The Expression of the Interleukin 6 Gene Is Induced by the Human Immunodeficiency Virus 1 TAT Protein

By Giuseppe Scala,* Maria R. Ruocco,* Concetta Ambrosino,†
Massimo Mallardo,* Vincenzo Giordano,* Francesca Baldassarre,*
Emilia Dragonetti,* Ileana Quinto,*† and Salvatore Venuta†

From the *Department of Biochemistry and Biomedical Technology, Medical School, University
“Federico II,” 80131 Naples, Italy; and the †Department of Clinical and Experimental Medicine,
Medical School, University of Reggio Calabria, 88100 Catanzaro, Italy

Summary
Human immunodeficiency virus 1 (HIV1) infection is associated with severe psoriasis, B cell lymphoma, and Kaposi’s sarcoma. A deregulated production of interleukin 6 (IL-6) has been implicated in the pathogenesis of these diseases. The molecular mechanisms underlying the abnormal IL-6 secretion of HIV1-infected cells may include transactivation of the IL-6 gene by HIV1. To test this hypothesis, we used the plL6Pr-chloramphenicol acetyltransferase (CAT) plasmid, an IL-6 promoter-CAT construct, as a target of the transactivating function of the HIV1 TAT protein. By cotransfecting the plL6Pr-CAT and the tat-expressing pSVT8 plasmid in MC3 B-lymphoblastoid or in HeLa epithelial cells, we observed that TAT transactivates the human IL-6 promoter. These results were confirmed when plL6Pr-CAT was transfected in MC3 or HeLa cells that constitutively expressed the tat gene in a sense (pSVT8 cells) or antisense (pSVT10 cells) orientation. 5’ deletion plasmids of plL6Pr-CAT, in which regions at -658, -287, and -172 were inserted 5’ to the cat gene, were transiently transfected in pSVT10 and pSVT8 cells and showed that TAT-induced activation of the IL-6 promoter required a minimal region located between -287 and -54 bp. Moreover, experiments with plasmids carrying the -658, -287, and -172 bp regions of the IL-6 promoter inserted downstream to a TAR-deleted HIV1-LTR identified the sequence of -172 to -54 as the minimal region of the IL-6 promoter required for TAT to transactivate the TAR-deleted HIV1-LTR. By DNA-protein binding experiments, tat-transfected cells expressed a consistent increase in κB and nuclear factor (NF)-IL-6 binding activity. Accordingly, the pDRCAT and IL-1REK9CAT, carrying tandem repeats of NF-κB or NF-IL6 binding motifs, respectively, were activated in TAT-expressing cells. The biological relevance of the TAT-induced IL-6 secretion was addressed by generating 7TD1 cells, an IL-6-dependent mouse cell line, stably expressing the tat gene. These tat-positive cells expressed the endogenous IL-6 gene, secreted high amounts of murine IL-6, and grew efficiently in the absence of exogenous IL-6. Moreover, the tat-positive 7TD1 cells sustained the growth of parental 7TD1 cells and showed a dramatic increase in their tumorigenic potency. These results suggest that TAT protein may play a role in the pathogenesis of some HIV1-associated diseases by modulating the expression of host cellular genes.

HIV1 infection causes various clinical and immunological abnormalities, including lymphadenopathy, activation of polyclonal B cells that manifests as hypergammaglobulinemia and auto-antibody production, Kaposi’s sarcoma, and lymphoma of the B cell phenotype (1–3). In HIV1-infected subjects, there is a deregulated production of cytokines, that affects the growth and differentiation of lymphoid and mesenchymal cells, and that may contribute to the development of the clinical features of AIDS. IL-6 has a broad range of biological effects (4) and plays a major role in the development of B cell malignancies and Kaposi’s sarcoma (5–7). IL-6 gene transcription is induced in cells infected by HIV1, and increased levels of IL-6 have been reported in serum and cerebral spinal fluid of HIV1-infected patients (8–10). These findings suggest that HIV1 may transactivate the host cellular IL-6 gene.

The TAT protein of HIV1 is required for efficient viral gene expression (11). TAT increases the initiation of transcription from the HIV1-LTR (12) and affects RNA processing and utilization by interacting with a TAT-responsive element
(TAR) located between nucleotide +1 and +44 with respect to the initiation site (+1) of viral transcription (13). In addition, TAR interacts with upstream regulatory DNA sequences circumscribed within the nuclear factor (NF)-xB/Sp1 region of the HIV1 promoter (14) and with host cell proteins (15–19). TAR could affect the transcription of cell genes such as IL-6, whose regulatory sequences, like HIV1-LTR, possess NF-xB and NF-IL6 enhancer elements (20, 21). To test this hypothesis, we used the plL6Pr-CAT, an IL-6 promoter-CAT construct, as a target of the transactivating function of the HIV1 TAR proteins. By cotransfecting the plL6Pr-CAT and the tat-expressing pSVT8 plasmids (22) in MC3 B-lymphoblastoid or in HeLa epithelial cells, we observed that TAR transactivated the human IL-6 promoter. These results were confirmed when plL6Pr-CAT was transfected in MC3 or HeLa cells that constitutively expressed the tat gene. The biological relevance of the tat-induced IL-6 secretion was addressed by generating 7TD1 cells, an IL-6-dependent mouse cell line (23), stably expressing the tat gene. These tat-positive cells expressed the endogenous IL-6 gene, grew efficiently in the absence of exogenous IL-6, and showed a dramatic increase in their tumorigenic potency.

Materials and Methods

Animals and Reagents. 4-wk-old female nude mice were purchased from Charles River Breeding Laboratories (Como, Italy). LPS Escherichia coli 055:B55 was obtained from Difco Laboratories (Detroit, MI). G418 (Geneticin, 658 μg of G418 base per mg) was purchased from Sigma Chemicals (Milan, Italy). Recombinant TAT protein and the rabbit anti-TAT antibody were obtained from the National Institutes of Health-AIDS Research and Reference Agent Program. Rat monoclonal and goat polyclonal antibodies to mouse IL-6 were purchased from Boehringer Mannheim (Milan, Italy) and from Genzyme (Cambridge, MA), respectively. Elisa assays specific for human or mouse IL-6 were purchased from Genzyme. HPLC-purified human rIL-6 (sp act, 2.7 × 10^9 U/mg) was provided by G. Ciliberto (Istituto di Ricerche di Biologia Molecolare, Pomezia, Italy).

Plasmids. To generate the plL6Pr-CAT plasmid, the 1.2 Kbp BamHI-Xho1 5' upstream sequences of the IL-6 gene were excised from the pGEMB672A plasmid (24) and inserted into compatible sites (BamHI-Sall) of the pEMBL-CAT plasmid using standard techniques (25). The 5' deletion mutants of plL6Pr-CAT were generated by digesting the plL6Pr-CAT with Ball, NheI, or HaelIII, followed by fill-in with Klenow polymerase. The resulting constructs contained regions of -658, -287, and -172 relative to the first nucleotide of the ATG codon of the IL-6 genomic sequence (24). To generate the HIV1-LTR-IL-6 promoter fusion plasmids, the TAR-deleted EcoR1-BglII fragment of plLC-CAT (26) was isolated, filled-in, and inserted at the SstI site (filled) of the various IL-6 promoter mutants. The correct 5'-3' orientation was analyzed by multiple restriction digests and by sequencing using the Sanger's method (25). The pSVT8 and pSVT10 plasmids, expressing the tat gene in a sense or antisense orientation, respectively (22), were obtained from A. Caputo (University of Ferrara, Ferrara, Italy). The β-galactosidase expressing pmls-lacZ plasmid was obtained from A. Weisz (University of Naples, Naples, Italy). The pDRCAT plasmid, carrying the HIV1-xB enhancer upstream to a thymidine

1 Abbreviations used in this paper: CAT, chloramphenicol acetyltransferase; NF, nuclear factor; nt, nucleotide.
to sustain the growth of the IL-6-dependent 7TD1-pSV2neo cells, 7TD1-pSVneo cells were cultured (4 x 10^3 cells/well in 0.2 ml cultures) with 25% vol/vol of conditioned medium from 7TD1-pSVT8 cells in the absence of exogenous IL-6. Cell growth was evaluated after 72 h by cell counting and [3H]thymidine incorporation as previously reported (6). In other experiments, 7TD1-pSVneo cells were cocultured at 2 x 10^3 cells/ml with 7TD1-pSVT8 cells (2 x 10^4 cells/ml) in transwell plates, where 7TD1 cells (upper level) were separated by a 0.45-µm membrane from the tat-transfected 7TD1-pSVT8 cells (lower level). In these experiments, 7TD1 cells were counted daily. In other experiments, 7TD1 cells were cultured in the presence of 50 ng/ml of human IL-6 or with amounts of recombinant Tat protein ranging from 0.3 to 10 µg/ml. In parallel experiments, tat-positive 7TD1-pSVT8 cells were cultured in the presence of a rabbit polyclonal antibody to Tat at concentrations ranging from 1:100 to 1:1,000 final dilution. The proliferative rate was analyzed at 72 h as described (6).

For transient expression experiments, cells were transfected by electroporation at 5 x 10^6 cells in 0.8 ml of D-PBS as detailed above and cultured for 48 h in complete DMEM medium. The amounts of transfected DNA were equalized either with the p2ptSVneo control plasmid or with pUC18 plasmid DNA. Moreover, in preliminary experiments, we found that cell transfections generated CAT activity that was linear in the range of 5 to 70 µg/ml of pSV2-CAT, after which it declined. Under these conditions, CAT activity in cellular extracts from duplicate transfections varied by <15%. Transfection efficiency was monitored by cotransfecting the cells with 5 µg of plnS-lacZ plasmid. β-Gal activity was assayed using 80–100 µg of protein cell extract as described (25). A random 25% variation of the β-Gal levels was observed throughout this study. The transient expression experiments were performed at least five times with different plasmid preparations. Primer extension was carried out as described (25). 20 µg of total RNA was annealed to the oligonucleotides 5'-CAACGCGTGGTATATCCAGTGY-3' (for tat RNA), and 5'-CAGATACTAGTTAATAAT3' (for U2 RNA). RNA was elongated with reverse transcriptase, digested with RNase A, and separated over a 6% denaturing 7 M urea-acrylamide gel.

**CAT Assays.** 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 previously described (27, 29). Proteins were measured in each cell extract with an assay kit (Bio-Rad) 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 [3H]-thymidine ([3H]dThd) (New England Nuclear, Boston, MA) 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 silica gel plates (Polygram Sil G; Macherey-Nagel, Duren, Germany). Plates were run in a tank containing a mixture of chloroform/methanol (95:5). After a 16-h autoradiography, the plates were cut and samples were counted in a scintillation counter (LS5000TD; Beckman Instruments, Inc., Palo Alto, CA).

**Electrophoretic Mobility Shift Assays (EMSAs).** Nuclear extracts and gel shift assays were performed as described elsewhere (27, 29). After incubation, cells were harvested, washed once in cold PBS, and transferred to 1.7-ml microfuge tubes for a second wash in cold PBS. 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 PMSF, and 0.2% vol/vol NP-40 for 5 min. Nuclei were collected by centrifugation (500 g for 5 min), rinsed with NP-40–free lysing buffer, and resuspended in 150 µl of buffer containing 250 mM Tris HCl, pH 7.8, 20% glycerol, 0.42 M NaCl, 60 mM KCl, 1 mM DTT, and 1 mM PMSF. Nuclei were then subjected to three cycles of freezing and thawing. The suspension was cleared by centrifugation (7,000 g for 15 min), and aliquots were immediately tested in a gel retardation assay or stored in liquid phase N2 until use. Oligonucleotide probes used included 5'-GATCGGACGTCACATTGGAACACTCTCATTATAAT3' (NF-IL-6), 5'GGACGTCACACTACAAACTCTATTATAA3' (mutant NF-IL-6), 5'TGGGATTTCCCA-3' (kb), and 5'TATAATTITCCCA3' (mutant kb). Each oligonucleotide was annealed to its complementary strand and end-labeled with γ[32P]-ATP (Amersham Corp., Arlington Heights, IL) using polynucleotide kinase (New England Biolabs, Beverly, MA). Equal amounts of cell extracts were incubated in a reaction mixture consisting of 20 µl buffer containing 20 mM Hepes, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 2 µg poly (d[I-C]) (Boehringer Mannheim), and 5 µg of extract for 5 min on ice. 1 µl of γ[32P]-labeled double-stranded probe (0.2 ng, 4–6 x 10^6 cpm) was then added with or without a 100-fold excess of competitor wild type or mutant oligonucleotide. The reactions were incubated at room temperature for 30 min and run on a 5% acrylamide/bis-acrylamide (30:1) gel in 22.5 mM Tris-borate, 0.5 mM EDTA. Gels were dried and autoradiographed.

**Results and Discussion**

The HIV1 TAT Protein Transactivates the Promoter of the IL-6 Gene. The pIL6Pr-CAT was cotransfected with either the pSVT8 or the pSVT10 plasmid (22) that carry the tat gene in sense and antisense orientation, respectively, and allow G-418 selection by expressing the neo gene. As shown in Fig. 1, a and b, the tat gene product efficiently increased the IL-6 promoter–dependent CAT activity in both B lymphoblastoid MC3 and epithelial HeLa cells. In these experiments LPS, a likely contaminant of plasmid preparations and a possible inducer of IL-6 promoter activity (30), exerted no effect. These data suggested that the tat gene, transiently expressed in both B lymphoid and epithelial cells, transactivated the promoter of IL-6.

In further experiments, MC3 and HeLa cells were stably transfected with either the pSVT8 or the pSVT10 plasmid. After selection in G-418, we obtained stable bulk cultures of MC3-pSVT8, MC3-pSVT10, HeLa-pSVT8, and HeLa-pSVT10 cells. The integration of the tat gene was tested by Southern blots of genomic DNA, and the tat transcription was analyzed by PCR amplification of tat-specific transcripts. To ascertain the presence of TAT proteins, we exploited the transactivating effects that TAT exerts on HIV1-LTR (11), and conducted transient expression experiments in which pSVT8 or pSVT10–positive cells were transfected with the pILC-CAT, an HIV1-LTR–carrying plasmid (26). These experiments showed that the transfected MC3 and HeLa cells (a) harbored the tat gene in a random genomic integration with an average of one copy per cell genome (data not shown); (b) efficiently expressed tat-mRNAs (data not shown); and (c) had a high constitutive activation of the HIV1-LTR–CAT plasmid, which indicated the endogenous production of functional TAT proteins (Fig. 2 a). We used transient expression experiments to test whether pSVT8– or pSVT10–transfected
Figure 1. The tat gene product transactivates the IL6 promoter. (a) HeLa cells were transiently transfected with 10 µg of pIL6Pr-CAT plasmid together with the indicated amounts of the tat-expressing pSVT8 plasmid. The percent specific acetylations, calculated by directly counting the acetylated spots, were: 0.3 (baseline), 2.2 (1 µg), 6.8 (5 µg), and 16.8 (10 µg). In parallel experiments, cells were transfected with 10 µg of pIL6Pr-CAT plasmid and with similar amounts of pSVT10 (anti-tat) plasmid with no significant variations over the baseline pIL6Pr-CAT expression (data not shown). The data are representative of several independent experiments. Similar results were obtained by transfecting MC3 cells (data not shown). (b) MC3 B-lymphoblastoid cells were transfected with 10 µg of pIL6Pr-CAT and with 10 µg of the indicated plasmids. At 24 h after transfection, cell aliquots were stimulated with *Escherichia coli* LPS (1 µg/ml) for additional 24 h. Percent acetylations were: pSV2 neo, 0.80 (LPS-), 0.75 (LPS+); pSVT10, 1.10 (LPS-), 1.15 (LPS+); pSVT8, 10.5 (LPS-), 10.8 (LPS+). The data are representative of several independent experiments. Similar results were obtained by transfecting HeLa cells (data not shown).

cells transactivated the pIL6Pr-CAT plasmid. Both MC3-pSVT8 and HeLa-pSVT8 produced a higher IL-6 promoter-driven CAT activity than did the control pSVT10-transfected cells (Fig. 2 b).

When the tat-transfected MC3 and HeLa cells were tested for the secretion of IL-6 by specific ELISA assay, pSVT8-transfected cells secreted a higher amount of IL-6 than the pSV2neo- or pSVT10-transfected cells (Table 1). These data indicate that stable expression of the HIV tat gene results in the activation of the endogenous IL-6 gene.

Identification of the Region of the IL-6 Promoter Responsive to TAT. To gain further insights into the molecular mechanisms of the TAT-induced activation of the IL-6 gene, we performed a primer extension analysis of cat mRNA in pSVT10- and pSVT8-cells transfected with the pIL6pr-CAT plasmid. As shown in Fig. 3, HeLa-pSVT8 cells expressed higher amount of cat mRNA than the anti-tat-transfected pSVT10-cells. The elongated product extended for 98 bp, locating the transcription start site at -63 bp, corresponding to the major start site of the IL-6 gene (24). Similar results were obtained by primer extension analysis of the IL-6 mRNA transcribed from the endogenous IL-6 gene in tat- or anti-tat-transfected MC3 and HeLa cells (data not shown).

To address the question of whether TAT could interact with IL-6 promoter sequences located upstream to the transcription start site, we constructed 5’ deletion mutants of pIL6Pr-CAT in which regions at -658, -287, and -172 were inserted 5’ to the cat gene (Fig. 4 a). These plasmids were transiently transfected in pSVT10 and pSVT8 cells and showed that the pIL6Pr(-287)-CAT plasmid was efficiently transac-
Figure 3. Primer extension analysis of pIL6Pr-CAT mRNA in pSVT10- or pSVT8-transfected cells. Anti-tat (pSVT10)- or tat (pSVT8)-expressing HeLa cells were transfected with 10 μg of pIL6Pr-CAT plasmid. 36 h later, total RNA was isolated and analyzed by primer extension. Protected bands of cat and U2 mRNAs are indicated. A 24 h exposure of U2 band is shown, while cat mRNA band is a 4-d exposure. (a) HeLa-pSVT10; (b) HeLa-pSVT8.

activated by TAT, whereas the pIL6Pr(-172)-CAT construct was unresponsive to TAT (Fig. 4 b). This suggests that TAT-induced activation of the IL-6 promoter required a minimal region located between -287 and -54 bp (at the XhoI site). Next, we generated plasmids where the -658, -287, and -172 bp regions of the IL-6 promoter were inserted downstream to a TAR-derived HIV1-LTR sequence. The resulting pΔILC-IL6Pr-CAT plasmids (Fig. 4 a) were transiently expressed in tat or anti-tat-transfected cells. In these experiments, the sequence of -172 to -54 of the IL-6 promoter was unresponsive to TAT (pIL6Pr(-172)-CAT, Fig. 4 b). This sequence, however, conferred TAT responsiveness to the TAR-deleted HIV1-LTR promoter (compare pΔILC-CAT and pΔILC-IL6Pr(-172)-CAT plasmids in Fig. 4 b).

A primer extension analysis of cat mRNA transcribed by the pΔILC-IL6Pr(-172)-CAT identified a major protected

Figure 4. (a) Schematic representation of pIL6Pr-CAT and HIV1-LTR-IL6 promoter fusion plasmids. Restriction sites are indicated as: (B) BamHI; (Bl) Ball; (Bg) BglII; (N) NheI; (H) HaelII; (X) XhoI.
(b) Induction of mutant pIL6Pr-CAT and of HIV1-LTR-pIL6Pr-CAT fusion plasmids by TAT. 10 μg of the indicated plasmids, carrying 5' deletions of pIL6Pr-CAT or discrete regions of the IL-6 promoter fused to a TAR-deleted HIV1-LTR sequence, were transiently transfected in pSVT10- or pSVT8-HeLa cells. CAT activity was assayed at 48 h as detailed in Materials and Methods.
Table 1. Secretion of IL-6 Molecules by MC3, HeLa, and 7TD1 Cells

| Cells          | IL-6 secretion (pg/ml) |
|---------------|------------------------|
| MC3-pSV2neo   | 14.0                   |
| MC3-pSVT10    | 16.6                   |
| MC3-pSVT8     | 318                    |
| HeLa-pSV2neo  | 336                    |
| HeLa-pSVT10   | 384                    |
| HeLa-pSVT8    | 1,913                  |
| 7TD1-pSV2neo  | <3                     |
| 7TD1-pSVT8    | 216                    |
| Mo + LPS      | 2,350                  |

pSV2neo-transfected control cells and tat- or anti-tat-transfected cells were cultured for 48 h. Supernatants were then collected and tested for IL-6 content by ELISA specific for human or murine IL-6. Data express the amounts of IL-6 secreted by 10^6 cells over 48-h culture time. Human monocytes were isolated by centrifugation over Ficoll-Hypaque followed by 46% Percoll gradients and stimulated with LPS at 1 μg/ml, as described elsewhere (45).

Figure 5. Primer extension analysis of cat mRNA in pSVT10- or pSVT8-transfected cells. Anti-tat (pSVT10)- or tat (pSVT8)-expressing HeLa cells were transfected with 10 μg of pIL6Pr(-287)-CAT or pAILC-IL6Pr(-172)-CAT. Total RNA was isolated and analyzed by primer extension. The 98-nt and the 248-nt cat messages were generated by transfecting the pIL6Pr(-287)-CAT or the pAILC-IL6Pr(-172)-CAT, respectively. HeLa cells transfected with the pAILC-IL6Pr(-172)-CAT (a) or with the pIL6Pr(-287)-CAT (b); HeLa-pSVT8 cells transfected with the pAILC-IL6Pr(-172)-CAT (c) or with the pIL6Pr(-287)-CAT (d). A 24-h exposure is shown. Similar results were obtained in the case of transfected MC3 lymphoblastoid cells.

The Expression of the HIV1 tat Gene Induces an Increase in NF-kB and NF-IL6 Binding Factors. The data shown in Fig. 5 b, where Tat activated the pIL6Pr(-287)-CAT plasmid while the pIL6Pr(-172)-CAT was unresponsive to Tat, suggested that Tat-mediated activation of the IL-6 gene required transcription factors binding sequences located within -287 and -54 bp of the IL-6 promoter. In this region, functional NF-kB and NF-IL6 enhancers have been identified (20, 21). Therefore, we tested whether Tat might induce nuclear factors binding to the NF-kB or NF-IL6 sequences of the IL-6 promoter. As shown in Fig. 6, tat-transfected cells expressed a consistent increase in NF-kB and NF-IL6 binding activity. Accordingly, the IL-1REK9CAT and pDRCAT, carrying tandem repeats of NF-IL6 or NF-kB binding motifs, were consistently activated in Tat-expressing cells (Table 2). In other experiments, we also found that suboptimal amounts of the tat-expressing pSVT8 plasmid synergized with the pCMV-NFIL6 expression vector (21) in inducing a full activation of the IL-6 promoter (data not shown).

The Expression of the HIV1 tat Gene in the IL-6-dependent 7TD1 cells Confers Growth Independency from IL-6 and Enhanced Tumorigenicity. Deregulated expression of the IL-6 gene has been associated with the abnormal growth and tumorigenic phenotype of a variety of lymphoid and mesenchymal cells,

Figure 6. RT-PCR analysis of cat mRNA in pSVT10- or pSVT8-transfected cells. Anti-tat (pSVT10)- or tat (pSVT8)-expressing HeLa cells were transfected with 10 μg of pIL6Pr(-287)-CAT or pAILC-IL6Pr(-172)-CAT. Total RNA was isolated and analyzed by RT-PCR. The 98-nt and the 248-nt cat messages were generated by transfecting the pIL6Pr(-287)-CAT or the pAILC-IL6Pr(-172)-CAT, respectively. HeLa cells transfected with the pAILC-IL6Pr(-172)-CAT (a) or with the pIL6Pr(-287)-CAT (b); HeLa-pSVT8 cells transfected with the pAILC-IL6Pr(-172)-CAT (c) or with the pIL6Pr(-287)-CAT (d). A 24-h exposure is shown. Similar results were obtained in the case of transfected MC3 lymphoblastoid cells.

band of 248 nucleotides (nt), corresponding to the start site (+1) of HIV1-LTR (Fig. 5, lanes a and c). Moreover, consistent with the data shown in Fig. 3, the amount of cat mRNA in tat-expressing cells was significantly higher than the cat mRNA transcribed by anti-tat-transfected cells. In fact, both the 98-nucleotide cat band generated by transfecting pIL6Pr(-287)-CAT (Fig. 5, lanes b and d), and the 248 nt cat band generated by the pAILC-IL6Pr(-172)-CAT (Fig. 5, lanes a and c) were highly activated in TAT-positive cells. A longer exposure of the primer extension experiments revealed the presence of a 98-nt additional cat band in cells transfected with the pAILC-IL6Pr(-172)-CAT, indicating that the start sites of the IL-6 promoter and of the HIV1-LTR were both functional. A densitometer analysis of the cat bands revealed that the HIV1-LTR start site was preferentially utilized, with a minimal transcription from the IL-6 promoter start site (data not shown). This suggests that both the pIL6Pr(-287)-CAT and the pAILC-IL6Pr(-172)-CAT plasmids were responsive to Tat. Results shown in Fig. 4 b identified the sequence of -172 to -54 (at the XhoI site) as the minimal region of the IL-6 promoter required for Tat to transactivate the TAR HIV1-LTR. This suggests that the -172/-54 bp region could function as a Tat-responsive sequence, possibly allowing Tat to be directed close to the TATA box of the IL-6 promoter.

The Expression of the HIV1 tat Gene Induces an Increase in NF-kB and NF-IL6 Binding Factors. The data shown in Fig. 4 b, where Tat activated the pIL6Pr(-287)-CAT plasmid while the pIL6Pr(-172)-CAT was unresponsive to Tat, suggested that Tat-mediated activation of the IL-6 gene required transcription factors binding sequences located within -287 and -54 bp of the IL-6 promoter. In this region, functional
including EBV-positive B cell lymphoma and Kaposi’s sarcoma (4–7) which are common malignancies among HIV-1-infected patients. In vitro, IL-6 molecules enhance the survival and the growth potential of neoplastic B cells and Kaposi’s sarcoma cells and they induce a tumorigenic phenotype in immortalized EBV-positive cells (5, 7). To gain further insight into the biological relevance of the TAT-mediated induction of IL-6, we transfected the IL-6-dependent 7TD1 cells (23) with pSVT8, pSVT10, or pSV2neo control plasmids. These cells, which are strictly dependent on exogenous IL-6 for their growth, were cultured in the presence of optimal amounts (20 ng/ml) of human rIL-6 and G-418 for 15 d.

Isogenic 7TD1-pSVT98 (TAT positive) and 7TD1-pSVT10 (TAT negative) cells were then cultured without exogenous IL-6. Only the tat-expressing 7TD1-pSVT8 cells survived and grew efficiently without exogenous IL-6 (shown in Fig. 7a). pSVT8- and pSVT10-transfected cells expressed tat-specific mRNA (Fig. 7b). Moreover, tat-transfected cells showed a constitutive activation of pILC-CAT plasmid higher than the anti-tat-transfected cells, indicating that functional TAT proteins were produced by the tat-expressing cells (Fig. 7c). These tat-positive cells expressed IL-6 mRNAs, whereas control 7TD1 cells did not express any detectable IL-6 mRNAs (Fig. 7d). Moreover, 7TD1-pSVT8 cells constitutively secreted consistent amounts of murine IL-6 (Table 1).

The IL-6–dependent 7TD1-pSV2neo and 7TD1-pSVT10 cells could grow both in the presence of conditioned medium of 7TD1-pSVT8 cells and when they were cocultured with TAT-positive 7TD1-pSVT8 cells in transwell culture plates where cell contacts were prevented (data not shown). These effects were probably caused by the IL-6 secreted by tat-transfected cells, rather than by TAT proteins released by pSVT8-transfected cells. In fact, a polyclonal antibody to TAT protein did not inhibit the IL-6–independent growth of TAT-positive 7TD1-pSVT8 cells, whereas antibodies to mouse IL-6 consistently decreased the growth of 7TD1-pSVT8 cells (data not shown). Furthermore, recombinant TAT was unable to substitute for IL-6 in the growth of 7TD1 parental cells (data not shown). These data argue against a direct role of TAT as growth factor.

The expression of the tat gene and the subsequent IL-6–independent growth of 7TD1-pSVT8 cells would be expected to induce a modulation of the tumorigenic phenotype of the TAT-positive 7TD1 cells. This possibility was tested by subcutaneously injecting either the control 7TD1-pSV2neo or the TAT-positive 7TD1-pSVT8 cells in athymic nude mice.

### Table 2. Constitutive Expression of pDR CAT and pIL-1REK9CAT in tat- or Anti-tat-transfected HeLa Cells

| Cells              | Transfected plasmid | CAT activitya | Fold inductionb |
|--------------------|---------------------|---------------|-----------------|
|                    | pDR CAT      | pIL-1REK9CAT | pILC-CAT        | Acetylation |   |
| HeLa pSVT10*      | +           | −             | −               | 3.2        |   |
| HeLa pSVT8        | +           | −             | −               | 55.4       | 17.3 |
| HeLa pSVT10       | −           | +             | −               | 0.3        |   |
| HeLa pSVT8        | −           | +             | −               | 5.7        | 19.0 |
| HeLa pSV2neo      | −           | −             | +               | 62.0       | 22.1 |
| HeLa pSVT8        | −           | −             | +               | 2.8        |   |

The data are representative of five independent experiments. Similar results were obtained in the case of MC3-pSVT10 and MC3-pSVT8 cells.

* HeLa-pSVT10 (anti-tat-transfected) or HeLa-pSVT8 (tat-transfected) were transiently transfected with 10 μg of the pDR CAT (carrying two kB motifs) or 10 μg of the pIL-1REK9-CAT (carrying three NF-IL6 binding motifs). Cells were also transfected with 10 μg of pILC-CAT, a HIV1-LTR-CAT construct.

1 Determined at 48 h after transfection by using 50 μg of cell extract.

2 Expressed as the ratio of percentages acetylated.
Tat-transfected 7TD1-pSVT8 cells caused a dramatic increase in both the number of mice with tumors and in the number of tumors per animal (Table 3). These data suggest that TAT proteins could play a role in the generation of a tumorigenic phenotype by sustaining the autocrine growth of susceptible cells.

In this paper we show that the HIV1 tatl gene, either transiently expressed or stably transduced in MC3 lymphoblastoid cells and in HeLa epithelial cells, transactivates the human IL-6 promoter. Moreover, the stable expression of the HIV1 tatl gene resulted in the activation of the endogenous IL-6 gene and in the secretion of consistent amounts of IL-6 molecules. This finding may be of relevance because IL-6 molecules are secreted by HIV1-infected cells and can increase HIV1 gene expression (8, 31), and because a variety of cell types, including B and epithelial cells, can be infected by HIV1 (32, 33). The foregoing data suggest that there is a potent autocrine or paracrine mechanism of stimulation of HIV1 gene expression in which HIV1 infection results in a TAT-mediated secretion of IL-6 molecules, which in turn enhance HIV1 gene expression. Interestingly, IL-6 gene expression can also be triggered by the HIV1 envelope glycoproteins gp120 and gp160 (34, 35), suggesting that a deregulated production of IL-6 could take place in the early phase of HIV1 infection, and could result in the stimulation of different bystander cell types. A recent paper (36) reported that HIV1 infection resulted

Table 3. In Vivo Tumorigenecity of tat-transfected 7TD1 Cells

| Cells          | No. of mice with tumors/ Average of tumors | No. of inoculated mice per positive mouse |
|---------------|-------------------------------------------|----------------------------------------|
| 7TD1-pSV2neo  | 1/6/1                                     | 6/6/3                                   |
| 7TD1-pSVT8    |                                           |                                        |

Tumorigenecity in nude mice was assayed by a single subcutaneous injection of 2 × 10^6 cells in 0.2 ml of PBS (6). Typically, tumors >5 mm in diameter developed after 10–15 d of latency at the challenge site and in the regional LN and showed a histological picture of infiltrating blast cells (data not shown). Data are the numbers of tumors recorded through 6 wk of observation.
in the activation of TNF-β, whose gene product is a potent inducer of IL-6 gene (4), likely through the TAT protein. In the same study, the IL-6 production was not detected, suggesting that TAT may selectively activate specific promoters in different cell phenotypes.

The molecular mechanisms whereby TAT activates the IL-6 gene are at present unknown. The TAT protein of HIV1 is required for efficient viral gene expression (12). TAT increases the initiation of transcription from the HIV1 LTR (13) and affects RNA processing and utilization by interacting with a TAT-responsive element (TAR) located between nucleotide +1 and +44 with respect to the start site (+1) of viral transcription (11). TAT could activate IL-6 transcription by interacting with RNA stem-loop structures in the 5' untranslated region of the IL-6 promoter. At least three functional motifs are at present known (24). A sequence analysis of these regions, carried out using the energy minimizing algorithm of Zuker (37), defines a RNA stem-loop structure at the 5' untranslated region of IL-6 mRNA potentially able to bind to TAT. Here we report that the region of the IL-6 promoter located at -172 to -54 can substitute for the TAR sequence, allowing TAT to transactivate the TAR-deleted HIV1-LTR. Our data suggest that TAT may be directed at this region of the IL-6 promoter by binding to a RNA structure, and could induce the transcription of the IL-6 gene by possibly cooperating with transcriptional factors such as NF-κB and NF-IL6. This possibility is currently under investigation. Alternatively, TAT may bind to cell transcription factors, as recently suggested (15, 17), and ultimately activate IL-6 transcription. Another possibility is that TAT could increase the stability of a baseline constitutive transcription of IL-6 gene in MC3 and HeLa cells. However, such posttranscriptional activity cannot apply to 7TD1 cells because they do not express any detectable IL-6 mRNA.

Recent reports suggest that TAT cooperates with upstream regulatory DNA sequences circumscribed within the NF-κB/Spl region of the HIV1 promoter (14) and with host cell proteins (15-19). TAT could affect the transcription of cell genes such as IL-6, whose regulatory sequences, like HIV1-LTR, possess NF-κB and NF-IL6 enhancer elements (20, 21). In support of this possibility, we found that expression of tat leads to an increase in κB and NF-IL6 binding factors. In addition, since the pIL6p(-172)-CAT plasmid, which lacks a NF-IL6 binding motif but retains a κB site, was not induced by TAT, our data suggest that in tat-transfected cells NF-κB and NF-IL6 could function as a unique transcriptional complex, as recently suggested (38), in activating the IL-6 promoter.

A variety of clinical lesions are associated with HIV1 infection. This includes B lymphomas, psoriasis, and Kaposi's sarcomas, where HIV1 transcripts have been detected (1-3, 39). There is evidence that abnormal secretion of IL-6 plays a major role in the pathogenesis of these diseases by cooperating with such other cytokines as oncostatin M and IL-1, which in turn are potent inducers of IL-6 secretion (4, 40-42). In this paper we showed that TAT is a major stimulant of IL-6 secretion and could induce autocrine growth and enhanced tumorigenicity of the IL-6-dependent 7TD1 cells. These data are consistent with and support the recent observations that TAT can activate the transcription of genes from heterologous viral and cellular promoters (36, 43, 44), and suggest that TAT proteins may directly participate in the pathogenesis of HIV1-associated diseases by modulating the expression of host cellular genes.

We thank A. Caputo for providing pSVT8 and pSVT10 plasmid; A. Rabson for the gift of pSVT12 and pLC-CAT plasmids; A. Weisz for providing the puls-lacZ plasmid; W. Greene for the pDRCAT plasmid; and T. Kishimoto and T. Hirano for the gift of mouse IL-6 cDNA, pGEMB672A plasmids, and IL-1REK9CAT plasmids. We are grateful to G. Ciliberto for providing the purified human IL-6 and to the National Institutes of Health AIDS Research and Reference Reagent Program for the gift of the recombinant TAT protein and of the rabbit anti-TAT antibody used in this study. We acknowledge the careful review of the manuscript by A. Rabson, J. Guardiola, T. Russo, and G. N. Pavlakis. We also thank C. Maresca and F. Dello Stritto for preparing the manuscript, and Jeane Gilder for editorial revision.

This work was supported by grants from the Associazione Italiana per la Ricerca sul Cancro (A.I.R.C.), from the Consiglio Nazionale delle Ricerche (C.N.R.), and from A.I.D.S. project of the Istituto Superiore di Sanità. M. R. Ruocco was supported by a fellowship from the Istituto Superiore di Sanità. V. Giordano and F. Baldassarre were supported by fellowships from A.I.R.C. M. Mallardo was supported by a fellowship from C.N.R.

Address correspondence to Dr. Giuseppe Scala, Dipartimento di Biochimica e Biotecnologie Mediche, Via S. Pansini 5, 80131 Napoli, Italy.

Received for publication 14 June 1993 and in revised form 11 November 1993.
References

1. Selligman, M., L. Chess, J.L. Fahey, A.S. Fauci, P.J. Lachmann, J. L'Age-Stehr, J. Ngu, A.J. Pinching, F.S. Rosen, T.J. Spira, and J. Wybran. 1984. AIDS - An immunologic reevaluation. N. Engl. J. Med. 311:1286.

2. Fauci, A.S., A.M. Macher, D.L. Longo, H.C. Lane, A.H. Rook, H. Masur, and E.P. Gelman. 1984. Acquired immunodeficiency syndrome: epidemiologic, clinical, immunologic, and therapeutic considerations. Ann. Intern. Med. 100:92.

3. Levine, A.M. 1992. Acquired immuno deficiency syndrome-related lymphoma. Blood. 80:8.

4. Kishimoto, T., S. Akira, and T. Taga. 1992. Interleukin-6 and its receptor: a paradigm for cytokines. Science (Wash. DC). 258:593.

5. Kawano, M., T. Hirano, T. Matsuda, T. Taga, Y. Horii, K. Iwato, H. Asakoe, B. Tang, O. Tanabe, H. Tanaka, A. Kuramoto, and T. Kishimoto. 1988. Autocrine generation and requirement of BSF-2/IL6 for multiple myelomas. Nature (Lond.). 332:83.

6. Scala, G., I. Quinto, M.R. Ruocco, A. Arcucci, M. Mallardo, P. Caretto, G. Forni, and S. Venuta. 1990. Expression of an exogenous interleukin 6 gene in human Epstein Barr virus B cells confers growth advantage and in vivo tumorigenicity. J. Exp. Med. 172:61.

7. Miles, S.A., A.R. Rezai, J.F. Salzar-Gonzalez, M. Vander Meyden, R.H. Stevens, D.M. Logan, R.T. Mitsuysu, T. Taga, T. Kishimoto, and O. Martinez-Maza. 1990. AIDS Kaposi sarcoma-derived cells produce and respond to interleukin 6. Proc. Natl. Acad. Sci. USA. 87:4068.

8. Nakajima, N., O. Martinez-Maza, T. Hirano, E.C. Breen, P.G. Nishanian, J.F. Sazar-Gonzalez, J.L. Fahey, and T. Kishimoto. 1989. Induction of IL-6 (B cell stimulatory factor-2: IFN-t32) production by HIV. J. Immunol. 142:144.

9. Breen, E.C., A.R. Rezai, K. Nakajima, G.N. Beall, R.T. Mitsuysu, T. Hirano, T. Kishimoto, and O. Martinez-Maza. 1990. Infection of HIV is associated with elevated IL6 levels and production. J. Immunol. 144:480.

10. Gallo, P., K. Frei, C. Rordorf, J. Lazdins, B. Tavolato, and A. Fontana. 1989. Human immunodeficiency virus type 1 (HIV-1) infection of the central nervous system: an evaluation of cytokines in cerebrospinal fluid. J. Neuroimmunol. 23:109.

11. Rosen, C.A., J.G. Sodroski, and W.A. Haseltine. 1985. The location of cis-acting regulatory sequences in the human T cell lymphotrophic virus type III (HTLV-III/LAV) long terminal repeat. Cell. 41:813.

12. Rice, A.P., and M.B. Matthews. 1988. Transcriptional but not translational regulation of HIV-1 by the tat gene product. Nature (Lond.). 332:551.

13. Lapsia, M.F., A.P. Rice, and M.B. Matthews. 1989. HIV-1 Tat protein increase transcriptional initiation and stabilizes elongation. Cell. 59:283.

14. Berkhout, B., A. Gatignol, A.B. Rabson, and K. Jeang. 1990. Tat trans-activates the human immunodeficiency virus through a nascent RNA target. Cell. 62:757.

15. Nelbock, P.J., Dill, A. Perkins, and C.A. Rosen. 1990. A cDNA for a protein that interacts with the human immunodeficiency virus tat transactivator. Science (Wash. DC). 248:1650.

16. Gatignol, A., A. Buckler-White, B. Berkhout, and K. Jeang. 1991. Characterization of a human TAR RNA-binding protein that activated the HIV-1 LTR. Science (Wash. DC). 251:1597.

17. Desai, K., P.M. Loewenstein, and M. Green. 1991. Isolation of a cellular protein that binds to the human immunodeficiency virus Tat protein and can potentiate transactivation of the viral promoter. Proc. Natl. Acad. Sci. USA. 88:8875.

18. Shibuya, H., K. Irie, J. Ninomiya-Tsuji, M. Goebel, T. Taniguchi, and K. Matsumoto. 1992. New human gene encoding a positive modulator of HIV Tat-mediated transactivation. Nature (Lond.). 357:700.

19. Swaffield, J.C., J.F. Bromberg, and S.A. Johnson. 1992. Alterations in a yeast protein resembling HIV Tat-binding for an acidic activation domain in GAL4. Nature (Lond.). 357:698.

20. Libermann, T.A., and D. Baltimore. 1990. Activation of interleukin-6 gene expression through the NF-kB transcription factor. Mol. Cell. Biol. 10:2327.

21. Akira, S., H. Ishikii, T. Sugita, O. Tanabe, S. Kinoshita, Y. Nishino, T. Nakajima, T. Hirano, and T. Kishimoto. 1990. A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. EMBO (Eur. Mol. Biol. Organ.) J. 9:1897.

22. Caputo, A.J., J.G. Sodroski, and W.A. Haseltine. 1992. Constitutive expression of HIV-I tat protein in human Jurkat T cells using a BK virus vector. J. Acquired Immune Defic. Syndr. 3:372.

23. Van Snick, J., S. Capayhas, A. Vink, C. Uyttenhove, P.G. Coule, M.R. Rubira, and R.J. Simpson. 1986. Purification and NH2-terminal amino acid sequence of a T-cell-derived lymphokine with growth factor activity for B-cell hybridomas. Proc. Natl. Acad. Sci. USA. 83:9679.

24. Yasukawa, K., T. Hiranro, Y. Watanabe, K. Muratani, T. Matsuda, and T. Kishimoto. 1987. Structure and expression of human B cell stimulatory factor 2 (BSF-2) gene. EMBO (Eur. Mol. Biol. Organ.) J. 6:2939.

25. Maniatis, T., E.F. Fritsch, and J. Sambrook. 1990. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 545 pp.

26. Leonard, J., C. Parrott, A.J. Buckler-White, A. Turner, E.K. Ross, M.A. Martin, and A.B. Rabson. 1989. The NF-kB binding sites in the human immunodeficiency virus type 1 long terminal repeat are not required for virus infectivity. J. Virol. 63:4919.

27. Scala, G., I. Quinto, M.R. Ruocco, M. Mallardo, C. Ambrosino, B. Squitieri, P. Tassone, and S. Venuta. 1993. The Epstein-Barr Virus nuclear antigen 2 trans-activated the long terminal repeat of human immunodeficiency virus type 1. J. Virol. 67:2853.

28. Tanabe, O., S. Akira, T. Kamiya, G.G. Wong, T. Hirano, and T. Kishimoto. 1988. Genomic structure of the murine IL6 gene. High degree of conservation of potential regulatory sequences between mouse and human. J. Immunol. 141:3875.

29. Gorman, C.M., L.F. Moffat, and B.H. Howard. 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. 2:1044.

30. Ray, A.S., B.S. Tarr, L.R. May, and P.B. Sehgal. 1988. Activation of the human "32-interferon/hepatocyte-stimulating factor/interleukin 6" promoter by cytokines, viruses, and second messenger agonists. Proc. Natl. Acad. Sci. USA. 85:6701.

31. Poli, G., P. Bressler, A. Kinter, E. Duh, W.C. Timmer, A. Rabson, J.S. Justement, S. Stanley, and A.S. Fauci. 1993. The Tat protein increase transcriptional initiation and stabilizes elongation. Science (Wash. DC). 260:1597.
33. Pomerantz, R.J., S.M. de la Monte, S.P. Donegan, T.R. Rota, M.W. Vogt, D.E. Craven, and M.S. Hirsch. 1988. Human immunodeficiency virus (HIV) infection of the uterine cervix. Ann. Intern. Med. 108:321.

34. Clouse, K.A., L.M. Cosentino, K.A. Weih, S.W. Pyme, P.B. Robbins, H.D. Hochstein, V. Natarajan, and W.L. Farrar. 1991. The HIV-1 gp120 envelope protein has the intrinsic capacity to stimulate monokine secretion. J. Immunol. 147:2892.

35. Oyaizu, N., N. Chirmule, Y. Ohnishi, V.S. Kalyanaraman, and S. Pahwa. 1991. Human immunodeficiency virus type 1 envelope glycoproteins gp120 and gp160 induce interleukin-6 production in CD4+ T-cell clones. J. Virol. 65:6277.

36. Buonaguro, L., G. Barillari, H.K. Chang, C.A. Bohan, V. Kao, R. Morgan, R.C. Gallo, and B. Ensoli. 1992. Effects of the human immunodeficiency virus type 1 Tat protein on the expression of inflammatory cytokines. J. Virol. 66:7159.

37. Zuker, M., and P. Stiegel. 1981. Optimal computer folding of large RNA sequences using thermodynamics and auxiliary information. Nucleic Acids Res. 9:133.

38. Le Clair, K.P., M.A. Blanar, and P.A. Sharp. 1992. The p50 subunit of NF-kB associates with the NF-IL6 transcription factor. Proc. Natl. Acad. Sci. USA. 89:8145.

39. Mahoney, S.E., M. Duvic, B.J. Nickoloff, M. Minshall, L.C. Smith, C.E.M. Griffiths, S.W. Paddock, and D.E. Lewis. 1991. Human immunodeficiency virus (HIV) transcripts identified in HIV-related psoriasis and Kaposi's sarcoma lesions. J. Clin. Invest. 88:174.

40. Nair, B.C., A.L. De Vico, S. Nakamura, T.D. Copeland, Y. Chen, A. Patel, T. O'Neil, S. Oroszlan, R.C. Gallo, and M.G. Sarngadharan. 1992. Identification of a major growth factor for AIDS-Kaposi's sarcoma cells as oncostatin M. Science (Wash. DC). 255:1430.

41. Miles, S.A., O. Martinez-Maza, A. Rezai, L. Magnapantay, T. Kishimoto, S. Nakamura, S.F. Radka, and P.S. Linsley. 1992. Oncostatin M as a potent mitogen for AIDS-Kaposi's sarcoma-derived cells. Science (Wash. DC). 255:1432.

42. Brown, T.J., J.M. Rowe, J. Liu, and M. Shoyab. 1991. Regulation of IL-6 expression by oncostatin M. J. Immunol. 147:2175.

43. Kim, Y.S., and R. Risser. 1993. TAR-independent transactivation of the murine cytomegalovirus major immediate-early promoter by the tat protein. J. Virol. 67:239.

44. Tada, H., J. Rappaport, M. Lashgary, S. Amini, F. Wong-Staal, and K. Khalili. 1990. Trans-activation of the JC virus late promoter by the tat protein of type 1 human immunodeficiency virus in glial cells. Proc. Natl. Acad. Sci. USA. 87:3479.

45. Scala, G., P. Allavena, J.Y. Djeu, T. Kasahara, J.R. Ortaldo, R.B. Herberman, and J.J. Oppenheim. 1984. Human large granular lymphocytes are potent producers of interleukin-1. Nature (Lond.). 309:56.