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.

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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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 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 EcoRI-BglII fragment of pILIC-CAT was isolated, filled in, and inserted at the Xhol site (filled) of pILIC(-112/+15). The resulting pILIC(IL6(-112/+15)-CAT plasmid carries the IL6 promoter region from -112 to +15 that substitutes for TAR. To generate pILIC(IL6(-112/+67)-CAT plasmid, the EcoRI fragment of pILIC, IL6(-112/+15)-CAT fusion was filled in and digested with SspI. The EcoRI-SspI fragment, containing the TAR-deleted LTR fused to the -112/-67 region of IL6 promoter, was cloned in pEMBL-CAT digested with BamHI-HindIII (filled). The SspI-EcoRI fragment, formed by the -67/+15 region of IL6 promoter fused to a part of the cat gene, was recovered and HindIII-digested. The -67/+15 region of IL6 promoter, the cat gene, and the HindIII digestion site, was replaced in pILIC(IL6(-112/+15)-CAT from which the -112/15 IL6 fragments were removed by KpnI-HindIII 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 PsrI. The following oligonucleotides were used (the mutated bases are underlined): 5′-CTGAGGCCTGTCGAACCTGCGGAATC-3′ for pIL6(-596/+15) M1-CAT (bulge mutant); 5′-ATTTCTGCGAACAGGACACCCTG-3′ for pIL6(-596/+15) M2-CAT (stem mutant); and 5′-CTGAGGACCTTACGACCTGACCCAGGAT-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 pEGX-TAT plasmid was obtained from M. Giccia (International Center for Genetic Engineering and Biotechnology, Trieste, Italy). In this plasmid, the first exon of the tat gene is cloned in pSP64 (obtained from S. Akira, Institute for Molecular and Cellular Biology, Osaka University, Osaka, Japan). The pGAL4-TAT plasmids, used for reporter gene assays, were a gift of B. Cullen (36). In these plasmids, the expression of GAL4-Tat fusion sequences, consisting of wild-type or truncated tat gene fused to its complementary strand and end-labeled with [γ-32P]ATP (Amer sham Life Science, Inc.) by using polynucleotide kinase (New England Biolabs). One microgram of nuclear extracts were incubated in a reaction mixture consisting of 20 μl of bacterial cell containing 10% glycerol, 60 μg of KCl, 1 μl of bacterial cell containing 50 μg/ml of cat (0.5 μCi) and 1 μg of DNA (0.5 μg/ml of T7 RNA polymerase) 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- and RSV-RNA Assays—To produce the IL6 RNA [α-32P]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′-GATCATTAATACGACTCACTATTAGGGGACACAGAGGACAAAC-3′ (IL6 wild type sense), 5′-GATCATTAATACGACTCACTATTAGGGGACACAGAGGACAAAC-3′ (IL6 M1 sense), 5′-GATCATTAATACGACTCACTATTAGGGGACACAGAGGACAAAC-3′ (IL6 M2 sense), and 5′-GATCATTAATACGACTCACTATTAGGGGACACAGAGGACAAAC-3′ (IL6 M3 sense). For antisense sequences, the following transcription primer was used: 5′-GATCATTAATACGACTCACTTTAGGGGACACAGAGGACAAAC-3′ (IL6 M1 antisense), 5′-GATCATTAATACGACTCACTTTAGGGGACACAGAGGACAAAC-3′ (IL6 M2 antisense), and 5′-GATCATTAATACGACTCACTTTAGGGGACACAGAGGACAAAC-3′ (IL6 M3 antisense).
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, 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 in which 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. 2A), corresponding to the major transcription start site of the IL6 gene (40). 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. 2A). 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. 2B). A densi-
CAT activity was determined 48 h after transfection by using 50 μg of pSV-T8 or pSV-T10 plasmids. Similar results were obtained with two different plasmid preparations in which two different plasmid preparations were used. Similar results were obtained by transient expression of pSV-T8 and pSV-T10 plasmids.

A secondary structure 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 pILIC:IL6(−112/+15)-CAT were stronger in Tat-positive than in Tat-negative cells (Fig. 2A). These results identified the sequence of −112/+15 as the minimal region of the IL6 promoter required for Tat to transactivate the ATAR 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 pILIC:IL6(−112/+15)-CAT hybrid plasmid. This allowed the construction of the pILIC: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.
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The Basic Region of Tat Is Required for Tat-Mediated Expression of the IL6 Gene—A basic domain of Tat, encompassing 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 pILIC:IL6(−112/+15)-CAT, pILIC:IL6(−112/+67)-CAT, or pILIC:IL6(−67/+15)-CAT plasmids. 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 pILIC:IL6(−112/+15)-CAT and pILIC:IL6(−67/+15)-CAT). It is noteworthy that Tat(1–49) was unable 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 induction of the IL6 promoter, we tested 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 an antisense orientation) were tested for binding to an oligonucleotide corresponding to the C/EBP cis sequence of IL6 promoter. As shown in Fig. 5A, tat expression leads to a significant increase in C/EBP DNA binding activity. Moreover, an
antiserum to C/EBPβ supershifted the C/EBP complex, while an antiserum to C/EBPα was ineffective. In parallel experiments, cytosolic extracts from tat- or anti-tat-transfected cells expressed equal levels of C/EBP DNA binding activity (Fig. 5B). 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. 6A). 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/EBPα 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 GST-Tat proteins as detailed under “Materials and Methods.” 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
readily revealed in transfected cells, and that C/EBP
b 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/EBP
b cDNA was inserted in frame with the sequence of GAL4 coding for the GAL4
activation domain (amino acid residues 768–881). The result-

TABLE I

| Transfected plasmids | Acetylation (%) | Fold induction |
|----------------------|----------------|---------------|
| pILIC:IL6(1–72)CAT  | 8.0 (0.5)      | 9.4 (1.3)     |
| pILIC:IL6(1–49)CAT  | 0.7 (0.1)      | 2.3 (0.3)     |
| pILIC:IL6(1–49)CAT  | 1.3 (0.3)      | 2.1 (0.4)     |
| pILIC:IL6(1–49)CAT  | 0.8 (0.1)      | 5.6 (0.7)     |
| pILIC:IL6(1–49)CAT  | 11.7 (1.5)     | 13.3 (2.0)    |
| pILIC:IL6(1–49)CAT  | 1.4 (0.3)      | 1.7 (0.3)     |
| pILIC:IL6(1–49)CAT  | 0.8 (0.1)      | 1.5 (0.2)     |
| pILIC:IL6(1–49)CAT  | 1.3 (0.2)      | 1.5 (0.2)     |
| pILIC:IL6(1–49)CAT  | 1.3 (0.3)      | 2.1 (0.4)     |
| pILIC:IL6(1–49)CAT  | 0.7 (0.1)      | 2.3 (0.3)     |
| pILIC:IL6(1–49)CAT  | 1.7 (0.2)      | 2.1 (0.4)     |
| pILIC:IL6(1–49)CAT  | 1.3 (0.2)      | 2.3 (0.3)     |
| pILIC:IL6(1–49)CAT  | 1.3 (0.3)      | 2.1 (0.4)     |
| pILIC:IL6(1–49)CAT  | 0.8 (0.1)      | 2.1 (0.4)     |
| pILIC:IL6(1–49)CAT  | 1.7 (0.2)      | 2.1 (0.4)     |
| pILIC:IL6(1–49)CAT  | 1.3 (0.3)      | 2.1 (0.4)     |
| pILIC:IL6(1–49)CAT  | 0.8 (0.1)      | 2.1 (0.4)     |
| pILIC:IL6(1–49)CAT  | 1.7 (0.2)      | 2.1 (0.4)     |
| pILIC:IL6(1–49)CAT  | 1.3 (0.3)      | 2.1 (0.4)     |
| pILIC:IL6(1–49)CAT  | 0.8 (0.1)      | 2.1 (0.4)     |
| pILIC:IL6(1–49)CAT  | 1.7 (0.2)      | 2.1 (0.4)     |
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| pILIC:IL6(1–49)CAT  | 0.8 (0.1)      | 2.1 (0.4)     |
| pILIC:IL6(1–49)CAT  | 1.7 (0.2)      | 2.1 (0.4)     |
| pILIC:IL6(1–49)CAT  | 1.3 (0.3)      | 2.1 (0.4)     |
| pILIC:IL6(1–49)CAT  | 0.8 (0.1)      | 2.1 (0.4)     |
| pILIC:IL6(1–49)CAT  | 1.7 (0.2)      | 2.1 (0.4)     |
| pILIC:IL6(1–49)CAT  | 1.3 (0.3)      | 2.1 (0.4)     |
| pILIC:IL6(1–49)CAT  | 0.8 (0.1)      | 2.1 (0.4)     |
| pILIC:IL6(1–49)CAT  | 1.7 (0.2)      | 2.1 (0.4)     |
| pILIC:IL6(1–49)CAT  | 1.3 (0.3)      | 2.1 (0.4)     |
| pILIC:IL6(1–49)CAT  | 0.8 (0.1)      | 2.1 (0.4)     |
| pILIC:IL6(1–49)CAT  | 1.7 (0.2)      | 2.1 (0.4)     |
| pILIC:IL6(1–49)CAT  | 1.3 (0.3)      | 2.1 (0.4)     |
| pILIC:IL6(1–49)CAT  | 0.8 (0.1)      | 2.1 (0.4)     |
| pILIC:IL6(1–49)CAT  | 1.7 (0.2)      | 2.1 (0.4)     |
| pILIC:IL6(1–49)CAT  | 1.3 (0.3)      | 2.1 (0.4)     |
| pILIC:IL6(1–49)CAT  | 0.8 (0.1)      | 2.1 (0.4)     |
| pILIC:IL6(1–49)CAT  | 1.7 (0.2)      | 2.1 (0.4)     |
| pILIC:IL6(1–49)CAT  | 1.3 (0.3)      | 2.1 (0.4)     |
| pILIC:IL6(1–49)CAT  | 0.8 (0.1)      | 2.1 (0.4)     |
| pILIC:IL6(1–49)CAT  | 1.7 (0.2)      | 2.1 (0.4)     |
| pILIC:IL6(1–49)CAT  | 1.3 (0.3)      | 2.1 (0.4)     |
| pILIC:IL6(1–49)CAT  | 0.8 (0.1)      | 2.1 (0.4)     |
| pILIC:IL6(1–49)CAT  | 1.7 (0.2)      | 2.1 (0.4)     |
| pILIC:IL6(1–49)CAT  | 1.3 (0.3)      | 2.1 (0.4)     |
| pILIC:IL6(1–49)CAT  | 0.8 (0.1)      | 2.1 (0.4)     |
| pILIC:IL6(1–49)CAT  | 1.7 (0.2)      | 2.1 (0.4)     |
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| pILIC:IL6(1–49)CAT  | 0.8 (0.1)      | 2.1 (0.4)     |
| pILIC:IL6(1–49)CAT  | 1.7 (0.2)      | 2.1 (0.4)     |
| pILIC:IL6(1–49)CAT  | 1.3 (0.3)      | 2.1 (0.4)     |

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.
tional activation of the integrated \( \text{lacZ} \) 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/EBP \( \beta \), indicating that the N-terminal, cysteine-rich, and core regions of Tat represent the minimal region of Tat required for 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/EBP \( \beta \) (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 non-progressors 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-

**FIG. 7.** *In vitro* interaction of Tat with C/EBP\( \beta \). C/EBP\( \beta \) 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/EBP\( \beta \) 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/EBP\( \beta \) 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/EBP\( \beta \) (lane b). Lane a shows the amount of C/EBP\( \beta \) expressed by 30 \( \mu \)g 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.

**FIG. 9.** *In vivo* interaction of Tat and C/EBP\( \beta \) proteins. \( \beta \)-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/EBP\( \beta \).
HIV-1 Tat Activates the Interleukin-6 Gene Transcription

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fures 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 plL6−596/+151-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, TFIIID factors, RNA polymerase II, and RNA polymerase II-associated factors (50, 73–78). In addition, we now support the possibility that Tat may associate with specific transcription factors. This possibility warrants 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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REFERENCES

1. Fauci, A. S., Machler, A. M., Longo, D. L., Lane, H. C., Rook, A. H., Masur, H., and Gelman, E. P. (1984) Annu. Intern. Med. 100, 92–103

2. Beral, V., Peterman, A., Berkley, R. L., and Jaffé, H. W. (1990) Lancet 335, 123–128

3. Levine, A. M. (1992) Blood 80, 8–15

4. Nowak, M. A., Anderson, R. M., McLean, A. R., Wolfs, T. F. W., Goudsmit, J., and May, R. M. (1991) Science 254, 963–969

5. Biggar, R. J. (1990) AIDS 4, 1059–1065

6. Nakajima, N., Martinez-Maza, O., Hirano, T., Breen, E. C., Nishanian, P. G., Szazar-Gonzalez, J. F., Fahey, J. L., and Kishimoto, T. (1989) J. Immunol. 142, 144–155

7. Kishimoto, T., Akira, S., and Taga, T. (1992) Science 256, 593–597

8. Breen, E. C., Rezlai, A. R., Nakajima, K., Beall, G. N., Mitsuayasu, R. T., Hirano, T., Kishimoto, T., and Martinez-Maza, O. (1990) J. Immunol. 144, 480–487

9. Gallo, R. C., Frei, K., Rordorf, C., Lanzina, J., Tavolato, U., and Fontana, A. (1989) J. Neuroimmunol. 24, 109–115

10. Rosen, C., Sodroski, J. G., and Haseultine, W. A. (1985) Cell 41, 813–823

11. Sharp, P. A., and Marciniak, R. A. (1989) Cell 59, 229–230

12. Gagelto, A., Buerker-White, A., Berkhour, B., and Jeang, K. T. (1991) Science 251, 1597–1600

13. Cullen, B. R. (1993) Cell 73, 417–420

14. Riee, P. A., and Matthews, M. B. (1988) Nature 332, 551–555

15. Berkhour, B., Silvermann, R. H., and Berkhour, B. (1989) Cell 39, 273–282

16. Lapsia, M. F., Rice, A. P., and Matthews, M. B. (1989) Cell 59, 283–292

17. Arya, S. K., Guo, C., Josephs, S. F., and Wong-Staal, F. (1988) Science 239, 89–73

18. Sodroski, J., Patarca, R., Rosen, C., Wung-Staal, F., and Haseultine, W. A. (1985) Science 229, 74–77

19. Garcia, J. A., Harrich, D., Sozianakis, E. W. P., Mitsuayasu, R., and Raynor, G. B. (1989) EMBO J. 8, 765–778

20. Frankel, A. D., and Pabo, C. O. (1988) Cell 55, 1189–1193

21. Dingwall, C., Ehrlich, I., Gatt, M. J., Green, S. M., Hephy, K., Karr, J., Lowe, A. D., Singh, M., Steinman, R. M., and Valerio, R. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 6925–6929

22. Roy, S., Delling, U., Chen, C. H., Rosen, C. A., and Sunzenberg, N. (1990) Genes & Dev. 4, 1365–1373

23. Nelbock, P., Dillon, P. J., Perkins, A., and Rosen, C. A. (1990) Science 248, 1650–1653

24. Desai, K., Laewteit, P. M., and Green, M. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 8875–8879

25. Berkhour, B., Gagelto, A., Rabson, A. B., and Jeang, K. T. (1990) Cell 62, 757–767

26. Shibuya, H., Irie, K., Ninomiya-Tsuji, J., Goebel, M., Taniguchi, T., and Matsuda, K. (1992) Nature 357, 700–704

27. Dang, C. V., and Lee, W. M. F. (1989) J. Biol. Chem. 264, 18019–18023

28. Siomi, H., Shida, H., Makii, M., and Hatanaka, M. (1990) J. Virol. 64, 1803–1807

29. Weeks, K. M., Ampe, C., Schultz, S. C., Steitz, T. A., and Crothers, D. M. (1990) Science 240, 1281–1285

30. Scala, G., Rusco, M. R., Ambrosino, C., Mallardo, M., Giordano, V., Baldassarre, F., Dragoniht, E., Quentin, I., and Venuta, S. (1994) J. Exp. Med. 179, 961–971

31. Sastry, K. J., Reddy, R. R. H., Pandita, R., Totpal, K., and Aggarwal, B. B.
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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