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HIV-1 Tat Induces the Expression of the Interleukin-6 (IL6) Gene by Binding to the IL6 Leader RNA and by Interacting with CAAT Enhancer-binding Protein β (NF-IL6) Transcription Factors*

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Human immunodeficiency virus type 1 (HIV-1) infection is associated with severe psoriasis, B cell lymphoma, and Kaposi's sarcoma. A deregulated production of interleukin-6 (IL6) has been implicated in the pathogenesis of these diseases. The molecular mechanisms underlying the abnormal IL6 secretion of HIV-1-infected cells may include transactivation of the IL6 gene by HIV-1. Here we report the molecular mechanisms of Tat activity on the expression of the IL6 gene. By using 5′ deletion mutants of pIL6Pr-CAT and using IL6:HIV-1-LTR hybrid constructs where discrete regions of the IL6 promoter replaced the TAR sequence in HIV-1 LTR, we identified a short sequence of the 5′-untranslated region of the IL6 mRNA that is required for Tat to trans-activate the IL6 promoter. This sequence acquires a stem-loop structure and includes a UCU sequence that binds to Tat and is necessary for full trans-activation. In addition, we provide the evidence that Tat can function by enhancing the CAAT enhancer-binding protein (C/EBP) DNA binding activity and is able to complex with *in vitro* translated C/EBPβ, which is a major mediator of IL6 promoter function. By using the yeast two-hybrid system and immunoprecipitation, we observed that the interaction of Tat with C/EBP proteins also occurred *in vivo*. The data are consistent with the possibility that Tat may function on heterologous genes by interacting with RNA structures possibly present in a large number of cellular and viral genes. In addition, Tat may function by protein-protein interactions, leading to the generation of heterodimers with specific transcription factors.

Human immunodeficiency virus type 1 (HIV-1)[1] is the etiologic agent for acquired immunodeficiency syndrome (AIDS) and causes various clinical and immunological abnormalities, including activation of polyclonal B cells that manifests as hypergammaglobulinemia and autoantibody production, lymphadenopathy, Kaposi's sarcoma, and lymphoma of the B cell phenotype (1–3). Studies on small cohorts of subjects who were exposed to HIV-1 and did not develop HIV-1 infection and individuals who harbored HIV-1 but remained disease-free for long periods (4, 5) strongly suggest that the development of AIDS may depend on a dynamic interplay between viral and host cellular gene products. Accordingly, in HIV-1-infected subjects there is a deregulated production of cytokines, including the proinflammatory interleukin-6 (IL6) (6), which affects the growth and differentiation of lymphoid and mesenchymal cells (7) and may contribute to the development of the clinical features of AIDS. Accordingly, IL6 gene transcription is induced in cells infected by HIV-1 (8), and increased levels of IL6 have been reported in serum and cerebral spinal fluid of HIV-1-infected patients (9).

The Tat protein of HIV-1 is required for efficient viral gene expression (10–15). Tat increases the initiation of transcription from the HIV-1 LTR (14) and affects RNA processing and utilization by interacting with a transactivating responsive element (TAR) located between nucleotides +1 and + 44 with respect to the initiation site (+1) of viral transcription (16, 17). TAR contains a 6-nucleotide loop and a 3-nucleotide pyrimidine bulge that are essential for Tat activity (18–21). Tat binds to the bulge and appears to require cellular factors binding to the loop sequence to efficiently transactivate the HIV-1 LTR (22–24). In addition, Tat interacts with upstream regulatory DNA sequences circumscribed within the NF-κB/Sp1 sites of the HIV-1 promoter (25) and with host cell proteins (12, 24). The 86-amino acid-long Tat contains a highly conserved cysteine-rich region, which mediates the formation of metal-linked dimers *in vitro* and is essential for Tat function (16–18). A conserved basic region with 6 arginines and 2 lysines in nine residues, stretching from amino acid 47 to 58, is crucial for nuclear localization, mediates the specific binding of Tat to TAR RNA, and is required for the full activity of Tat (26–29).

In addition to its role in HIV-1 transcription, Tat may participate in the development of AIDS by modulating the expression of heterologous genes. In support of this possibility, Tat has been shown to increase the expression of cellular genes, such as the IL6 (30) and tumor necrosis factor-β genes (31, 32), and to activate the life cycle of some AIDS-associated viruses (33). The mechanisms of the Tat-mediated activation of non-HIV-1 genes are obscure. Here, we describe the mechanisms for Tat-mediated induction of the IL6 gene expression. We find that Tat is tethered to the IL6 transcription start site by specific binding to a UCU sequence present in the stem-loop

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[1] The abbreviations used are: HIV-1, human immunodeficiency virus type 1; IL6, interleukin-6; TAR, transactivating responsive element; C/EBP, CAAT enhancer-binding protein; EMSA, electrophoretic mobility shift assay; CAT, chloramphenicol acetyltransferase; LTR, long terminal repeat; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; x-gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside.

structure of IL6 leader RNA. Tat physically interacts with C/EBPβ and increases selectively the nuclear pool of C/EBP factors binding to the C/EBP *cis* sequence in the IL6 promoter. This interaction was confirmed to occur *in vivo* by immunoprecipitation and by using the yeast two-hybrid system.

MATERIALS AND METHODS

Plasmids and Cloning Strategies—pILIC-CAT (34), a HIV-LTR-CAT plasmid was obtained from A. Rabson (MBCL, Piscataway, NJ). The 5′ deletion mutants of IL6 promoter, pIL6(−596/+15)-CAT, pIL6(−225/+15), and pIL6(−112/+15)-CAT plasmids, were generated as reported (30). To generate the HIV-1-LTR:(−112/+15) IL6 promoter fusion plasmid, the TAR-deleted *Eco*RI-*Bgl*II fragment of pILIC-CAT was isolated, filled in, and inserted at the *Sst*I site (filled) of pIL6(−112/+15). The resulting pΔILIC:IL6(−112/+15)-CAT plasmid carries the IL6 promoter region from −112 to +15 that substitutes for TAR. To generate pΔILIC:IL6(−112/−67)-CAT plasmid, the *Eco*RI fragment of pΔILIC:IL6(−112/+15)-CAT was filled in and digested with *Ssp*I. The *Eco*RI-*Ssp*I fragment, containing the TAR-deleted LTR fused to the −112/−67 region of IL6 promoter, was cloned in pEMBL-CAT digested with *Bam*HI-*Hin*dIII (filled). The *Ssp*I-*Eco*RI fragment, formed by the −67/+15 region of IL6 promoter fused to a part of the *cat* gene, was recovered and *Hin*dIII-digested. The −67/+15 region of the IL6 promoter, the *Ssp*I-*Hin*dIII fragment, was recovered and cloned in pΔILIC:IL6(−112/+15)-CAT from which the −112/+15 IL6 fragment was removed by *Kpn*I-*Hin*dIII digestion. pIL6(−596/+15) mutants were produced with the Transformer™ site-directed mutagenesis kit, as instructed by manufacturer (CLONTECH Laboratories, Inc., Palo Alto, CA), with minor modifications. In fact, one oligonucleotide was used to introduce the desired mutations in IL6 promoter and to create the site for *Pst*I. The following oligonucleotides were used (the mutated bases are underlined): 5′-CTGAGGCTCATTGGGCCCTCGACCTGCAGGCAT-3′ for pIL6(−596/+15) MI-CAT (bulge mutant); 5′-ATTCTGCCCTAGGCCCGCAGGCATGC-3′ for pIL6(−596/+15) M2-CAT (stem mutant); and 5′-CTGAGGCTCATTCTGAGCTCGACCTGCAGGCAT-3′ for pIL6(−596/+15)M3-CAT (loop mutant).

The pSVT8 and pSVT10 plasmids, expressing the *tat* gene in a sense or antisense orientation, respectively (35), were obtained from A. Caputo. pCMV-TAT plasmid, expressing the first exon of the *tat* gene, and pCMV-TAT 49 were a gift of K. T. Jeang (Laboratory of Molecular Microbiology, NIAID, NIH, Bethesda, MD). The pGEX-TAT plasmid was obtained from M. Giacca (International Center for Genetic Engineering and Biotechnology, Trieste, Italy). In this plasmid, the first exon of the *tat* gene is cloned in pGEX-2T, an isopropyl-1-thio-β-D-galactoside-inducible expression vector (Pharmacia, Uppsala, Sweden), which allows the production of GST-Tat fusion proteins. The pBlue610 plasmid expressing the C/EBPβ (obtained from S. Akira, Institute for Molecular and Cellular Biology, Osaka University, Osaka, Japan) was used for *in vitro* transcription and translation of C/EBPβ. The pSP6:BSF2.5 plasmid, which allows for the *in vitro* production of IL6, was obtained from T. Kishimoto (Institute for Molecular and Cellular Biology, Osaka University, Osaka, Japan). The pGAL4-TAT plasmids, used in yeast transfections, were a gift of B. Cullen (36). In these plasmids, the expression of GAL4-Tat fusion sequences, consisting of wild-type or truncated Tat protein fused to GAL4 DNA-binding domain (amino acids 1–117), is directed by the yeast alcohol dehydrogenase promoter, while the yeast selectable marker is HIS3. To generate pGAD424-C/EBPβ, the c/EBPβ cDNA was cloned downstream the GAL4 activation domain (amino acids 768−881). The C/EBPβ cDNA was excised from pBlue610 by *Sal*I-*Eco*RI digestion and cloned in compatible sites of pGAD-424 vector (37). In this yeast expression vector, the selectable marker is LEU2, while the production of the fusion protein is driven by the alcohol dehydrogenase constitutive promoter. The correct insertion of the plasmids was verified by multiple restriction digestion and by sequencing using the Sanger method (38).

Cell and Transfection Procedures—HeLa-T8 and HeLa-T10, expressing the *tat* gene in a sense or antisense orientation, respectively, have been described (30). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Flow Laboratories, Milan, Italy), 3 mM glutamine, and 10 mM Hepes buffer, pH 7.2 (Life Technologies, Inc., Milan, Italy). For transient expression experiments, cells were transfected by electroporation using a Bio-Rad apparatus (Bio-Rad, Milan, Italy). 3×10^6 cells were resuspended in 0.3 ml of RPMI 1640 supplemented with 20% fetal calf serum and subjected to a double electrical pulse (0.2 V, 960 microfarads) in the presence of the indicated amounts of plasmid DNA. After electroporation, cells were washed and plated in complete medium. Transfection efficiency was monitored by cotransfecting the cells with 5 μg of pnls-LacZ plasmid. β-Galactosidase activity was assayed using 50 μg of protein extracts as described (39).

CAT Assays and Primer Extension Analysis—48 h after transfection, cells were harvested and washed once with PBS. Cell extracts were prepared by three cycles of freeze-thawing in 0.25 M Tris, pH 7.8, and CAT assays were performed as described previously (39). Proteins were measured in each cell extract with the Bio-Rad protein assay kit, and equal amounts of proteins were analyzed for each sample. Each assay contained 50 μg of cell extract, 20 μl of 4 mM acetyl-coenzyme A (Boehringer Mannheim), 1 μl (0.5 μCi) of D-threo-[1,2-^{14}C]-chloramphenicol (DuPont NEN) in a final volume of 150 μl of 0.25 M Tris, pH 7.8. Reactions were incubated for 3 h at 37 °C, extracted with ethyl acetate, dried, and spotted on Polygram Sil G silica gel plates (Macherey-Nagel, Düren, Germany). Plates were run in a TLC tank containing a mixture of chloroform:methanol (95:5). After a 16-h autoradiography, the TLC plates were cut, and samples were counted in a Beckman LS5000TD scintillation counter.

Primer extension was carried out as described (38). 20 μg of total RNA was annealed to the oligonucleotides 5′-CAACGGTGGTATATCCAGTG-3′ (for *cat* RNA) and 5′-CAGATACTACACTTG-3′ (for U2 RNA). RNA was elongated with reverse transcriptase, digested with RNase A, and separated over a 6% denaturing 7 M urea-acrylamide gel.

Electrophoretic Mobility Shift Assays (EMSAs)—Nuclear and cytosolic extracts were isolated as described elsewhere (39−41). Cells were harvested, washed once in cold PBS, and transferred to 1.7-ml microcentrifuge tubes for a second wash. The supernatant was removed, and the cell pellet was resuspended in lysing buffer (10 mM HEPES, pH 7.9, 1 mM EDTA, 60 mM KCl, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 0.2% (v/v) Nonidet P-40). After a 5-min incubation on ice, nuclei were collected by centrifugation (500 × *g*, 5 min). The supernatant (cytosolic proteins) was recovered and stored at −80 °C. Nuclei were rinsed with Nonidet P-40-free lysing buffer, resuspended in 300 μl of Nonidet P-40-free lysing buffer, and layered on the top of 300 μl of the same buffer containing 30% sucrose. After centrifugation at 2,900 × *g* for 10 min, the pelletted nuclei were resuspended in 150 μl of buffer containing 250 mM Tris-HCl, pH 7.8, 60 mM KCl, 1 mM DTT, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Nuclei were then subjected to three cycles of freezing and thawing. The suspension was cleared by centrifugation (135,000 × *g*, 15 min), and aliquots were immediately tested in a gel retardation assay or stored at −80 °C until use.

For EMSAs, the following oligonucleotide probes were used: 5′-GATCGGACGTCACATTGCACAATCTTAATAAT-3 (IL6 C/EBP binding site), 5-GGACGTCACACTACAAACTCTTAATAA-3′ (mutant IL6 C/EBP binding site), 5′-TCGAGTTGCCTGGACTTGCCTGGCCTTGCCTTTTC-3′ (p53 binding site), 5′-CATTCTGCCCTCGAG-3′ (IL6, nucleotide +1/+15 coding strand), and 5′-CTCGAGGGCAGAATG-3′ (IL6, nucleotide +1/+15 minus strand). Each oligonucleotide was annealed to its complementary strand and end-labeled with [γ-^{32}P]ATP (Amersham Life Science, Inc.) by using polynucleotide kinase (New England Biolabs, Beverly, MA). Equal amounts of nuclear extracts were incubated in a reaction mixture consisting of 20 μl of buffer containing 10% glycerol, 60 mM KCl, 1 mM EDTA, 1 mM DTT, 2 μg of poly(dI-dC) (Boehringer Mannheim), and 5 μg of extract for 5 min on ice. 1 μl of γ-^{32}P-labeled double-stranded probe (0.2 ng, $4-6 \times 10^4$ cpm) was then added with or without a 100-fold molar excess of competitor wild-type or mutant oligonucleotide. Where indicated, 2 μg of antibody to C/EBP (Santa Cruz Biotechnology Inc., Santa Cruz, CA), or preimmune serum were added. The reactions were incubated at room temperature for 20 min and run on a 5% acrylamide/bisacrylamide (30:1) gel in 22.5 mM Tris borate, 0.5 mM EDTA, and 3.7 mM β-mercaptoethanol. Gels were dried and autoradiographed.

Tat-RNA EMSAs—To produce the IL6 RNA [α-^{32}P]UTP-labeled probes, we used oligonucleotides encompassing the sequence of T7 promoter and either the wild type or mutant IL6 untranslated sequences in sense or antisense orientation. These were as follows: 5′-GATCTAATACGACTCACTATAGGGCATTCTGCCCTCGAGCCACCGGGAAC-3′ (IL6 wild type sense), 5′-GATCTAATACGACTCACTATAGGGCATTGGGCCCTCGAGCCACCGGGAAC-3′ (IL6 MI sense), 5′-GATCTAATACGACTCACTATAGGGCATTCTGCCCTAGGGCCACCGGGAAC-3′ (IL6 M2 sense), and 5′-GATCTAATACGACTCACTATAGGGCATTCTGAGCTCGAGCCACCGGGAAC-3′ (IL6 M3 sense). For antisense transcripts, the following oligonucleotides were used: 5′-GATCTAATACGACTCACTATAGGGCAAGGGCCACCGAGCTCCCGTCTTAC-3′ (IL6 wild type antisense), 5′-GATCTAATACGACTCACTATAGGGCAAGGGCCACCGAGCTCCCGGGTTAC-3′ (IL6 M1 antisense), 5′-GATCTAATACGACTCACTATAGGGCAAGGGCCACCGGGATC-

CCGTCTTAC-3′ (IL6 M2 antisense), 5′-GATCTAATACGACTCACTATAGGGCAAGGGCCACCGAGCTCGATCTTAC-3′. TAR RNA was obtained by transcribing the *Hin*dIII-linearized pTAR plasmid with T7 RNA polymerase. ^{32}P-labeled transcripts were resolved in an 8 M urea, 10% polyacrylamide gel and eluted from the gel slides by a 37 °C overnight incubation in TE buffer (10 mM Tris/Cl, pH 8.0, 1 mM EDTA, pH 8.0). Eluted RNAs were extracted with phenol-chloroform and precipitated with ethanol. The standard binding reaction was performed in 20 μl of reaction mixture containing 1 × binding buffer (25 mM Tris/HCl, pH 8.0, 1 mM $MgCl_2$, 0.5 mM DTT, 50 mM NaCl, 5% glycerol), 1 μg of sonicated salmon sperm DNA, 0.4 μg of tRNA, 15 units of RNase inhibitor, 2.5 μg of recombinant Tat (AIDS Research and Reference Reagents) or of GST or GST-Tat, and competitors. After a 10-min incubation in ice, 1 μl of ^{32}P-labeled probe (about 0.3 ng, 70,000 cpm) was added. The samples were incubated for 20 min at room temperature and run on 6% polyacrylamide gel, 0.5 × TBE at 4 °C, and 180 V. Gels were dried and autoradiographed.

In Vitro Tat-C/EBP Protein Interactions—To produce GST-Tat proteins, the plasmid pGEX-TAT was introduced in *Escherichia coli* strain SF_8. Bacteria containing the plasmid were grown to 0.4 A_{600} and induced with 0.5 mM isopropyl-1-thio-β-D-galactoside, Inalco, Milan, Italy) for 2 h. Cells were then collected by centrifugation at 4 °C (3,000 × *g* for 15 min) and resuspended in RE buffer containing 50 mM Tris/HCl, pH 8.0, 120 mM NaCl, 0.5% Nonidet P-40, 5 mM DTT, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM PMSF. Cells were broken with a French press apparatus, and the lysates were clarified by centrifugation at 4 °C and 27,000 × *g* for 30 min. Proteins were recovered and added to glutathione-Sepharose beads (Pharmacia), previously equilibrated in RE buffer. After an overnight incubation, the beads were extensively washed, and GST-Tat was eluted in RE buffer with 10 mM glutathione. The ^{35}S-labeled C/EBPβ proteins were *in vitro* translated by using TNT™ coupled reticulocyte lysate systems (Promega, Madison, WI) according to the instructions of the manufacturer. For protein interaction studies, 10 μg of GST and GST-Tat proteins were incubated with 15 μl of translation mixture in buffer A (20 mM Hepes, pH 7.9, 10 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 150 mM NaCl, 5% (v/v) glycerol, and 0.05% Nonidet P-40). The samples were incubated for 2 h at room temperature. At the same time, the glutathione-Sepharose beads were washed, blocked in buffer A with 1 mg/ml BSA for 2 h, and washed again. These beads were added to the samples. After 3 h, the beads were collected by centrifugation (2,000 × *g* for 10 s) and washed 10 times with buffer A. The pellets were then resuspended in sample buffer (70 mM Tris/HCl, pH 6.8, 7 mM EDTA, 0.01% bromphenol blue, 13% sucrose, 1% SDS, 7 M urea, and 10% (v/v) β-mercaptoethanol) and resolved on 12% SDS-polyacrylamide gel. Gels were treated with the Entensify kit (DuPont NEN), dried, and exposed.

Immunoprecipitation and Immunoblotting Analysis—Total cell extracts were prepared as described (38). Briefly, 2 × 10^7 transfected cells were harvested, washed twice with cold PBS, and resuspended in 0.5 ml of lysis buffer containing 50 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EGTA, 1.5 mM $MgCl_2$, 100 mM NaF, 10 mM sodium pyrophosphate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 5 mM DTT. Samples were incubated for 1 h in ice and lysed by gently passing through a 21-gauge needle. After centrifugation at (500 × *g*, 15 min), the proteins were recovered and stored at −80 °C. For immunoprecipitation, 1 mg of protein was incubated overnight at 4 °C with 20 μg of mouse anti-Tat monoclonal antibody (ABT, Milan, Italy). The immunocomplexes were precipitated with protein A-Sepharose (Sigma-Aldrich, Milan, Italy) by centrifugation at 720 × *g* for 10 min. The immunoprecipitates were washed several times in buffer containing 20 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, 1 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 5 mM DTT. Immunoprecipitates were resuspended in sample buffer, run on 12% SDS-polyacrylamide gel, and analyzed by immunoblotting with anti-C/EBPβ polyclonal antibody (Santa Cruz Biotechnology), or anti-Tat antibody (AIDS Research and Reference Reagents).

Immunoblotting analysis was performed as described (39). Total cell extracts, nuclear proteins, or cytosolic proteins (10 μg) were separated by SDS-10% polyacrylamide gel electrophoresis, transferred onto a membrane filter (Cellulosenitrate, Schleicher & Schuell), and incubated with the indicated first antibody in PBS plus 5% dry milk for 2 h at room temperature. Filters were washed three times in PBS and incubated with peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (Boehringer Mannheim) at a 1:2000 dilution for 1 h. The proteins were revealed by using the enhanced chemiluminescence system (ECL) (Amersham).

In Vivo Protein Interaction: Two-hybrid System—Saccaromyces cerevisiae CTY2 strain was grown in YPD medium (20 g/liter peptone, 10 g/liter yeast extract, 20 g/liter glucose, pH 5.8) and transformed by electroporation. Yeast were inoculated at 3 × 10^6 cells/ml and grown up to 3 × 10^7 cells/ml. Cells were harvested by centrifugation at room temperature (3,000 × *g*, 15 min) and rinsed with H_2O. Yeast pellet was resuspended in prepulse buffer (10 mM Tris/HCl, pH 7.5, 1 M sorbitol) and left for 30 min at room temperature. Cells were collected and resuspended in YPD, 1 M sorbitol at 6 × 10^9 cells/ml. 3 × 10^8 cells were added to the DNA mix consisting of 20 μg of each plasmid in YPD, 1 M sorbitol to a 10-ml final volume. After 10 min, the mixture was electroporated with a Bio-Rad apparatus set at 1,100 V, 600 ohms, and 25 microfarads in 0.2-cm gap cuvettes. Cells were transferred in 1 ml of 1 M sorbitol and plated on 5-bromo-4-chloro-3-indolyl β-D-galactoside (x-gal)-selective minimal medium containing 6.7 g of yeast nitrogen base, 20 g of agar, 20 g of glucose, 1 M sorbitol, 1 × amino acids minus leucine and histidine, 0.1 M KPO_4, pH 7, 20 μg/ml of x-gal and H_2O to 1 liter. Colonies were visible in 3 days and blue-positive after 4–5 days. The expression of fusion proteins was assayed by immunoblotting, as previously reported (39). For this purpose, 50 μg of cell extracts of transfected yeast cells were probed with polyclonal antibodies to Tat or to C/EBP proteins, which were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (Bethesda, MD) and from Santa Cruz Biotechnology, respectively.

RESULTS

Identification of the Region of the IL6 Promoter Responsive to Tat—We have recently reported that *tat* expression in epithelial HeLa cells and in MC3 lymphoblastoid cells resulted in the activation of endogenous IL6 gene transcription, as well as in the transcriptional induction of pIL6-CAT plasmid, an IL6 promoter-CAT construct (30). To gain further insight into the molecular mechanisms of the Tat-mediated activation of the IL6 gene, we constructed 5′ deletion mutants of pIL6-CAT in which the region from −596 to +15, −225 to +15, or −112 to +15 (29, 41), was inserted 5′ to the *cat* gene (shown in Fig. 1). These plasmids, hereafter referred to as pIL6(−596/+15)-CAT, pIL6(−225/+15)-CAT, and pIL6(−112/+15)-CAT, respectively, were transiently transfected in HeLa cells stably expressing the *tat* gene in a sense (HeLa-T8) or antisense (HeLa-T10) orientation. Results from these experiments showed that pIL6(−596/+15)-CAT and pIL6(−225/+15)-CAT plasmids were efficiently transactivated by Tat, while the pIL6(−112/+15)-CAT construct was unresponsive to Tat (Fig. 1). This suggested that Tat-induced activation of the IL6 promoter required a region located between −225 and −112 bp. Indeed, this region harbors a C/EBP (NF-IL6) enhancer necessary for efficient IL6 promoter function (30, 43). Next, we generated a plasmid where the −112/+15 base pair region of the IL6 promoter was inserted downstream to a TAR-deleted HIV-1 LTR sequence (pΔILIC-CAT). The resulting pΔILIC:IL6(−112/+15)-CAT plasmid (shown in Fig. 1) was transiently expressed in Tat-positive or Tat-negative HeLa cells. The −112/+15 sequence of the IL6 promoter, (see pIL6(−112/+15)-CAT in Fig. 1), conferred Tat responsiveness to the TAR-deleted HIV-1 LTR promoter (compare pΔILIC-CAT and pΔILIC:IL6(−112/+15)-CAT plasmids in Fig. 1). This indicated that the −112/+15 region, which was unresponsive to Tat in the context of the IL6 promoter (see pIL6(−112/+15)-CAT in Fig. 1), could act as a TAR-like element when placed in the context of the HIV-1 promoter.

A primer extension analysis of *cat* mRNA transcribed from pIL6(−596/+15)-CAT revealed a protected band of 98 nucleotides (Fig. 2*A*), corresponding to the major transcription start site of the IL6 gene (42). The pΔILIC:IL6(−112/+15)-CAT generated a major band of 248 nucleotides, corresponding to the transcription start site of the HIV-1 LTR (shown in Fig. 2*A*). Moreover, we observed the presence of a 98-nucleotide additional *cat* band in cells transfected with the pΔILIC:IL6(−112/+15)-CAT, indicating that the start sites of the IL6 promoter and of the HIV-1 LTR were both functional (Fig. 2*B*). A densi-

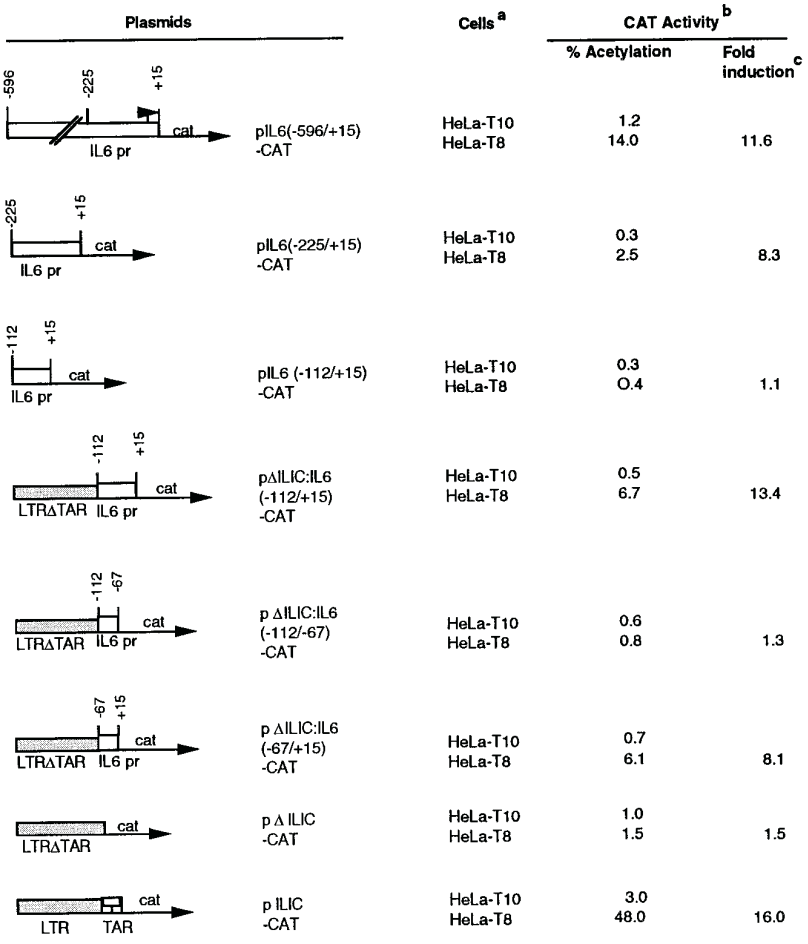


FIG. 1. **Expression of pIL6-CAT and pΔILIC:IL6-CAT plasmids in *tat* (HeLa-T8) or anti-*tat* (HeLa-T10) transfected HeLa cells.** *a*, HeLa-T10 or HeLa-T8 were transiently transfected with 10 μg of the indicated plasmids. *b*, CAT activity was determined 48 h after transfection by using 50 μg of whole cellular extract. *c*, expressed as the ratio of the percentages acetylated. Transfection efficiency was monitored by co-transfecting the cells with 5 μg of pnls-LacZ plasmid. β-Galactosidase activity was assayed using 50 μg of protein extracts as described (39). The data are representative of five independent experiments in which two different plasmid preparations were used. Similar results were obtained by transient expression of pSV-T8 and pSV-T10 plasmids.

tometric analysis of the *cat* bands confirmed that the HIV-1 LTR start site was preferentially utilized, with a minimal transcription originating from the IL6 promoter start site (not shown). The amount of *cat* mRNA in *tat*-expressing cells was 8–10-fold higher than the *cat* mRNA transcribed by anti-*tat*-transfected cells. In fact, both the 98-nucleotide *cat* band generated by transfecting pIL6(−596/+15)-CAT and the 248-nucleotide *cat* band generated by the pΔILIC:IL6(−112/+15)-CAT were stronger in Tat-positive than in Tat-negative cells (Fig. 2*A*). These results identified the sequence of −112/+15 as the minimal region of the IL6 promoter required for Tat to transactivate the ΔTAR HIV-1-LTR. This suggested that the −112/+15 base pair region could function as a Tat-responsive sequence, possibly allowing Tat to be directed close to the TATA box of the IL6 promoter.

HIV-1 Tat Interacts with the IL6 Leader RNA—The primer extension results shown in Fig. 2, *A* and *B*, indicated that two transcription start sites were active in pΔILIC:IL6(−112/+15)-CAT hybrid plasmid. This allowed the construction of the pΔILIC:IL6(−112/−67)-CAT plasmid, where the region of −67/+15, encompassing both the transcription start site and the 5′-untranslated region of the IL6 gene, was deleted (shown in Fig. 1). The resulting pΔILIC:IL6(−112/−67)-CAT plasmid was transiently transfected in HeLa-T10 (Tat-negative) and HeLa-T8 (Tat-positive) cells to address the question of whether the IL6 leader RNA was required for Tat-induced activation. As shown in Fig. 1, the pΔILIC:IL6(−112/−67)-CAT plasmid was unresponsive to Tat, indicating that a discrete region of IL6 leader RNA is strictly required for Tat. Accordingly, the −67/+15 region, encompassing the transcription start site and the 5′-untranslated region of the IL6 gene, restored the responsiveness of the Tat-deleted pΔILIC-CAT plasmid (see pΔILIC:IL6(−67/+15)-CAT in Fig. 1). A secondary structure analysis of this region according to the energy-minimizing algorithm of Zuker (44) defines an RNA stem-loop structure at the 5′-untranslated region of the IL6 mRNA (shown in Fig. 3). This RNA contains a UCU stretch that fulfills the sequence requirements for Tat binding to an RNA structure (45) and is potentially able to bind to Tat. To test this possibility, point mutations affecting the secondary RNA structure of the IL6 leader RNA at the bulge, stem, or loop were introduced into pIL6(−596/+15)-CAT. The resulting mutant plasmids (shown in Fig. 3) were tested for responsiveness to Tat in transient expression experiments. As shown in Fig. 3, mutations that affect the bulge and the stem RNA (mutant M1 and M2, respectively) led to a drastic decrease in Tat responsiveness, while mutations of the loop were ineffective (mutant M3). In these experiments, the pIL6(−596/+15)M1-CAT and pIL6(−596/+15)M2-CAT plasmids did show a significant activation in Tat-positive (HeLa-T8) cells, suggesting that Tat can function, albeit at lower efficiency, in the absence of an RNA tethering structure. Indeed, Tat is able to activate the transcription of HIV-1 genes in a TAR-independent way, as recently reported (46–47).

To test for the physical binding of Tat to the leader IL6 RNA, oligonucleotides corresponding to the wild-type IL6 leader RNA and to the relative mutants M1, M2, and M3 (shown in Fig. 3), were placed under the transcriptional control of the T7 promoter in a sense or antisense orientation and *in vitro* transcribed. Labeled RNAs were then tested for binding to Tat in an RNA-protein EMSA. Results shown in Fig. 4 indicate that Tat is able to specifically bind to the wild-type IL6 leader RNA. The Tat RNA binding was not displaced by the M1-RNA or M2-RNA

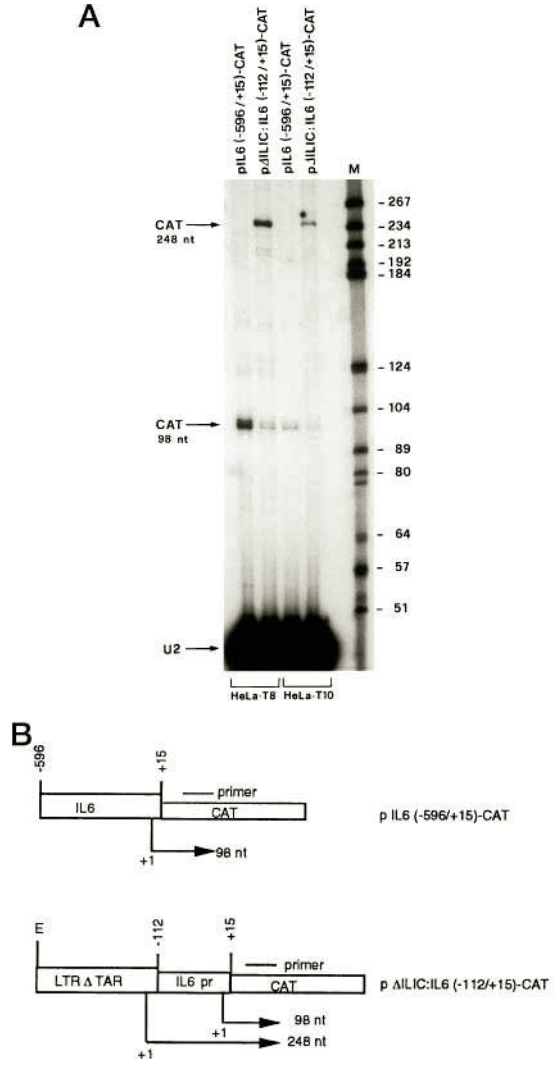
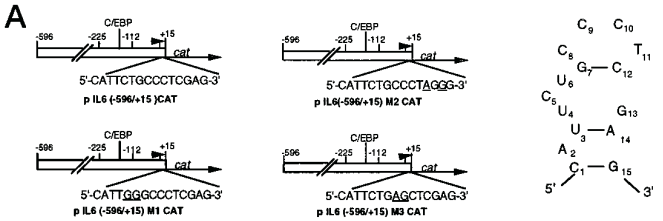


FIG. 2. **Primer extension analysis of *cat* mRNA in HeLa-T10 or HeLa-T8 cells transfected with pIL6(−596/+15)-CAT or with pΔILIC:IL6(−112/+15)-CAT plasmids.** *A*, HeLa-T10 (Tat-negative) or HeLa-T8 (Tat-positive) cells were transfected with 10 μg of pIL6(−596/+15)-CAT or pΔILIC:IL6(−112/+15)-CAT. 24 h later, total RNA was isolated and analyzed by primer extension. The 98- and 248-nucleotide *cat* transcripts are shown after a 24-h exposure. *B*, schematic representation of the transcription start sites of pIL6(−596/+15)-CAT and of pΔILIC-IL6(−112/+15)-CAT plasmids. Nucleotides are numbered from the first transcribed nucleotide, according to the results shown in *A*.



B

Plasmids	Cells[a]	CAT activity[b] % Acetylation	Fold induction[c]
pIL6(-596/+15)-CAT	HeLa-T10	1.2 (0.1)	
	HeLa-T8	16.7 (1.4)	13.5 (1.6)
pIL6(-596/+15)M1 CAT	HeLa-T10	0.9 (0.2)	
	HeLa-T8	4.4 (0.5)	4.7 (0.9)
pIL6(-596/+15)M2 CAT	HeLa-T10	0.8 (0.2)	
	HeLa-T8	6.0 (0.5)	6.5 (0.7)
pIL6(-596/+15)M3 CAT	HeLa-T10	1.2 (0.2)	
	HeLa-T8	14.5 (2.0)	14.6 (1.7)
pILC-CAT	HeLa-T10	4.0 (0.5)	
	HeLa-T8	60.3 (5.5)	15.2 (1.3)

FIG. 3. **Expression of wild-type pIL6(−596/+15)-CAT and of the mutant plasmids in Tat-positive or Tat-negative HeLa cells.** *A*, schematic representation of pIL6(−596/+15)-CAT and pIL6(−596/+15)M1-CAT, pIL6(−596/+15)M2-CAT, or pIL6(−596/+15)M3-CAT plasmids. The predicted secondary structure of the IL6 5′-untranslated region is also shown. M1 and M2 mutants carry mutations affecting the bulge and the stem loop, respectively. M3 mutant carries mutations affecting the loop of the structure. *B*, Tat responsiveness of pIL6(−596/+15)-CAT and of pIL6(−596/+15)M1-CAT, or pIL6(−596/+15)M2-CAT, or pIL6(−596/+15)M3-CAT plasmids. Cells were transfected with 10 μg of the indicated plasmids and tested for CAT activity as detailed under "Materials and Methods." *a*, HeLa-T10 or HeLa-T8 cells were transiently transfected with 10 μg of the indicated plasmids. *b*, CAT activity was determined 48 h after transfection by using 50 μg of whole cellular extract. *c*, expressed as the ratio of the percentages acetylated. The data are the mean ± S.D. (shown in parentheses) of five independent experiments.

(affecting the bulge and stem IL6 RNA, respectively), while M3-RNA, affecting the loop IL6 RNA, was able to compete for the binding to Tat. In these experiments, wild-type TAR RNA competed specifically with IL6 RNA for the binding to Tat (Fig. 4*A*). Accordingly, the binding of Tat to TAR RNA was displaced by wild-type IL6 RNA and by M3-RNA, while M1-RNA and M2-RNA were substantially ineffective (Fig. 4*B*). In parallel experiments, the antisense RNA sequences were unable to bind to Tat (not shown). Moreover, Tat did not bind to either the single or to the double-stranded oligonucleotides corresponding to the IL6 leader RNA (shown in Fig. 4*C*).

The Basic Region of Tat Is Required for Tat-mediated Expression of the IL6 Gene—A basic domain of Tat, encompassing the amino acid residues 47–58, has been shown to significantly contribute to the capacity of Tat to bind to HIV-1 TAR RNA through an arginine fork (16, 17, 28, 49). To test whether the arginine-rich domain of Tat is required to transactivate the IL6 promoter, we transfected HeLa cells with plasmids expressing either Tat amino acid residues 1–72 or a truncated form of Tat (residues 1–49, lacking the basic domain), together with pΔILIC:IL6(-112/+15)-CAT, pΔILIC:IL6(-112/−67)-CAT, or pΔILIC:IL6(−67/+15)-CAT plasmid. Results from these transient expression experiments showed that the Tat protein lacking the basic domain was unable to significantly transactivate the IL6 promoter (compare the results for pΔILIC:IL6(−112/+15)-CAT in Table I). Consistent with the results shown in Fig. 1, pΔILIC(−112/−67)-CAT, lacking the IL6 leader RNA, was unresponsive to both of the *tat*-expressing plasmids, while pΔILIC:IL6(−67/+15)-CAT was fully responsive to Tat-(1–72). It is noteworthy that the residue 1–49-truncated Tat was still able to activate the wild-type HIV-1 LTR, albeit at a lower level than the wild-type Tat, suggesting that the amino-terminal domain of Tat can function as a transcription factor in the absence of TAR binding. Under these circumstances, Tat is possibly tethered to the HIV-1 LTR by a strong interaction with transcription factors binding to HIV-1 LTR *cis* sequences. This possibility is supported by the observation that Tat cooperates with transcription factors binding upstream regulatory DNA sequences circumscribed within the NF-κB/Sp1 region of the HIV-1 promoter and with host cell proteins (12, 24, 25). Indeed, a binding of Tat to Sp1 factors has been reported (50).

HIV-1 Tat Induces an Increase in C/EBP Binding Activity and Interacts with C/EBP Transcription Factors—To gain further insights into the molecular mechanisms of the Tat-mediated activation of the IL6 promoter, we tested whether Tat might induce an increased DNA binding activity of C/EBP (NF-IL6) transcription factors, which are major stimulants of the IL6 promoter (43). Nuclear extracts from HeLa cells transfected with pSVT8 (*tat*-expressing) or with pSVT10 (expressing *tat* in a antisense orientation) were tested for binding to an oligonucleotide corresponding to the C/EBP *cis* sequence of IL6 promoter. As shown in Fig. 5*A*, *tat* expression leads to a significant increase in C/EBP DNA binding activity. Moreover, an

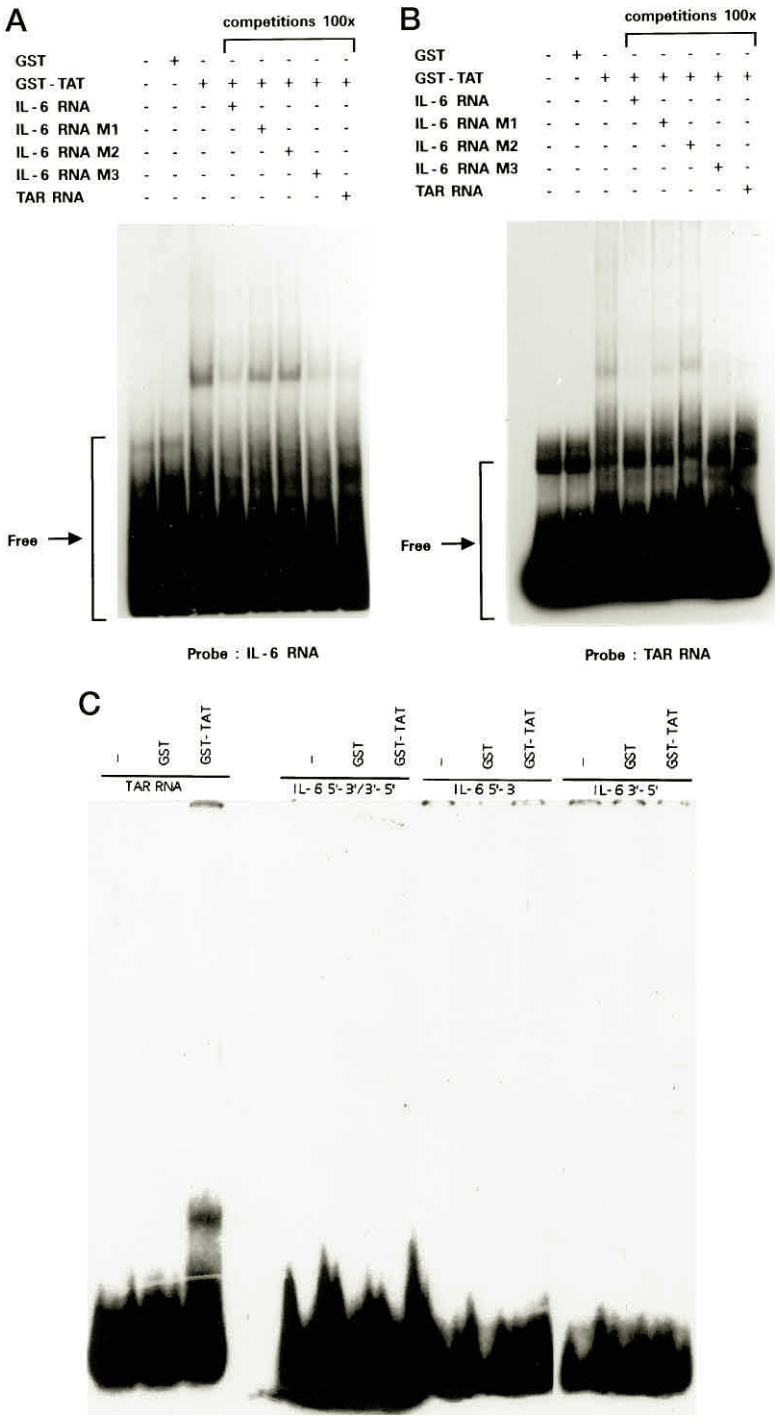


FIG. 4. **HIV-1 Tat binds to the IL6 leader RNA sequence.** *A*, double-stranded oligonucleotides corresponding to either wild type IL6 leader RNA or to the relative mutant RNAs (shown in Fig. 3*A*) were *in vitro* transcribed in the presence of [^{32}P]UTP and tested for binding to recombinant Tat. *B*, the HIV-1 TAR sequence (+1/+44) was *in vitro* transcribed in presence of [^{32}P]-UTP and tested for binding to recombinant Tat. Cross-competitions were tested at 100-fold molar concentrations of unlabeled IL6 or TAR RNAs. *C*, single- or double-stranded oligonucleotides corresponding to the region of +1 to +15 of the IL6 promoter were labeled with [γ-^{32}P]ATP and tested for binding to GST or to GST-Tat proteins as detailed under "Materials and Methods." In control experiments, the HIV-1 TAR sequence (+1/+44) was *in vitro* transcribed in the presence of [^{32}P]UTP and tested for binding to GST or to GST-Tat proteins.

antiserum to C/EBPβ supershifted the C/EBP complex, while an antiserum to C/EBPδ was ineffective. In parallel experiments, cytosolic extracts from *tat*- or anti-*tat*-transfected cells expressed equal levels of C/EBP DNA binding activity (Fig. 5*B*). Aliquots of nuclear or cytosolic extracts were assayed for p53 DNA binding activity to monitor for protein concentrations (data not shown).

Immunoblot analysis of cell extracts of HeLa cells transfected with either pSVT8 or pSVT10 plasmid revealed equal amounts of total or cytosolic C/EBPβ in both *tat*- and anti-*tat*-transfected cells, while a consistent increase in C/EBPβ proteins was observed in the nuclear fraction of *tat*-transfected cells (shown in Fig. 6*A*). The increase in the nuclear C/EBPβ was detectable at 36 h post-transfection and declined thereafter (not shown). These data indicate that Tat specifically increases the nuclear levels of C/EBPβ factors, resulting in an enhanced binding activity to C/EBP *cis* sequence. Under the same conditions, C/EBPδ proteins were undetectable (data not shown).

To address the question of whether Tat could interact with C/EBP transcription factors, C/EBPβ was *in vitro* translated and tested for binding to a GST-Tat fusion protein. As shown in Fig. 7, Tat physically associated with C/EBPβ. Under the same conditions, Tat did not bind to IL6 control protein *in vitro* produced from pSP6:BSF2.5 plasmid (not shown).

To test the possibility that Tat-C/EBP complexes could form *in vivo*, HeLa cells were transiently transfected with pSVT8 plasmid and subjected to immunoprecipitation with a Tat-specific monoclonal antibody followed by immunoblotting with antibodies to C/EBP proteins. We observed that Tat was

TABLE I
The basic domain of Tat is required for Tat-induced expression of the IL6 gene

Transfected plasmids[a]	Acetylation[b]	Fold induction[c]
	%	
pΔILIC:IL6(−112/+15)-CAT	0.9 (0.1)	
pΔILIC:IL6(−112/+15)-CAT + pCMV-TAT(1–72)	8.0 (0.5)	9.4 (1.3)
pΔILIC:IL6(−112/+15)-CAT + pCMV-TAT(1–49)	1.3 (0.2)	1.5 (0.2)
pΔILIC:IL6(−112/−67)-CAT	0.7 (0.1)	
pΔILIC:IL6(−112/−67)-CAT + pCMV-TAT(1–72)	1.7 (0.2)	2.3 (0.3)
pΔILIC:IL6(−112/−67)-CAT + pCMV-TAT(1–49)	1.3 (0.3)	2.1 (0.4)
pΔILIC:IL6(−67/+15)-CAT	0.8 (0.1)	
pΔILIC:IL6(−67/+15)-CAT + pCMV-TAT(1–72)	11.7 (1.5)	13.3 (2.0)
pΔILIC:IL6(−67/+15)-CAT + pCMV-TAT(1–49)	1.4 (0.3)	1.7 (0.3)
pILIC-CAT	0.8 (0.1)	
pILIC-CAT + pCMV-TAT(1–72)	19.1 (2.3)	23.8 (3.1)
pILIC-CAT + pCMV-TAT(1–49)	4.5 (0.6)	5.6 (0.7)

[a] pCMV-TAT(1–72) or pCMV-TAT(1–49), expressing the first exon of Tat or a truncated form of Tat lacking the basic domain, respectively, was co-transfected with the indicated reporter CAT plasmids in HeLa cells. Trans-activation by Tat was determined by assaying CAT activity in cell extracts, as detailed under "Materials and Methods."
[b] Determined by scintillation counting of acetylated spots.
[c] Expressed as the ratio of the percentages acetylated. Data are expressed as mean ± S.D. (shown in parentheses) of five independent experiments.

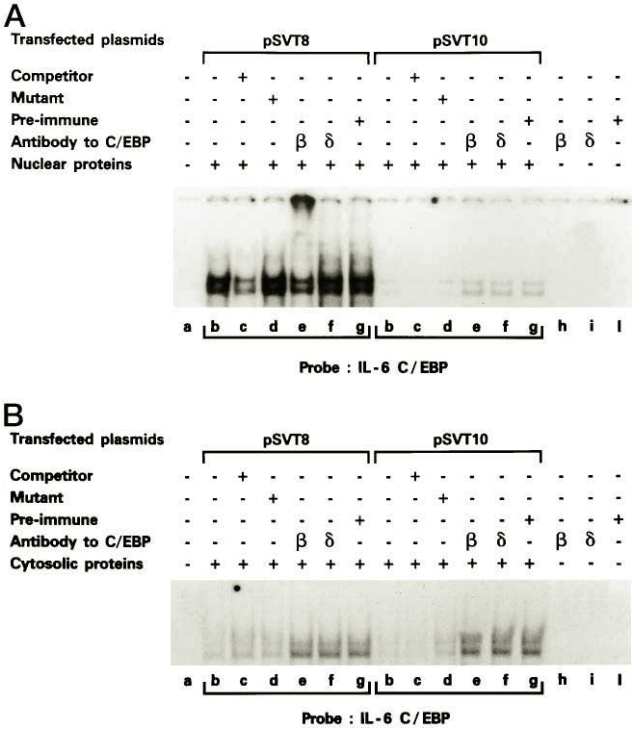


FIG. 5. **Induction of C/EBP DNA binding activity by Tat.** Cells were transiently transfected with 10 μg of the indicated plasmids. 24 h post-transfection, 5 μg of nuclear (*A*) or cytosolic (*B*) extracts were analyzed for binding to the C/EBP *cis* element of IL6 promoter. Competitions were tested with 50-fold molar concentrations of unlabeled C/EBP or mutant C/EBP oligonucleotides. 1 μl of preimmune serum or of an antiserum to C/EBPβ or C/EBPδ was added to the reaction mixtures. Aliquots of nuclear or cytosolic extracts were analyzed for equal protein content by testing their binding to a double-stranded oligonucleotide corresponding to the p53 DNA-binding region (Ref. 85, data not shown). The data are representative of three independent experiments.

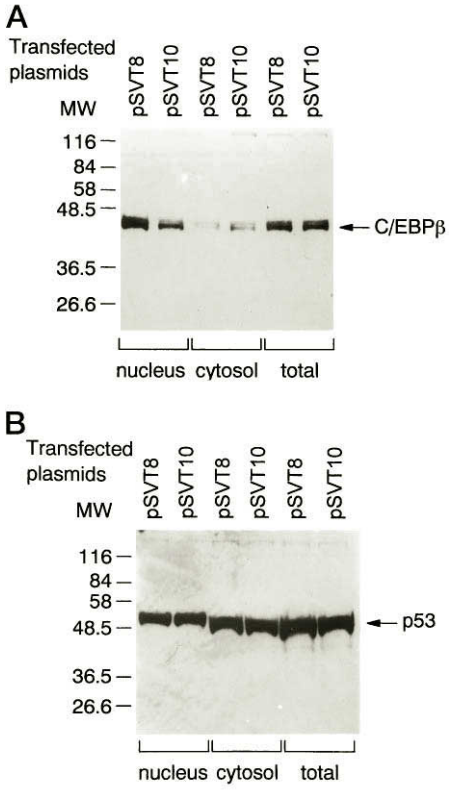


FIG. 6. **Immunoblot analysis of cell extracts of *tat*- or anti-*tat*-transfected cells.** HeLa cells were transiently transfected with the indicated plasmids and harvested at 36 h post-transfection. 10 μg of nuclear, cytosolic, or total cell extracts were analyzed by using a rabbit antiserum to C/EBPβ (*A*). Under the same conditions, C/EBPδ proteins were undetectable (data not shown). The filter was stripped and probed with a control monoclonal antibody to p53 (*B*). Detection was achieved by ECL. Results of a 1-min exposure are shown.

readily revealed in transfected cells, and that C/EBPβ was specifically detected in immunoprecipitates of *tat*-expressing cells (Fig. 8, *A* and *B*).

To test whether Tat could functionally cooperate with C/EBP factors, we took advantage of the yeast genetic two-hybrid system (37). For this purpose, the C/EBPβ cDNA was inserted in frame with the sequence of GAL4 coding for the GAL4 activation domain (amino acid residues 768–881). The resulting pGAD424-C/EBPβ plasmid was cotransfected with pGAL4-TAT plasmids carrying the full-length *tat* sequence or truncated sequences of *tat*, fused to the GAL4 DNA-binding domain (shown in Fig. 9) in the CTY2 yeast strain, which carries an integrated copy of *lacZ* gene. Blue colonies grown on x-gal-selective medium were evaluated as indicative of *in vivo* interaction between C/EBPβ and discrete regions of Tat. Results shown in Fig. 9 indicate that Tat interacted *in vivo* with C/EBPβ and that this interaction resulted in the transcrip-

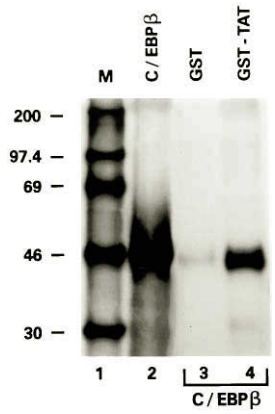


FIG. 7. ***In vitro* interaction of Tat with C/EBPβ.** C/EBPβ proteins were *in vitro* translated and tested for binding to GST-Tat fusion proteins, as detailed under "Materials and Methods." *Lane 2* shows the translated labeled proteins. The amount of C/EBPβ proteins shown in *lane 2* was applied to GST or GST-Tat proteins (*lanes 3* and *4*, respectively).

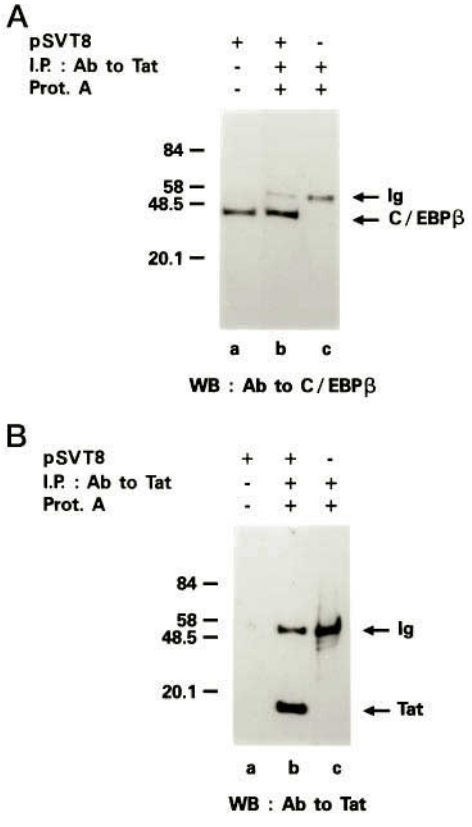


FIG. 8. **Co-immunoprecipitation of Tat and C/EBPβ in *tat*-transfected HeLa cells.** *A*, whole lysate (1 mg) of pSVT8-transfected cells was subjected to immunoprecipitation with a monoclonal antibody to Tat, followed by immunoblotting with a rabbit polyclonal antibody to C/EBPβ (*lane b*). *Lane a* shows the amount of C/EBPβ expressed by 30 μg of cell lysate. In *lane c*, lysate (1 mg) from untransfected HeLa cells is shown. Results of a 1-min exposure are shown. *B*, the filter shown in *A* was stripped and stained with the antibody to Tat. Detection was achieved by ECL. Results of a 5-min exposure are shown.

tional activation of the integrated *lacZ* reporter gene. This activation was comparable with the transcription induced by Tat homodimerization (Ref. 36, shown in the *first line* of Fig. 9 as the interaction of Tat fused to GAL4 binding domain with Tat fused to VP16 activation domain). Moreover, this activation also occurred when Tat-(1–47) was used as a partner of C/EBPβ, indicating that the N-terminal, cysteine-rich, and core regions of Tat represent the minimal region of Tat required for

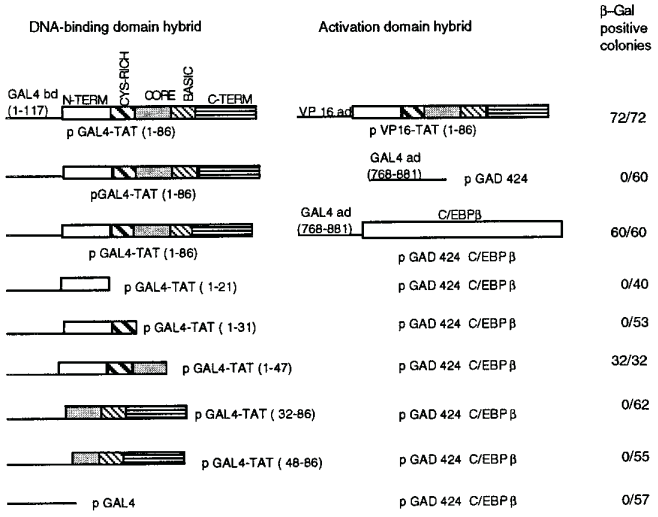


FIG. 9. **In vivo interaction of Tat and C/EBPβ proteins.** β-Galactosidase-positive colonies were determined after transformation of yeast strain CTY2 with the indicated combinations of plasmids, as detailed under "Materials and Methods." Data show the number of blue colonies/number of double transformants grown on x-gal-, leucine-, and histidine-selective media. The number of the blue colonies grown on the appropriate selective medium was 0 in the case of pGAD-424, pGAL4, and all of the pGAL4-TAT mutants transfected alone. No blue colonies were detected in the case of pGAL4-TAT, pVP16-TAT, and pGAD-424-C/EBPβ.

an efficient heterodimerization, while the entire protein is required for a full transcriptional activation. In these experiments, comparable amounts of Tat or C/EBP proteins were produced by transfected yeast cells, as seen by immunoblotting of cell extracts, using antibodies to Tat or to C/EBPβ (data not shown).

DISCUSSION

Despite the intensive investigation of the immunopathogenesis of AIDS, many questions concerning the molecular mechanisms of HIV-1 primary infection and progression remain unanswered (5, 51, 52). Recently, the identification of cohorts of HIV-exposed individuals who remain free of infection over a long period of viral exposure (53) as well as the existence of a small subgroup of HIV-1-infected subjects who are long term nonprogressors were described (54). Together with recent reports on viral life cycle (55, 56), the above evidence argues that HIV infection and disease progression may ultimately result from a complex interplay between viral and host cellular factors involved in the immunological response to the viral infection and in the clinical evolution of AIDS.

HIV-1 Tat is a potent transactivator of HIV-1 LTR, acting on nascent TAR RNA and promoting full-length gene transcription (10–13). Accordingly, Tat-defective HIV-1 is not viable (57, 58). Emerging evidence shows that, in addition to its role on HIV-1 gene expression, Tat may exert additional functions. Tat is released in some extent extracellularly (20, 59) and can function as a cytokine. In fact, Tat promotes the growth of endothelial cells and Kaposi's sarcoma cells directly or synergistically with basic fibroblast growth factor (Ref. 60 and references therein) and enhances cell survival in *tat*-expressing cells (61). Constitutive expression of *tat* in transgenic mice results in tumor development, including Kaposi's-like sarcomas and B cell lymphomas (62). Accordingly, stable expression of *tat* in IL6-dependent cells results in growth factor-independent growth and in tumorigenicity (30). Moreover, data in support of a nontranscriptional function of Tat in virion infectivity has been reported (63). The above evidence strongly suggests that Tat may participate in the establishment of HIV-1 infection and in the development of AIDS clinical fea-

tures by promoting the expression of host cellular genes. In support of this possibility, Tat has been shown to activate the expression of the proinflammatory cytokines IL6 and tumor necrosis factor-β (30–32) and to increase interleukin-2 and collagen gene expression (64, 65). Tat was also shown to suppress promoter activity of major histocompatibility complex class I genes (66) and to exert immunosuppressive activity on antigen-induced T cell proliferation (67–69). Moreover, Tat has been shown to promote apoptosis by up-regulating CD95 ligand expression (70) or by activating cyclin-dependent kinases (71).

The mechanisms of Tat function on the expression of heterologous genes are unknown. In this paper, we address in molecular detail the mechanisms of Tat activity on the expression of IL6, a cytokine with a broad biological activity (7, 72) whose expression is deregulated in HIV-infected subjects (6, 8, 9). By using 5′ deletion mutants of pIL6-CAT plasmid, and IL6:HIV-1-LTR hybrid plasmids where discrete regions of the IL6 promoter replaced the TAR sequence in HIV-1 LTR, we identified a short sequence of the 5′-untranslated region of IL6 mRNA that is required for Tat to transactivate the IL6 promoter. This region can acquire a stem-loop structure including a UCU trinucleotide bulge. Point mutations of the UCU bulge or of the stem resulted in a drastic decrease in Tat responsiveness (shown in Fig. 3) and in the inability of Tat to bind to the IL6 leader RNAs (Fig. 4). The IL6 RNA structure, with an estimated structure energy of −9.1 kcal/mol, is expected to be less stable than the TAR RNA structure. This suggests that Tat could bind with a low affinity to heterologous RNA sequences and may account for the ability of Tat to regulate the expression of multiple genes. Interestingly, Tat was still able to induce a low but significant activation of the bulge mutant pIL6(−596/+15)M1-CAT plasmid (shown in Fig. 3), suggesting that Tat can function, albeit at a lower efficiency, without binding to an RNA tethering structure. In this case, Tat could be directed to the transcription start site of IL6 promoter by associating with specific transcription factors. This possibility is supported by the reports showing that Tat may associate with Sp1, TFIID factors, RNA polymerase II, and RNA polymerase II-associated factors (50, 73–78). In addition, we now provide evidence that Tat can function by cooperating with C/EBP transcription factors. In fact, we observed an increase in the C/EBP DNA binding activity of *tat*-expressing cells with a selective increase in the amounts of nuclear C/EBPβ factors (Figs. 5 and 6). This raises the possibility that Tat may increase the nuclear levels of C/EBP transcription factors by inducing post-translational modifications of C/EBP factors through the activation of specific kinases. Indeed, Tat activity on HIV-1 LTR-driven gene expression requires protein kinase C (79). Moreover, specific interaction of Tat with a cellular protein kinase has been reported (80), and serine and threonine phosphorylations of C/EBPβ are required for IL6 promoter activation (81). The above data are consistent with and extend the recent observation that Tat enhances the tumor necrosis factor-induced activation of NF-κB binding activity by possibly inducing protein phosphorylation (82). Since C/EBP and NF-κB factors associate as heterodimers (83), which are potent activators of HIV-1 LTR (84), the above data suggest that Tat may also promote HIV-1 gene expression by up-regulating the cellular levels of transcription factors acting on the viral LTR. Moreover, Tat was able to complex with *in vitro* translated C/EBPβ, which is a major mediator of IL6 promoter function (Fig. 7). By immunoprecipitation and by taking advantage of the yeast two-hybrid system, this interaction was proved to occur also *in vivo* and to result in transcriptional activation of a reporter *lacZ* gene (shown in Figs. 8 and 9). The Tat association with C/EBPβ suggests that Tat may increase the DNA binding activity of C/EBP dimers by enhancing their affinity for the target DNA. This mechanism accounts for the Tax activity on transcription mediated by bZip proteins (86, 87). In the EMSA experiments shown in Fig. 5*A*, Tat could not be detected in the C/EBP-DNA complexes with an anti-Tat antibody (data not shown). This suggests either that Tat does not directly participate in the C/EBP-DNA complexes or that Tat dissociates from the DNA-binding complexes due to the electrical field of EMSAs. These possibilities warrant further studies.

The data are consistent with the possibility that Tat may function on heterologous genes by interacting with RNA structures possibly present in a large number of cellular and viral genes, as recently reported (30–33). In addition, Tat may function by forming heterodimers with specific transcription factors. These possibilities dramatically enhance the capacity of Tat to modulate the expression of heterologous genes and to play a major role in the pathogenesis of HIV-associated diseases.

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