

# Upregulation of c-Fos in Activated T Lymphoid and Monocytic Cells by Human Immunodeficiency Virus-1 Tat Protein

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The regulatory Tat protein of the human immunodeficiency virus type-1 (HIV-1) is essential for viral replication and also shows pleiotropic activities on various cell functions. To get further insights into the molecular mechanisms underlying the biological activity of Tat, we investigated the effect of endogenous and exogenous Tat protein on *c-fos* gene expression in T lymphoblastoid (Jurkat) and monocytic (U937) cell lines, as well as in primary peripheral blood mononuclear cells (PBMC). Transient cotransfection of *tat* cDNA in sense orientation (*tat/S*), together with a plasmid containing the *c-fos* promoter (FC3, from -711 to +42) in front of the bacterial chloramphenicol acetyltransferase (CAT) gene significantly enhanced CAT activity in Jurkat cells activated by the addition of 15% fetal calf serum (FCS) or 5  $\mu\text{g}/\text{mL}$  phytohemagglutinin plus  $10^{-7}$  mol/L phorbol myristate acetate (PMA) and U937 cells activated by 15% FCS or  $10^{-7}$  mol/L PMA. This effect was specifically due to Tat, since Jurkat and U937 cells cotransfected either with *tat* cDNA in antisense orientation (*tat/AS*), *tat* carrying a mutation in the amino acid  $\text{cys}^{22} - \text{gly}^{22}$  (*tat*<sup>22/S</sup>) or with the backbone vector alone (pRPneo-SL3) did not show any significant difference in *c-fos* promoter activity as compared to cells transfected with

FC3 plasmid alone. By using deletion mutants of the *c-fos* promoter, we found that the minimal DNA sequence required for Tat activity was located between nucleotides -404/-220 and that the serum responsive element (SRE, -317/-288), present within this region, was still responsive to Tat. A single point mutation in the SRE completely abrogated the responsiveness to *tat/S*. Exogenous recombinant Tat protein was also able to upregulate *c-fos* promoter activity in serum-activated Jurkat and U937 cells, as well as endogenous *c-fos* mRNA expression and c-Fos protein synthesis in both serum-activated cell lines and primary PBMC. c-Fos protein was shown essential for an optimal transactivation of the HIV-1 long terminal repeat (LTR) by Tat: incubation of Jurkat cells with antisense, but not sense, *c-fos* oligonucleotides significantly reduced either the Tat-enhanced expression of an LTR-CAT reporter construct or the levels of gag p24 in the culture supernatants of Jurkat cells and PBMC acutely infected with HIV-1. Our data suggest that the *c-fos* upregulation mediated by Tat might play a significant role in the control of viral gene transactivation.

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**T**HE HUMAN immunodeficiency virus-type 1 (HIV-1) 86-101 amino acid Tat protein is encoded by two exons and comprises five distinct domains: namely the N-terminal, cysteine-rich, core, basic, and C-terminal sequences.<sup>1</sup> The cysteine-rich region is responsible for the formation of intramolecular disulphide bonds,<sup>2</sup> whereas the basic region contains nuclear localization signals<sup>3</sup> and the binding site for the transactivation response element (TAR).<sup>4</sup> The transcriptional activation domain of Tat (amino acids 1 through 48) includes the amino-terminal region, the conserved cysteine-rich and core domains.<sup>5</sup> Tat plays a pivotal role for HIV-1 gene expression and replication,<sup>1</sup> by interacting with the TAR sequence, located at the 5' end of all viral mRNAs. However, increasing experimental evidence suggests that Tat can also control viral replication via TAR-independent mechanisms.<sup>6,7</sup>

Besides its transactivating function on viral gene expression, Tat affects the survival, growth, and function of a variety of infected and uninfected cells.<sup>8-17</sup> These biological effects are at least partially mediated by the upregulation or downregulation of genes involved in the control of cell survival/proliferation<sup>10,18-22</sup> or coding for cytokines.<sup>23-29</sup>

Tat can be actively secreted by HIV-1 infected and *tat*-

transfected cells,<sup>30</sup> and can easily enter different cell types both in vitro<sup>31-32</sup> and in vivo,<sup>33</sup> contributing to the transcellular transactivation of HIV-1 long terminal repeat (LTR) promoter in latently infected cells.<sup>34-36</sup> Moreover, the addition of an anti-Tat neutralizing antibody in culture blocks an autocrine/paracrine loop of extracellular Tat in *tat*-transfected cell lines,<sup>37</sup> and reduces the levels of gag p24 in the culture supernatant of HIV-1 infected lymphoid T-cell lines and primary peripheral blood mononuclear cells (PBMCs).<sup>38-39</sup> These findings add further complexity to the mechanisms of action of Tat protein, strongly suggesting that extracellular Tat may also significantly contribute to the pathogenesis of HIV-1 disease.

It is noteworthy that c-Fos-responsive sequence motifs have been identified in HIV-1 LTR,<sup>1</sup> as well as in many promoters of genes encoding for cytokines transactivated by Tat.<sup>26,28</sup> In this study, we investigated whether Tat is able to modulate c-Fos protein expression as a part of its mechanism of action. To do this, we selected as model systems the Jurkat human CD4<sup>+</sup> T and the U937 monocytic cell lines, which are representative of the main targets of HIV-1 infection in vivo.<sup>40</sup> Induction of the *c-fos* gene promoter, *c-fos* mRNA expression and c-Fos protein synthesis were evaluated in both cell lines, either (1) transiently transfected with *tat* cDNA, or (2) treated with recombinant Tat protein, whether alone or in combination with various stimuli known to activate *c-fos* expression. The effect of extracellular Tat on endogenous *c-fos* gene expression was also evaluated on primary PBMCs. Finally, to evaluate the physiological relevance of these results to HIV-1 infection, we performed experiments in which antisense and sense *c-fos* oligonucleotides were added to Jurkat cells either transfected with *tat* cDNA plus constructs containing the LTR region of HIV-1 genome fused with a chloramphenicol acetyltransferase (CAT), or acutely infected with the IIIB strain of HIV-1.

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Submitted May 9, 1996; accepted October 10, 1996.

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0006-4971/97/8905-0116\$3.00/0

## MATERIALS AND METHODS

**Cell lines and PBMCs.** Jurkat CD4<sup>+</sup> lymphoblastoid T and U937 monocytic cell lines were obtained from the American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 (GIBCO Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum (FCS; GIBCO) at an optimal cell density of 0.3 to 1.5 × 10<sup>6</sup> cells/mL.

PBMCs were taken from 11 normal blood donors, who gave their informed consent to this research according to the Helsinki declaration of 1975. Briefly, heparinized (20 U/mL) blood samples were diluted 1:3 with RPMI 1640, layered over Ficoll Histopaque (d = 1.077 g/mL, Pharmacia, Uppsala, Sweden) and centrifuged at 1,500 rpm for 30 minutes. Light-density mononuclear cells were collected, washed twice, counted, and treated with various agonists.

**Plasmids, oligonucleotides, and viral proteins.** Plasmids pRPneo-C/SL3/tat/S, pRPneo-C/SL3/tat<sup>22</sup>/S, pRPneo-C/SL3/tat/AS containing the HIV-1 *tat* cDNA, respectively in sense (*tat*/S), carrying a mutation in the aminoacid cys<sup>22</sup> - gly<sup>22</sup> (*tat*<sup>22</sup>/S) or in antisense (*tat*/AS) orientation under the control of the LTR region of the SL3 murine leukemia virus were derived from the backbone plasmid, as previously described.<sup>29</sup>

Antisense and sense *c-fos* oligonucleotides were represented by 18 residues starting from and including the start codon for *c-fos*: gene: ATG ATG TTC TCG GGT TTC AA (sense *c-fos*, FOS-OLIGO); TTT AAG CCC GAG AAC ATC AT (antisense *c-fos*, SOF-OLIGO).

The *c-fos*/CAT plasmid constructs (FC2, FC3, FC4, FC8) containing the bacterial CAT gene under the control of various *c-fos* promoter sequences from position -1450 (FC2), -711 (FC3), -404 (FC4), -220 (FC8) to position +42 were described by Deschamps et al.<sup>41</sup> The wild-type serum responsive element (SRE)/CAT plasmid contained *c-fos* promoter sequences from -317 to -288 base pairs (bp). The mutated SRE/CAT plasmid contained two point mutations (CC-GG) in positions -309/-308. All the *c-fos*/CAT plasmids were a generous gift of Dr Sassone-Corsi (IGBMC, Illkirch, France). The  $\beta$ -actin/CAT and the HIV-1 LTR were described previously.<sup>29</sup>

Recombinant Tat and p24 proteins, produced in *Escherichia coli* (ABT, Cambridge, MA), were found free of endotoxin by a Limulus Amebocyte Lysate test (Whittakers, Walkersville, MA).

**Transfections.** Transient cotransfection experiments were carried out using the DEAE-dextran method as previously described by Zauli et al.<sup>37</sup> Briefly, 10 × 10<sup>6</sup> of exponentially growing Jurkat or U937 cells were maintained in RPMI 1640 plus 1% FCS for 24 hours and then transfected in 3 mL volume with 15  $\mu$ g of each plasmid DNA in 500  $\mu$ g DEAE-dextran for 90 minutes. After transfection, cells were seeded again in 10 mL of RPMI 1640 plus 1% FCS for 12 hours. Jurkat cells were supplemented with 15% FCS or 5  $\mu$ g/mL phytohemagglutinin (PHA; Difco, Detroit, MI) plus 10<sup>-7</sup> mol/L phorbol myristate acetate (PMA, Sigma Chemicals, St Louis, MO), while U937 cells were supplemented with 15% FCS or 10<sup>-7</sup> mol/L PMA for an additional 24 hours. Cells were then lysed and the clarified lysates were assayed for CAT activity using volumes of extract corresponding to equal amounts of proteins as determined by the Bio-Rad protein assay system (Bio-Rad Laboratories, Richmond, CA).

In other experiments, 10 × 10<sup>6</sup> Jurkat cells were transiently transfected with 15  $\mu$ g of *tat*/S plus 5  $\mu$ g of LTR-CAT plasmids plus either sense *c-fos* (FOS-OLIGO, 25  $\mu$ mol/L final concentration) or antisense *c-fos* (SOF-OLIGO, 25  $\mu$ mol/L final concentration) oligonucleotides, then treated as described above.

To assay the effect of extracellular Tat on the *c-fos* promoter activity, 5 × 10<sup>6</sup> Jurkat or U937 cells were transiently transfected with the FC3 vector as described above. Twelve hours after transfection, 1  $\mu$ g of recombinant Tat or recombinant p24 plus 15% FCS

was added to 5 mL of medium, and incubation was allowed to proceed for an additional 24 hours. In blocking experiments, before its addition to the culture medium, recombinant Tat was preincubated for 2 hours at 37°C with 20  $\mu$ g/mL of an anti-Tat monoclonal antibody (MoAb, ABT), which recognizes the N-terminus of Tat or with 20  $\mu$ g/mL of an anti-p24 MoAb (ABT).

**RNA analysis.** To study the effect of extracellular Tat on endogenous *c-fos* mRNA expression, 30 × 10<sup>6</sup> Jurkat were cultured in RPMI plus 1% FCS for 24 hours and then supplemented with 15% FCS or PMA plus or minus 5  $\mu$ g of recombinant Tat or p24 in 5 mL of medium for 30 minutes. Total RNA was extracted, using the RNazol kit (Biotech, Galveston, TX) according to the manufacturer's instructions. 5  $\mu$ g of poly(A<sup>+</sup>) RNA were then purified from each sample by using the poly(A<sup>+</sup>) RNA purification kit (Qiagen, Hilden, Germany) and processed for Northern blot as previously described.<sup>29</sup> *c-fos* and control probes were represented by the *Xho* I/*Nco* I 2,700 bp *c-fos* fragment<sup>42</sup> and by the *Xba* I/*Pst* I 780 bp fragment of glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH),<sup>29</sup> respectively. Both probes were digoxigenin-labeled using a random primer labeling kit (Boehringer Mannheim, Postfach, Germany), according to the manufacturer's instructions.

For RNase protection analysis, 30  $\mu$ g of total RNA were processed using a RNase protection kit (Boehringer Mannheim) according to the manufacturer's instructions. A 340 nucleotide complementary digoxigenin-labeled RNA from FC3 was used as a probe for *c-fos* CAT mRNA.<sup>43</sup>

**Analysis of c-Fos protein by flow cytometry and confocal microscopy.** 5 × 10<sup>6</sup> Jurkat cells or PBMC were cultured in RPMI plus 1% FCS for 24 hours and then supplemented with 15% FCS alone or in combination with Tat or p24 for 45 minutes. Cells were then fixed and permeabilized with cold 70% ethanol for 30 minutes at 4°C, washed twice with phosphate-buffered saline (PBS), and treated for 90 minutes at 37°C with a 1:100 dilution of anti-c-Fos rabbit polyclonal serum (Santa Cruz Biotech, Santa Cruz, CA) in PBS plus 1% bovine serum albumin (BSA). 2.5% of nonspecific blocking guinea pig serum was present during each antibody incubation. Cells were washed twice and incubated for 30 minutes at 37°C with a 1:100 dilution of goat anti-rabbit serum conjugated to fluorescein (GAR-FITC, Sigma) in PBS plus 1% BSA. Negative controls consisted of normal rabbit serum followed by identical second layer labeling as above. The presence and subcellular distribution of c-Fos protein was investigated by FACScan using the Lysis II software (Becton Dickinson, San José, CA). For confocal microscopy, the cells were first spun on coverslips, then fixed and stained with the anti-Fos antibody as described above. The cells were analyzed by means of a MCR-1000 confocal microscopy (Bio-Rad Microscience Ltd, Hemel, Hempstead, UK) equipped with a krypton/argon ion laser emitting at 488 nm. The signal was achieved through an Epide-tector filter (passing band 522/35 nm), analyzed by CoMOS software (Bio-Rad) and printed on a Ektachrome 64T-Kodak film (Eastman Kodak, Rochester, NY) by a Focus Imagecorder Plus (Focus Graphics, Inc, Foster City, CA).

**Acute HIV-1 infection of Jurkat cells and primary PBMC.** Virus stock was represented by the supernatant of H9 lymphoblastoid T cells cultured at optimal cell density (0.5 to 1.5 × 10<sup>6</sup> cells/mL) and obtained 14 days after infection with HIV-1 (strain IIIB). It contained a reverse transcriptase activity of 1.5 × 10<sup>6</sup> cpm/mL with an infectivity of 3 × 10<sup>6</sup> TCID<sub>50</sub> (tissue culture infectious dose 50%) equivalents for lymphocytes, determined as previously described.<sup>44</sup> One milliliter of HIV-1 was added to either Jurkat cells or primary PBMC for 2 hours at 37°C. Control (mock-treated) cultures were run in parallel by challenging Jurkat cells or PBMC with 1 mL of the supernatant of uninfected H9 lymphoblastoid T cells cultured under optimal conditions. After virus absorption, Jurkat and PBMC were washed and adjusted to the concentration of 1 × 10<sup>6</sup>/mL and seeded

in 1 mL of RPMI containing 15% FCS. SOF-OLIGO and FOS-OLIGO were added at a final concentration of 25  $\mu\text{mol/L}$  at the beginning of culture. Every other day, cultures were demi-populated and supplemented with 0.5 mL of fresh medium containing 25  $\mu\text{mol/L}$  of oligonucleotides. The harvested medium was centrifuged and the supernatants were monitored for the presence of gag p24 antigen by means of a commercial immunoplate assay (Du Pont de Nemours, Les Ulis, France), while cell pellet was resuspended and viable cells were counted by Trypan blue dye exclusion.

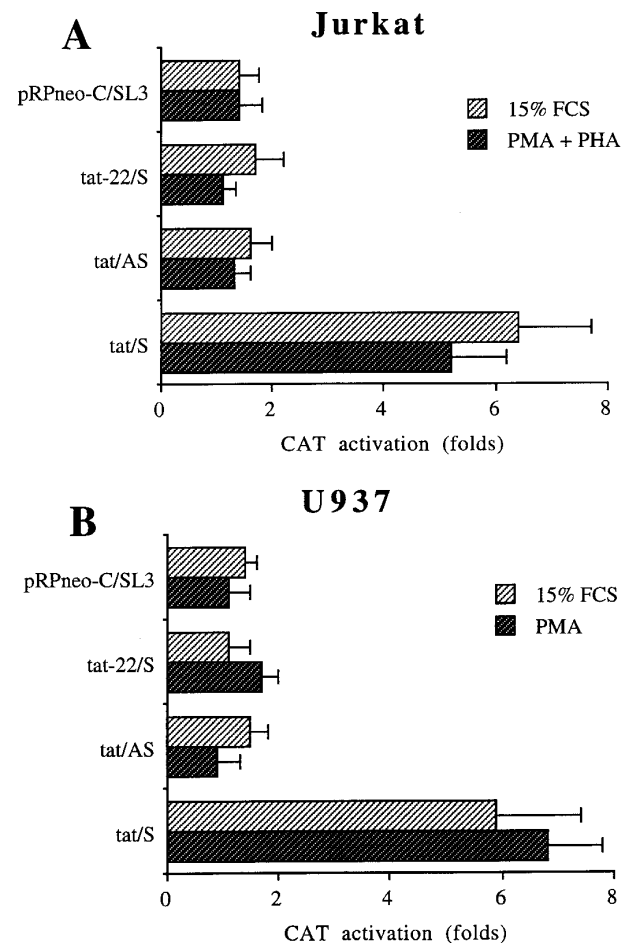
**Statistical analysis.** The results are expressed as means  $\pm$  standard deviations (SDs) of at least three separate experiments performed in duplicate. Two-tailed Student's test for unpaired data was used for statistical comparison.

## RESULTS

**HIV-1 Tat specifically upregulates *c-fos* promoter activity in serum-stimulated Jurkat and U937 cells.** Since Tat protein is predominantly produced during the early phases of *de novo* HIV-1 infection or during reactivation from viral latency,<sup>1</sup> the effect of Tat on the *c-fos* promoter expression was first examined in transient transfection experiments. Jurkat and U937 cell lines were cotransfected with the FC3 plasmid, where CAT gene expression is driven by the *c-fos* promoter, together with an HIV-1 *tat* expression plasmid (*tat/S*). Control samples were represented by cells transfected with the FC3 plasmid alone as well as cells cotransfected with FC3 and a *tat* antisense plasmid (*tat/AS*), *tat* carrying a mutation in the amino acid cys<sup>22</sup> - gly<sup>22</sup> (*tat*<sup>22/S</sup>) or FC3 and the pRPneo-SL3 backbone vector.

When Jurkat or U937 cells were cultured in the presence of low serum concentrations (RPMI + 1% FCS), Tat was unable to upregulate CAT activity over the levels observed in the presence of control vectors (data not shown). The effect of Tat expression was then evaluated in the presence of additional stimuli: 15% FCS or PHA (5  $\mu\text{g/mL}$ ) + 10<sup>-7</sup> mol/L PMA for Jurkat, 15% FCS, or 10<sup>-7</sup> mol/L PMA for U937 cells. Under these conditions, CAT activity showed a fivefold to sevenfold increase ( $P < .01$ ) in both Jurkat (Fig 1A) and U937 (Fig 1B) cells cotransfected with *tat/S* as compared to cells cotransfected with *tat/AS*, *tat*<sup>22/S</sup>, pRPneo-SL3 or cells transfected with FC3 alone. The enhancing effect of *tat/S* on FC3 activity was promoter specific, as Tat did not significantly upregulate CAT expression when the reporter CAT gene was under the transcriptional control of the  $\beta$ -actin promoter ( $\beta$ -actin/CAT) (data not shown).

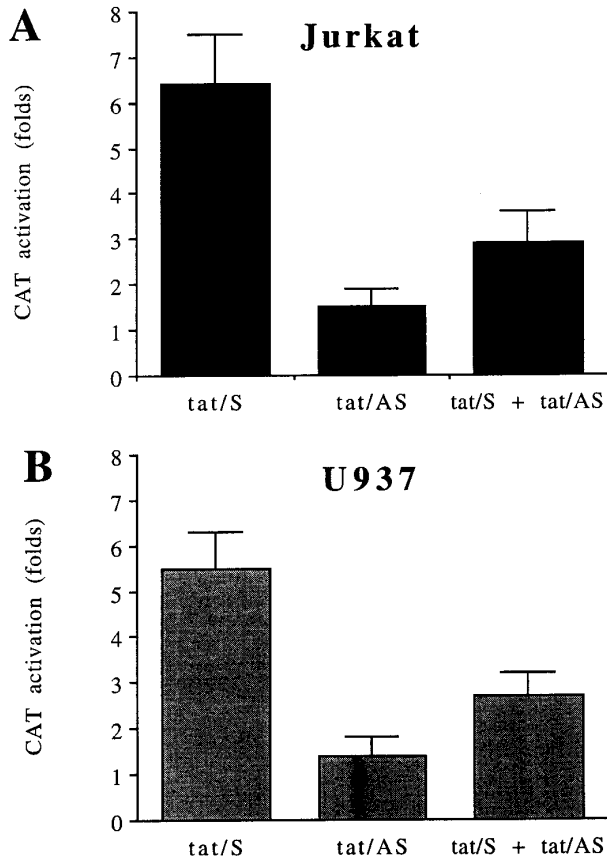
To provide an additional proof that the increased *c-fos* promoter activity was specifically mediated by Tat, transient cotransfection assays were performed with *tat/S*, *tat/AS*, and FC3 vectors in both Jurkat and U937 cell lines cultured in the presence of 15% FCS. *tat/AS* significantly ( $P < .01$ ) reduced the activation of FC3 mediated by *tat/S* in both Jurkat (Fig 2A) and U937 (Fig 2B) cells from fivefold-sevenfold to twofold-threefold. Moreover, to exclude the possibility that Tat activates the transcription of the CAT gene in an unusual start site, RNase protection analysis was performed using an RNA probe complementary to the CAT gene (Fig 3). After RNase digestion, the protected fragment resulted of 310 nucleotides as expected. An increased level of correctly initiated RNA was observed in Jurkat cells co-



**Fig 1. Upregulation of the *c-fos* promoter activity by Tat in (A) Jurkat cells activated by 15% FCS or 10<sup>-7</sup> mol/L PMA plus 5  $\mu\text{g/mL}$  PHA and (B) U937 cells activated by 15% FCS or 10<sup>-7</sup> mol/L PMA 24 hours after serum-starvation (1% FCS). Cells were transiently cotransfected with FC3 plus *tat/S* or *tat/AS* or pRPneo-SL3 control vector. CAT activity is expressed as folds of activation with respect to cell lines transfected with FC3 alone. Data are reported as means  $\pm$  SDs of four independent transfection experiments performed in duplicate.**

transfected with FC3 and *tat/S* as compared to cells cotransfected with FC3 and *tat/AS*.

**The -317/-288 serum responsive element of the *c-fos* promoter is required for Tat transactivation in activated Jurkat cells.** In the next series of cotransfection assays we used different 5' deletion mutants of the *c-fos* promoter linked to the CAT gene (Fig 4A and B) together with *tat* expressing vectors, to determine which sequences of the *c-fos* promoter were required for Tat function. Figure 4C shows the CAT analysis in Jurkat cells cultured in 15% FCS after cotransfection with various *c-fos* promoter/CAT deletion mutants plus *tat/S* or *tat/AS*. In the presence of FC2, FC3, and FC4 plasmids, *tat* was able to significantly enhance CAT activity with respect to *tat/AS*, whereas *tat/S* was unable to activate CAT expression from the FC8 mutant over the level achieved in the presence of *tat/AS*. Therefore, the majority of the Tat-induced activation was mediated by *c*-



**Fig 2. Selective blocking of the effect of Tat on the *c-fos* promoter activity.** (A) Jurkat and (B) U937 cells were transiently cotransfected with *tat/S*, *tat/AS* and FC3. Controls are represented by cells cotransfected with FC3 together with *tat/S* or *tat/AS*. CAT activity is expressed as folds of activation with respect to cell lines transfected with FC3 alone. Data are reported as means  $\pm$  SDs of three independent transfection experiments performed in duplicate.

*fos* promoter sequences located between positions  $-404$  and  $-220$  bp.

In this region, different cis-acting regulatory elements exist (Fig 4A) which may be responsible for the transactivation activity mediated by Tat. In fact, this 184 bp region contains multiple promoter elements including the dyad symmetry element that encompasses the SRE, an AP-1 site, the platelet-derived growth factor responsive sequence, a CRE element and an E1A-inducible region.<sup>45</sup> Since it has previously been shown that the SRE is essential for the transactivation of *c-fos* mediated by transactivating Tax protein of human T-cell leukemia virus type 1 (HTLV-1),<sup>43</sup> in the next series of experiments, we cotransfected *tat/S* and *tat/AS* with plasmids containing the wild-type SRE in front of CAT (Fig 4B and C). Tat significantly activated the SRE/CAT plasmid (a threefold increase of CAT activity with respect to *tat/AS*), although to a lesser extent than FC3 (a fivefold to sevenfold increase). On the other hand, *tat/S* was unable to stimulate CAT activity when an SRE mutated was used (data not shown). These data suggest that a functional wild-type SRE

was involved in the Tat-mediated transactivation of the *c-fos* promoter.

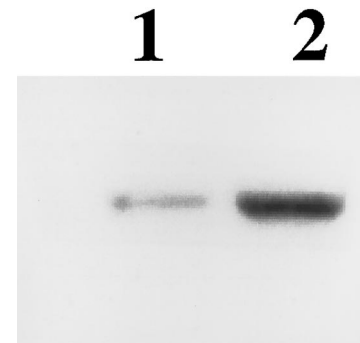
Extracellular recombinant Tat is able to upregulate *c-fos* promoter activity and induce nuclear *c-Fos* accumulation in both Jurkat cells and primary PBMC. It has been well-documented that Tat can be secreted in a biologically active form either by acutely infected cells or by *tat*-transfected cell lines, and be taken up by neighboring uninfected cells, thus contributing to the transactivation of the HIV-1 LTR and heterologous cellular gene promoters. To test the effect of extracellular Tat protein on the activity of the *c-fos* promoter, recombinant Tat or p24 were added to Jurkat and U937 cells 12-hours posttransfection with FC3, and cultures were allowed to proceed for an additional 24 hours.

As shown in Fig 5, CAT activity was significantly ( $P < .01$ ) enhanced by the addition of recombinant Tat but not by recombinant p24. Moreover, Tat activity was specifically neutralized by an anti-Tat MoAb. These results indicate that also in its extracellular form Tat was able to upregulate the activity of the *c-fos* promoter.

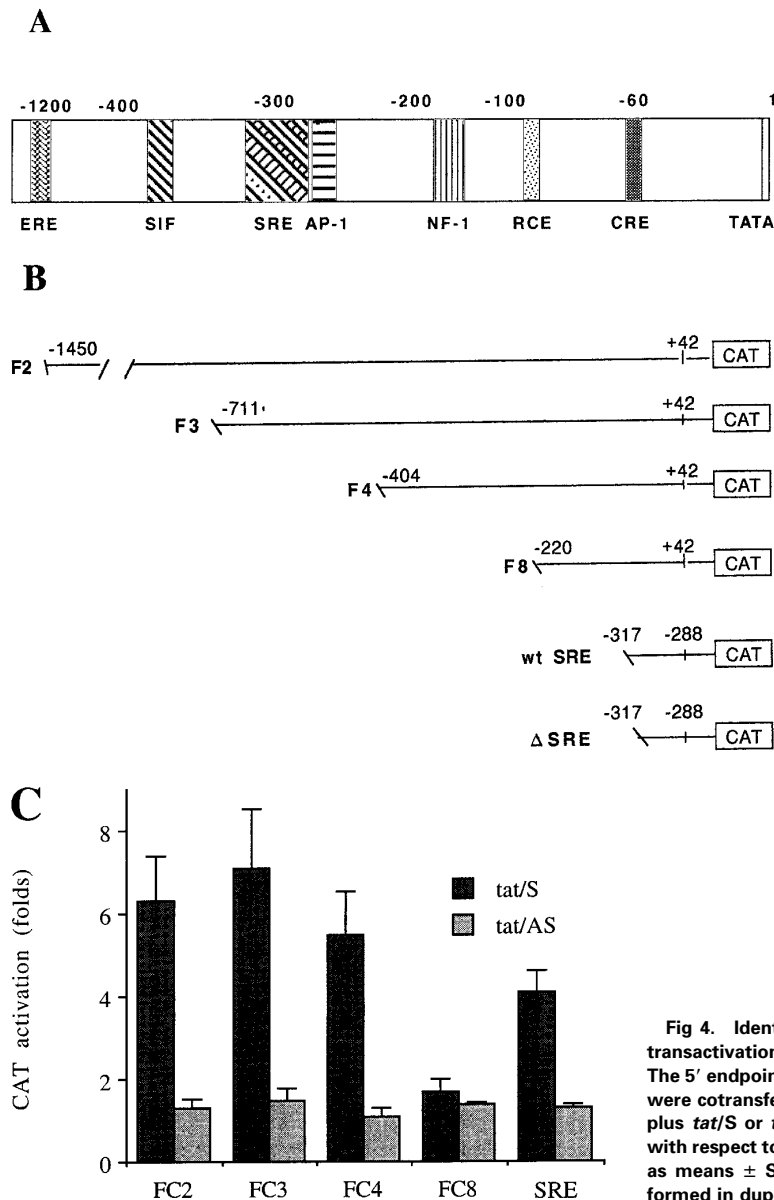
An episomal *c-fos* promoter/CAT fusion construct differs from the endogenous *c-fos* gene with respect to the conformation and sequences of the flanking DNA. Therefore, in the next group of experiments we evaluated the effect of extracellular Tat on endogenous *c-fos* gene expression in Jurkat cells.

Northern blot analysis showed that the endogenous *c-fos* mRNA expression was markedly higher in Jurkat treated with 15% FCS plus recombinant Tat as compared to cells treated with 15% FCS alone or 15% FCS plus recombinant p24 (Fig 6). The enhancing effect mediated by Tat on *c-fos* mRNA was specifically blocked by an anti-Tat neutralizing antibody.

In parallel experiments, the amount of c-Fos protein was then evaluated by flow cytometry (Fig 7A through N) in both serum-starved Jurkat and freshly isolated PBMC treated with the same agonists described above for the *c-fos* mRNA studies. PBMC were examined taking into account that primary T cells and monocytes differ from tumor cell lines in growth control and requirement for growth factors. The flow cytometry approach was chosen, since it has been previously



**Fig 3. RNase protection analysis of *c-fos* promoter/CAT transcription mediated by Tat in U937 cells cotransfected with FC3 and *tat/AS* (lane 1) or *tat/S* (lane 2). The protected fragment was 310 nucleotides long as expected. One of three separate experiments is shown.**



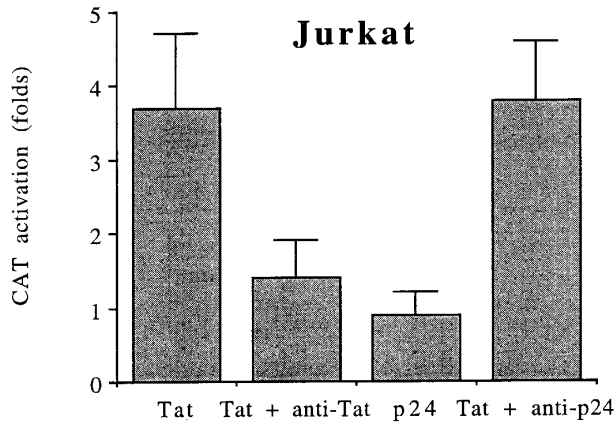
**Fig 4.** Identification of the *c-fos* promoter region required for Tat transactivation. (A) Schematic map of the *c-fos* promoter structure. (B) The 5' endpoints of the deletion mutants are indicated. (C) Jurkat cells were cotransfected with the indicated *c-fos* promoter/CAT constructs plus *tat/S* or *tat/AS*. CAT activity is expressed as folds of activation with respect to cell lines transfected with FC3 alone. Data are reported as means  $\pm$  SDs of four independent transfection experiments performed in duplicate.

demonstrated that it represents the most sensitive and reliable method for the detection of nuclear oncoproteins, including c-Fos, in hematopoietic cells.<sup>46</sup> c-Fos protein was first examined in 24-hour serum-starved Jurkat cells (Fig 7A and D) and freshly isolated PBMC (Fig 7G and L). The level of c-Fos protein significantly increased in both Jurkat and PBMC (Fig 7B and H) after a 45-minute treatment with 15% FCS or PHA plus PMA (Fig 7E and M) and showed a further increase when recombinant Tat was added to 15% FCS or PHA plus PMA (Fig 7C, F, I, and N).

The presence of c-Fos protein was also analyzed in Jurkat cells by indirect immunofluorescence and confocal microscopy (Fig 8A, B, and C). While Jurkat showed weak or absent staining after 24 hours of serum starvation (Fig 8A), the addition of 15% FCS for 45 minutes induced an intense

green-yellow positive staining (Fig 8B), which further increased in the presence of Tat (Fig 8C).

*Addition of antisense c-fos oligonucleotides in culture diminishes the Tat-mediated transactivation activity on HIV-1 LTR and the gag p24 levels in the culture supernatants of acutely IIIB infected Jurkat cells and PBMC.* The experiments described thus far suggested that Tat protein was able to upregulate c-Fos nuclear protein in both T lymphoid and monocytic cells. If this elevation in c-Fos protein is relevant to the ability of Tat to transactivate HIV-1 LTR, preventing this increase in *c-fos* expression should diminish the expression of HIV-1 LTR and be detrimental for a productive viral infection. Such an approach has been used successfully to analyze the role of c-Fos in regulating the expression of stromelysin by epidermal growth factor,<sup>47</sup> and of collagenase



**Fig 5. Upregulation of the *c-fos* promoter activity by extracellular Tat.** Twenty-four hour serum-starved (1% FCS) Jurkat cells were transfected with FC3 then cultured with recombinant Tat or recombinant p24. Alternatively cells were incubated with recombinant Tat pretreated with a MoAb to Tat or p24. CAT activity is expressed as folds of activation with respect to cell lines transfected with FC3 alone. Data are reported as means  $\pm$  SDs of three independent transfection experiments performed in duplicate.

by integrin-derived signals.<sup>48</sup> Accordingly, we used synthetic DNA oligonucleotides that were complementary to *c-fos* mRNA sequences (antisense *c-fos* oligonucleotides, SOF-OLIGO) or identical to *c-fos* mRNA sequences (sense *c-fos* oligonucleotides, FOS-OLIGO), introducing them by along with *tat/S* and an LTR-CAT construct.

When cultures were kept in 15% FCS for the last 24 hours, transfection of Jurkat with SOF-OLIGO but not with FOS-OLIGO markedly decreased the expression of HIV-1 LTR in the presence of *tat/S* (Fig 9). On the other hand, no significant differences in the levels of LTR-CAT activity were observed when cultures were kept for 36 hours in 1% FCS after transfection (data not shown). These results suggested that c-Fos protein is involved in the Tat-mediated regulation of HIV-1 LTR. On the other hand, activation of LTR-CAT in the absence of *tat* expression was very low and did not allow to establish whether c-Fos was also involved in the basal HIV-1 LTR-CAT transcription.

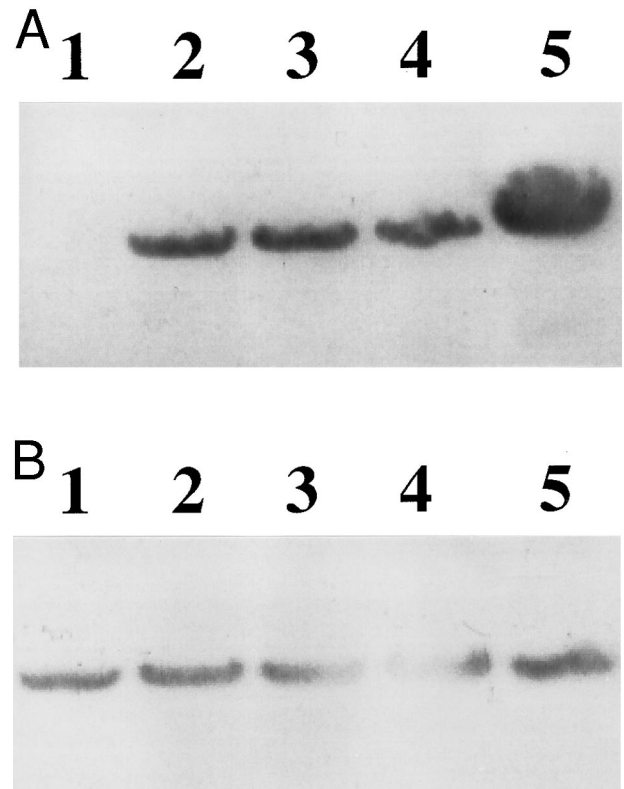
We then analyzed the levels of gag p24 antigen in the culture supernatants of Jurkat (Fig 10A) or PBMC (Fig 10B) acutely infected with high doses of infectious HIV (IIIB) and continuously supplemented with SOF- and FOS-OLIGO. The addition of SOF-OLIGO but not FOS-OLIGO to cultures caused a specific decrease of the gag 24 levels evaluated over a 15-day culture period in either Jurkat cells or PBMC. This effect was not due to a toxic effect of SOF-OLIGO, since the number of viable cells did not significantly change in Jurkat cells that were left untreated with respect to cells treated with SOF- or FOS-OLIGO (data not shown).

#### DISCUSSION

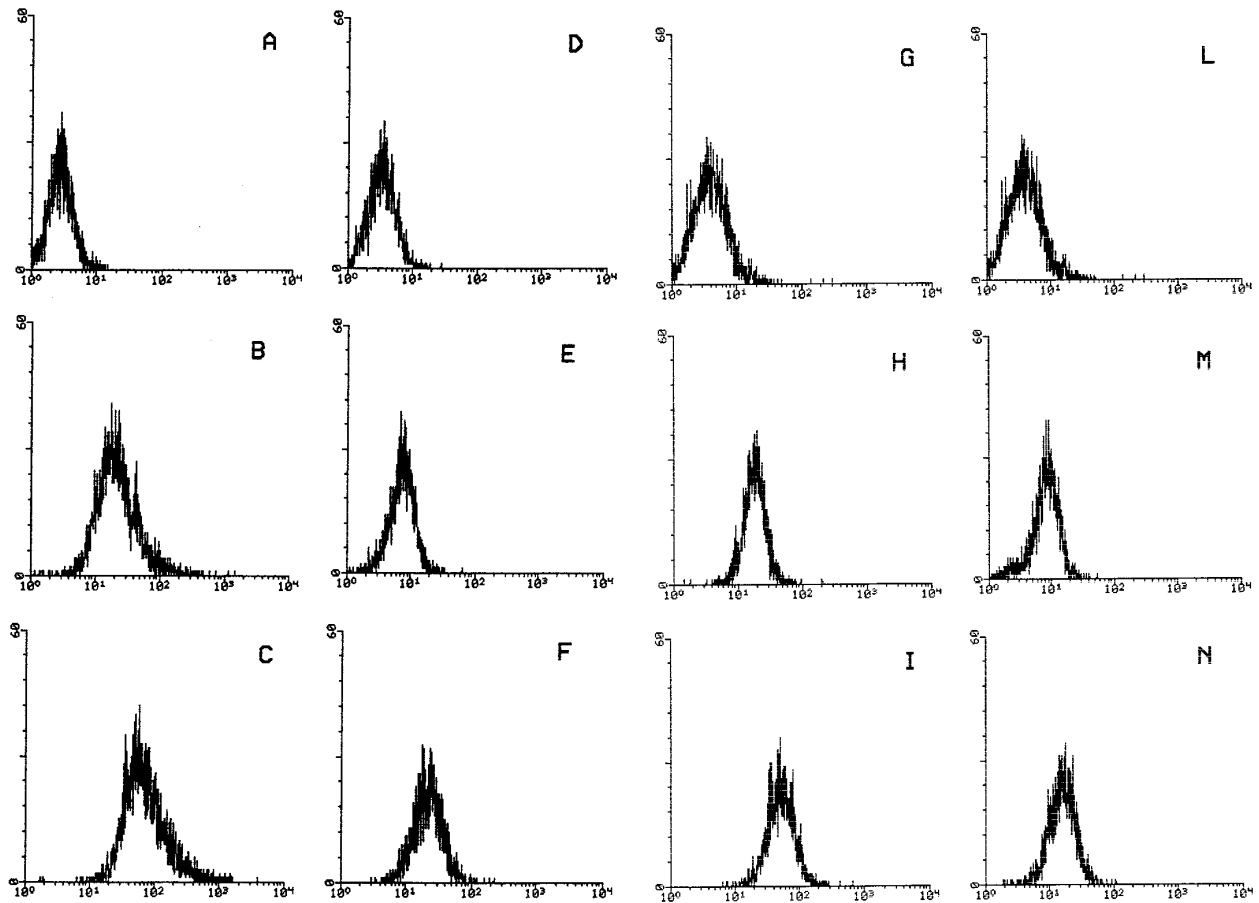
The c-Fos protein is a component of the AP-1 transcription factor complex and is a member of a multigene family, including the *fos*-related (*fos* B, *fra-1*, *fra-2*) and *jun*-related (*c-jun*, *jun* B, *jun* D) genes. The AP-1 binding site is recog-

nized by dimeric protein complexes composed of Jun homodimers or Fos/Jun heterodimers. The Fos/Jun heterodimer complex shows greater affinity than Jun/Jun homodimer for the AP-1 binding site.<sup>49-51</sup> Moreover, c-Fos protein can also heterodimerize with some components of the NF- $\kappa$ B<sup>52</sup> or ATF/CREB<sup>53</sup> families, thus allowing a cross-talk between different transduction pathways, and providing an important route for different intracellular signals to be integrated at the level of transcriptional control.

Our data indicate that the transactivator Tat protein of HIV-1 upregulates *c-fos* gene expression in both activated Jurkat and U937 cell lines, as well as in freshly isolated activated PBMC. The effects of Tat on promoter activity, mRNA expression and protein synthesis suggested that *c-fos* upregulation was primarily caused at the transcriptional level. Moreover, the structural integrity of the activation domain of Tat was required for the promotion of the *c-fos* promoter in both Jurkat and U937 cells, since a single mutation in the aminoacid cys<sup>22</sup> was sufficient to completely block the upregulation of *c-fos* expression. Since the mutation in cys<sup>22</sup> results in the complete loss of the transactivating activity of Tat on HIV-1 LTR,<sup>44</sup> our findings suggest that Tat-mediated activation of the *c-fos* promoter and the HIV-1 LTR are likely dependent on the same Tat function. According to previous findings on the transactivation of the IL-



**Fig 6. Northern blot analysis of (A) *c-fos* and (B) GAPDH mRNA in Jurkat cells.** RNA was extracted from cells serum-starved (1% FCS) for 24 hours (lane 1) and then treated for 30 minutes with 15% FCS (lane 2), 15% FCS plus recombinant p24 (lane 3), 15% FCS plus recombinant Tat preincubated with an anti-Tat MoAb (lane 4), and 15% FCS plus recombinant Tat (lane 5).



**Fig 7.** Analysis of *c-Fos* protein expression by indirect immunofluorescence and flow cytometry in Jurkat (A through F) and primary PBMC (G through N). *c-Fos* protein was determined in 24-hour serum-starved cells before (A, D, G, L) and after a 45-minute treatment with 15% FCS (B, H), 5  $\mu\text{g}$  PHA plus  $10^{-7}$  mol/L PMA (E, M), Tat plus 15% FCS (C, I), Tat plus 5  $\mu\text{g}$  PHA plus  $10^{-7}$  mol/L PMA (F, N). Horizontal axis, relative *c-Fos* expression detected by fluorescence intensity (logarithmic scale). Vertical axis, relative number of cells. Nonspecific fluorescence (not shown) was monitored by staining cells with normal rabbit serum plus GAR-FITC.

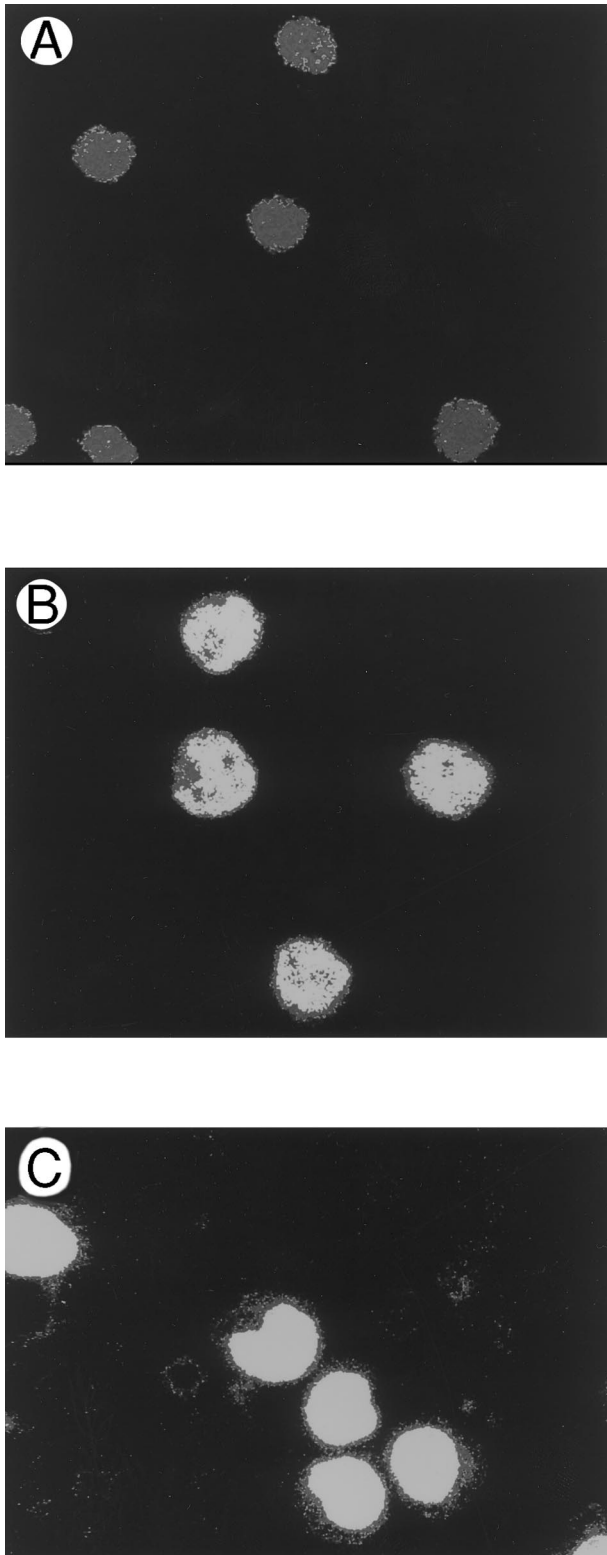
2 gene mediated by Tat,<sup>28</sup> we found out that the transient expression of *tat* was unable in itself to enhance *c-fos* expression in either tumoral or primary unstimulated cells; however, Tat significantly improved the effect of other stimuli such as high serum concentrations.

Experiments performed with *c-fos* promoter deletion mutants indicated that the responsive region required for Tat activity was set between  $-404$  and  $-220$  bp and that the SRE was critical for Tat transactivation. Interestingly, the *c-fos* activation mediated by Tat revealed both clear differences and definite similarities with the *c-fos* transactivation induced by the transactivating Tax protein of HTLV-1.<sup>43</sup> Both transactivators synergized with high serum concentrations, PHA or PMA in stimulating *c-fos* gene expression. Moreover, the analysis of deletion mutants underlined the central role of SRE in the transactivation activity of both Tat and Tax. However, Tax is a stronger inducer of *c-fos* expression, as it is able to transactivate *c-fos* promoter even in the presence of a low serum concentration.

The mechanism by which Tat affects *c-fos* gene expression remains to be fully elucidated. Bulge (AUCUG) and

stem-loop (CUGGGA) structures similar to the Tat-binding site on HIV-1 LTR RNA have been identified downstream of the transcriptional start site of the tumor necrosis factor- $\beta$ <sup>54</sup> and JC virus<sup>55</sup> promoters, and have been implicated in the Tat-induced activation of these genes. However, sequence comparison of the *c-fos* mRNA and TAR revealed no similarities between the TAR region of LTR and *c-fos* mRNA. Similarly, other authors failed to show the presence of stem-loop sequences in different cellular genes activated by Tat.<sup>26,28</sup> Therefore, the transactivation of *c-fos* gene expression may be the consequence of a TAR-independent mechanism of transcriptional activation mediated by Tat. Moreover, the ability of Tat to enhance *c-fos* transcription only when additional stimuli are present, suggests a primary role in elongation and transcription processivity rather than in the transcription initiation of *c-fos* gene, as previously described for the HIV-1 LTR.<sup>1</sup>

It is remarkable that even in its extracellular form Tat was able to upregulate *c-fos* expression in activated primary and tumoral cells. The effect of extracellular Tat may imply the uptake and nuclear localization of Tat protein,<sup>33</sup> which may



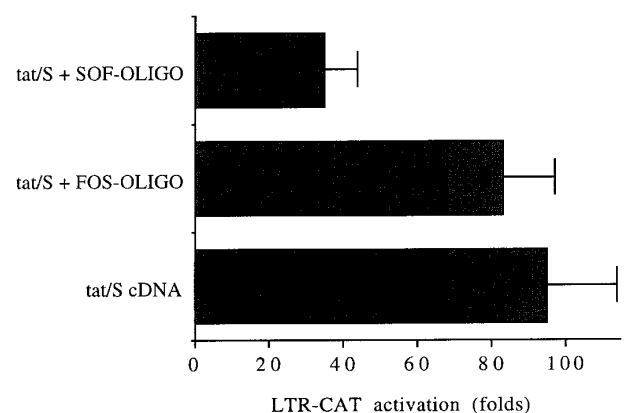
**Fig 8.** Analysis of c-Fos protein performed in Jurkat cells by indirect immunofluorescence and revealed by confocal microscopy. The presence of c-Fos was evaluated in 24-hour serum-starved cells before (A) and after a 45-minute treatment with 15% FCS (B) or Tat plus 15% FCS (C).

act directly on *c-fos* promoter. Alternatively, extracellular Tat might trigger an intracellular signal transduction pathway after specific membrane interactions with different types of integrin receptors present on those cells sensitive to its biological activity.<sup>38,56-58</sup> A similar mechanism has been proposed to explain the Tat-induced proliferation of Kaposi's sarcoma spindle cells and normal endothelial cells preactivated by cytokines.<sup>59</sup> If this model is correct, extracellular Tat could mimic the effect of extracellular matrix proteins activating *c-fos* expression through an intracellular signal cascade.<sup>60</sup>

Our data indicate that c-Fos induction by Tat may have a role in the activation of HIV-1 LTR. In fact, SOF-OLIGO significantly inhibited CAT activity in both Jurkat and U937 cells transiently transfected with *tat/S* and an LTR-CAT reporter plasmid, and were able to specifically decrease the *gag* p24 levels in the culture supernatants of either Jurkat cells or primary PBMC acutely infected with HIV-1.

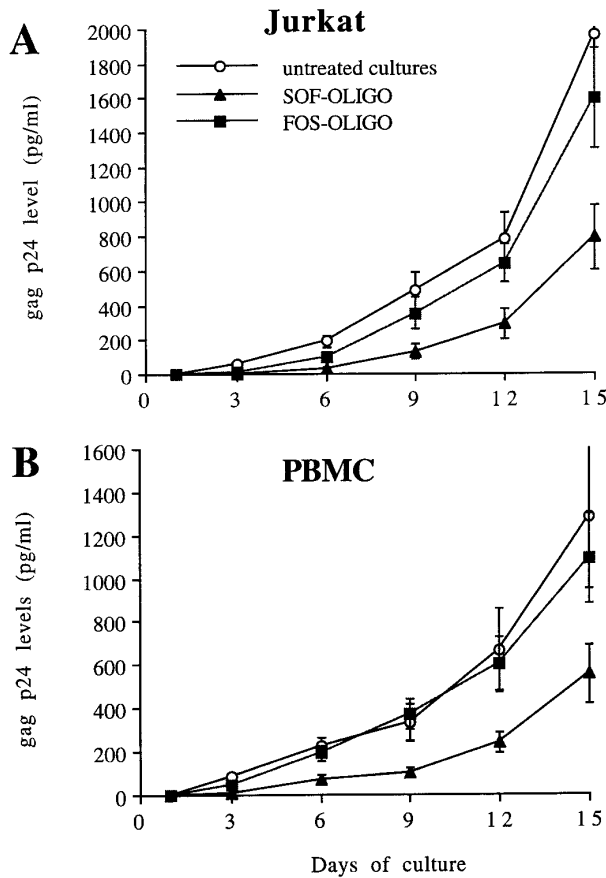
The upregulation of c-Fos induced by Tat may influence HIV-1 genome expression and HIV-1 replication in several ways. Firstly, the activation of c-Fos contributes to cell cycle progression of hematopoietic cells, acting throughout all the phases of the cell cycle.<sup>61</sup> In this respect, it has been established that HIV-1 infects more efficiently activated cells than the quiescent ones.<sup>41</sup> Secondly, transcriptional studies of the HIV-1 genome have shown that the HIV-1 LTR promoter can be subdivided into three functional domains: a basal core promoter, which is made up by the transcriptional start site, a TATA box, and three tandem Sp1 binding sites (GC-boxes); an upstream enhancer element containing two adjacent binding sites for nuclear factor  $\kappa$ B (NF $\kappa$ B); a far upstream modulatory region.<sup>62</sup> The HIV-1 LTR promoter also contains AP-1 motifs both upstream<sup>1</sup> and downstream<sup>63</sup> of the transcriptional start site.

These AP-1 sites can function independently of or in concert with the NF $\kappa$ B-binding enhancer to activate HIV-1 transcription. Moreover, a functional and physical interplay of



**Fig 9.** Inhibition of the Tat-mediated transactivation of HIV-1 LTR-CAT by SOF-OLIGO in Jurkat T cells, activated by 15% FCS 24 hours after serum-starvation (1% FCS). Cells were transiently cotransfected with LTR-CAT plus *tat/S* plus SOF-OLIGO or FOS-OLIGO. CAT activity is expressed as folds of activation with respect to Jurkat cells transfected with LTR-CAT alone. Data are reported as means  $\pm$  SDs of three independent transfection experiments performed in duplicate.





**Fig 10.** Quantitative levels of gag p24 levels in the culture supernatants of (A) Jurkat cells and (B) PBMC acutely infected with HIV-1 (IIIIB) and then cultured in the absence or presence of 25  $\mu$ mol/L SOF-OLIGO or FOS-OLIGO added every other day. Data are reported as means  $\pm$  SDs of three independent transfection experiments performed in duplicate.

the AP-1 and NF- $\kappa$ B families of transcription factors has recently been described.<sup>52</sup>

In conclusion, we have clearly shown that HIV-1 transactivating Tat protein upregulates the expression of *c-fos* gene and the enhanced c-Fos protein production may represent an important intermediate step for the Tat-dependent HIV-1 genome transactivation. In the light of the positive synergy between NF- $\kappa$ B p65 and c-Fos, the ability of Tat to stimulate both factors has important implications for the promotion of viral replication, and, possibly, for the control of both immune and inflammatory responses through the production of various cytokines. In an evolutionary perspective, it may provide the virus with a mechanism to broaden its LTR response to extracellular stimuli and activate viral gene transcription under a wide variety of cellular conditions. Functional redundancy is a common feature of viral as well as cellular regulatory regions and in this case it may represent a mechanism to increase virus host range and virus replication potentiality.

#### ACKNOWLEDGMENT

We are grateful to Dr Paolo Sassone-Corsi for providing all the *c-fos*/CAT plasmids and to Dr Enzo Lalli for helpful discussion.

This research was supported by "AIDS project" of the Italian Ministry of Health and MURST 40 and 60%.

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