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A Tat-Induced Auto-Up-Regulatory Loop for Superactivation of the Human Immunodeficiency Virus Type 1 Promoter

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The virus-encoded Tat protein strongly activates transcription of human immunodeficiency virus (HIV). A well-recognized mechanism involves interaction of Tat with the nascent RNA transcript of the viral *tar* gene; mutation of *tar* greatly decreases activation by Tat. However, Tat still provides a low level of activation, demonstrating that it also has a *tar*-independent mode of action. We propose that this *tar*-independent mode of Tat action is through activation of gene transcription to produce tumor necrosis factor alpha. This cytokine and other compounds that activate NF-κB up-regulate the HIV promoter at a low level, similarly to the second Tat action. Through this mechanism, they also activate promoters of tumor necrosis factor alpha and other cytokines and thereby establish an auto-up-regulatory loop. Activated NF-κB motifs in the HIV promoter synergize with Tat/*tar*. Mutations of these motifs decrease activation by Tat to a few percent of the wild-type value. In cooperation, the two modes of activation by Tat (*tar* dependent and cytokine based) set up positive up-regulatory loops which greatly superactivate transcription of HIV. Agents that block these synergistic pathways at three different steps and are more inhibitory in combination than is any one alone have been found. Thereby, multidrug modalities for transcription of HIV are proposed for virus suppression.

Tat, a regulatory protein encoded by the HIV genome, plays a unique role in the emergence of the virus from the latent state. Viruses with a defective Tat gene cannot proliferate and do not show cytopathic effects. Tat stimulates HIV-1 gene expression by increasing the rate of transcription and length of transcripts (20, 24, 54, 57).

Tat-induced activation of HIV-1 promoter action is mediated via a virus sequence known as the *trans*-activator response (*tar*) element, extending from −17 to +42 bp (17, 57). Inactivation of *tar* greatly decreases the activation by Tat. The minimum Tat-responsive element is reported to be the sequence from +19 to +42 (32). Tat binds to *tar* RNA and then interacts with a transcriptional initiation complex (9, 16–18) composed of both DNA- and RNA-binding proteins, which regulates transcription from the LTR (5, 25, 28, 42, 54).

Tat has other modes of action. Expression of Tat in human cells in culture led to overexpression of cellular genes encoding cytokines (12, 48, 51, 55). Tat also *trans*-activated the *tar*-less TNF promoter (12) and potentiated TNF action (61). Cytokine overproduction by HIV-infected, Tat-producing cultured $CD4^+$ human T lymphocytes and monocytes was reported (12, 48, 51). Consistent with these results, HIV-1-infected, Tat-producing patients have significant elevations of cytokine levels in plasma (11, 21, 37, 38, 50) and cytokine-specific mRNAs (21). These cytokines increase virus replication in HIV-1-infected cells and thereby play an important role in the pathogenesis of the disease (45–47). These results suggest that this viral protein could influence the HIV-1 promoter, by a *tar*-independent mechanism.

Cellular gene products can activate HIV replication, as well as cytokine production. A low level of activation is caused by compounds such as TNF-α, a cytokine that activates the NF-κB sequences in the HIV promoter. We propose that an important part of Tat activation of HIV is through *tar*-independent activation of cytokine gene transcriptions, producing cytokines such at TNF-α which activate NF-κB in the HIV promoter.

Different cellular transactivators and LTR enhancer elements demonstrate various levels of interaction with Tat and *trans*-activation of the HIV-1 promoter (5, 33, 36, 60). "Superactivation" of HIV-1 LTR-driven gene expression is induced by the concerted interaction of cellular *trans*-activator NF-κB and viral protein Tat (4, 6, 15, 34, 35, 39). The interaction between SP1 and Tat similarly superactivates HIV-1 gene expression (31, 33). We have reported that NF-κB and Tat/*tar* cooperate to superactivate HIV-1 LTR-driven reporter gene expression in cultured human $CD4^+$ Jurkat T lymphocytic cells. This superactivation was observed in the absence of an externally added activator of NF-κB and was somewhat increased by these activators (6). These results suggest that Tat activates NF-κB. Mutations of these motifs decrease activation to a few percent of the wild-type value.

We have also defined roles of cellular genes and of cytokines such as TNF-α in the Tat/NF-κB-induced superactivation of HIV-1 promoter and virus replication by using specific inhibitors of NF-κB, Tat, and TNF-α actions (6). Compounds that interfere with such multifactorial interplay inhibit viral gene expression and replication (reference 7 and unpublished data). The *tar*-dependent and cytokine-based activations together set up positive-feedback loops. Agents have been found that block these pathways at three different steps and that are more inhibitory in combinations than is any one.

Whether and how Tat can activate the HIV-1 promoter by a *tar*-independent mechanism is unclear. These results prompt us to make three postulations. (i) One action of Tat in inducing HIV-1 promoter activation is mediated via a pathway which is independent of the viral element *tar*. (ii) By this pathway, Tat induces overexpression of cellular cytokine genes that activate NF-κB, which exists as an inactive complex in the cytoplasm of

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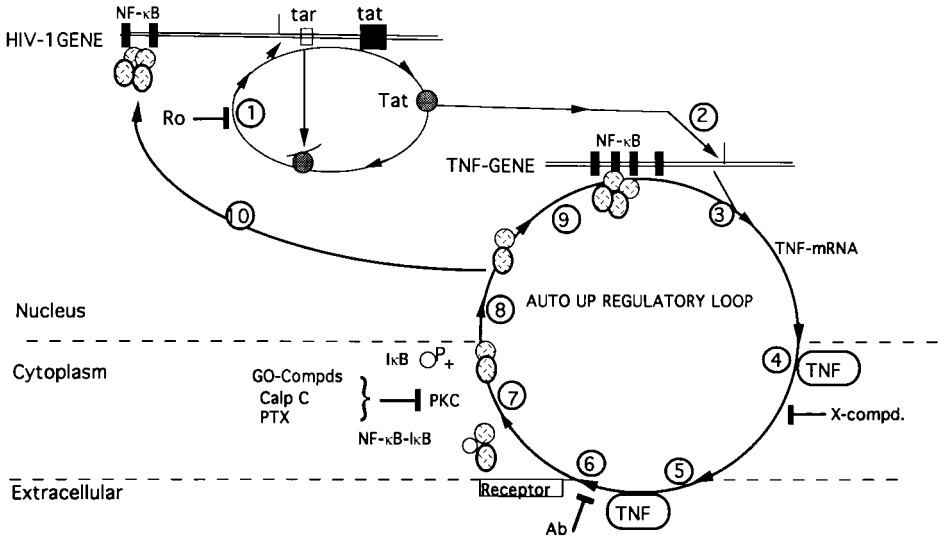

FIG. 1. Schematic description of a Tat-induced auto-up-regulatory loop, and two modes of Tat action in superactivating the HIV-1 promoter. Steps of the *tar*-dependent and *tar*-independent pathways of Tat action in superactivating the HIV-1 promoter function are indicated by numerals. The HIV-1 LTR sequence is shown at the top left, and the cytokine (TNF) promoter is shown to the right. Steps: 1, production of Tat protein from the Tat gene and its activation of the HIV-1 promoter via *tar*; 2, up-regulation of the cytokine promoter by Tat protein; 3, elevation of cytokine mRNA levels; 4, increased cytokines in the cytoplasm; 5, secretion of cytokines; 6, interaction of cytokines with receptors and signal transduction pathway; 7, protein kinase C (PKC)-mediated release of active NF-κB from the inactive complex; 8, translocation of NF-κB into the nucleus; 9, up-regulation of the cytokine promoter and completion of the *tar*-independent loop; 10, interaction of NF-κB with its motifs in the LTR and Tat/NF-κB-mediated superactivation of the HIV-1 promoter. Abbreviations: Ab, antibody; GO-Compds, Gö-6976; Calp C, calphostin C.

cells other than B cells (26, 56; for a review, see reference 27). (iii) NF-κB in turn activates the HIV-1 promoter and also cytokine promoters and establishes an auto-up-regulatory loop (Fig. 1).

The experimental goal of this study is to examine these possibilities by establishing a *tar*-independent pathway of Tat-induced *trans*-activation of the HIV-1 promoter and thereby to define the in vivo relevance of the two modes of action of Tat, i.e., *tar* dependent and *tar* independent, in the context of Tat/NF-κB-induced superactivation of the HIV-1 promoter. We provide evidence to support two separate pathways of action of Tat. We postulate that their synergism induces superactivation of the viral promoter and of virus replication.

MATERIALS AND METHODS

Abbreviations. HIV-1, human immunodeficiency virus type 1; Tat, *trans*-activator of transcription; *tar*; *trans*-activating response element; LTR, long terminal repeat; TNF-α, tumor necrosis factor alpha; PMA, phorbol 12-myristate 13-acetate; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; PTX, pentoxifylline, IL-1 and IL-6, Interleukin-1 and interleukin-6.

Cells. Jurkat CD4$^+$ T lymphocytes and a stable transfectant of Jurkat cells in which the simian virus 40 promoter-driven *tat* gene is constitutively expressed (Jurkat-Tat; obtained from J. Sodroski, Dana-Farber Cancer Institute, Boston, Mass.) were maintained in RPMI 1640 (GIBCO/BRL, Gaithersburg, Md.) tissue culture medium supplemented with 10% fetal bovine serum at 37°C in an atmosphere of 5% CO_2 and 95% air. G418 (Sigma Chemical Co., St. Louis, Mo.) at 400 μg/ml (100% potency) was added to the culture medium of Jurkat-Tat cells.

Reagents. PTX, Ro24-7429, and Gö-6976 were gifts from Hoechst-Roussel Pharmaceuticals, Sommerville, N.J., Hoffman-La Roche, Nutley, N.J., and Parke-Davis Co., Ann Arbor, Mich., respectively. DEAE-dextran, acetyl coenzyme A, Geneticin (G418), and PMA were from Sigma Chemical Co., St. Louis, Mo. TNF-α and anti-human TNF-α antibody were from Endogen, Cambridge, Mass. [^{14}C]chloramphenicol was from DuPont/NEN. The complementary strands of the oligonucleotide (5′-TCGAC**AGGGACTTTCC**GAGAG-3′) containing the NF-κB motif (in boldface type) were custom synthesized in the core facilities of Dana-Farber Cancer Institute.

Plasmids. The plasmids pHIV-1 LTR-CAT and pHIV-1 LTR-mut-NF-κB-CAT (43) were gifts from G. Nabel, Howard Hughes Medical Institute, University of Michigan Medical Center, Ann Arbor. The fusion plasmid pHIV-LTR-Δ*tar*-CAT (54) and the expression plasmid pSV-Tat (14) were gifts from J. Sodroski, Dana-Farber Cancer Institute. The fusion gene constructs with 1,087 and 109 bp of 5′-flanking region of mouse TNF promoter ligated to the CAT gene (pTNF-CAT-TNF-1087 and pTNF-CAT-109) were constructed by Bazzoni et al. (3). These and other fusion gene constructs with different lengths of 5′ TNF promoter were gifts from B. Beutler, University of Texas Southwestern Medical Center, Dallas, and C. V. Jongeneel, Ludwig Institute for Cancer Research, Lausanne, Switzerland.

Transfections. Transient transfections of Jurkat cells with the specified fusion plasmids were performed by the DEAE-dextran procedure as described previously (8). Results of all the transient-transfection studies were normalized for transfection efficiency by cotransfection of pSV-*lacZ* plasmid and measurement of β-galactosidase activity along with CAT activity in the control and treated cell extracts (40). Simian virus 40 promoter-driven β-galactosidase activity was not influenced by the treatment conditions described in this study. The procedures for extract preparation, protein content (10), CAT activity, and β-galactosidase activity were described previously (7, 8).

EMSA. Nuclear extracts from control and treated cells were prepared as described by Dignam et al. (23). Reaction conditions with ^{32}P-labeled double-stranded oligonucleotide carrying the NF-κB motif and EMSA conditions were the same as described previously (7). Details of the quantitation of CAT activity, β-galactosidase activity, and autoradiographic signals of EMSA and Northern (RNA) blot analysis are described in the figure legends.

RESULTS

Activation of HIV LTR by NF-κB and by Tat. Basal reporter CAT activity, using our standard assay conditions (7, 8) and the unmutated pHIV-1 LTR-CAT plasmid, was stimulated only about three- to fourfold by PMA or TNF-α (Fig. 2A). A dramatically greater induction of CAT was observed when Tat was provided to the wild-type HIV promoter construct as reported previously (6). Tat induced an up-regulation of HIV-1 promoter with functional NF-κB (Fig. 2B) that was 70-fold higher than the basal level in unstimulated cells. PMA or TNF-α further stimulated this Tat-induced superactivation by less than twofold, to a lesser degree than the activation by these compounds observed in the absence of Tat (Fig. 2A). These results suggest that when both pathways of activation by Tat are functional they synergize and that the second pathway

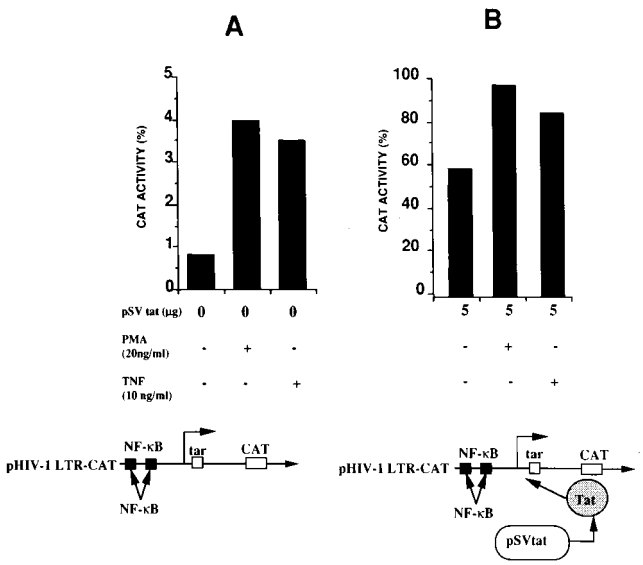

FIG. 2. Up-regulation of the HIV-1 promoter in the absence or presence of activators of NF-κB. Jurkat cells were transiently transfected (6) with 10 μg of plasmid pHIV-1 LTR-CAT (A) plus indicated amounts of pSV-Tat (B). Some cells were treated with 20 ng of PMA per ml or 10 ng of TNF-α per ml for 18 and 4 h, respectively, before extracts were prepared 72 h after transfection, and enzyme assays (CAT and β-galactosidase) were performed as described previously (6, 8).

operates through production of a subsaturating level of TNF-α.

A second, *tar*-independent, pathway of activation of HIV-1 promoter by Tat. The two pathways of activation by Tat are shown schematically in Fig. 1. The Tat/*tar*-mediated pathway is designated as *tar* dependent (Fig. 1, step 1). The *tar*-independent, cytokine-mediated pathway is designated the auto-up-regulatory loop (Fig. 1, steps 2 to 9).

The *tar*-dependent first pathway was studied by transient transfection of Jurkat cells with the fusion plasmid pHIV-1 LTR-mut-NF-κB-CAT (43, 44) cotransfected with the Tat-expressing vector pSVTat (14) followed by measurements of CAT reporter gene expression (6) (Fig. 3A). Activation of the HIV-1 promoter via NF-κB transcription was not operative in this assay because of the specific mutations in the LTR. HIV-1 LTR-driven reporter CAT activity was undetectable in control transfections with pSV-*lacZ* (lane designated by 0 Tat) or the plasmid backbone without the insert (data not shown), suggesting that contributions by other *trans*-activators such as SP1 were very minimal and undetectable in this system. The input expression vector pSV-Tat demonstrated a concentration-dependent stimulation of reporter gene expression, which correlated with elevated levels of Tat-specific mRNA measured in transiently transfected Jurkat cells. A stimulation of the basal (0.9%) reporter gene expression following transfection of the cells with pSVTat was observed, ranging from three- to fivefold in three separate transfection experiments. TNF-α or PMA did not further stimulate HIV-1 LTR-driven reporter gene expression in these three experiments (Fig. 3A), demonstrating that these cytokines were not directly involved in this Tat/*tar*-mediated pathway of activation of the NF-κB-defective HIV-1 promoter.

We propose a second pathway for Tat in up-regulating the HIV-1 promoter, which does not involve interaction with the nascent RNA product of the viral gene *tar*. This *tar*-independent pathway was studied by transiently cotransfecting Jurkat cells with a fusion plasmid lacking the *tar* element (pHIV-LTR-Δ*tar*-CAT) and pSV-Tat and measuring LTR-driven reporter CAT activity. The results (Fig. 3B) demonstrated a pSV-Tat plasmid concentration-dependent 3.5-fold stimulation of *tar*-less HIV-1 LTR-driven reporter gene expression above a low basal activity detected in the absence of Tat. This influence of Tat was observed in three separate experiments, and stimulation varied from three- to fivefold. About two- to threefold further stimulation of reporter gene expression by PMA and TNF-α was detected in all of these experiments, indicating a modest activation of HIV-1 promoter via the NF-κB motifs. The stimulations by these substances of wild-type plasmid (Fig. 2A) were also similar to those observed with pHIV-1 LTR Δ*tar*-CAT plus or minus 10 μg of pSVTat expression (Fig. 3B). Both constructs have wild-type NF-κB motifs. These results are consistent with the concept of production of TNF-α under the influence of Tat, which activates NF-κB by the *tar*-independent pathway.

The influence of Tat, expressed from the indicated concentrations of input plasmid, on cell viability was examined in all of these three experiments. Tat at these concentrations was not cytotoxic to the host cells (Fig. 3C).

Tat induces overexpression of cytokine genes. Since overexpression of genes encoding cytokines were detected in Tat-producing HIV-1-infected patients (22, 37) and cytokines are physiological activators of NF-κB (26, 56), the expression of cellular cytokine genes under the influence of Tat was the next focus of this study. We examined the level of expression of three cytokine genes, TNF-α, IL-6, and IL-1, in cells in which Tat was either expressed from a transiently transfected expression vector pSVTat or in a transfectant of Jurkat cells that stably express Tat protein (14). Figure 4A shows the levels of TNF-α-, IL-6-, and IL-1-specific mRNA species as determined by Northern blot analysis under the influence of Tat protein, expressed from the indicated concentrations of the input pSV-Tat plasmid. The autoradiographic signals (Fig. 4A) were quantitated by densitometric scanning as described in the legend to Fig. 4, and the results are presented in Fig. 4B. The levels of the TNF-α-, IL-6-, and IL-1-specific mRNAs were elevated six-, three-, and twofold, respectively, concurrent with elevated Tat mRNA levels. Similar elevated levels of specific mRNAs under the influence of Tat were observed in three separate experiments. The ^{32}P-labeled unrelated gene 36B4 cDNA was used as the loading control (52). To establish that the Tat gene was expressed in pSV-Tat-transfected cells, Tat-specific mRNA was measured; elevated levels of Tat mRNA corresponding to the increased concentrations of the input plasmid were observed (Fig. 4A and B). No Tat-specific mRNA was detected in untransfected Jurkat cells. Elevated levels of TNF-α-specific mRNA were also detected in stable transfectants of Jurkat cells (Jurkat-Tat) that constitutively express Tat from a stably integrated plasmid (14) (Fig. 4C). The level of actin mRNA was used as a loading control in this experiment.

Tat stimulates TNF promoter activity. The above results suggest that Tat either directly or indirectly stimulates TNF gene expression. To further examine this influence of Tat, we measured expression of the CAT gene in a fusion gene construct (pTNF-CAT-TNF) with 1,087 bp of 5′ upstream region of mouse TNF gene, which has four NF-κB motifs along with one SP1 motif, the TATA box, and is fused to the CAT gene (3). This flanking region is similar to that of the human TNF gene. The plasmid was cotransfected into Jurkat cells with different amounts of the Tat gene expression vector pSVTat. The results showed a concentration-dependent Tat stimulation of CAT activity (Fig. 5A). In the middle part of each panel,

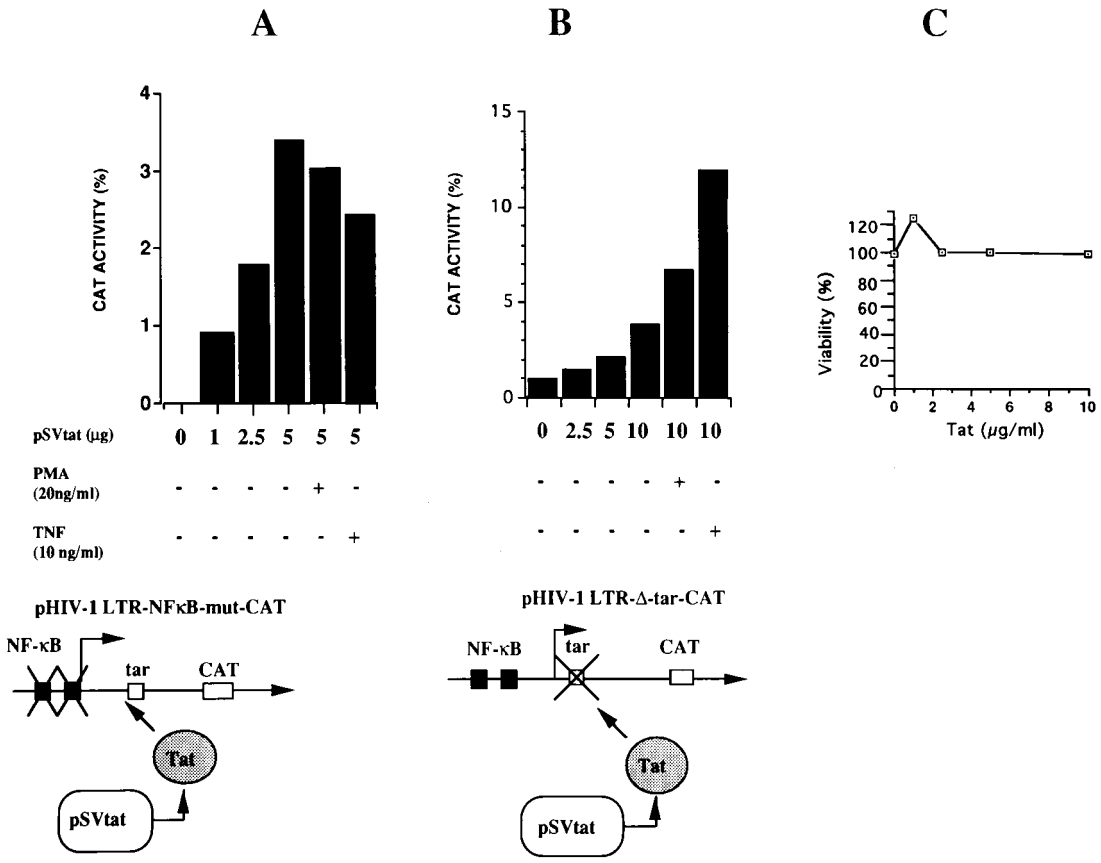

FIG. 3. Two pathways of up-regulation of the HIV-1 promoter by Tat. (A) The activation of HIV-1 promoter by viral protein Tat was measured by CAT assays after 72 h of transient transfection of Jurkat cells by the DEAE-dextran procedure (6) with 10 μg of the fusion plasmid pHIV-1 LTR NF-κB-mut-CAT (43), cotransfected with different amounts of pSV-Tat expression plasmid (14). The NF-κB motifs were mutated in the LTR sequence, so that activation by NF-κB was not operational. Treatment of cells with PMA (20 ng/ml) and TNF-α (10 ng/ml) was carried out for 18 and 4 h, respectively. Preparation of cell extracts and measurement of CAT and β-galactosidase activity in 10-μg protein aliquots were determined as described previously (8). The results were quantitated by scanning the thin-layer chromatography paper in a Betascope 603 blot analyzer (Betagen, Waltham, Mass.). Normalized CAT activity is expressed as the percentage of [^{14}C]CAP derivatives produced per hour. (B) Activation of *tar*-less HIV-1 promoter (pHIV-1 LTR-Δ*tar*-CAT) under the influence of Tat was determined. Jurkat cells were transiently cotransfected with 10 μg of the fusion plasmid pHIV-1 LTR-Δ*tar*-CAT and pSV-Tat. Transfection and treatment conditions were as for panel A. (C) The Tat plasmid has no effect on cell viability as measured by the 3-[4, 5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium (MTT) assay (41).

results on the level of CAT activity in duplicate samples are shown, and these are quantitated by scanning of the thin-layer chromatography paper, which is shown as bar graphs in the lower part of these panels. This experiment was repeated three times, and similar stimulation, ranging from six- to eightfold, was detected. These results are in agreement with those reported by Buonaguro et al. (12), who observed 1.4- to 4-fold stimulation of TNF promoter under the influence of Tat. These results with a *tar*-less TNF promoter confirm the concept that Tat can activate a promoter in the absence of a *tar* element, in accord with the concept of the second pathway of Tat action.

To examine the role of NF-κB in this Tat activity, a fusion plasmid (pTNF-109-CAT-TNF) in which the 978-bp region of the promoter which includes the four NF-κB motifs was deleted (3) was cotransfected with pSVTat (Fig. 5B). The basal level of TNF promoter activity was somewhat higher in experiments carried out with fusion plasmid with a deleted (−978 bp) 5′ end of the promoter. However, the stimulatory influence of Tat on TNF promoter was lost with the deletion of the NF-κB motifs in this region. This result suggests that the NF-κB motifs are important; however, it is also possible that deleted sequences other than the NF-κB motifs are also involved in the Tat-induced activation of TNF promoter.

Anti-TNF-α antibody inhibits Tat-induced up-regulation of the HIV-1 promoter. The above results suggest involvement of TNF-α in the Tat-induced activation of genes carrying NF-κB, as mediated via a *tar*-independent second pathway. Evidence for TNF-α involvement was obtained by adding anti-TNF-α antibody to Jurkat cells transiently cotransfected with pHIV-1 LTR-Δ*tar*-CAT and pSV-Tat. Tat-induced activation of this *tar*-less HIV-1 promoter was reduced to the basal level by the presence of anti-TNF-α antibody, in a concentration-dependent fashion (Fig. 6A). Similarly, NF-κB/Tat-induced superactivation of the HIV-1 promoter, examined by transiently cotransfecting Jurkat cells with fusion plasmid pHIV-1 LTR-CAT and with pSV-Tat, was inhibited in the presence of even lower concentrations of anti-TNF-α antibody (Fig. 6B). These results strongly suggest involvement of autocrine TNF-α, overexpressed under the influence of Tat via the *tar*-independent pathway, in activation of the HIV-1 promoter. It is not clear at this stage how superactivation of HIV-1 promoter is more sensitive to anti-TNF-α antibody (Fig. 6B). Results in Fig. 6C demonstrate that anti-TNF-α antibody was not cytotoxic at these concentrations.

Tat induces activation of NF-κB. To verify that activation of NF-κB is a result of Tat action via the second pathway, we examined the levels of active NF-κB in Jurkat cells by using

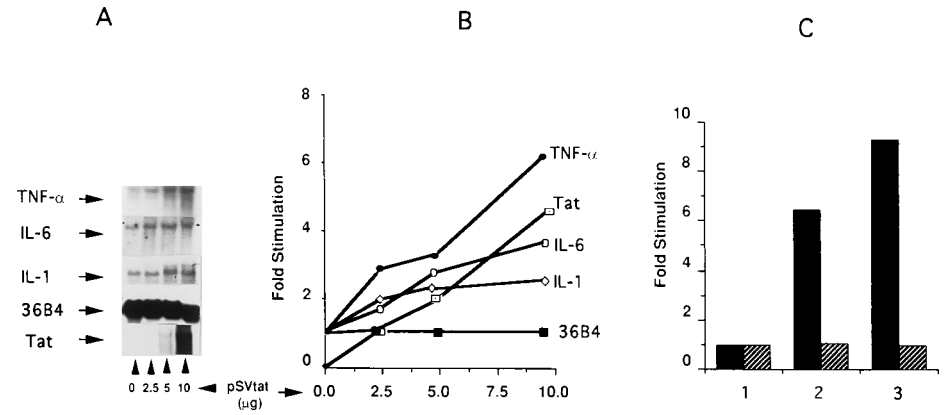

FIG. 4. Tat-induced overexpression of genes encoding cytokines. (A) Expression of TNF-α, IL-6, and IL-1 genes was measured by Northern blot analysis of total cellular RNA (25 μg per lane). TNF-α (59), IL-1β (2), and IL-6 (58) cDNA inserts were ^{32}P labeled by using a random-primed DNA-labeling kit (Boehringer Mannheim Biochemicals, no. 1004760) and were used for hybridization to identify specific mRNA species. ^{32}P-labeled 36B4-cDNA Northern blots served as a loading control. (B) The intensity of the autoradiographic signals as shown in panel A was quantitated by densitometric scanning and integration of the specific autoradiographic signals on a model GS-670 imaging densitometer (Bio-Rad, Richmond, Calif.). The integrated peak areas are plotted. The basal-level expression of the genes was defined as 1, and the fold stimulation under the influence of Tat, expressed from the indicated concentrations of pSVTat, is shown. The level of Tat-specific mRNA was undetectable in control Jurkat cells that were transfected only with control plasmid pSV-*lacZ*. Equal amounts of total cellular RNA from control and Tat-expressing cells were loaded as shown by the same level of expression of the 36B4 gene (52), which served as a loading control. (C) The level of TNF-α-specific mRNA (■) in Jurkat cells (bar graph 1), in Jurkat cells transiently transfected with pSV-Tat (bar graph 2), and in Jurkat-Tat cells in which the *tat* gene is stably integrated and constitutively expressed (bar graph 3). ^{32}P-labeled cDNA-actin probe (▨) served as the loading control in this experiment.

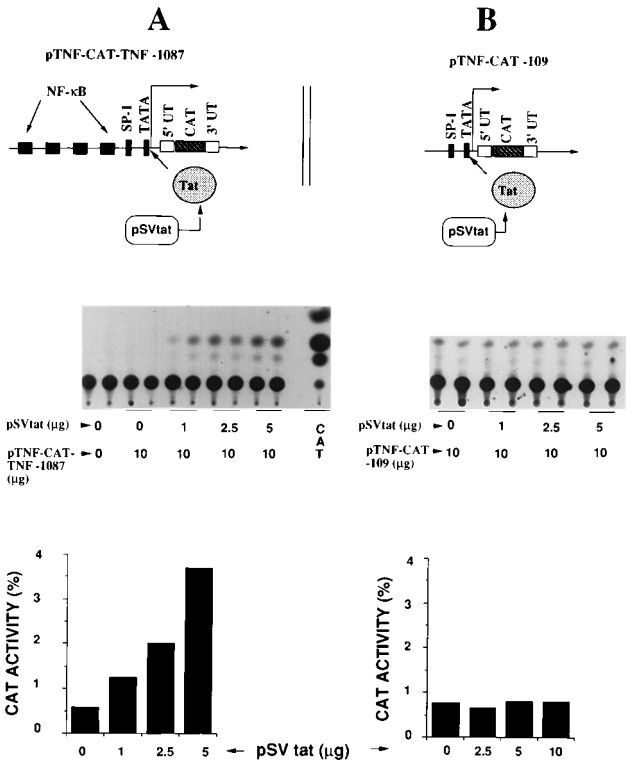

FIG. 5. Influence of Tat on the TNF promoter. Jurkat cells were transfected with 10 μg of fusion plasmids pTNF-CAT-TNF-1087 (A) or pTNF-CAT-109 (B) (3). The indicated concentrations of pSV-Tat were cotransfected with each of these TNF promoter fusion plasmids. At 72 h following transfections, the cell extracts were prepared and CAT activity was measured and quantitated. The top of each panel shows the structural organizations of the promoter regions of plasmid p-TNF-CAT-TNF-1087 (A) and pTNF-CAT-109 (B). The middle of each panel shows the CAT assays performed in duplicates. The bottom of each panel shows quantitation of these averaged CAT activities plotted as bar graphs. UT, untranslated.

EMSA to determine interaction between nuclear extracts and the ^{32}P-labeled motif. The retarded DNA-protein complex formed by interaction of the active NF-κB with its motif within the ^{32}P-labeled synthetic oligonucleotide is indicated by the arrow (Fig. 7). The specificity of this DNA-protein interaction was previously established in this laboratory by competition experiments with nonradioactive oligonucleotide carrying wild-type or mutated NF-κB motifs (8). The lower retarded complex that is routinely observed in this assay is apparently formed by the interaction of p50 dimers in the nuclear extracts with the radioactive oligonucleotide (53).

A weak interacting band was detected from nuclear extract of Tat-nonexpressing Jurkat cells (Fig. 7A, lane 1). Active NF-κB in transiently transfected Jurkat cells was increasingly elevated by larger amounts of pSV-Tat (lanes 2 to 4). This Tat-induced stimulation was substantially reduced in the presence of anti-TNF-α antibody (lanes 5 to 8), suggesting a role of the secreted cytokine in Tat-induced activation of NF-κB. Externally added TNF-α reversed this inhibitory influence of anti-TNF-α antibody (lanes 9 and 10). As shown previously (8), the results in lanes 11 and 12 demonstrate the stimulatory influence of TNF-α, by itself, on activation of NF-κB. Figure 7B shows a quantitative representation of the results in Fig. 7A by densitometric scanning of the NF-κB specific binding activity. Anti-TNF-α antibody did not influence HIV-1 LTR-driven reporter gene expression in cells in the presence of PMA or of TNF-α (data not shown).

Differential influences on the two modes of Tat-induced *trans*-activation of HIV-1 promoter. We used conditions that preferentially activate or block the two separate pathways of Tat activation of the HIV-1 promoter to distinguish between them. The results presented in Table 1 summarize the influence of seven agents on the HIV-1 promoter acting via (i) the *tar*-dependent pathway, (ii) the NF-κB-dependent pathway (*tar* independent), and (iii) both pathways. The agents characterized as inhibitors of NF-κB action are Gö-6976 (49) and PTX (7), which act via inhibition of protein kinase C. Ro24-7429 and Ro5-3335 have been characterized as inhibitors of Tat action (19, 29, 30) and also of Tat/NF-κB-induced superacti-

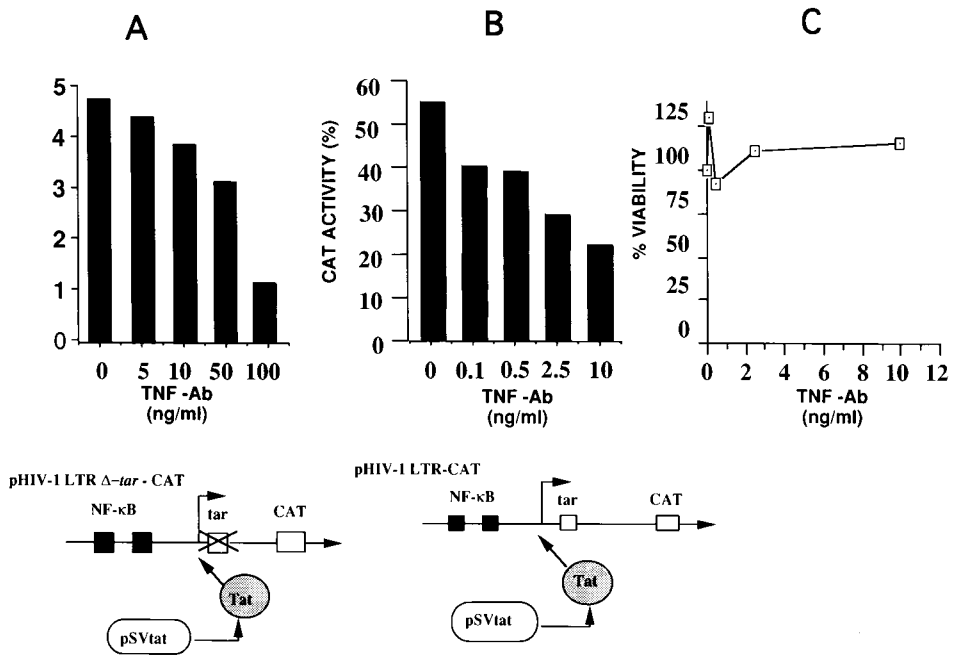

FIG. 6. Influence of anti-TNF-α antibody on activation of HIV-1 promoter by Tat via the *tar*-independent pathway and on Tat/NF-κB-induced superactivation. Jurkat cells were cotransfected with *tar*-less fusion plasmid pHIV-1 LTR-Δ*tar*-CAT (10 μg) and pSV-Tat (10 μg) (A) or pHIV-1 LTR-CAT (5 μg) and pSV-Tat (5 μg) (B). At 24 h later, the indicated amounts of anti-TNF-α antibody (TNF-Ab) were added. Cell extracts were prepared 72 h after transfection and 48 h after antibody treatments. (C) Cell viability at different concentrations of anti-TNF-α antibody as measured by the MTT procedure (41).

vation of the HIV-1 promoter (6). Anti-TNF-α antibody blocks interaction of TNF-α with its receptor.

Influences of these substances on HIV-1 LTR-driven reporter gene expression were studied by transient transfection of Jurkat cells with LTR-CAT fusion gene constructs having either mutated, deleted, or wild-type motifs. Neither TNF-α nor PMA stimulated *tar*-dependent Tat action (with mutated NF-κB motifs), which was inhibited by only the Tat inhibitor Ro24-7429, suggesting that TNF-α and NF-κB are not involved in the *tar*-dependent mode of action of Tat. The *tar*-independent action of Tat (on cells transfected with the fusion plasmid with its *tar* element deleted) was stimulated by Tat and also by TNF-α and PMA. This pathway was inhibited by PTX, Gö-6976, and anti-TNF-α antibody but not by Ro24-7429, suggesting that protein kinase C inhibitors such as PTX and Gö-6976, and also the antibody, inhibit only the *tar*-independent pathway and do not influence the *tar*-dependent pathway of activation by Tat. Ro24-7429, the Tat inhibitor, had just the opposite effect.

As reported previously (6), Tat expressed from cotransfected plasmid pSV-Tat greatly elevated CAT levels when the LTR sequence of the transfected fusion plasmid (pHIV-1 LTR-CAT) carried both wild-type NF-κB motifs and the *tar* element. All these inhibitors blocked this NF-κB/Tat-induced superactivation (Table 1). These results demonstrated that inhibitors of either NF-κB or Tat were effective when both pathways of Tat action were operative, a cellular state which is comparable to the HIV-1-infected one.

DISCUSSION

We postulate that the viral protein Tat initiates two chains of reactions following infection with HIV (Fig. 1). Synergism between these two actions of Tat, mediated via separate pathways, is responsible for the superactivation of the HIV-1 promoter, viral gene expression, and virus replication. One involves the Tat/*tar* interaction, and the other starts a *tar*-independent pathway which overexpresses genes encoding cytokines such as TNF-α, which activates NF-κB through the specific receptor-mediated signaling mechanism. Active NF-κB in turn closes the loops by *trans*-activating the HIV-1 and TNF promoters. Each pathway alone has minimal effects.

The end result of Tat action via the *tar*-independent cytokine pathway is the activation of NF-κB, which plays a central role in linking the two pathways of Tat action by stimulating both HIV-1 and cytokine promoters. A recent study with normal T lymphocytes and a lymphoblastoid cell line (J-JHAN) suggests that Tat-mediated stimulation of the HIV-1 promoter is abso-

TABLE 1. Influence of Tat, NF-κB, and TNF-α modulators on HIV-1 promoter activity[a]

Pathway	NF-κB Motif	Tar	Effect of[b]:						
			Activators			Inhibitors			
			Tat	TNF	PMA	Gö	PTX	TNF antibody	Ro
1	mut	wt	+	0	0	0	0	0	−
2	wt	del	+	+	+	−	−	−	0
1+2	wt	wt	+++	+	+	−	−	−	−

[a] Jurkat cells were transfected with 10 μg of the fusion plasmid pHIV-1 LTR-mut-NF-κB-CAT and 5 μg of pSV-Tat (pathway 1), 10 μg of pHIV-1 LTR-Δ*tar*-CAT and pSV-Tat (pathway 2), and 5 μg each of pHIV-1 LTR-CAT and pSV-Tat (pathway 3). At 24 h after transfection, the cells were treated with TNF-α (10 ng/ml) for 4 h or PMA (20 ng/ml) for 18 h.

[b] Treatments with Ro24-7429 (300 nM), Gö-6976 (300 nM), PTX (300 μM), and anti-TNF-α antibody (10 ng/ml) were performed for 48 h. At 72 h after transfection and 48 h after drug treatments, the cell extracts were prepared and CAT activity in 10 μg of protein from each extract was measured as described previously (6). +++, strong activation; +, activation, −, inhibition, 0, does not activate or inhibit; Gö, Gö-6976; Ro, Ro24-7429.

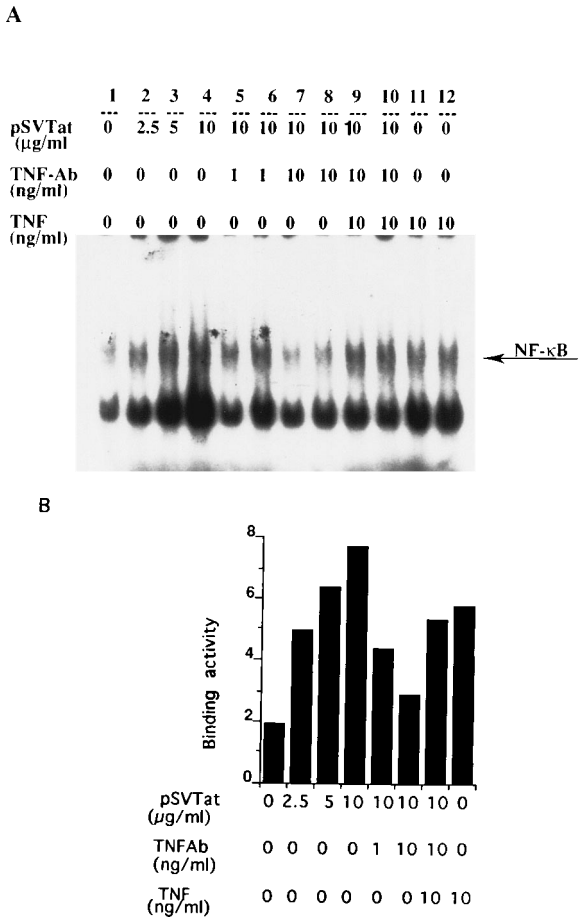

FIG. 7. Influences of Tat anti-TNF-α antibody on nuclear NF-κB. Nuclear extracts from control, pSV-Tat-transfected, and anti-TNF-α antibody (TNF-Ab)-treated Jurkat cells were prepared (23), and levels of active NF-κB were determined (8). (A) Autoradiographic signals of retarded DNA-protein complexes formed by the interaction of active NF-κB with its motif (arrow). The basal level (0 pSV-Tat, 5 μg of pSV-*lacZ*) of active NF-κB in 5 μg of nuclear extract protein is shown in lane 1. Nuclear extracts from untransfected cells showed similar basal active NF-κB. Levels of active NF-κB in Jurkat cells following transient transfection with 2.5 μg (lane 2), 5 μg (lane 3), and 10 μg (lane 4) of pSV-Tat are shown. Levels of active nuclear NF-κB in Jurkat cells transfected with 10 μg of pSV-Tat in the presence of anti-TNF-α antibody at 1 ng/ml (lanes 5 and 6) and 10 ng/ml (lanes 7 and 8) (duplicates) are shown. Lanes 9 and 10 (duplicates) show the level of active NF-κB in nuclear extracts of Jurkat cells transfected with 10 μg of pSV-Tat and with 10 ng of anti-TNF-α antibody per ml for 48 h and 10 ng of TNF-α per ml for 6 h. Lanes 11 and 12 (duplicates) show the effect of TNF-α alone (10 ng/ml). (B) Densitometric quantitation of the specific interaction of active NF-κB (arrow in panel A). The binding activity (numerals on the *y* axis) represents the relative intensity of the autoradiographic signals quantitated as described in the legend to Fig. 4.

lutely dependent on NF-κB response elements (1). We postulate that this requirement for Tat action is fulfilled by Tat-induced overexpression of cytokine genes via the *tar*-independent pathway, which turns on NF-κB *trans*-activation.

The mechanism of the observed up-regulation of TNF promoter by Tat is not clearly understood. Buonaguro et al. (13) implicated a *tar*-like sequence immediately downstream of TATA in Tat-induced up-regulation of the TNF-β promoter, but the role of such a DNA sequence in the TNF-α gene has not been demonstrated. Furthermore, our results suggest that the remaining region of the TNF promoter lacking the NF-κB motifs in a fusion gene construct that still contains SP1 and other immediate downstream sequences of the basic transcriptional complex is not sufficient to stimulate TNF promoter activity (Fig. 5B). Activated NF-κB is central to superactivation by Tat of the HIV-1 promoter and of cytokine promoters. These results are consistent with the concept that Tat-induced up-regulation of cytokines and the HIV-1 promoter operates via *tar*-independent and NF-κB-dependent transcriptional activation. As both Tat-induced activation of NF-κB and superactivation of the HIV-1 promoter are partly blocked by anti-TNF-α antibody, it may be concluded that Tat-induced TNF-α generated via the auto-up-regulatory loop plays a major role in the NF-κB/Tat-induced superactivation of the HIV-1 promoter.

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