocyte/macrophage colony-stimulating factor (GM-CSF). No significant change in CAT activity was seen except for an 8-fold increase in the presence of TNF-α (Fig. 1*A*). This induction increased in response to the concentration of TNF-α and was inhibited by specific antibodies to TNF-α (Fig. 1*B*). The dose-response curve was approximately logarithmic in a physiologic range of 1–100 units/ml.

To examine whether this stimulation is dependent on the κB sequences, we transfected cells with a mutant HIV–CAT plasmid that contains 6-base-pair changes that abolish NF-κB binding (1). Jurkat cells containing the *tat*-I gene were used for this analysis because they allow more efficient expression of these plasmids but do not differ in their regulation from normal Jurkat cells (18). Although both plasmids can be activated by a herpesvirus transactivator, infected cell protein O(ICPO) (Fig. 2, see also ref. 18), the κB mutant plasmid is not induced by TNF-α (Fig. 2), indicating a requirement for the κB sites.

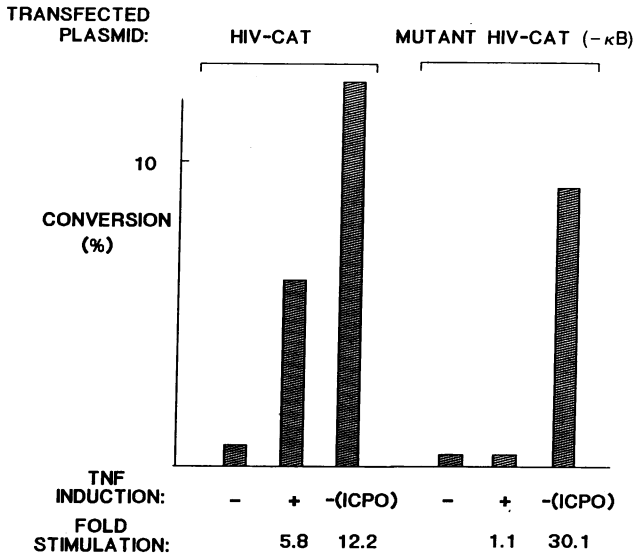
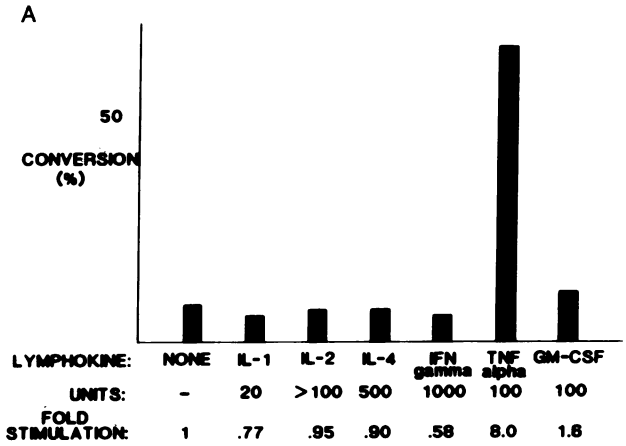


FIG. 2. TNF-α activation of the HIV enhancer is dependent on κB sites. Jurkat *TAT*-III cells (10^7) (18, 19) were transfected with HIV-CAT (10 μg) or a mutant plasmid (10 μg) altered in both κB sites (1). Twenty-four hours after transfection, an aliquot of cells was incubated for an additional 20 hr in the presence of recombinant TNF-α (100 units/ml). Positive controls were obtained by cotransfection with an ICPO-containing plasmid (5 μg), pSHZ (18), in the absence of TNF-α.

Specificity of TNF-α-Inducible NF-κB and Superinduction. The ability of TNF-α to induce NF-κB binding was analyzed by using an electrophoretic mobility-shift assay with Jurkat cell extracts incubated with or without TNF-α. A TNF-α-inducible complex comigrated with the PMA-inducible complex (Fig. 3*A*, lanes 1 and 3) and was specifically competitive with the unlabeled κB site fragment but not with an unrelated fragment (Fig. 3*A*, lanes 4–7). This effect was seen in other cell types, including the 70Z pre-B and U937 promonocytic leukemia lines (Fig. 3*B*).



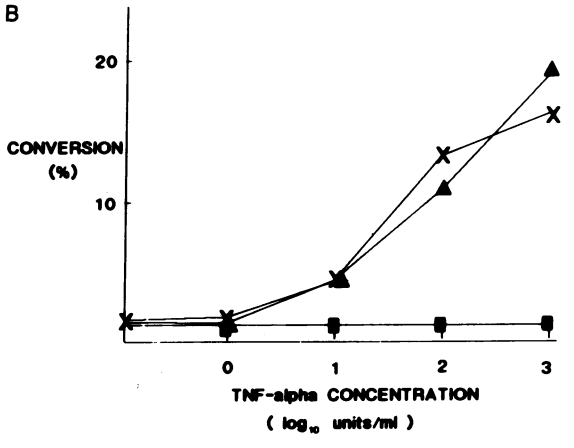


FIG. 1. Activation of HIV–CAT expression by cytokines in Jurkat cells and specificity of induction by recombinant TNF-α. (*A*) Jurkat cells (10^7) were transfected with HIV–CAT plasmid (10 μg) using DEAE-dextran (1) and divided into groups; 24 hr after transfection, the indicated amounts of each recombinant lymphokine or cytokine (Genzyme) were added, and incubation was continued for 20 more hr. IFN, interferon. (*B*) Jurkat cells were transfected with HIV–CAT as above and incubated with increasing amounts of human TNF-α (X) (Genzyme) 24 hr after transfection for an additional 20 hr. To verify the specificity of the stimulant a preimmune serum (▲) or antiserum (■) prepared against human TNF-α (1:100) was added to the culture medium 10 min before addition of the indicated amounts of recombinant TNF-α.

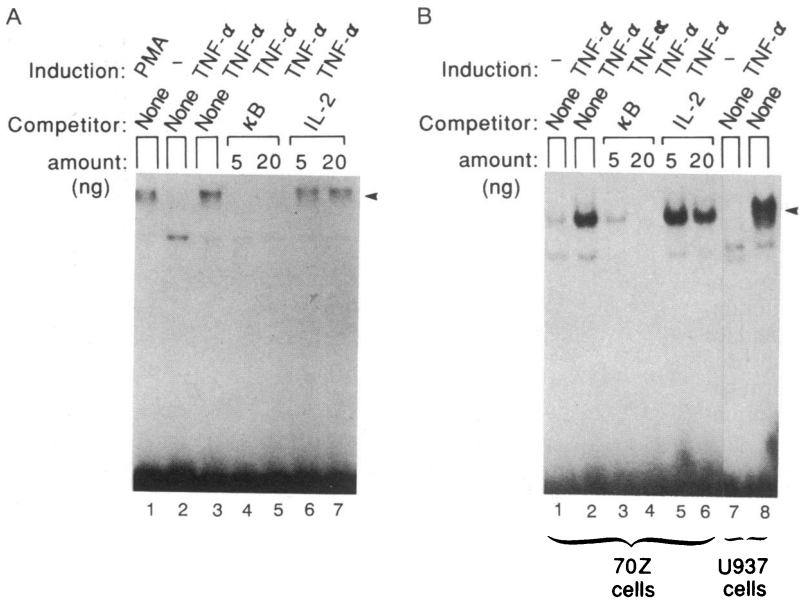


FIG. 3. Induction and specificity of NF-κB binding by TNF-α in Jurkat, 70Z, or U937 cells. (*A*) Analysis of TNF-α-inducible complexes in Jurkat cells with the electrophoretic mobility-shift assay. Cellular extracts were prepared from cells incubated for 2–4 hr in the presence of 40 nM PMA (Sigma) (lane 1), medium alone (lane 2), or recombinant TNF-α at 100 units/ml (lane 3). Competition studies were performed using extracts from TNF-α-stimulated cells (100 units/ml) and the indicated amounts of double-stranded oligonucleotide competitor containing κB site (6) (lanes 4 and 5) or an unrelated IL-2 promoter fragment (lanes 6 and 7) as described (6, 20). Arrows in *A* and *B* denote specific inducible complexes competitive with double-stranded κB oligonucleotide. (*B*) TNF-α induction of NF-κB in 70Z and U937 cells. Cellular extracts were prepared from 70Z cells incubated with medium alone (lane 1) or with TNF-α at 100 units/ml (lane 2). Competition studies were done using extracts from cells stimulated for 2–4 hr with TNF-α (100 units/ml) in the presence of κB fragment (lanes 3 and 4) or an unrelated IL-2 fragment (lanes 5 and 6) as above. Cellular extracts from U937 cells, unstimulated (lane 7), or induced with TNF-α at 100 units/ml (lane 8), were also analyzed.

To examine whether NF-κB activation by TNF-α occurs through a PMA- or a cycloheximide-related pathway, extracts were prepared from cells treated with these agents. Incubation in the presence of either TNF-α or cycloheximide alone induced comparable binding activity in Jurkat cells (Fig. 4); a combination of both stimulated little additional binding in Jurkat cells. In contrast, addition of TNF-α in the presence of optimal amounts of PMA caused superinduction of NF-κB similar to that seen in the presence of PMA and cycloheximide (Fig. 4), suggesting that TNF-α acts through a pathway similar to that of cycloheximide.

TNF-α Effect on Lymphokine Production. To further compare TNF-α and PMA stimulation, we analyzed expression of T-cell growth factors synthesized by EL-4 cells. In EL-4 cells, lymphokine secretion is induced by PMA (Fig. 5*B*, ref. 21). Both TNF-α and PMA stimulated NF-κB binding in these cells (Fig. 5*A*); however, TNF-α did not stimulate secretion of T-cell growth factor (Fig. 5*B*). Thus, induction of NF-κB by TNF-α occurs through a cellular activation pathway that differs from PMA. The effect of TNF-α is probably unrelated to its cytotoxic effect on some cell types: it did not inhibit lymphokine secretion when added in the presence of PMA (Fig. 5*B*), and no morphologic changes occurred in cells treated with TNF-α at concentrations that induced NF-κB.

NF-κB Activation in Known Cytokine-Responsive Cells. TNF-α is distinctive among the cytokines examined in this study because it activates NF-κB expression in Jurkat cells. Because Jurkat cells may not bear surface receptors for some of the cytokines tested, we have tested several known factor-responsive cell lines. Stimulation by IL-2, IL-3, IL-4, or GM-CSF in the relevant factor-dependent lines did not stimulate NF-κB binding activity (Fig. 6, lanes 10, 11, 14, 17). In contrast, stimulation by IL-1 in the 70Z pre-B leukemia cell strongly activated NF-κB binding (Fig. 6, lane 3). Because 70Z cells have shown clonal variation in IL-1 and TNF-α responsiveness and are not easily transfected, we have

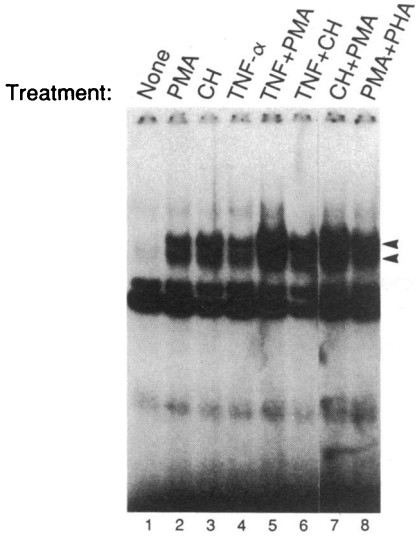


FIG. 4. TNF-α activates NF-κB binding activity through a PMA-independent mechanism. Extracts were prepared from Jurkat cells incubated alone or for 4 hr in the presence of 40 nM PMA, cycloheximide (CH) at 10 μg/ml, TNF-α at 100 units/ml, TNF-α plus PMA, TNF-α plus CH, CH plus PMA, or PMA plus phytohemagglutinin at 2 μg/ml (Sigma). Results are representative of at least three independent experiments. Arrows designate NF-κB binding activity that competes specifically with excess unlabeled probe.

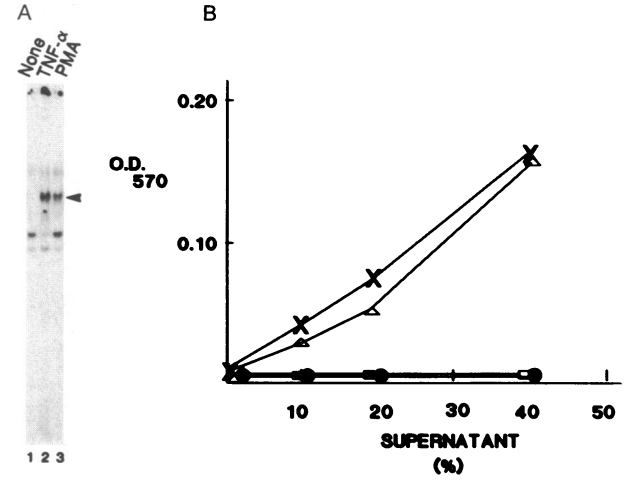


FIG. 5. Activation of NF-κB binding is not associated with lymphokine production in EL-4 cells. (*A*) Extracts were prepared from EL-4 cells incubated alone or for 4 hr in the presence of TNF-α at 100 units/ml or PMA. Electrophoretic mobility-shift assay was performed using a κB probe (6). Arrows denote specific inducible complex competitive with the double-stranded κB oligonucleotide. (*B*) EL-4 cells were incubated for 24 hr in medium alone (●) or in the presence of 40 nM PMA (X), TNF-α at 100 units/ml (□), or PMA and TNF-α (△). Cell proliferation of HT-2 cells was determined using a colorimetric assay as described (17).

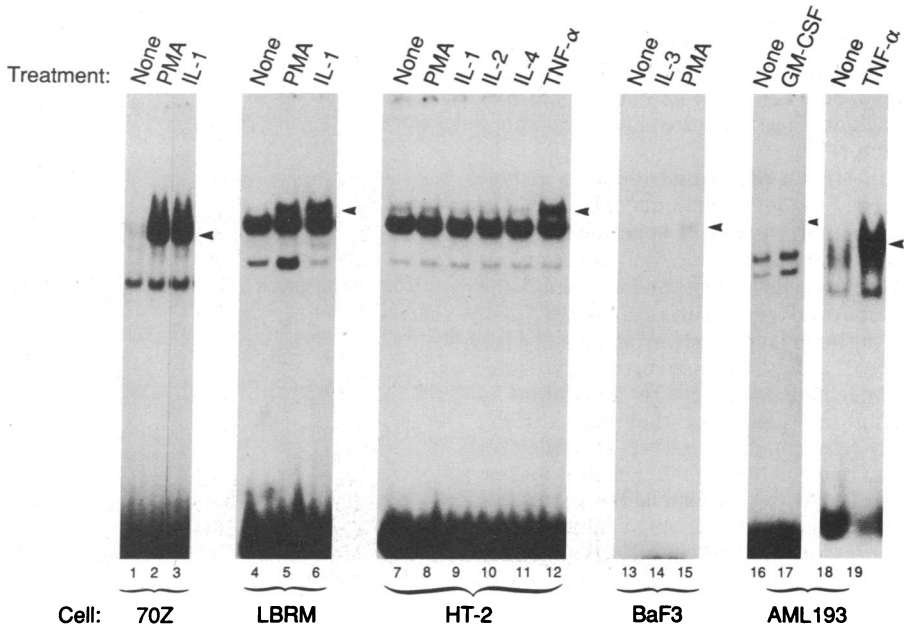


FIG. 6. Cytokine activation of NF-κB binding in various factor-responsive cells. Cells were treated with the indicated cytokines from the appropriate species for 2–4 hr. Nuclear extracts were prepared from 70Z cells, a mouse pre-B cell leukemia responsive to IL-1 (lanes 1–3); LBRM cells, a mouse T-cell line that secretes IL-2 in response to stimulation with IL-1 (lanes 4–6) (22); HT-2 cells, a mouse helper T cell that proliferates in the presence of IL-2 or IL-4 (lanes 7–12); BaF3 cells, a mouse lymphoid cell dependent on IL-3 (lanes 13–15) (23); or AML 193, a human granulocytic leukemia cell, provided by S. Emerson (University of Michigan Medical Center), which proliferates in response to GM-CSF and other cytokines (lanes 16 and 17). Proliferation in the presence of these lymphokines was determined as described for Fig. 5 at the same time that nuclear extracts were prepared for analysis by the electrophoretic mobility-shift assay. Arrows denote specific inducible complex that is competitive with double-stranded κB oligonucleotide.

examined other IL-1-responsive cells. NF-κB binding may be stimulated by IL-1 in other lines, including LBRM (Fig. 6, lane 6), a mouse T-cell leukemia line (22). HIV–CAT activity is also stimulated by IL-1 in these cells and is dependent on the presence of intact κB sites (Fig. 7). In the HT-2 cell line, NF-κB binding activity was not stimulated by PMA but was markedly increased by TNF-α (Fig. 6, lanes 8 and 12), providing further evidence of distinct activation pathways.

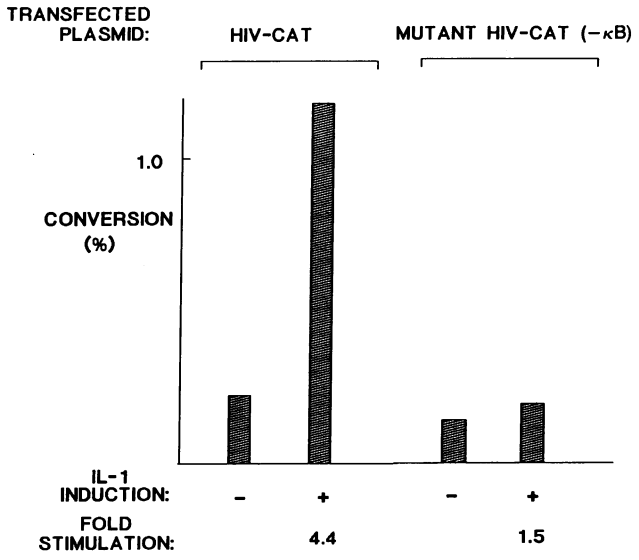


FIG. 7. IL-1 activation of the HIV enhancer is dependent on κB sites. LBRM cells (10^7) (22) were transfected using DEAE-dextran with HIV–CAT (10 μg) or a mutant plasmid (10 μg) altered in both κB sites (1). Twenty-four hours after transfection, an aliquot of cells was incubated for an additional 20 hr in the presence of recombinant IL-1α (50 units/ml; Genzyme).

DISCUSSION

TNF-α and IL-1 are distinctive among the eight cytokines tested in this study in their ability to stimulate the HIV enhancer. Because activation of the HIV enhancer can occur through multiple independent sites (18), it was necessary to define the cis-acting target sequences in the enhancer. Our results demonstrate that stimulation by both cytokines is dependent on the κB sites. Although NF-κB is a PMA-inducible transcription factor known to activate the HIV enhancer in T cells, physiologic activators of this transcription factor have not, to our knowledge, been identified. These cytokines, both products of activated macrophages, thus represent specific physiologic activators of NF-κB and the HIV enhancer. TNF-α, in particular, is apparently more specific in its transcriptional activation than PMA. For example, lymphokine secretion by EL-4 cells, which can be stimulated by PMA, is unaffected by TNF-α (Fig. 5). TNF-α activation thus appears to act through a set of cellular transcription factors different, and probably more restricted, from those stimulated by PMA. Analysis of transcription factors stimulated by peptide hormones may provide the basis to define pathways in lymphoid cells.

It has recently become apparent that there is heterogeneity among DNA-binding proteins that recognize κB-like sites. Although the H2TF1 site (7, 8, 24) and the IL-2 receptor α κB site (6, 10, 11) compete for binding to NF-κB, evidence suggests differences among these proteins. For example, H2TF1 activity may be found in mouse erythroleukemia or HeLa nuclear extracts that lack NF-κB binding activity (7, 8). The κB site for the IL-2 receptor α subunit gene is also different from the canonical κB site because it is not PMA responsive (6). The cytokine-inducible κB binding activity reported here, however, is indistinguishable from NF-κB: it binds to the κB site, is specifically competitive with κB, activates the HIV enhancer, and does not stimulate expression dependent on the IL-2 receptor α κB site (data not

shown). Although multiple bands were noted occasionally (Fig. 4), no consistent patterns emerged to suggest a distinct TNF-inducible factor. Nonetheless, because it is not possible presently to distinguish between all potential NF-κB-like factors, we cannot exclude that TNF-α induces a distinct NF-κB-like binding factor.

TNF-α stimulation of NF-κB binding is similar to activation by cycloheximide in Jurkat T-lymphoma cells. IL-1, in contrast, gives no consistent pattern of superinduction in combination with PMA or cycloheximide (data not shown). Although cycloheximide has been suggested to inhibit the synthesis of a labile inhibitor, or repressor, of NF-κB (12–14), whether this mechanism might function physiologically has been unknown. TNF-α, a naturally occurring cytokine, activates through a mechanism similar to activation by protein synthesis inhibitors, suggesting that this pathway may be physiologically relevant. Because NF-κB binding is stimulated by TNF-α in one cell line, HT-2, which shows no increase in response to PMA, this mechanism is apparently independent. Whether this activation occurs by inhibiting the synthesis of a labile repressor is unknown. NF-κB activation may involve both dissociation from an inhibitor and, possibly, translocation of the binding protein to the nucleus (13, 14). It remains possible that TNF-α may regulate either removal of an inhibitor, localization to the nucleus, activation of the binding protein, or some combination.

Our findings also have implications for the pathogenesis of HIV disease. It has been found recently that HIV replication can be stimulated by TNF-α in a chronically infected T-cell line (25). Although previous studies had shown that HIV replication increases after T-cell activation (26, 27), whether this activation was obligately linked to lymphokine secretion was unclear. Our data suggest that activation of latent virus might occur through TNF-α activation of NF-κB without any lymphokine secretion. These findings suggest that HIV replication might be blocked by TNF-α antagonists in cells that do not secrete lymphokines.

Both IL-1 and TNF-α appear to induce NF-κB in multiple cell types. In particular, TNF-α activates NF-κB in T and promonocytic (U937) cell lines (Figs. 3 and 6), cell types that may both be relevant to latent HIV infection (28, 29). Although more evidence accrues that TNF-α plays a role in normal lymphocyte differentiation and function (30, 31), the role of TNF-α in HIV activation is less clear. Early studies showed no effect on HIV production *in vitro* (32, 33); however, the presence of TNF-α receptors or NF-κB stimulation was not determined in these cell lines. A recent study has shown that TNF-α activates HIV expression in a chronically infected CEM T-leukemia cell (25), raising the possibility that TNF-α plays a role in activation of HIV replication through NF-κB. Because TNF-α is synthesized actively in a variety of clinical infections (34–38) and elevated levels of circulating TNF-α have been detected in AIDS patients (39), the role of TNF-α and IL-1 in the activation of HIV deserves further clinical evaluation.

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