# HIV-1 Tat Inhibits IL-2 Gene Transcription Through Qualitative and Quantitative Alterations of the Cooperative Rel/AP1 Complex Bound to the CD28RE/AP1 Composite Element of the IL-2 Promoter[1]

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**Dysregulation of cytokine secretion plays an important role in AIDS pathogenesis. Here, we demonstrate that expression of HIV-1 Tat protein in Jurkat cells induces a severe impairment of IL-2 but not TNF gene transcription. Interestingly, this inhibition correlates with the effect of the viral protein on the transactivation of the CD28RE/AP1 composite element (−164/−154), but not with that observed on the NFAT/AP1 site of the IL-2 gene promoter, neither with the effect on NF-κB- nor AP1-independent binding sites. Endogenous expression of Tat induced a decrease in the amount of the specific protein complex bound to the CD28RE/AP1 probe after PMA plus calcium ionophore stimulation. This effect was accompanied by qualitative alterations of the AP1 complex. Thus, in wild-type Jurkat cells, c-jun was absent from the complex, whereas in Tat-expressing cells, c-jun was increasingly recruited overtime. By contrast, similar amounts of c-*rel* and a small amount of NFAT1 were detected both in wild type and in Jurkat Tat$^+$ cells. Furthermore, Tat not only induced the participation of c-jun in the cooperative complex but also a decrease in its transactivation activity alone or in combination with c-*rel*. Thus, the interaction of Tat with the components of this rel/AP1 cooperative complex seems to induce quantitative and qualitative alterations of this complex as activation progresses, resulting in a decrease of IL-2 gene transcription. Altogether our results suggest the existence of tuned mechanisms that allow the viral protein to specifically affect cooperative interactions between transcription factors.** ***The Journal of Immunology,*** **2001, 166: 4560–4569.**

The pathogenic mechanisms underlying HIV-1 infection and disease are extremely complex, and virological as well as immunological factors contribute to pathogenesis. Among the viral factors involved in the induced immunological dysregulation, the transactivator protein Tat has been widely studied (1). Tat is a small (72–101 aa) regulatory viral protein required for efficient transcription and viral replication. Besides its interaction with the RNA stem-loop structure-denominated *trans* activation response, Tat has been shown to interact with several members of the transcriptional machinery during the process of initiation and elongation of viral transcription. This is the case of RNA polymerase II, the kinase complex P-TEFb, and the transcriptional coactivators cAMP response element binding protein binding protein (CBP)[3]/p300 (2, 3).

Furthermore, the presence of Tat does not only affect viral transactivation but also mediates alterations in multiple cellular processes. Thus, Tat has been involved in both apoptotic (4–6) and survival (7, 8) mechanisms in the alteration of T cell proliferation (9) and in the aberrant expression of several cytokine genes: TNF-α (10), TGF-β (11), IL-6 (12), IL-2 (13–16), IFN-α (17), and IL-8 (18). Many of these effects are thought to be mediated by alterations of cellular gene expression by Tat. In this regard, it has been demonstrated a direct interaction of the viral protein with several transcription factors including Oct, Sp1, and NFAT (19, 20). Besides, indirect mechanisms have been proposed to explain alterations of the transcriptional activity of NF-κB (5, 21) and AP1 (22) transcription factors.

In the immune response, IL-2 is considered to play a pivotal role. This cytokine is strongly regulated at the level of transcription, and the regulatory sequences conferring its inducible expression in T cells are localized in a region of ~300 bp 5′of the transcription start site (23). Within this region, the existence of binding sites for different ubiquitous and cell-specific transcription factors (NF-κB, AP1, NFAT, and Oct1, among others) has been reported (reviewed in Ref. 24). Many of these sites are noncanonical, differing in one or several base pairs from the corresponding consensus sequences and, consequently, the existence of cooperative interactions between transcription factors binding to adjacent sites becomes necessary to obtain an efficient transactivation (25). In this regard, the CD28RE/AP1 (−164/−145) and NFAT/AP1 distal (−286/−268) sites of the human IL-2 promoter are very representative examples of these cooperative interactions between the rel and AP1 families in the former case (26) and NFAT and AP1 families in the latter (27). The contribution of the different regulatory elements to IL-2 gene expression is controversial (28, 29), and it seems to be different in normal and Jurkat T cells (30).

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[3] Abbreviations used in this paper: CBP, cAMP response element binding protein binding protein; Io, calcium ionophore A23187; LTR, long terminal repeat; CONA, conalbumin; JNK, c-Jun N-terminal kinase.

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Transcriptional transactivation regulated through composite elements is further complicated by the interplay between these families of transcription factors. Thus, binding of NFAT proteins to κB-like sites takes place on several promoters (20, 31–33). Moreover, NF-κB and AP1 transcription factors are regulated not only by transcriptional and posttransductional mechanisms, but also by the qualitative composition of the dimers. This determines the transcriptional activity, the DNA sequence specificity, and the interactions with transcriptional coactivators (34–36).

As the Tat viral protein has been shown to affect cytokine expression and interact with different transcription factors, we consider of interest the study of how this viral protein can alter the cooperative interactions determining the transactivation of these composite elements. Here, we demonstrate that Jurkat cells stably expressing Tat have a drastic inhibition in CD28RE/AP1-mediated transactivation, concomitantly with a severe impairment of IL-2-induced transcription. Furthermore, we show that this viral protein acts on this composite element inducing quantitative and qualitative changes in the transactivating complex as activation progresses, possibly explaining the inhibition of IL-2 gene transcription observed in Jurkat $Tat^+$ cells.

## Materials and Methods

### *Cell cultures and reagents*

Jurkat Jhan cells (both wild type and stably expressing the full-length HIV-1 Tat of 86 aa) were a gift from J. Alcamí and were grown in complete RPMI 1640 medium supplemented with 10% FCS. Tat-expressing cells were obtained by transfection of pLTat SN plasmid (a gift from O. Schwartz, Institute Pasteur, Paris, France). This plasmid contains Tat under long terminal repeat (LTR) of Moloney virus control. The stable expressing cells were selected by resistance to geneticin. COS cells were obtained from the American Type Culture Collection (Manassas, VA) and were maintained in complete DMEM supplemented with 10% FCS. Cells were stimulated with anti-CD3 (1 μg/ml) plus anti-CD28 (1 μg/ml) Abs, or with PMA (25 ng/ml; Sigma, St. Louis, MO) and ionophore A23187 (1 μM; Sigma) as indicated. Anti-Tat polyclonal Ab was obtained from B. Cullen (Duke University, Durham, NC) through AIDS Research and Reference Program (37). None of the agents affect the viability of the cells at the concentrations used, as confirmed by the trypan blue dye exclusion test.

### *Cytokine measurement*

The concentration of IL-2 was quantified in supernatants of Jurkat $Tat^-$ or Jurkat $Tat^+$ cell cultures ($7 \times 10^5$ cells/ml) harvested after 24 or 48 h of cultivation in the absence or presence of PMA+calcium ionophore A23187 (PMA+Io). Commercially available ELISA kits were used according to manufacturer's instructions (IL-2; R&D Systems, Minneapolis, MN). Cytokine concentration was assayed in duplicate.

### *Plasmid constructs*

The reporter plasmid pIL2luc containing the sequences from −326 to +46 of the human IL-2 gene directing transcription of the firefly luciferase gene has already been described (23). The plasmid TK-luc contains the herpes simplex I thymidine kinase promoter (38). The pCD28RE/AP1luc plasmid contains four copies of the oligonucleotide corresponding to the CD28RE/NF-IL-2B of the human IL-2 gene promoter and was a gift from A. Weiss (University of California, San Francisco, CA) (26). The plasmid pκB-conalbumin (CONA)-luc containing three tandem copies of the κB site of the Ig κ-chain promoter cloned upstream of the CONA transcription start site was provided by J. Alcamí (Hospital 12 de Octubre, Madrid, Spain) (39). The reporter plasmid pLTRxluc was a gift from J. L. Virelizier (Institute Pasteur). (40). It carries the U3′UTR of the LTR of the LAI strain of HIV-1 from nucleotide −644 to +78. The pNFATluc plasmid was a gift from G. Crabtree (Stanford University Medical School, Stanford, CA). It contains three tandem copies of the NFAT distal site of the human IL-2 promoter fused to the minimal human IL-2 promoter (23). The pTNF-luc plasmid contains a region 1311 bp upstream from the transcriptional initiation site of the human TNF-α promoter (41). The p-73col-luc plasmid including the AP1-responsive (−73/+63 bp) regions of the human collagenase promoter fused to the luciferase gene has been previously described (42). The pGAL4-luc reporter plasmid includes five GAL4 DNA binding sites fused to the luciferase gene (43). The pRSV-GAL4-c-Jun (wild type) and pRSV-GAL4-c-Jun $S_1+S_2$ constructs encode the wild-type transactivation domain of c-jun, and this domain mutated in its phosphorylation sites (Ser 63 and Ser 73), respectively, in frame with the GAL4 DNA binding domain (44). The pGAL4-c-*rel* (309–588) and pGAL4-c-*rel* (309–318) express a chimera containing the GAL4 DNA binding domain together with the transactivation domain of c-*rel* or 10 aa without transactivating capacity, respectively, and were cloned by A. García in our laboratory (45). The pRSV-cJ-expressing c-jun was a gift from A. Muñoz (Instituto de Investigaciones Biomedicas, Madrid, Spain) and was previously described (44) and the pRc-hc-*rel* expression plasmid resulting from the cloning of the cDNA of c-*rel* in the *Hin*dIII-*Xba*I restriction sites of pRcCMV (Invitrogen, San Diego, CA) was provided by N. Rice (National Cancer Institute, Frederick, MD) (45). The plasmid pEF-BOS-NFAT1 bears the gene encoding the influenza virus hemagglutinin-tagged NFAT1 and was a gift from J. M. Redondo (Centro de Biología Molecular, Madrid, Spain). The CMVTat was a gift from J. Alcamí and contains full-length HIV Tat (86 aa) under control of CMV-immediate early promoter (40). The pcDNA3 plasmid (Invitrogen) is a cloning vector containing the CMV promoter. It was used in our experiments as a control in the transfection of expression plasmids or to adjust the quantities of DNA transfected. To evaluate transfection efficiency, pRL-tk-luc plasmid (Promega, Madison, WI) was used. It contains the herpes simplex virus thymidine kinase (HSV-TK) promoter to provide low to moderate levels of Renilla luciferase expression in cotransfected mammalian cells.

### *EMSAs*

Nuclear extracts were obtained from Jurkat cells essentially by the previously described method (46). Gel retardation assays were performed as described (47) with the only modification that in the competition experiments, the unlabeled homologous oligonucleotides were used at 80-fold molar. The sequences of the oligonucleotides (5′ to 3′) used in EMSAs were tcgaGTTTAAAGAAATTCCAAAGAGTCATCAG (CD28RE/AP1 of human IL-2 promoter), gatcGGAGGAAAAACTGTTTCATACA GAAGGCGT (distal NFAT site of human IL-2 promoter), gatcATA AAATTTTCCAATGTAAA (mouse P sequence of the IL-4 promoter), and CGCTTGATGAGTCAGCCGGAA (AP1 consensus oligonucleotide).

The pairs of complementary synthetic oligonucleotides were annealed and labeled with the Klenow fragment of the DNA polymerase I using [$\alpha$-$^{32}$P]dCTP and [$\alpha$-$^{32}$P]dGTP with the exception of the AP1 oligonucleotide (Promega) that was labeled with the T4-PNK using [$\gamma$-$^{32}$P]dATP. To purify the labeled oligonucleotides, Quick Spin Columns Sephadex G25 (Boehringer Mannheim, Heidelberg, Germany) equilibrated with Tris-EDTA were used.

When using serum to supershift or to inhibit the induced binding, the Abs were added to the binding reactions before the addition of the radiolabeled probe. The serum used in EMSAs were 672 anti-NFAT1 (gift from J. M. Redondo), anti-junB, and anti-fos family (provided by A. Corbí, Centro de Investigaciones Biológicas, Madrid, Spain), anti c-jun (sc-45-G; Santa Cruz Biotechnology, Santa Cruz, CA), and the anti-c-*rel* (1136) (48) and anti-p65 (1226) (49) sera were gifts from N. Rice.

### *RT-PCR analysis*

Total RNA was prepared from Jurkat cells by the TRIzol reagent RNA protocol (Life Technologies, Paisley, U.K.). Total RNA (1 μg) was reverse transcribed into cDNA and used for PCR amplification with either human IL-2 or HIV-1-Tat or GAPDH-specific primers by the RNA PCR Core Kit (Perkin-Elmer, Norwalk CT) as previously described (50). Briefly, the PCR was amplified by 20–35 repeat denaturation cycles at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. Amplified cDNAs were separated by agarose gel electrophoresis, and bands were visualized by ethidium bromide staining.

### *Transient transfections*

Transcriptional activity in Jurkat and COS cells was measured in reporter gene assays after transient transfection of cells with the corresponding plasmid using Lipofectamine as recommended by the manufacturer (Life Technologies). Briefly, exponential growing Jurkat cells ($2 \times 10^6$) were transfected with 4 μl of Lipofectamine, 6 μl of Plus Reagent (Life Technologies) and 0.5 μg of the firefly luciferase reporter plasmid (containing the regulatory region under test) together with 10 ng of the pRL-tk-luc in a final volume of 1 ml of OptiMem (Life Technologies). Following 4 h of incubation, 3 ml of complete medium was added to cells, and they were incubated overnight to complete transfection. Cells were then counted by trypan blue exclusion and treated ($7 \times 10^5$ cells/ml) with different stimuli for 6 h. In the case of COS cells, exponential growing cells were transfected in suspension. For $5 \times 10^5$ cells, 2 μl of Lipofectamine, 3 μl of Plus Reagent, and 1 μg of the firefly luciferase reporter plasmid plus 20 ng of

the pRL-tk-luc and 0.25 $\mu$g of the corresponding expression plasmid were incubated for 3 h in a final volume of 1 ml of OptiMem. COS cells were then pelleted and resuspended in 4 ml of complete medium. Cells were plated on multidish 24-well plates and, after 24 h, stimuli were added for an additional 6-h period.

### *Luciferase assays*

After the stimulation period, cells were harvested, lysed, and measured for 10 s in a luminometer following the instructions in the Dual-Luciferase Assay System Kit (Promega). Data are represented in relative firefly luciferase units normalized by the relative renilla luciferase units obtained in the control samples of every transfection (RLUff/ren). Every experiment was conducted in duplicate. All of the experiments shown are representative of at least three performed to guarantee the reproducibility of the results.

## Results

### *Expression of Tat in T cells down-regulates IL-2 synthesis*

To study the effect of Tat on IL-2 synthesis, we compared the activation response of Jurkat wild-type (Tat$^-$) cells to those stably expressing the viral protein (Jurkat Tat$^+$). Cells were stimulated with phorbol esters in combination with calcium ionophore, and IL-2 secretion into the supernatant was evaluated by bioassays (data not shown) or by specific ELISA (Fig. 1*A*). Normal Jurkat Tat$^-$ cells secreted IL-2 upon stimulation by PMA+Io. By contrast, Jurkat Tat$^+$ cells had a drastic if not complete blockade in their ability to produce IL-2. Furthermore, no IL-2 mRNA could be detected by RT-PCR techniques in Jurkat Tat$^+$ cells at any time after activation although it was already observed at 2 h of stimulation in wild-type Jurkat cells (Fig. 1*B*). These data suggested an inhibitory effect of Tat at the level of IL-2 gene transcription.

### *Tat decreases IL-2 gene transcription*

To further confirm that Tat was affecting IL-2 gene expression, we analyzed the expression of a reporter gene under the control of the −326/+45 region of the human IL-2 gene promoter. Transactivation of the IL-2 promoter was increased in Jurkat cells upon PMA+Io stimulation, but this induced transcription was strongly inhibited in Jurkat Tat$^+$ cells. By contrast, the transactivation of the TNF promoter was up-regulated in Jurkat Tat$^+$ cells.

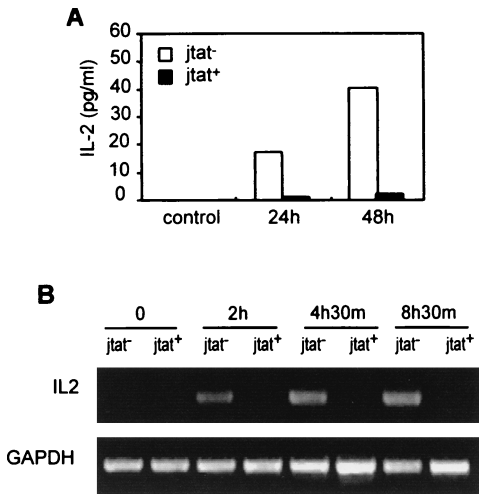


**FIGURE 1.** Inhibition of IL-2 gene expression in Tat-expressing cells. *A*, Jurkat Tat$^-$ and Tat$^+$ cells were cultured ($7 \times 10^5$ cells/ml) in absence (control) or presence of PMA+Io. Supernatants were harvested after 24 or 48 h of treatment, and the presence of IL-2 was evaluated by ELISA. *B*, Total RNA (1 $\mu$g) from Jurkat Tat$^-$ and Jurkat Tat$^+$ cells stimulated as mentioned in *A* for the indicated times was analyzed by RT-PCR to measure IL-2 and GADPH mRNA levels. An aliquot of the amplified DNA was separated on an agarose gel and stained with ethidium bromide for qualitative comparison.

It is well known that IL-2 gene expression is dependent on the coordinated activity of several transcription factors activated after T cell stimulation (24). Among the DNA sequences present in the IL-2 promoter, the most relevant in transcriptional regulation are those recognized by AP1, NF-$\kappa$B, and the composite elements NFAT/AP1 (NFAT distal site) and CD28RE/AP1, where Rel and AP1 proteins transactivate in a cooperative way (26). Therefore, we decided to analyze which of those factors was altered in Jurkat Tat$^+$ cells by transiently transfecting plasmids that express the luciferase reporter gene under the control of the corresponding response elements. As shown in Fig. 2*B*, PMA+Io-induced transcription controlled by CD28RE/AP1 was severely abolished in Jurkat Tat$^+$ cells. By contrast, transcription dependent on pure $\kappa$B or AP1 sites was not significantly altered, whereas NFAT/AP1-dependent transcription was enhanced 2-fold in Jurkat Tat$^+$ cells.

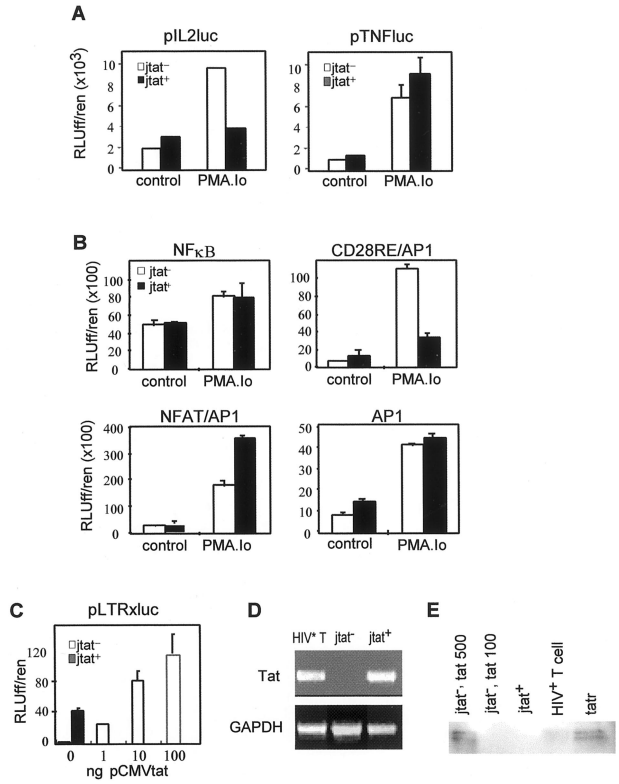


**FIGURE 2.** Regulation of transcriptional transactivation in Jurkat Tat$^-$ and Jurkat Tat$^+$ cells. *A*, Effect of Tat on IL-2 and TNF promoters was evaluated by transiently transfecting Jurkat Tat$^-$ and Jurkat Tat$^+$ cells with pIL-2luc and pTNFluc plasmids, respectively. *B*, Transactivation controlled by the response elements mentioned at the *top* of each diagram was evaluated after transient transfection of the cells with the luciferase reporter plasmids p-$\kappa$B-CONA-luc (NF-$\kappa$B), pNFATluc (NFAT/AP1), p-73col-luc (AP1), and pCD28RE/AP1luc. In both *A* and *B*, transiently transfected cells were cultured in the absence of stimulation (control) or treated with PMA+Io for 6 h. Transfection, stimulation, and luciferase activity measurements were performed as described in *Materials and Methods. C*, Comparison of the ability of Jurkat Tat$^+$ cells with transiently transfected Jurkat Tat$^-$ cells to activate HIV LTR. Cells were transfected with pLTRxluc and with the indicated amounts of pCMVTat. Luciferase activity was measured after 6 h of culture. *D*, Expression of HIV-Tat mRNA in Jurkat Tat$^+$ cells. Total mRNA (1 $\mu$g) from Jurkat Tat$^+$ or Tat$^-$ cells as well as from T lymphocytes from an HIV-1-infected patient was analyzed by RT-PCR to measure HIV-Tat or GADPH mRNA levels. *E*, Expression of HIV Tat by Western blot. Cell extracts from $10^7$ Jurkat Tat$^+$ or Jurkat Tat$^-$ transfected with 100 or 500 ng of pCMVTat as well as from $10^8$ T lymphocytes from an HIV-infected patient were run on SDS-PAGE and blotted with anti-Tat Ab. Recombinant Tat was added as a control.

Thus, the global effect of Tat on IL-2 promoter regulation seemed to correlate with the effect on the CD28RE/AP1 element, indicating its prominent role in controlling IL-2 transcription.

The expression of Tat in Jurkat Tat$^+$ cells was difficult to detect by Western blot with specific anti-Tat antisera (Fig. 2*E*). However, by RT-PCR, Tat mRNA was detected (Fig. 2*D*). Besides, by transfection of Jurkat Tat with pCMVTat, only cells transfected with 500 ng, but not with 100 ng, of plasmid allow a clear detection of Tat by Western blot. This indicates that the amount of Tat in Jurkat Tat cells was rather low. However, the presence of active Tat could be inferred by a very strong up-regulation of the activity of the HIV-LTR promoter. Furthermore, if we compared the level of LTR transactivation in Jurkat cells transiently and stably expressing Tat, we can tentatively assume that Jurkat Tat cells should express very low levels of Tat, comparable to those obtained in transfections of 1–10 ng of Tat plasmid DNA per million cells (Fig. 2*C*). Similarly, Tat was difficult to detect by Western blot in PBMC from an infected HIV patient, although a faint doublet could be observed (Fig. 2*E*).

To further corroborate that this effect was exclusively to Tat and not to some artifact produced during the generation of stable transfected cells, Jurkat wild-type cells were transfected with different doses of a pCMVTat-expressing plasmid together with CD28RE/AP-1 reporter. As shown in Fig. 3, the transfection of low amounts of the Tat-expressing plasmid significantly decreased the transactivation of the CD28RE/AP-1 reporter induced by anti-CD3 plus CD28 PMA+Io induced a stronger up-regulation of this reporter than anti-CD23 plus anti-CD28, but this induction was also inhibited by Tat, although it required higher amounts. As a control, TK-luc was unaffected by Tat expression (Fig. 3). Similar results were found in another human T cell line, MOLT-4 (data not shown).

Contrasting effects of Tat depending on whether it acts intracellularly or extracellularly have been reported (13–16). To address this point, we evaluated the activation of the CD28RE/AP1 reporter in Jurkat Tat$^+$ cells activated by PMA+Io in the presence of a neutralizing anti-Tat Ab (Fig. 4). No effect of anti-Tat was observed despite the fact that Tat was secreted. Tat secretion was demonstrated by the fact that cocultures of Jurkat Tat$^+$ with Jurkat cells transfected with HIV LTR up-regulated the transcription from this promoter, and this effect was blocked by the anti-Tat Ab.

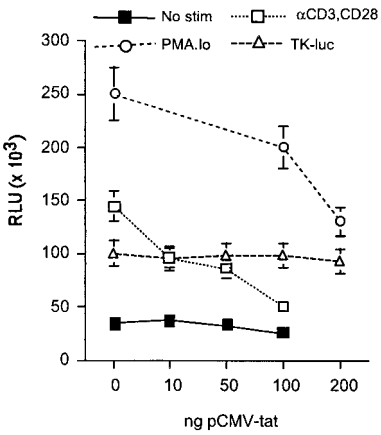


**FIGURE 3.** Effect of transiently expressed Tat on CD28RE/AP1-dependent transcription. Jurkat Tat$^-$ cells were transiently transfected with pCD28RE/AP1luc or pTKluc together with the indicated doses of pCMVTat/$10^6$ cells. After transfection the cells were cultured in absence of stimulation or treated with anti-CD3 plus CD28 or PMA+Io for 6 h as indicated.

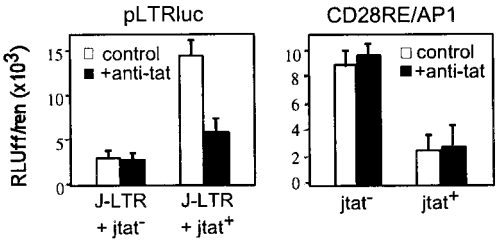


**FIGURE 4.** Endogenous Tat expression is responsible for the down-regulation of CD28RE/AP1-dependent transcription. In the *left panel*, Jurkat Tat$^-$ ($5 \times 10^5$ cells) transfected with the pLTRxluc (J-LTR) were cocultured with Jurkat Tat$^+$ ($5 \times 10^5$ cells) in absence (control) or presence of anti-Tat Ab. In the *right panel*, Jurkat Tat$^+$ or Jurkat Tat$^-$ cells were transfected with pCD28RE/AP1luc and cultured in the absence or in presence of neutralizing anti-Tat Abs. Cells were cultured for 6 h in the presence of PMA+Io.

As expected, anti-Tat antiserum did not affect CD28RE/AP1-dependent transcription in Jurkat Tat$^-$ cells. Taken together, those results indicate that extracellular Tat was not responsible for the observed inhibition of CD28RE/AP1 in Jurkat Tat$^+$ cells.

### *Effect of Tat on the kinetics of binding of transcription factors to the CD28RE/AP1 composite element*

To study the underlying mechanism of Tat-mediated inhibition on CD28RE/AP1 transactivation, we analyzed the effects of the viral protein on the binding of activated transcription factors to this composite element. For that purpose, we performed EMSAs with nuclear extracts of Jurkat Tat$^-$ and Jurkat Tat$^+$ cells using the corresponding oligonucleotide-labeled probe. As shown in Fig. 5,

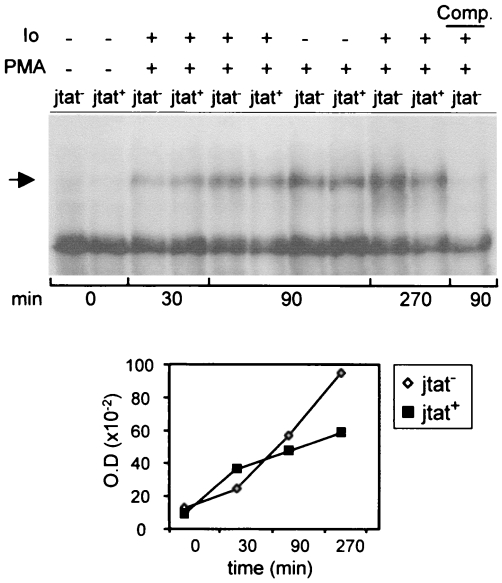


**FIGURE 5.** Kinetics of the binding of transcription factors to the CD28RE/AP1 element of the human IL-2 gene promoter in activated Jurkat Tat$^-$ and Jurkat Tat$^+$ cells. Nuclear extracts of cells stimulated for 0, 30, 90, or 270 min with PMA alone or in combination with Io were analyzed by EMSA using the radiolabeled probe corresponding to the CD28RE/AP1 site of the human IL-2 promoter. An 80-fold molar excess of unlabeled homologous oligonucleotide was added to the binding reaction mixture where indicated to test the specific binding. At the bottom, the results of the densitometric analysis of the specific complex bound after PMA+Io stimulation (indicated by the arrow) in both types of cells are shown.

activation of Jurkat cells led to the appearance of a specific complex bound to the CD28RE/AP1 oligonucleotide that increased with time after stimulation. In Jurkat Tat$^+$ cells, the amount of this complex was about the same as that in Jurkat Tat$^-$ up to 90 min and only at longer times after activation it was significantly decreased.

### *Effect of Tat on the NFκB composition at the CD28RE/AP1 element*

Both the apparent discrepancies between the strong inhibition observed in Jurkat Tat$^+$ cells in CD28RE/AP1 transactivation and the partial effect observed in EMSAs were suggestive of the existence of additional inhibitory mechanisms. As the transactivating function of both NF-κB and AP1 transcription factors is regulated by the composition of the dimers (34–36), a possible Tat-induced qualitative alteration in the transactivating complex could not be excluded.

To test this hypothesis, we investigated which one of the components in the binding complex could be altered, because the CD28RE/AP1 site of the IL-2 promoter is known to bind transcription factors from the NF-κB and AP1 families (26). Within this composite element, the CD28RE has been described as a nonconsensus κB site (51) recognized by diverse members of the NF-κB transcription factor family (49). Therefore, we evaluated whether the κB composition was being altered by Tat using Abs against the p65 and c-*rel* proteins of the κB family in the EMSAs. Both in nuclear extracts of PMA+Io-stimulated Jurkat Tat$^-$ and Jurkat Tat$^+$ cells, the presence of c-*rel* was predominant when compared with p65, but no significant differences were detected in the composition of the complex either at 90 (Fig. 6) or 270 min of stimulation (data not shown).

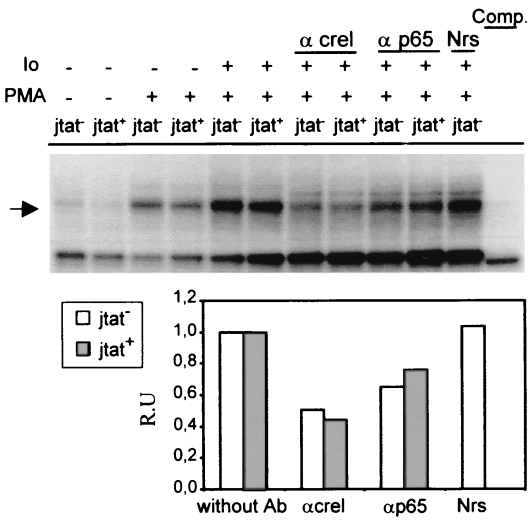


**FIGURE 6.** Endogenous expression of Tat in Jurkat cells does not alter c-*rel* and p65 binding to the CD28RE/AP1 element. EMSA analysis was performed with nuclear extracts from Jurkat Tat$^-$ and Jurkat Tat$^+$ cells either unstimulated or activated with PMA alone or in combination with Io for 90 min. One microliter of anti-c-*rel* (1136), anti-p65 (1226), or normal rabbit serum (Nrs) was added to the reaction mixture before the addition of the radiolabeled CD28RE/AP1 oligonucleotide where indicated. In the last lane, competition with an 80-fold molar excess of unlabeled homologous oligonucleotide was conducted. The amount of bound complexes was analyzed by densitometric analysis of the specific band (indicated by the arrow). The values obtained were normalized to those corresponding to the binding reaction performed without antiserum and are represented as relative units at the bottom of the diagram.

### *Effect of Tat on the binding and transcriptional transactivation of CD28RE/AP1 by NFAT*

It has already been shown that Tat interacts with NFAT altering its transactivating capacity (20) and at present there is a controversy about the implication of NFAT in the regulation of the CD28RE/AP1 response element (28, 31). Therefore, it could be possible that the effects of Tat on this responsive element were caused by Tat-NFAT interaction, somehow altering the transactivating κB-AP1 complex. To test this hypothesis, first we analyzed whether NFAT was present in the complex bound to the CD8RE/AP1 probe by using specific Abs against NFAT1 in the EMSAs. As shown in Fig. 7*A*, a small amount of NFAT was able to bind to this probe. However, almost identical supershifting results were observed in Jurkat Tat$^-$ and Jurkat Tat$^+$, indicating a similar participation of NFAT1 in the complex in both types of cells. The participation of NFAT was also confirmed by competition of the binding using unlabeled probes containing NFAT binding sites. The result of this competition evidenced the presence of AP1 in the complex and, to a minor extent, the participation of NFAT (competition with the NFAT binding site of the mouse IL-4 promoter) both in Jurkat Tat$^-$ and Jurkat Tat$^+$ cells. However, we could not exclude the possibility that the presence of Tat in the complex could be inducing a competition between the NFAT and the c-*rel* proteins bound to the κB-like site, altering their cooperation with AP1 and, therefore, the transactivating activity. To evaluate this possibility, we transfected COS cells, which do not endogenously express NFAT but express high basal levels of AP1 (52) with the pCD28RE/AP1luc plasmid together with different combinations of the expression plasmids of c-*rel*, Tat and/or NFAT1. NFAT1 did not inhibit the transactivation of the CD28RE/AP1 element induced by c-*rel* either in the presence or in the absence of Tat. Actually, even an additive effect in transactivation was detected when NFAT and c-*rel* proteins were cotransfected (Fig. 7*B*). These results excluded the interaction between NFAT and Tat as the main cause of the inhibition on the CD28RE/AP1 element by the viral protein.

### *Effect of Tat on the binding of AP1 transcription factors to CD28RE/AP1*

It is well known that AP1-dependent transcriptional regulation is determined by the composition of the AP1 dimers (36). To test whether this component of the complex was qualitatively affected in Jurkat Tat$^+$ cells, the participation of some of the AP1 factors was evaluated by analyzing the inhibition of the specific binding after incubation with Abs in the EMSAs. As shown in Fig. 8*A*, the presence of c-jun, junB, and fos proteins in the complex bound to the CD28RE/AP1 element was very similar when comparing nuclear extracts from 90-min stimulated wild type and Tat-expressing Jurkat cells. However, when we evaluated nuclear extracts from cells stimulated for 270 min, qualitative differences, in addition to the previously shown reduction in the amount of the complex bound, could be observed (Fig. 8*B*). Thus, although in both Jurkat Tat$^-$ and Jurkat Tat$^+$ the AP1 dimers contained a similar proportion of fos proteins both at 90 and 270 min, the participation of c-jun at different times of stimulation was different in Jurkat Tat$^-$ and Jurkat Tat$^+$. c-jun was very poorly represented in both types of cells at 90 min of stimulation, but at 270 min its participation in the complexes bound to the CD28RE/AP1 site significantly increased in Jurkat Tat$^+$ cells. This effect of Tat was selective on c-jun, as the participation of junB in the complex was very similar in Jurkat Tat$^-$ and Jurkat Tat$^+$ at both 90 and 270 min of stimulation.

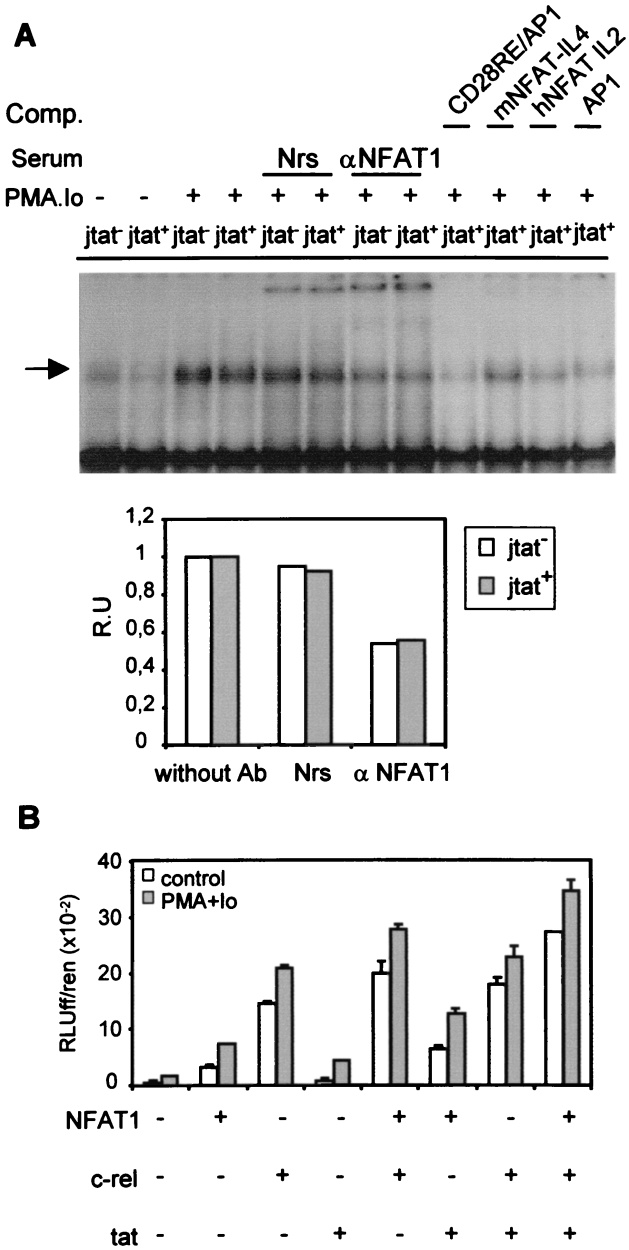


**FIGURE 7.** NFAT is not responsible for the inhibitory effects of Tat on the CD28RE/AP1 element. *A*, The participation of NFAT in the complexes bound to the CD28RE/AP1 element from nuclear extracts of Jurkat Tat$^-$ and Jurkat Tat$^+$ cells was evaluated in EMSAs using the corresponding labeled probe. Competition with the indicated unlabeled oligonucleotides was performed in an 80-fold molar excess. Nuclear extracts of unstimulated or 90-min PMA+Io-activated cells were assayed, and 1 $\mu$l of anti-NFAT1 672 serum or normal rabbit serum (Nrs) was added to the binding reactions where indicated to evaluate the inhibition of the induced band (indicated by the arrow). Data in the diagram below represent the densitometric values normalized to the corresponding value in the sample without Ab. *B*, COS cells were cotransfected with the pCD28RE/AP1luc and the pRL-tk-luc reporter plasmids together with the expression plasmids pEF-BOS-NFAT1 and/or pCMVTat and/or pRc-hc-*rel*. The plasmid pcDNA3 was used to equal the amount of DNA used in every transfection. Transfected cells were cultured in the absence or presence of PMA+Io for 6 h, and luciferase activity was evaluated as indicated in *Materials and Methods*.

### *Effect of Tat on the transcriptional activity of c-jun and c-*rel

Altogether, the above mentioned results indicated that Tat expression in Jurkat cells was increasing the participation of c-jun in the CD28RE/AP1 binding complex as activation progresses. This effect was associated with a quantitative decrease in protein binding and an inhibition of the transactivating function of the complex. To test the existence of a direct effect of Tat on the transactivating activity of c-*rel* and c-jun, Jurkat Tat$^-$ cells were cotransfected with plasmids expressing GAL4 chimeric proteins (containing the transactivation domain under test and the DNA binding domain of GAL4) together with pGAL4luc and different doses of a Tat expression plasmid (Fig. 9). c-*rel* transactivation activity was not decreased by coexpression of Tat. In fact, we could observe some increase when very low doses of pCMVTat were used (Fig. 9*A*). In contrast, the activity of c-jun was inhibited in a dose-dependent manner by pCMVTat transfection (Fig. 9*B*). These data were suggestive of an inhibitory effect of Tat on the transactivation of the CD28RE/AP1 element mediated through a direct inhibition of the c-jun component.

In contrast, overexpression of neither c-*rel* nor c-jun allowed Jurkat Tat$^+$ cells to reach the values of transactivation obtained in Jurkat Tat$^-$ after stimulation, despite substantial increases in CD28RE/AP1 transactivation (20- to 50-fold). Only when both proteins were coexpressed, similar levels of luciferase activity were obtained in the presence and absence of Tat (Fig. 10*A*). These data were indicative of a direct effect of Tat on the interactions maintained between NF-$\kappa$B and AP1 at the CD28RE/AP1 element and, thus, the inhibition of transcription was only abolished when the presence of Tat in the Jurkat Tat$^+$ cell was insignificant compared with the functional overexpressed NF-$\kappa$B/AP1 complexes. To corroborate this hypothesis, Jurkat Tat$^-$ or COS cells were cotransfected with c-jun + c-*rel* and pCMVTat. As shown in Fig. 10*B*, expression of c-jun + c-*rel* increased CD28RE/AP1-dependent transactivation ~1000-fold in Jurkat Tat$^-$ cells. However, the cotransfection of low amounts of pCMVTat (50 ng) significantly down-regulated both the basal and the PMA+Io-induced transactivation of the CD28RE/AP1 reporter. Similar results were found in COS cells. Inhibition of CD28RE/AP1-dependent transactivation was dependent of the amount of pCMVTat cotransfected, being c-*rel* + c-jun-mediated transactivation completely abrogated by high doses of pCMVTat (Fig. 10*C*). Furthermore, these experiments excluded the alteration of the endogenous expression of c-jun and c-*rel* by Tat as the cause of the inhibition of CD28RE/AP1 transactivation, as it should have been bypassed by overexpression of these transcription factors.

## Discussion

Here we have shown that the constitutive expression of HIV-Tat in T cells is able to drastically reduce IL-2 but not TNF gene expression induced upon activation. Despite the large number of existing reports about the IL-2 transcriptional regulation, the exact contribution of the different response elements existing at the promoter is not clear. Mutagenesis and footprinting analysis provide good evidence that interdependent transcription complexes govern IL-2 transcription and, thus, stable occupancy of the promoter may require simultaneous binding to all sites (23, 24, 53).

Our results suggest that HIV1-Tat affects IL-2 transcription mainly by inhibiting the transactivation of the CD28RE/AP1 site. The preponderant role proposed for the CD28RE/AP1 complex in the regulation of IL-2 transcription would be in consonance with the data described in normal T cells about IL-2 promoter activity (30) and with the impaired IL-2 expression observed in c-*rel* knockout mice (54). However, we cannot exclude that within the entire promoter the inhibition of this composite element could induce alterations on other transactivating complexes bound at different sites of the promoter.

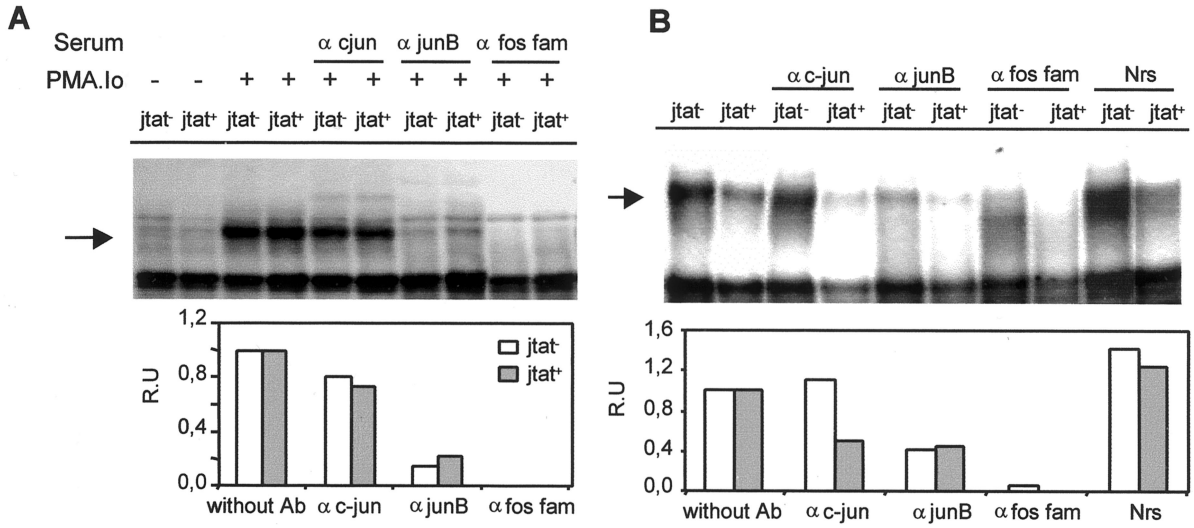


**FIGURE 8.** Qualitative analysis of the AP1 component bound to the CD28RE/AP1 composite element. EMSAs were performed and analyzed as described in Fig. 4. Nuclear extracts of unstimulated or 90-min PMA+Io-treated cells in *A* or nuclear extracts of 270-min stimulated cells in *B* were assayed. The indicated antiserum (1 $\mu$l) was added to the binding reaction to analyze its inhibitory effect on the induced binding complex (indicated by the arrow). The normalization of the densitometric values was performed as in Figs. 4 and 5*A*, and it has been represented in the diagrams of bars below the corresponding EMSA.

The drastic inhibition observed on the transactivation of the CD28RE/AP1 element in Jurkat Tat$^+$ cells is likely the result of a combination of a quantitative reduction of the binding of transcription factors to this site and also of subtle qualitative changes that may alter the transcriptional activity of the factors bound. Because Tat decreases in a dose-response manner c-jun + c-*rel*-induced CD28RE/AP1 transactivation, it is also plausible that Tat also down-regulates the transcriptional activity of the NF-$\kappa$B/AP-1 complexes bound to the CD28RE/AP-1 site of the IL-2 promoter without affecting the individual activity of NF-$\kappa$B and AP-1. Interestingly, our results indicate that c-jun minimally participates in AP1 dimers bound to the CD28RE/AP1 site in activated T cells. As activation progresses, an alteration of the relative participation of c-jun and junB in the binding complex is observed when comparing Jurkat Tat$^+$ cells to Jurkat Tat$^-$. In this regard, it has been demonstrated that heterodimerization of AP1 proteins not only determines their ability to bind specific DNA sequences and to interact with the basal transcriptional machinery but also influences their recognition by protein kinases and transcriptional coactivators (reviewed in Ref. 36). Thus, phosphorylation of jun proteins by c-Jun N-terminal kinase (JNK) is dependent on the presence of a JNK-docking site in the jun proteins, but efficient phosphorylation also requires certain specificity-conferring residues, such as a proline at the P + 1 position flanking the phosphate acceptor site (55). c-jun fulfils both requirements but junB, although having an efficient JNK-docking site is not phosphorylated by JNK as it lacks the necessary proline. An additional difference between c-jun and junB is that only the former interacts with the transcriptional coactivator JAB1 (jun activation domain binding protein 1), which enhances the ability of c-jun to activate transcription through stabilization of the AP1-DNA complex (56). Bearing in mind all these data, the existence of subtle alterations of the AP1 composition in the transactivating complex may lead to have amplified functional consequences. Furthermore, we show Tat does not only favor the participation of c-jun in the binding complex but besides, and, probably more importantly, it interacts with c-jun inhibiting its transcriptional activity. This is, to our knowledge, the first description of a Tat-mediated inhibition of c-jun transactivating activity and may have important consequences due the importance of this transcription factor in T cell activation.

The importance of the AP1 component on the complete function of the NF-$\kappa$B/AP1 complex has already been demonstrated (57). Significantly, the CD28-induced costimulation is based not only on the activation of the NF-$\kappa$B component bound to the CD28RE but also on the complete activation of AP1, both at the level of the composition of the dimer (58) and at the posttransductional modifications that alter the transactivating function of c-jun (59). Therefore, it is possible that the recruitment of c-jun to the CD28RE/AP1 element by Tat may be the main cause of the alteration observed on the binding characteristics of the NF-$\kappa$B/AP1 complex and subsequently in its decreased transactivating activity in Jurkat Tat$^+$ cells.

The existence of a direct interaction between a transactivator viral protein and AP1 has already been proposed for the Tat protein of the Visna virus (60) and for the human T cell leukemia virus transactivator tax (61, 62). Our results show that the effects of HIV-Tat on AP1 seem specific of composite elements as transactivation controlled by consensus AP1 sites was not affected by endogenous expression of Tat in the cell. Although not addressed in this study, this lack of effect could be due to the lack of alteration of the AP1 dimers bound to pure AP1 sites. Besides, this could indicate the existence of a direct contact of Tat with other components of the complex, somehow allowing a more efficient Tat/c-jun interaction. In this regard, cotransfection experiments of pGal4-c-*rel* and low doses of pCMVTat in Jurkat Tat$^-$ cells evidence an alteration of the transactivating function of c-*rel* by Tat and suggest the existence of an interaction between these two proteins. The dependence of this effect on the presence of a restricted Tat concentration (low levels) is intriguing. A possible explanation could result from the fact that from certain levels of Tat expression, dimerization of the viral protein could be taking place, thus avoiding its interaction with c-*rel*. The idea of an alteration on the dimer composition of AP1 in a manner dependent on Tat-rel contacts is reinforced by reports that demonstrate that c-*rel* may alter AP1-mediated transactivation (63). Altogether, the effect of Tat on the transactivating complex may be more complex than the sum of

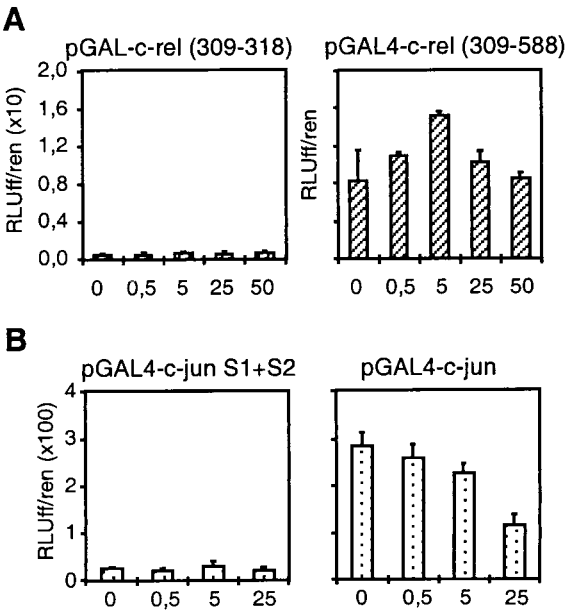


**FIGURE 9.** Effect of Tat on the transactivating function of c-*rel* and c-jun. Jurkat Tat$^-$ cells were transiently transfected with the reporter plasmids pGAL4-luc and pRL-tk-luc together with 2.5 ng/2 $\times$ 10$^6$cells of the plasmids pGAL4-c-*rel* (309–318) or pGAL4-c-*rel* (309–588) in *A* or 50 ng/2 $\times$ 10$^6$cells of the plasmids pRSV-GAL4-c-Jun (wild type) or pRSV-GAL4-c-Jun $S_1$+$S_2$ in *B*. In both *A* and *B*, these plasmids were cotransfected with the indicated amounts of the plasmid pCMVTat (0, 0.5, 5, 25, or 50 ng/10$^6$cells). Luciferase activity was evaluated and normalized to the transfection efficiency as mentioned in *Materials and Methods*.

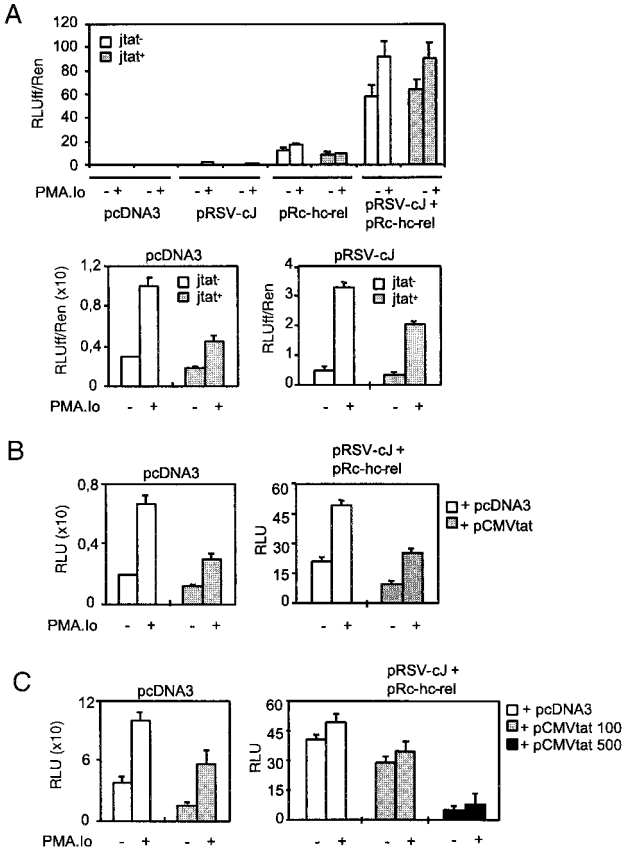


**FIGURE 10.** Effect of Tat on c-jun plus c-*rel*-dependent CD28RE/AP1 transactivation. *A*, Overexpression of both c-jun and c-*rel* is required to avoid the inhibitory effect of Tat on CD28RE/AP1 transactivation in Jurkat Tat$^+$ cells. Jurkat Tat$^-$ and Jurkat Tat$^+$ cells were transiently transfected with pCD28RE/AP1luc and pRL-tk-luc together with 250 ng/2 $\times$ 10$^6$ cells of the expression plasmids pRSV-cJ and/or pRc-hc-*rel*. Transfected cells were cultured for 6 h in the absence or presence of PMA+Io as indicated. At the bottom, the diagrams represent the luciferase values obtained in the cotransfections of the pcDNA3 plasmid or the pRSV-cJ plasmid in an amplified scale. *B*, Inhibition of c-jun plus c-*rel*. Mediated CD28RE/AP1 transactivation by Tat. Jurkat Tat$^-$ cells were transiently transfected with pCD28RE/AP-1 luc together with pRSV-cJ and pRC-hc-*rel* as above, with or without pCMVTat (50 ng/10$^6$ cells). *C*, COS cells were transiently transfected with pCD28RE/AP-1 luc together with pRSV-cJ and pRC-hc-*rel* (500 ng/2 $\times$ 10$^6$cells) with the indicated amounts of pCMVTat. Cells were stimulated or not with PMA+Io for 6 h. The amount of DNA transfected was adjusted in all the transfections using the plasmid pcDNA3 when necessary.

the effects of the viral protein on the individual components. Besides, taking into account that cooperative interactions are required on the CD28RE/AP1 element, it is possible that both the interactions of Tat with both c-*rel* and with c-jun are the cause of the selective recruitment of c-jun to this composite element. This idea is further supported by the fact that the effect of Tat on CD28RE/AP-1 transcription depends on the relative amounts of the three components as demonstrated by the transient transfection experiments in Jurkat Tat$^-$ or COS cells. In this regard, Lim et al. have recently found that Tat interaction with SP1 at the human monocyte chemoattractant protein 1 (hMCP-1) gene promoter may serve as a platform to recruit and stabilize the interaction of AP1 and NF-$\kappa$B proteins to this promoter (64). Thus, studying the effects of Tat on synergistic interactions among transcription factors is becoming increasingly relevant to fully understand its physiological role.

Although our results point toward a NF-$\kappa$B/Tat/AP1 tripartite interaction, the indirect disruption of interactions between these transcription factors and coactivators as a consequence of the presence of Tat in the cell cannot be excluded. Thus, the interaction of c-jun with the transcriptional coactivator CBP has been involved in the regulation of the transactivating function of c-jun (65), and Tat interacts in vivo and in vitro with CBP/p300 (2, 66). The possibility that c-jun-CBP interaction could be altered by the one maintained between Tat and CBP would also contribute to explain the inhibition of c-jun-transactivating activity by the viral protein.

In contrast, despite the highly controversial implication of NFAT in the regulation of the CD28RE/AP1 element (28, 31), our results demonstrate that NFAT is present in the induced binding complex, but in a similar amount both in Jurkat Tat$^-$ and Jurkat Tat$^+$ cells. Moreover, reporter gene experiments in COS-transfected cells evidence a cooperation between NFAT1 and c-*rel* in the transactivation of the CD28RE/AP1 element both in the absence or presence of Tat. These data are consistent with those suggesting that activation of the IL-2 gene expression through the CD28 costimulatory signal involves both NF-$\kappa$B/rel and NFAT functionally interacting with CD28RE (67) but exclude that the effects of HIV-Tat on the CD28RE/AP1 response element are mediated through the NFAT component of the complex, as it has been proposed for the HTLV-I-tax protein (32).

We have not detected a significant effect of constitutive Tat expression on the activity of $\kappa$B-dependent reporter genes. This is in contrast with several reports that have described effects of Tat on NF-$\kappa$B (5, 21) but in agreement with those by Ott et al. They suggest that Tat is able to alter the binding of NF-$\kappa$B to sequences recognized by this transcription factor with low affinity and with a strong participation of c-*rel*, not detecting any effect on "classical" $\kappa$B sites recognized mainly by p50-p65 dimers (18). The existing controversy about the enhancing or inhibiting effects of Tat on

IL-2 regulation (13–16) is also remarkable. All of these discrepancies are probably due to the use of different experimental conditions, as in many of those studies enforced expression of Tat (by transfecting large amounts of Tat expression plasmids) or extracellular recombinant Tat were used, whereas very low Tat intracellular concentrations (closer to physiological levels) exist in our Jurkat Tat cells. Despite the fact that Tat could be secreted and affects neighboring cells, extracellular Tat is not involved in the inhibition of the CD28RE/AP1-dependent transcription. Altogether, these results indicate that the effects of Tat may vary depending on its cellular location, a point not always taken into account in the literature.

In summary, we have shown that intracellular expression of HIV-Tat may affect transcription from the CD28RE/AP1 composite element of the IL-2 human promoter at various levels: reducing the binding of the transcription factors to the complex, altering their composition, and decreasing c-jun transcriptional activity. This causes a drastic inhibition of transcriptional activation of IL-2 gene. These data underline the importance of studying the multiple interactions maintained at composite response elements, as subtle distortions of one component of the complex may affect the binding and transactivating functions of complete transactivation complex.

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