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Activation of Transcription Factor NF-κB by the Tat Protein of Human Immunodeficiency Virus Type 1

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A recombinant Tat protein was used to investigate the molecular mechanisms of transcriptional activation of the human immunodeficiency virus type 1 long terminal repeat (LTR). Liposome-mediated delivery of this protein to responsive cells results in dose-dependent LTR activation. As evaluated by mRNA quantitation with competitive PCR, the activation response is rapid and transient, peaking at 5 h after the beginning of Tat treatment. In vivo footprinting experiments at the LTR showed that transcriptional activation is concomitant with a modification of the protein-DNA interaction pattern at the downstream κB site of the enhancer and at the adjacent Sp1 boxes. The effects of Tat on the enhancer are mediated by Tat-induced nuclear translocation of NF-κB, which parallels the kinetics of transcriptional activation. This induction results from degradation of the inhibitor IκB-α, is blocked under antioxidant conditions and by a protease inhibitor, and occurs as a rapid response in different cell types. The functional response to Tat is impaired upon cell treatment with a κB site decoy or with sodium salicylate, an inhibitor of NF-κB activation. These results show that NF-κB activation by Tat is important for LTR transcriptional activation. Furthermore, they suggest that some of the pleiotropic effects of Tat on cellular functions can be mediated by induction of NF-κB.

The regulation of human immunodeficiency virus type 1 (HIV-1) gene expression is mediated by the specific interactions of cellular transcription factors with the long terminal repeat (LTR) DNA sequence and of the product of the viral *tat* gene with the TAR RNA element, a 59-nucleotide stem-and-loop structure present at the 5′ ends of all viral mRNAs. The Tat protein is an ~8-kDa protein produced from different multispliced mRNAs and present at low abundance in HIV-infected cells. The protein modulates viral gene expression by increasing the rate of transcription initiation, elongation, and translation of TAR-containing mRNAs. The presence of both the protein and the responsive TAR element is essential for viral replication (for recent reviews, see references 16, 37, and 53).

In addition, Tat exerts a variety of effects on cell growth and metabolism. The protein has neurotoxic activity (58), acts as a growth factor for Kaposi's sarcoma cells (23), and induces immunosuppression and inhibits antigen-induced lymphocyte proliferation (14, 63, 73). Furthermore, the levels of expression of several cellular genes in different cell types are altered by the presence of the protein. Tat increases expression of extracellular matrix proteins (68), inflammatory cytokines (interleukin-1 [IL-1], IL-6, tumor necrosis factor alpha [TNF-α], and TNF-β) (11, 12, 60, 61), transforming growth factor β1 (17, 44, 79), the IL-4 receptor (54), glucose-6-phosphate dehydrogenase (71), and IL-10 (46). In contrast, the expression of other genes, including those for manganese-dependent superoxide dismutase (77), IL-2 (76), the HLA class I proteins (35), and p68 kinase, is downregulated by Tat (57). Finally, still-controversial findings about the ability of the protein to promote cell growth and survival (80) or, in contrast, to induce programmed cell death (42, 75) have been reported.

All of the above-mentioned effects are of particular relevance since the protein, even if lacking a recognizable signal sequence, can be secreted by the expressing cells and taken up by neighboring uninfected cells (24, 27, 32, 45).

Despite this large set of data about the functions of Tat, the actual molecular mechanisms by which most of these effects are exerted still remain elusive. As far as transcriptional activation of the HIV-1 LTR is concerned, Tat activity is probably mediated by the interaction with a number of cellular factors, including Sp1 (36), the TFIID components TBP (39) and TAF_{II} 55 (13), TAP (78), the kinase TAK (34), or some still-unidentified proteins (65, 81). From the functional standpoint, activation of transcription from the LTR requires the concerted action of Tat and of cellular proteins binding to the enhancer element (1, 9, 43, 77). Mutations of the LTR enhancer decrease or impair Tat-mediated transactivation (1, 7, 49). Accordingly, activation of transcription by Tat occurs even in the presence of mutations of TAR, provided that the enhancer element is intact (6, 31).

In the last few years, we have addressed the study of transcriptional control of the LTR in vivo by monitoring protein-DNA interactions at the LTR by genomic footprinting with ligation-mediated PCR (18, 19). These studies indicated that in systems in which transcription from the LTR is inducible, the major determinant for the transition from the latent to the activated state is the induction of factors binding to the LTR enhancer region. This element contains two tandemly arranged binding sites (κB sites) for the dimeric transcription factors composed of several combinations of members of the Rel/NF-κB family of polypeptides (for recent reviews, see references 4 and 62). The predominant complex binding to the LTR κB sites in activated cells is NF-κB (p50-p65 heterodimer). In unstimulated cells, NF-κB is held in the cytoplasm by the inhibitor protein IκB-α, which shields its nuclear translocation signal. Activation of NF-κB occurs through phosphorylation and proteolysis of IκB-α and subsequent translocation of the active factor into the nucleus where it can bind to its cognate binding sites (33).

The activation of NF-κB plays a major role in stimulating HIV-1 gene expression in response to a variety of stimuli of chemical, physical, and biological natures. Induction of HIV-1

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gene expression by TNF-α (2, 74), phorbol-12-myristate-13-acetate (PMA) (19, 74), UV irradiation (72), or cellular differentiation (30) is coupled to NF-κB activation. In addition, mutations in the NF-κB binding sites result in the loss of LTR activation in several cell lines (22, 48, 50) as well as in peripheral blood CD4 T lymphocytes (1). Moreover, a selective loss of NF-κB DNA-binding proteins confers resistance to HIV-1 infection to a T-cell line (55).

In order to understand the molecular details of the functional relationship between the Tat protein and the LTR enhancer region, we have recently obtained two recombinant Tat proteins that retain full transactivation activity and we have developed an efficient protocol for liposome-mediated protein delivery in tissue culture cells (unpublished data). In this work, we describe the utilization of these methods to study the kinetics of activation and the mechanism of action of Tat on LTR-mediated gene expression. The results obtained demonstrate that Tat activates NF-κB in different cell types and that the induction of NF-κB participates in the activation of the LTR promoter. The Tat-promoted NF-κB activation pathway acts through a change in the redox state of the cell and IκB-α degradation.

MATERIALS AND METHODS

Construction and purification of recombinant Tat. Plasmid pGST-Tat 2E was constructed by cloning the coding regions of both exons of HIV-1_{HXB2} Tat in the commercial vector pGEX2T (Pharmacia, Uppsala, Sweden). A detailed description of this plasmid and of the purification procedure for the glutathione-*S*-transferase (GST)-Tat fusion protein (Tat 2E) (see Results) will be presented elsewhere. Briefly, lysates from expressing bacterial cells were mixed with 1 ml of a 50% (vol/vol) slurry of glutathione-cross-linked agarose beads (Sigma, St. Louis, Mo.). The fusion protein was allowed to bind to the beads at 4°C on a rotating wheel for 1 h. The suspension was then loaded on an empty plastic column (Bio-Rad, Richmond, Calif.), letting the unbound proteins pass through, and the beads were subjected to a high-salt wash (0.8 M NaCl) to free the fusion protein from contaminating bacterial nucleic acids. The fusion protein was eluted in 1 ml 100 mM Tris containing 2 mM dithiothreitol (DTT) and 20 mM free glutathione (Sigma). By this procedure, 1 mg of protein per liter of medium was usually obtained. The purity and integrity of the protein were routinely checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining.

Cell culture and treatments. The HL3T1 cell line, a gift from B. Felber, is an HeLa derivative containing an integrated LTR-chloramphenicol acetyltransferase (LTR-CAT) construct (26). HL3T1 cells were grown in Dulbecco's modified Eagle's medium; Jurkat CD4 T cells and U937 monocytic cells were grown in RPMI 1640 medium. Both media were supplemented with 10% fetal calf serum, 2 mM glutamine, and 50 μg of gentamicin per ml.

For Tat treatment, HL3T1 cells were grown to about 60% confluence on 60-mm-diameter dishes in 5 ml of medium at 37°C. Nine micrograms of recombinant protein and 25 μl of lipofectin reagent (Gibco BRL Life Technologies LTD, Paisley, Scotland) were diluted in 300 μl of Optimem medium each; the two solutions were then combined, gently mixed, and incubated for 10 min at room temperature. Meanwhile, the culture medium was removed, the cells were washed with Optimem and covered with 2.4 ml of fresh Optimem, and the liposome-protein complex was overlaid on cells. Five hours after treatment, either cells were directly used for in vivo footprinting or protein or RNA extraction, or, if further incubation was required, 7 ml of complete medium was added to the plates. Exponentially growing Jurkat and U937 cells were washed with Optimem and seeded at a density of 3×10^6 cells per ml in Optimem on 10-cm-diameter dishes and treated with Tat as described above for HL3T1.

For CAT assays, cell extracts were prepared 20 h after Tat treatment, and the assay was performed according to standard protocols (59).

Stimulation of cultures with PMA (Sigma) was performed by incubation with 10^{-7} M PMA for 1 h. DTT treatment of cells was as described by Pahl and Baeuerle (51), using 2.5 mM (final concentration) DTT. Tosyl-Lys-chloromethylketone (TLCK) and dimethyl sulfoxide (DMSO) treatments were as described by Henkel et al. (33).

In vivo footprinting by ligation-mediated PCR. In vivo dimethyl sulfate (DMS) footprinting experiments were performed exactly as described previously (18, 19). For each in vivo DNase I footprinting experiment, 10^6 exponentially growing cells were washed three times with ice-cold phosphate-buffered saline and scraped off the plates with a rubber policeman. The cell pellet was lysed by incubation in lysis buffer (10 mM Tris-HCl [pH 7.5], 10 mM NaCl, 2 mM $MgCl_2$) with 0.5% Nonidet P-40 for 7 min on ice, and nuclei were isolated by a quick spin. The nuclear pellet was resuspended in 85 μl of lysis buffer, and the solution was adjusted to 3 mM $MgCl_2$ and 1 mM $CaCl_2$ and incubated with 0.5 U of DNase I (Boehringer, Mannheim, Germany) for 10 min on ice. Reactions were stopped with EDTA (25 mM final concentration), and DNA was extracted. In vitro control reactions were performed by incubating 50 μg of DNA in 100 μl of DNase I buffer with 0.01 U of DNase I for 10 min on ice. Reactions were stopped by phenol extraction, which was followed by a second phenol-chloroform extraction and DNA precipitation. DNase I-treated DNA was digested with *Hin*dIII to reduce viscosity and used for ligation-mediated PCR, as described previously (18).

At least three experiments were performed with each primer set to ensure reproducibility of the DMS methylation and DNase I cleavage patterns obtained.

Oligonucleotide treatment of cells. HL3T1 cells were plated on 60-mm-diameter dishes. When they were 60% confluent, cells were incubated with a double-stranded oligonucleotide (100-μg/ml final concentration) corresponding to a κB site or with the same amount of an unrelated oligonucleotide (8). After 3 h of incubation, the cells were washed and treated with Tat as described above, with the oligonucleotide concentration kept at 100 μg/ml. Six hours after Tat addition, fresh complete medium containing 100 μg of oligonucleotide per ml was added to the cells, and after an additional 18 h, CAT assays were performed.

The experiment was repeated three times to ensure reproducibility of the results.

Reverse transcription and competitive PCR. Cells were grown on 10-cm-diameter dishes and treated with Tat as described above. At different times after Tat treatment, total cellular RNA was harvested by the guanidium isothiocyanate method (15) and resuspended in 10 μl of water. One microgram of each RNA aliquot was reverse transcribed with random hexamers (Promega, Madison, Wis.) in a total volume of 25 μl. The levels of β-actin and CAT cDNAs were quantified by competitive PCR with the respective competitors. The competitor for β-actin quantification was plasmid pActinComp, containing a fragment of the β-actin cDNA in which an additional 20 bp had been added within the region amplified by primers BA1 and BA4. The construction and utilization of this competitor, as well as the sequences of primers BA1 and BA4, have already been described (29). The competitor for CAT cDNA quantification was plasmid pLTRΔCAT, obtained by deleting a 99-bp region of the CAT gene from plasmid pLTR-CAT (28). The wild-type and deleted CAT regions were amplified with primers CATupper (5′CCGTTGATATATCCCAATGGC3′) and CATlower (5′AATCGTCGTGGTATTCACTCC3′).

Competitive PCR experiments were performed by mixing of 1 μl of the cDNA obtained at each time point after Tat treatment with 10^4 copies of the β-actin competitor plasmid and amplification with primers BA1 and BA4 or by mixing of 2.5 μl of the cDNA with 10^6 copies of the CAT competitor plasmid and amplification with primers CATupper and CATlower. Amplification cycle profiles for both primer sets were as follows: denaturation at 95°C for 45 s, annealing at 60°C for 45 s, and extension at 72°C for 45 s, for 30 cycles of amplification. PCR products were resolved by PAGE and stained with ethidium bromide.

Gel retardation assays. Nuclear extracts from Jurkat, U937, and HL3T1 cells were prepared according to the microscale preparation protocol (41). Protein concentrations were determined by the Bradford assay with a commercial protein assay reagent (Bio-Rad). Oligonucleotides for gel retardation assays were synthesized by the International Centre for Genetic Engineering and Biotechnology Oligonucleotide Synthesis Service on an Applied Biosystem 380B synthesizer. Oligonucleotides corresponding to one strand were end labeled with [γ-^{32}P]ATP and T4 kinase and annealed to the complementary oligonucleotide prior to use.

The conditions for binding reactions with the NF-κB oligonucleotide probe (5′GAGTGGGGACTTTCCAGGCTC3′) and competition experiments were as previously described (19).

Supershift assays were performed with NF-κB p50 and p65 polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, Calif.) by preincubating the nuclear extracts with 2 μl of the antibody in the reaction buffer for 30 min and continuing with the gel retardation assay according to standard procedures.

Immunoblotting. Total cell extracts were prepared by lysing cells with a high-salt detergent buffer as described by Bauerle and Baltimore (3). Protein concentrations were measured by the Bradford assay and by analysis on Coomassie blue-stained protein gels. Proteins were transferred to 0.2-μm-pore-size nitrocellulose filters (Bio-Rad) by using a semidry blotting apparatus (Bio-Rad). The blotting buffer contained 15% methanol, 48 mM Tris, 39 mM glycine, and 0.00375% SDS. After being stained with Ponceau S to monitor transfer efficiency, the nitrocellulose sheets were saturated for 2 h in BLOTTO-Tween 20 (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.5% low-fat milk, 0.1% Tween 20) and incubated for 3 h at room temperature with a rabbit polyclonal anti-IκB-α/MAD3 (C15) antibody (Santa Cruz Biotechnology). The blots were then washed four times with BLOTTO-Tween 20 and incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G. After extensive washings in BLOTTO-Tween 20, the bands were visualized with the enhanced chemiluminescence detection system (Amersham International plc, Amersham, United Kingdom) and exposed to Kodak XAR films for 5 min. The same blot was also treated with a mouse monoclonal antitubulin antibody (a gift from C. Brancolini) essentially as described above for the anti-IκB-α antibody, except that the second antibody was an anti-mouse immunoglobulin G and the exposure time was 30 s.

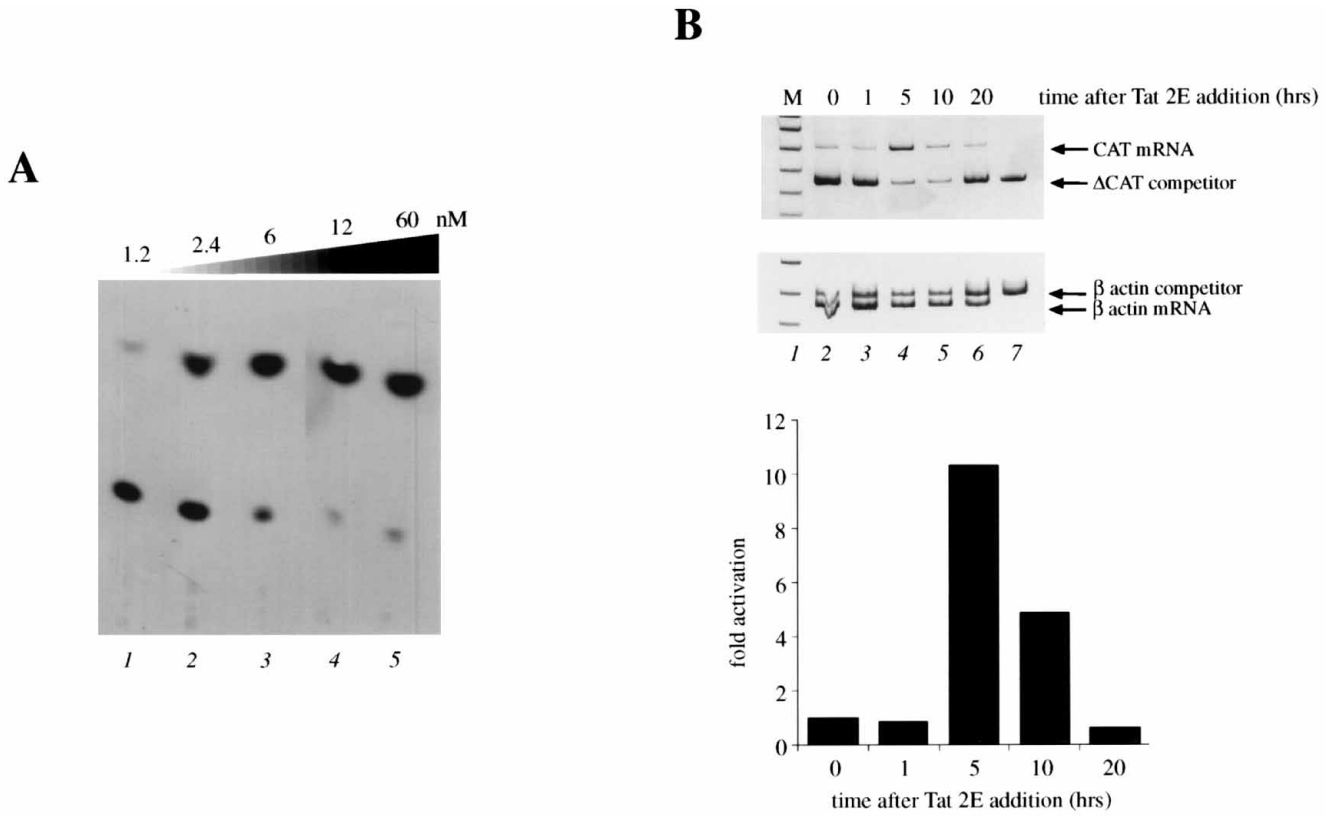

FIG. 1. Activation of the LTR promoter by recombinant Tat. (A) CAT assay with HL3T1 extracts after Tat treatment. Increasing concentrations of Tat 2E, as indicated, were delivered to HL3T1 cells by lipofection. The CAT assay was performed 20 h after transfection. (B) Kinetic analysis of LTR activation in Tat-treated HL3T1 cells. Tat 2E (3 μg/ml) was delivered to HL3T1 cells by lipofection. At 0, 1, 5, 10, and 20 h after the beginning of the lipofection procedure, total RNA was extracted and reverse transcribed with random hexameric primers. A fixed amount of the cDNA from each time point was mixed with 10^6 molecules of ΔCAT competitor (upper gel) or with 10^4 molecules of β-actin competitor (lower gel) and used for competitive PCR amplification with CAT- or β-actin-specific primers, respectively. The PCR products were resolved by 8% nondenaturing PAGE and stained with ethidium bromide. M, DNA molecular weight markers. The diagram at the bottom shows the results obtained by densitometric quantitation of the CAT PCR products, relative to the level in the absence of Tat treatment (time zero) and after standardization for β-actin cDNA content.

RESULTS

Activation of LTR-driven gene expression by recombinant Tat. We have obtained two recombinant Tat proteins by cloning the first exon or both exons of the *tat* gene from HIV-1 (clone HIV-1_{HXB2}) as fusion products with GST. These proteins are purified from *Escherichia coli* extracts by a procedure that removes bacterial nucleic acids and maintains the proteins in their reduced state. Both proteins are recognized by anti-Tat monoclonal antibodies, specifically bind to TAR RNA in gel retardation and filter binding assays, and activate transcription in in vitro transcription assays. A detailed description of these proteins will be presented elsewhere. In all of the experiments presented in this work, the recombinant protein containing the two-exon Tat protein fused to GST (Tat 2E) was used.

To study the transcriptional activity of Tat 2E in vivo, we used the HL3T1 cell line, a HeLa derivative containing an integrated CAT gene under the control of the HIV-1 LTR. Measurement of CAT activity from extracts of this cell line is a convenient indicator of inducible LTR promoter activity, since the level of transcription of the CAT gene is very low under basal conditions and is responsive to several different stimuli (21, 26).

Addition of Tat 2E to the medium of these cells in the presence of 100 μM chloroquine (27) (not shown) or liposome-mediated delivery of the protein results in activation of CAT expression. A representative lipofection experiment is shown in Fig. 1A, in which increasing amounts of recombinant Tat 2E in the nanomolar range trigger a proportional increase in CAT activity. Lipofection of GST alone or of a number of other GST fusion products obtained from *E. coli* extracts by similar purification procedures had no effect on the activation of CAT expression. No quantitative differences between the fusion proteins containing two-exon Tat or one-exon Tat, or with the same proteins after cleavage of the Tat moiety from GST by thrombin digestion, were observed in this assay (data not shown). All further data presented in this work were obtained by lipofection of the uncleaved two-exon Tat 2E protein.

Kinetic analysis of Tat-induced LTR-driven transcription. To determine the timing of LTR activation upon liposome-mediated delivery of Tat 2E, we studied the relative abundances of the CAT mRNA at different time points after lipofection. CAT mRNA was measured by a quantitative PCR procedure according to the principles of competitive PCR (5, 20). For this purpose, two competitors were constructed, one for the CAT cDNA, containing a 99-bp deletion within the CAT gene, and the other for the β-actin cDNA, containing a 20-bp insertion (29). The latter determination is used as a standard for the total cDNA obtained from each sample. PCR amplification of these competitors generates segments of different sizes with respect to the corresponding wild-type species

and allows the direct analysis of the PCR products on standard polyacrylamide gels. The ratio of the amounts of products obtained by the simultaneous amplification of target and competitor DNAs exactly reflects the ratio of the amounts of these two species initially present in the reaction (20).

Quantitation of CAT and β-actin mRNAs at different times (0, 1, 5, 10, 20 h) after Tat 2E lipofection is shown in Fig. 1B. Tat-treated and control cells were directly lysed in RNA extraction buffer. Total RNA was reverse transcribed by using random hexameric primers, and cDNA was amplified with the two different sets of primers for β-actin and CAT. Each reaction was performed in the presence of a fixed amount of the respective competitor for exact quantitation. While β-actin mRNA levels appear to be comparable in each sample, the relative abundance of CAT mRNA increases transiently after Tat treatment. In the lower part of Fig. 1B the results obtained by densitometric quantitation of PCR products and standardization with respect to β-actin mRNA are shown. As it appears from the diagram, at 5 h after addition of Tat there is an ~10-fold increase in CAT transcription, which progressively decreases at later times. These results show that activation of LTR-mediated transcription after Tat addition is rapid and transient.

Effects of Tat on protein-DNA interactions at the LTR. To explore the molecular triggers of Tat-mediated LTR activation, we monitored protein-DNA interactions by in vivo DMS and DNase I footprinting (47) at the integrated LTR of HL3T1 cells upon treatment with Tat 2E, as described in the previous paragraph. Six hours after Tat treatment, cells were treated with DMS or nuclei were prepared and incubated with DNase I. The two treatments produce complementary results, since DNase I produces discrete footprints at protein binding sites while DMS allows the detection of protein interactions at individual guanines. DNA extracted from DMS-treated cells was reacted with piperidine, and DNA from DNase I-treated nuclei was digested with *Hin*dIII to reduce viscosity. All samples were then subjected to ligation-mediated PCR with four sets of primers covering both strands of the LTR, as previously described (18).

Informative results were obtained with primer sets A and D, whose locations are schematically shown in Fig. 2A. These primer sets allow the study of the upper and lower strands, respectively, of the region encompassing the enhancer and Sp1 sites. These results are presented for DMS- and DNase I-treated DNA in Fig. 2B and C, respectively. Constitutive footprints were detected at the Sp1 and NF-κB boxes by both DMS and DNase I also in the absence of cell treatment or upon lipofection of GST alone (compare Fig. 1B, lanes 2 and 7 [in vitro-treated naked DNA], with lanes 3 and 8 [DNA from GST-treated cells]). Despite protein binding to these sites, transcription from the LTR is very low under both of these conditions. This situation resembles the one already described for unstimulated monocytic U1 cells (19).

In Tat-treated cells, the DMS sensitivity pattern clearly changes at the downstream enhancer repeat and at the adjacent Sp1 boxes, as indicated by gray circles in Fig. 2B. In particular, the enhanced reactivity of the guanine at position −92 with respect to the transcription start site, occurring upon Tat-mediated activation, resembles the pattern previously observed in U1 cells upon PMA stimulation (19) and in HL3T1 cells upon human herpesvirus 6 and HSV-1 infection (17a), suggesting that this pattern represents a marker of LTR activation. On the lower strands, the same regions show slightly, albeit reproducibly, increased protection from DNase I digestion upon addition of Tat (Fig. 2C, lane 7). Together, these genomic footprinting results further stress the importance of the Sp1 and enhancer regions in Tat-mediated transactivation of the LTR.

Tat-induced transcriptional activation is diminished upon pretreatment of cells with an NF-κB decoy or with sodium salicylate. To further study the functional role of enhancer-binding proteins in Tat-mediated transactivation of the LTR, we investigated the effects of inhibition of these proteins by cell treatment with a large excess of a specific oligonucleotide acting as an NF-κB decoy or by cell incubation with sodium salicylate, an inhibitor of NF-κB activation.

Treatment of HL3T1 cells with an excess of an oligonucleotide containing the NF-κB binding site has been shown to specifically block NF-κB-dependent gene expression, probably by competition with the genomic sites for NF-κB binding (8). HL3T1 cells were incubated for 2 h before Tat lipofection with a double-stranded oligonucleotide containing the downstream NF-κB sequence of the HIV-1 LTR or with a control oligonucleotide with an unrelated sequence. Twenty hours later, cell extracts were prepared and assayed for CAT activity. Figure 3A shows the results of a representative CAT experiment and the average results obtained in three different experiments. A net ~10-fold decrease of Tat-induced CAT activity was observed in cells treated with the NF-κB decoy compared with cells preincubated with the control oligonucleotide.

Treatment of cells with sodium salicylate was shown to significantly impair LTR activation in response to PMA (40). In order to verify whether a similar effect would also occur in response to Tat, HL3T1 cells were preincubated for 2 h with 2.5 or 5 mM sodium salicylate and then treated with Tat. Twenty hours later extracts were prepared and assayed for CAT activity. The results of a representative CAT experiment and the average results of three different experiments are presented in Fig. 3B. Treatment of cells with both concentrations of the drug results in a net decrease of CAT activity. In both sets of experiments presented in Fig. 3, the standard deviations of CAT activity were minor or equal to 10% for each experimental point. Together, these data demonstrate that κB-binding proteins play an important role in Tat-dependent induction of LTR-controlled gene expression.

Tat induces nuclear NF-κB binding activity in HL3T1 cells. The above-reported experiments have demonstrated that Tat delivery in HL3T1 cells determines a modification of protein-DNA interactions at the κB sites on the LTR and that inhibition of κB binding activity markedly reduces Tat-mediated LTR activation. On the basis of these results, we decided to investigate whether Tat could trigger the activation of κB-binding proteins in the nucleus. For this purpose, the recombinant Tat 2E protein was introduced into HL3T1 cells as described above, and 5 and 10 h later nuclear extracts were prepared and assayed for binding to a labeled NF-κB-specific oligonucleotide by mobility shift assays. Control reactions were performed with nuclear extracts from cells incubated for 5 h with lipofectin alone or with lipofectin and GST (Fig. 4A, lanes 1 and 2, respectively). As shown in Fig. 4A, lane 3, at 5 h after Tat addition there is a clear induction of a κB site binding activity (indicated by an arrow). This protein-DNA complex is absent in nuclear extracts from mock- or GST-treated cells (Fig. 4A, lanes 1 and 2, respectively). At 10 h after Tat treatment, this binding activity is reduced in accordance with the observed reduction of CAT mRNA levels, as shown in Fig. 1B.

The specificity of this binding activity was assessed by competition experiments. The retarded complex is specifically inhibited by a 150-fold excess of a cold specific oligonucleotide competitor but not by the same excess of an unspecific competitor (Fig. 4A, lanes 5 and 6, respectively).

To investigate the subunit composition of the induced com-

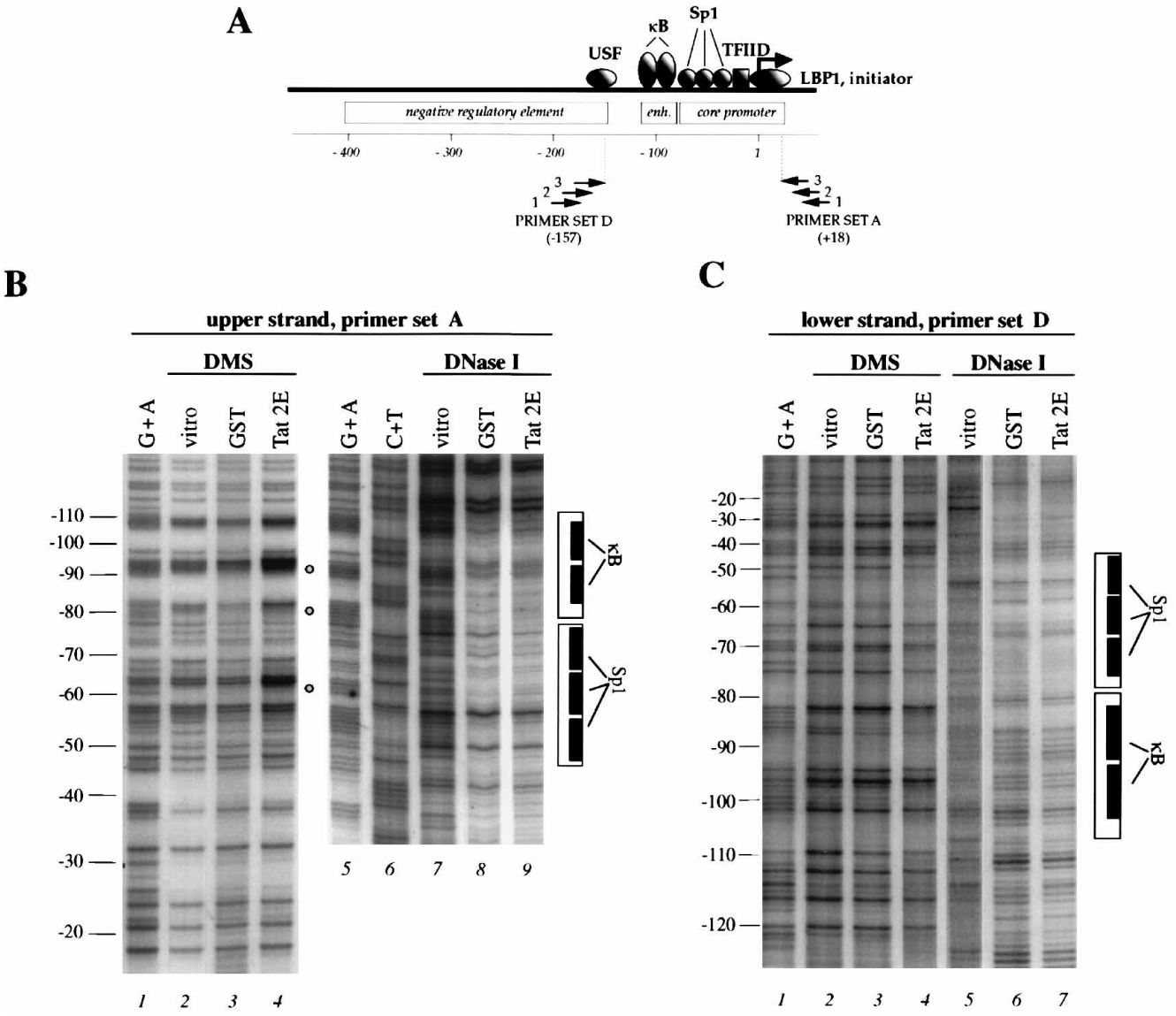

FIG. 2. Protein-DNA interactions at the HIV-1 LTR in HL3T1 cells before and after Tat lipofection, analyzed by in vivo DMS and DNase I footprinting. (A) Schematic representation of the LTR and some relevant protein binding sites in the 3′ portion. The LTR is divided in three functional regions as described previously (28). The arrows show the positions of the two primer sets used in the in vivo footprinting experiments shown in panels B and C. A detailed description of these primer sets was reported previously (18, 19). enh., enhancer region. (B) In vivo footprinting of the upper strand of the LTR with primer set A. Lanes 1 and 5, G+A sequencing control; lane 6, C+T sequencing control. Both sequencing ladders were also obtained by ligation-mediated PCR. Lane 2, DMS-treated naked DNA control; lane 3, in vivo DMS-methylated DNA from cells lipofected with GST; lane 4, in vivo DMS-methylated DNA from cells lipofected with Tat 2E; lane 7, DNase I-treated naked DNA control; lane 8, in vivo DNase I-digested DNA of nuclei from cells lipofected with GST; lane 9, in vivo DNase I-digested DNA of nuclei from cells lipofected with Tat 2E. The gray circles show guanines hypersensitive to DMS. On the right, the locations of the enhancer- and Sp1-binding regions are indicated by empty boxes and that of the consensus binding sequence is indicated by filled boxes. Numbers on the left indicate the location of the bands with respect to the transcription start site. (C) In vivo footprinting of the lower strand of the LTR with primer set D. Lanes and symbols are the same as in panel B.

plex, supershift assays were employed (Fig. 4B). Antibodies against the NF-κB subunits p50 and p65 were added to nuclear extracts of Tat-stimulated cells (Fig. 4B, lanes 3 and 4, respectively). Both antibodies are able to supershift the induced DNA binding complex, indicating the presence of both p50 and p65 in the κB site binding activity induced by Tat.

Tat-induced NF-κB activation is inhibited under antioxidant conditions and by protease inhibitors. All NF-κB-activating stimuli described to date use a common signal transduction pathway that acts through an increase in the cellular concentration of reactive oxygen intermediates and is blocked by antioxidative agents (for a review, see reference 4). In order to check whether Tat-mediated NF-κB induction also could be inhibited by antioxidants, HL3T1 cells were preincubated for 1 h with 2.5 mM DTT prior to Tat treatment, as described previously (51). As a control, cells were subjected to the same treatment before stimulation with PMA. Nuclear extracts were prepared 4 h after Tat addition or 1 h after PMA stimulation and used for mobility shift assays with an NF-κB probe. As shown in Fig. 5, cell incubation with 2.5 mM DTT inhibited NF-κB activation by both Tat and PMA (compare lane 2 with lane 5 and lane 6 with lane 9, respectively).

All NF-κB activation pathways converge to the phosphorylation of IκB-α and consequent proteolytic degradation (4, 62).

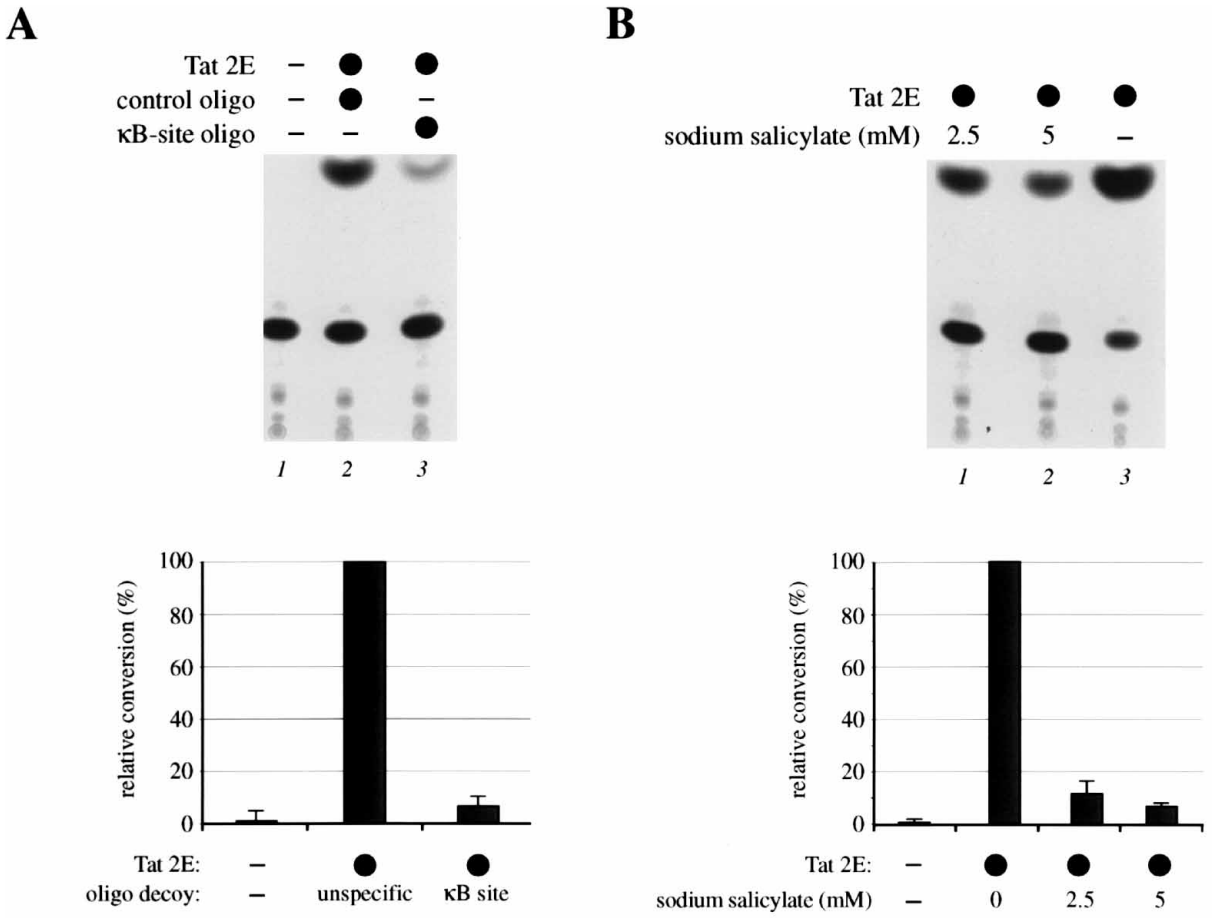

FIG. 3. Inhibition of Tat-mediated LTR activation by NF-κB by oligonucleotide decoys and sodium salicylate. The upper parts of both panels show a representative CAT experiment; the bottom parts show quantitation of results from three independent experiments (means ± standard deviations). (A) Effects of incubation of HL3T1 cells with an oligonucleotide (oligo) containing an NF-κB site or with an oligonucleotide with an unrelated sequence before lipofection of Tat 2E. Treatment with the κB site decoy results in a >10-fold reduction in LTR-driven CAT expression. (B) Effects of treatment of HL3T1 with sodium salicylate (2.5 and 5 mM, as indicated) before Tat 2E lipofection. Treatment with the drug results in a proportional decrease in CAT expression.

A number of protease inhibitors, such as TLCK, tosyl-Phe-chloromethylketone (TPCK), and calpain, have been proven to effectively block IκB-α degradation and thus NF-κB activation (33). As a first approach to test whether the pathway switched on by Tat also converges to IκB-α degradation, we used the protease inhibitor TLCK. Cells were preincubated for 30 min with TLCK dissolved in DMSO or with DMSO alone as a control and then were treated with either PMA or Tat. As shown in Fig. 6, TLCK, but not DMSO alone, prevents NF-κB activation by both PMA and Tat (compare lane 4 with lane 3 and lane 8 with lane 7, respectively).

Effect of Tat on the cellular levels of IκB-α. We have shown that the protease inhibitor TLCK can prevent the Tat-mediated induction of NF-κB, suggesting that IκB-α degradation could be a necessary step in the activation pathway triggered by Tat, similar to the case for other NF-κB activators. To further explore this possibility, we investigated IκB-α protein levels by immunoblotting at 0, 2, 4, and 6 h after treatment of cells with Tat 2E or GST as a control. As shown in Fig. 6, the amount of IκB-α is reduced in HL3T1 cells at 4 and 6 h after lipofection of Tat, as well as in PMA-treated cells. The same blot was also treated with a tubulin-specific antibody to verify whether comparable amounts of proteins were present in each lane (Fig. 6, lower blot).

Tat activates NF-κB binding activity in U937 and Jurkat cells. The above-reported experiments show that Tat treatment of the epithelial HL3T1 cell line induces NF-κB nuclear binding activity through IκB-α degradation. In order to study the effects of Tat in other cell types, which are more-physiological targets of HIV-1 infection, we analyzed NF-κB binding activity in U937 promonocytic and in Jurkat $CD4^+$ T cells upon lipofection of Tat 2E or GST as a control. For both cell lines, nuclear extracts were prepared 5 h after treatment. As shown in Fig. 7, Tat specifically activates NF-κB binding activity both in U937 cells and in Jurkat cells. This observation suggests that activation of NF-κB is not cell type restricted and that this event is likely to occur broadly in HIV infection and could possibly play a role in AIDS disease.

DISCUSSION

Despite apparent continuous virus replication in lymphoid tissues during the progression of HIV infection towards AIDS (52), viral gene expression at the cellular level is a tightly controlled process. One of the key regulators of this process is the viral Tat protein, which behaves as an extremely potent transcriptional activator at the viral LTR, acts at posttranscriptional levels on translation of viral mRNAs, exerts a number of

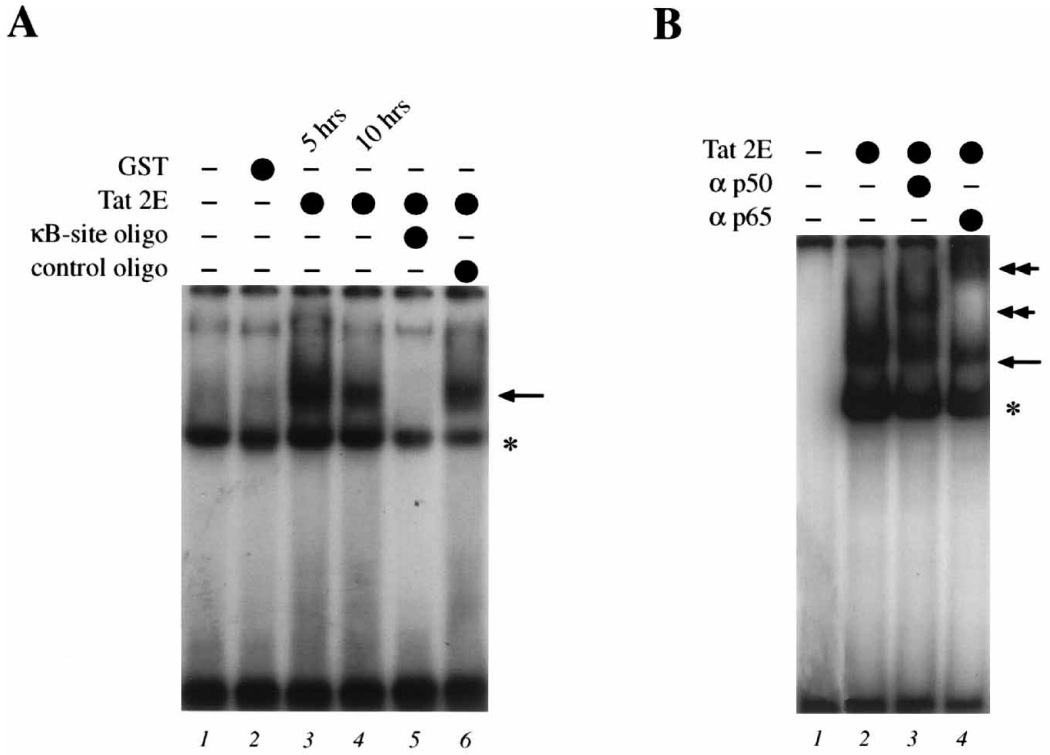

FIG. 4. Tat induces nuclear translocation of NF-κB. (A) Gel retardation assays with an oligonucleotide (oligo) probe corresponding to the downstream κB site of the LTR enhancer and nuclear extracts obtained from HL3T1 cells. Lane 1, no cell treatment; lanes 2 and 3, cells treated with Tat 2E at 5 and 10 h after the beginning of treatment, respectively; lanes 5 and 6, same as lane 3, with addition of a 150-fold molar excess of specific or nonspecific cold oligonucleotides, respectively. The arrow indicates the specific retarded complex, and the asterisk indicates a nonspecific complex (same as in reference 19). (B) Supershift of κB complexes by anti-p50 and anti-p60 antibodies. Gel retardation assays were performed with nuclear extracts from Tat-treated HL3T1 cells obtained at 5 h after the beginning of treatment (lanes 2 to 4). Lane 3, preincubation of nuclear extracts with anti-p50 antibodies; lane 4, preincubation with anti-p65 antibodies; lane 1, probe alone. The position of the specific complex bound by the κB site probe is indicated by an arrow, the positions of the supershifted complexes are indicated by double arrows, and the asterisk shows a nonspecific retarded complex. The results show that the κB site binding activity induced by Tat contains both the p50 and p65 proteins of the Rel family of transcription factors.

pleiotropic effects on cellular functions, and, by virtue of its property of crossing cellular membranes, is able to extend these effects also to uninfected neighboring cells. The understanding of the molecular details of these functions has long been hampered by the low levels at which the protein is produced within the cell (25). Furthermore, chemically synthesized Tat is very prone to oxidation and loss of activity (24), and it is difficult to obtain large amounts of recombinant protein because of its tendency to aggregate upon overexpression and purification (27, 56).

In this work, we have investigated the mechanisms by which Tat activates transcription from the HIV-1 LTR by using a recombinant GST-Tat protein (Tat 2E) that can be efficiently introduced into the cells by using liposomes as carriers. After lipofection, this protein is active in promoting LTR-driven transcription in the nanomolar range. Activity of the protein depends on its maintenance in a reduced state, since exposure to an oxidizing treatment impairs both transactivation (as judged by CAT assay) and NF-κB induction (as judged by gel retardation) (data not shown).

Since we were interested in understanding the early events of the activation process, we first studied the kinetics of the LTR-driven transcriptional activation upon addition of Tat. By analyzing the levels of CAT mRNAs originating from the LTR in HL3T1 cells by competitive PCR, we found that the increase in transcription is rapid and transient, peaking at 5 h after Tat addition. Similar results have been previously reported by Drysdale and Pavlakis (21) by fusion of HL3T1 cells with cells producing Tat. In that system, the rapid downregulation of transcriptional activity was found to be an active process that was insensitive to the continuous presence of Tat and could be blocked by protein synthesis inhibitors.

It has been shown that transcriptional activation of the LTR relies on the concerted action of Tat and factors binding to the Sp1 and enhancer regions (1, 38, 43, 66). We have addressed this issue by analyzing protein-DNA interactions in vivo at the LTR after Tat lipofection, using genomic footprinting. This is the technique of choice to detect the interactions between proteins and DNA which effectively occur in living cells. Most notably, it allows one to analyze the modifications in these reciprocal interactions occurring in response to external stimuli or metabolic changes. By this technique, we have previously studied protein footprints at the LTR in the H9 T-cell line, productively infected with HIV-1 (18), and in the U1 promonocytic cell line before and after HIV-1 induction by PMA (19).

The enhancer region of the LTR is engaged in protein binding also in the absence of appreciable transcriptional activity from the promoter, both in unstimulated U1 cells (19) and in untreated HL3T1 cells (Fig. 2). In both systems, transcriptional activation by PMA determines the nuclear translocation of transcription factor NF-κB and results in the same modification of the footprinting pattern. In particular, the guanine at position −92, at the downstream enhancer repeat, becomes

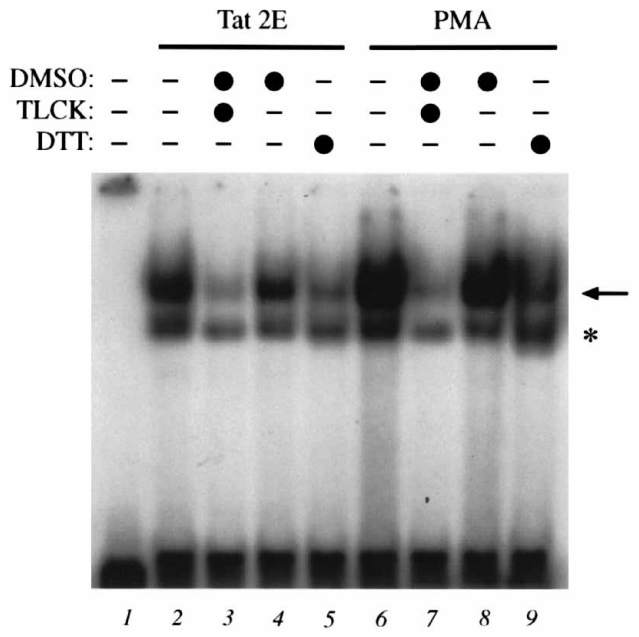

FIG. 5. Tat-induced NF-κB activation is inhibited by cell treatment with DTT or TLCK. HL3T1 cells were treated for 1 h with 5 mM DTT, with 100 μM TLCK dissolved in DMSO, or with an equivalent amount of DMSO, as indicated, and then lipofected with Tat 2E (lanes 2 to 5) or stimulated with PMA (lanes 6 to 9). Nuclear extracts were prepared 4 h after Tat lipofection or 1 h after PMA stimulation, and equal amounts of proteins were used for gel retardation assay with the κB probe. Lane 1, probe alone. Arrow, position of the specific retarded band; asterisk, position of a nonspecific complex.

strongly hypersensitive to DMS methylation. This finding is consistent with the substitution of a constitutive enhancer binding activity with NF-κB, with the latter causing more pronounced bending of the template DNA and subsequent increased sensitivity to DMS (19).

Comparing the DMS and DNase I sensitivity patterns at the LTR in HL3T1 cells before and after Tat transactivation, we detected major changes occurring at the Sp1 boxes adjacent to the enhancer region and at the downstream κB box of the enhancer. The modification of the footprinting pattern over the Sp1 sites could be explained by taking into account the existence of specific protein-protein interactions between Sp1 and Tat (36). In contrast, the change of the footprinting pattern over the κB site, with the increased DMS sensitivity of guanine −92, was more intriguing, especially considering that the footprinting analysis was performed only a few hours after Tat treatment, concomitantly with the observed peak in transcriptional activation. These results prompted us to look for the induction of NF-κB binding activity in response to Tat treatment. Indeed, we found that at 5 h after Tat treatment there is a consistent amount of NF-κB binding activity in the nuclei of HL3T1 cells. This effect is not cell type specific, since it also occurs in the U937 monocytic cell line and in the Jurkat lymphocytic T-cell line. Both of these cell types are in vivo targets of HIV-1 infection.

We have performed some preliminary experiments to try to understand the mechanisms of NF-κB induction by Tat. Induction of NF-κB usually results from the rapid degradation of IκB-α that follows stimulation of cells with various activators (10, 33). Accordingly, the levels of IκB-α appear to be reduced in Tat-treated HL3T1 cells. All stimuli causing degradation of IκB-α probably use a common signal transduction pathway, acting through the increase of the intracellular concentration of reactive oxygen intermediates (i.e., they elicit oxidative stress). In agreement with this model, activation of NF-κB by Tat also is inhibited by treatment of cells with the antioxidant DTT. Finally, degradation of IκB-α involves the proteolytic activity of a still-unidentified serine protease. Treatment of cells with TLCK, an inhibitor of trypsin-like serine proteases, is indeed able to block the induction of NF-κB by Tat. We are currently performing further experiments with an extended panel of serine protease inhibitors (33) for a better definition of the properties of the protease involved in this process.

Together, the data reported above suggest that Tat activates NF-κB by acting through the same biochemical pathways used by a variety of other NF-κB inducers (for a recent review, see reference 4). Similar findings have already been described for other viral proteins, including p40 Tax of human T-cell leukemia virus type 1, HBx and MHBs of hepatitis B virus, and EBNA-2 and LMP of Epstein-Barr virus (for references, see reference 4) and hemoagglutinin of influenza virus (51). Additionally, the possibility of a direct interaction between human T-cell leukemia virus type 1 Tax and IκB-α, with consequent destabilization of the NF-κB–IκB-α complex, has been demonstrated (67).

It has been recently shown that Tat treatment results in increased NF-κB inducibility by TNF-α (but not in an increased NF-κB basal level) by an indirect mechanism involving Tat-mediated inhibition of the manganese-dependent superoxide dismutase gene and subsequent induction of a pro-oxidative state (77). This effect (which requires full-length Tat) is complementary to the one described in this work (which can be obtained also with one-exon Tat [data not shown]), since it is exerted at later times after treatment with the protein. Taking these two results together, it appears that a complex series of biochemical changes occurs in Tat-treated cells, including a first transient event of NF-κB induction and transcriptional activation, with kinetics similar to that detected with other NF-κB inducers (64), followed by later changes caused by the action of Tat (or of NF-κB) on the expression of cellular genes. Both of these events are likely to involve a modification of the redox state of the cell.

What is the functional role of Tat-mediated activation of NF-κB? The finding that Tat activates NF-κB is in agreement with a number of observations that emphasize the cooperative effects of Tat and of different enhancer-binding factors—belonging to the Rel (1, 9, 43) and NFAT (49a) families—for LTR-controlled transcription. Accordingly, the enhancer element mediates TAR-independent induction of transcription by Tat (1, 43, 69). The importance of NF-κB induction by Tat is also demonstrated by the results presented in this work that show a diminished transcriptional activation of the LTR upon

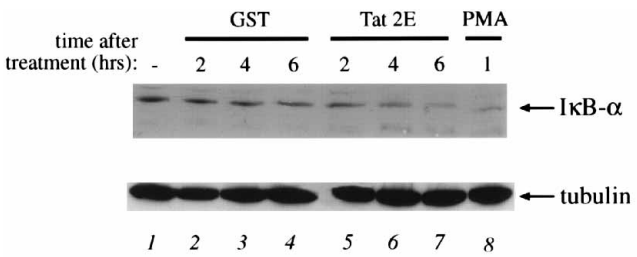

FIG. 6. Immunoblotting analysis of IκB-α in HL3T1 cells. Cell extracts were prepared at different times after lipofection with GST (lanes 2 to 4) or Tat 2E (lanes 5 to 7) or after treatment with PMA (lane 8), as indicated. IκB-α and tubulin (as a control for monitoring protein loading in each lane and transfer efficiency) were detected by Western blotting (immunoblotting) with specific antibodies. Tat and PMA treatments induce degradation of IκB-α.

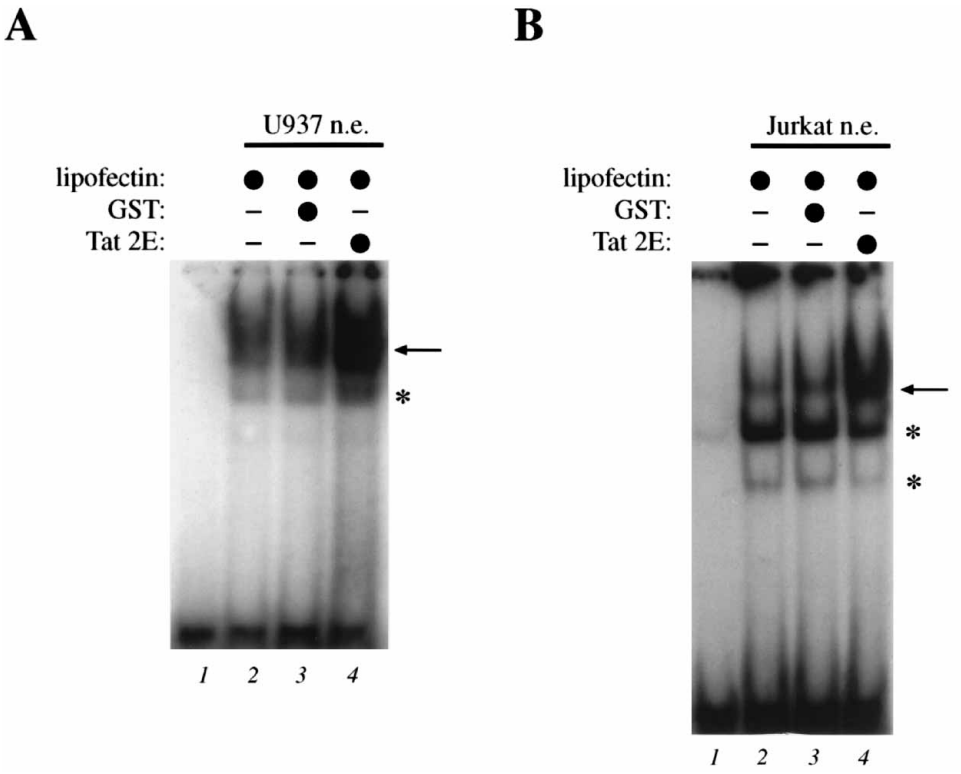

FIG. 7. Analysis of NF-κB activity in U937 cells (A) and Jurkat cells (B) upon lipofection with Tat 2E. Cells were treated with Tat 2E, GST, or lipofectin alone, as indicated. Nuclear extracts (n.e.) were prepared 5 h after lipofection and analyzed by gel retardation assays with a specific κB site probe. The arrows indicate the location of the specific retarded band; the asterisks indicate those of nonspecific complexes (as concluded from competition experiments [data not shown]).

cell treatment with a κB site oligonucleotide decoy or with sodium salicylate. Since nuclear translocation of NF-κB is an early T-cell activation signaling event (4) and since virus gene expression is exquisitely sensitive to the activated state of the cell (70), it is likely that the direct induction of NF-κB by Tat fulfills the requirement of T-cell activation for complete LTR induction.

Finally, several of the genes which have been shown to be regulated by Tat, including the major histocompatibility complex class I, TNF-α, TNF-β, Il-2, and Il-6 genes (62) and the glucose-6-phosphate dehydrogenase gene (71), contain NF-κB sites in their promoters. Moreover, it has been recently shown that Tat can induce apoptosis in lymphocytes (42, 75) and that NF-κB participates in the apoptosis signaling pathway (see references in reference 51). Therefore, it is tempting to speculate that at least some of the described effects of Tat on cellular gene expression and cell functions are indeed mediated by the pleiotropic actions of NF-κB.

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