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The Human Immunodeficiency Virus Type-1 Tat Protein Upregulates Bcl-2 Gene Expression in Jurkat T-Cell Lines and Primary Peripheral Blood Mononuclear Cells

By Giorgio Zauli, Davide Gibellini, Antonella Caputo, Alessandra Bassini, Massimo Negrini, Maria Monne, Meri Mazzoni, and Silvano Capitani

The regulatory Tat protein of human immunodeficiency virus type-1 (HIV-1) exerts a pleyotropic activity on the survival and proliferation of different cell types in culture. In this report, we investigated the effect of either endogenous or exogenous Tat on Bcl-2 proto-oncogene expression and cell survival in Jurkat T-cell lines and primary peripheral blood mononuclear cells. Stable and transient transfections of Jurkat cells with the cDNA of tat and a plasmid containing Bcl-2 promoter in front of CAT (Bcl-2 Pr/CAT) stimulated CAT activity and showed an increase of Bcl-2 mRNA and protein expression. This effect was specifically related to tat, because Jurkat cells transfected with the cDNA of tat in antisense orientation, tat carrying a mutation in the amino acid cys^{22}-gly^{22}, or the control vector alone (pRPneo-SL3) did not show any significant difference in Bcl-2 promoter activity with respect to parental Jurkat cells. We also observed a specific correlation between tat-induced Bcl-2 gene expression and inhibition of apoptosis induced by serum with-drawal. Our results suggest that the structural integrity of the activation domain of Tat was required for the promotion of the Bcl-2 promoter and Jurkat cell survival, because a single mutation in the aminoacid cys^{22} was sufficient to completely block the upregulation of Bcl-2 and inhibition of apoptosis. Moreover, picomolar concentrations of native or recombinant Tat were able to upregulate Bcl-2 expression both in Jurkat and primary peripheral blood mononuclear cells, suggesting that extracellular Tat, actively released by infected cells, may also play a significant role in suppressing apoptosis. An aberrant cell survival of lymphoid cells consequent to the upregulation of Bcl-2 may represent an additional pathogenetic mechanism that could help explain both the dysregulated immune response and the frequent occurrence of hyperplastic/neoplastic disorders in HIV-1–seropositive individuals.

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T HE Tat PROTEIN OF human immunodeficiency virus-type 1 (HIV-1) is essential for viral replication.[1] Tat is a protein of 86 to 104 amino acids encoded by two exons that acts as a positive regulator of HIV-1 gene expression mainly through a *cis*-acting sequence, the transactivation response element (TAR), located at the 5' end of all viral mRNAs.[2,3] Tat can be divided in five distinct domains called N-terminal, cysteine-rich, core, basic, and C-terminal sequences. Although the cysteine-rich region is responsible for the formation of intramolecular disulfide bonds,[4] the basic region contains nuclear localization signals[5] and the binding site to TAR RNA.[2] The activation domain of Tat (amino acids 1 to 48) includes the amino-terminal region and the conserved cysteine-rich and core domains.[1] Tat can also influence viral replication via TAR-independent[6] and posttranscriptional mechanisms.[7]

A number of reports have clearly shown that Tat displays several activities on cell survival, growth and function of uninfected cells.[8-16] Extracellular Tat can easily enter different cell types through aspecific endocytosis.[8,9] Conversely, both acutely HIV-1–infected and tat-transfected cells actively release Tat.[10-12] It has been shown that nanomolar concentrations (0.5 to 10 μg/mL) of extracellular Tat exert an inhibitory effect on the antigen-induced proliferation of isolated T lymphocytes,[13-15] whereas picomolar concentrations (0.1 to 10 ng/mL) of Tat promote the growth of adherent Kaposi's sarcoma and PC12 rat pheochromocytoma cells.[10-12] Futhermore, we have previously shown that various mammalian cell lines constitutively expressing endogenous Tat display higher resistance to the induction of apoptosis.[17]

The molecular bases of Tat activity on cell survival and proliferation are poorly understood. It has been suggested that extracellular Tat promotes the growth of Kaposi's sarcoma cells through specific interaction of the RGD motif, encoded by its second exon, with integrin receptors for fibronectin ($\alpha 5\beta 1$) and vitronectin ($\alpha_v\beta 3$).[18,19] Alternative receptors interacting with the basic region of Tat have also been described.[15,20-21] It is presently unclear whether the integrin-dependent promotion of cell proliferation is confined to KS cells or represents a general mechanism and which region of Tat is essential to elicit the growth response. The effects of Tat on cell survival/growth could also be exerted through transactivation of cellular genes encoding cytokines, such as transforming growth factor-$\beta 1$ (TGF-$\beta 1$), tumor necrosis factor-β (TNF-β), interleukin-6 (IL-6), and IL-2.[22-25]

In this study, we investigated whether Tat exerts its effects on lymphoid cell survival by affecting expression of cellular Bcl-2 proto-oncogene, because it was previously shown that Bcl-2 blocks programmed cell death in different tissues, particularly in lymphoid cells.[26] As a model system, we used the Jurkat lymphoblastoid CD4$^+$ cell line either transiently or permanently transfected with tat cDNA. To gain some insights into the physiologic relevance of the results obtained with Jurkat cells, the effects of extracellular Tat protein on peripheral blood mononuclear cells (PBMCs) were also evaluated.
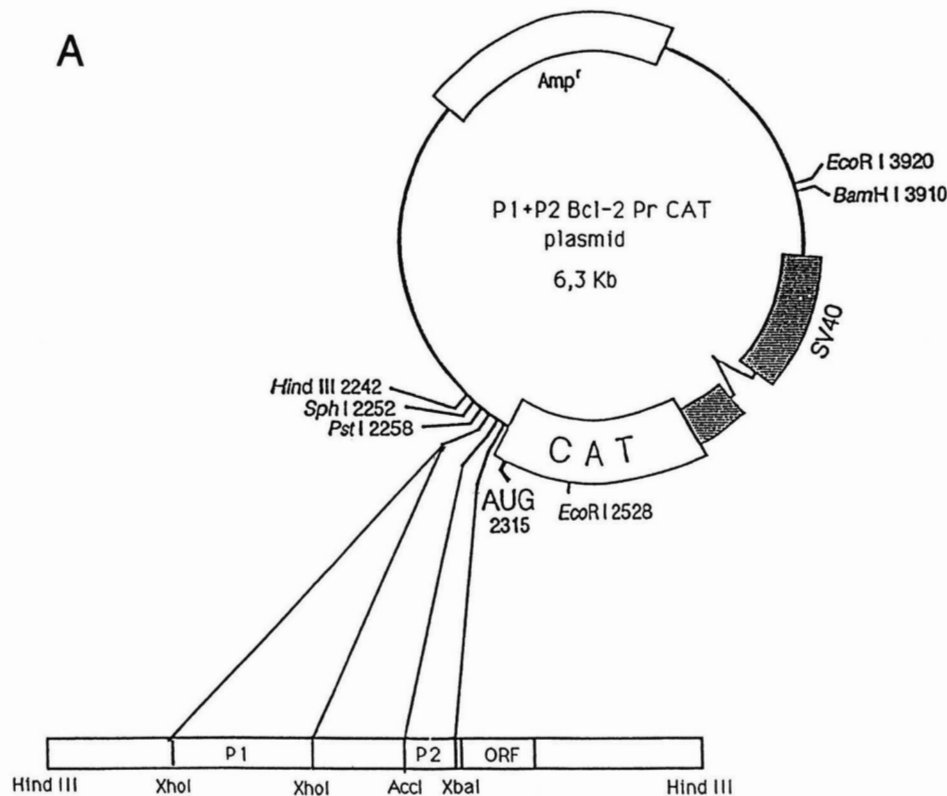
A

Fig 1. Upregulation of the Bcl-2 promoter activity in Jurkat T cells transiently transfected with tat. (A) Schematic diagram of P1+P2 Bcl-2 Pr/CAT. Cells were cotransfected with (B) P1 Bcl-2 Pr/CAT, (C) P1+P2 Bcl-2 Pr/CAT, or (D) β-actin Pr/CAT and tat/S, tat/AS, tat [22]/S, or pRPneo-SL3 control vector. Bcl-2 promoter activity was measured as the percentage of transacetylation in a CAT assay. Data are reported as the mean ± standard deviation of three independent transfection experiments performed in duplicate. (■) Parental Jurkat; (▨) Jurkat + pRPneo-SL3; (■) Jurkat + tat-22/S; (▨) Jurkat + tat/AS; (□) Jurkat + tat/S.

MATERIALS AND METHODS

Cell lines and PBMCs. Jurkat CD4+ lymphoblastoid T cells (0.3 to 1.2×10^6/mL) were cultured in RPMI 1640 (GIBCO, Grand Island, NY) plus 10% fetal calf serum (FCS; GIBCO).

PBMCs were obtained from 8 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 plus 10% FCS, 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 seeded in culture in RPMI plus 0.5% FCS.

Plasmids and transfections. Plasmids pRPneo-SL3, pRPneo-SL3/tat/S, and pRPneo-SL3/tat/AS containing the cDNA of tat of HIV-1 in sense (tat/S) or antisense (tat/AS) orientation under the control of the long terminal repeat region of the SL3 murine leukemia virus have been previously described.[27] Plasmid pRPneo-SL3/tat[22]/S was obtained by substitution of the cys[22] with glycine.[28] Jurkat human lymphoblastoid CD4+ T cells were permanently transfected by electroporation.[29] Stable clones were selected in the presence of 600 to 800 μg/mL of the aminoglicoside antibiotic geneticin (G418; Sigma, St Louis, MO).

Bcl-2 sequences have been previously described.[30-32] P1 Bcl-2Pr/CAT was constructed as follows: a 2.0-kb *Xho* I genomic fragment containing the human Bcl-2 P1 promoter (1.3 kb of the 5'-flanking region and 0.7-kb sequence of the 5'-untranslated region) was subcloned 5' to the CAT gene in the pCAT basic vector. The P1+P2 Bcl-2Pr/CAT contained, in addition, the P2 promoter.

Transient transfection experiments were performed using the diethyl aminoethyl (DEAE)-dextran method as described by Gorman.[33] Briefly, 10^7 cells were incubated with 15 μg of each plasmid DNA in 500 μg DEAE-dextran and 20 μg chloroquine/mL for 30 minutes.

Forty-eight hours after transfection, the clarified lysates were assayed for CAT activity as described,[34] using volumes of extract corresponding to equal amount of proteins.

Treatment of PBMC with recombinant or native Tat. Freshly isolated PBMCs were seeded in culture in Iscove's modified Dulbecco's medium (IMDM) containing 0.5% FCS and picomolar (5 to 10 ng/mL) amounts of recombinant Tat protein (American Biotechnologies, Cambridge, MA) or 50% (vol/vol) serum-free supernatant of rat pheochromocytoma PC12-tat cells, which actively release 1.8 to 15 ng/mL of Tat.[12] In blocking experiments, recombinant Tat or supernatant of PC12-tat was pretreated with neutralizing concentrations (5 μg/mL) of anti-Tat or anti-p24 control monoclonal antibody (MoAb), both purchased from American Biotechnologies.

Analysis of Bcl-2 RNA expression. Total RNA was extracted from 1×10^8 Jurkat T cells/experimental point using RNAzol (Biotecx, Galveston, TX) according to the manufacturer's instructions. Bcl-2 mRNA expression was evaluated by means of two separate procedures, ie, dot blot and RNase protection. For dot blot analysis, 30 μg of undiluted or diluted total RNA (dilution factor 2, from 1:8 to 1:128) from each sample was transferred to nylon membranes as described by Sambrook and Fritsch.[35] Filters were prehybridized for 2 hours at 42°C in 50% formamide, 5% blocking reagent (Boehringer Mannheim, Mannheim, Germany), 5× SSC, 0.02% sodium dodecyl sulfate (SDS), 0.1% sarkosyl. Hybridization proceeded for 16 hours at 42°C in the same solution containing a specific Bcl-2 DNA probe (100 ng/mL) corresponding to the B4 clone 5' end to *Bam*HI site (900 bp) previously described by Tsujimoto and Croce.[30]

The probe for Bcl-2 cDNA was digoxigenin-labeled using a random primer labeling kit (Boehringer Mannheim) according to manufacturer's instructions. Filters were washed with 2× SSC and 0.1% SDS for 15 minutes at room temperature; 0.5% SSC and 0.1% SDS at 55°C for 45 minutes, and 0.5× SSC, 0.1% SDS at room tempera-

ture for 10 minutes. The amount of RNA for each lane was normalized by stripping and reprobing the filters with a gliceraldeyde-3-phosphate dehydrogenase (GAPDH) probe represented by a *Xba* I/*Pst* I 780-bp fragment.[36]

For RNase protection analysis, 30 μg of RNA was processed
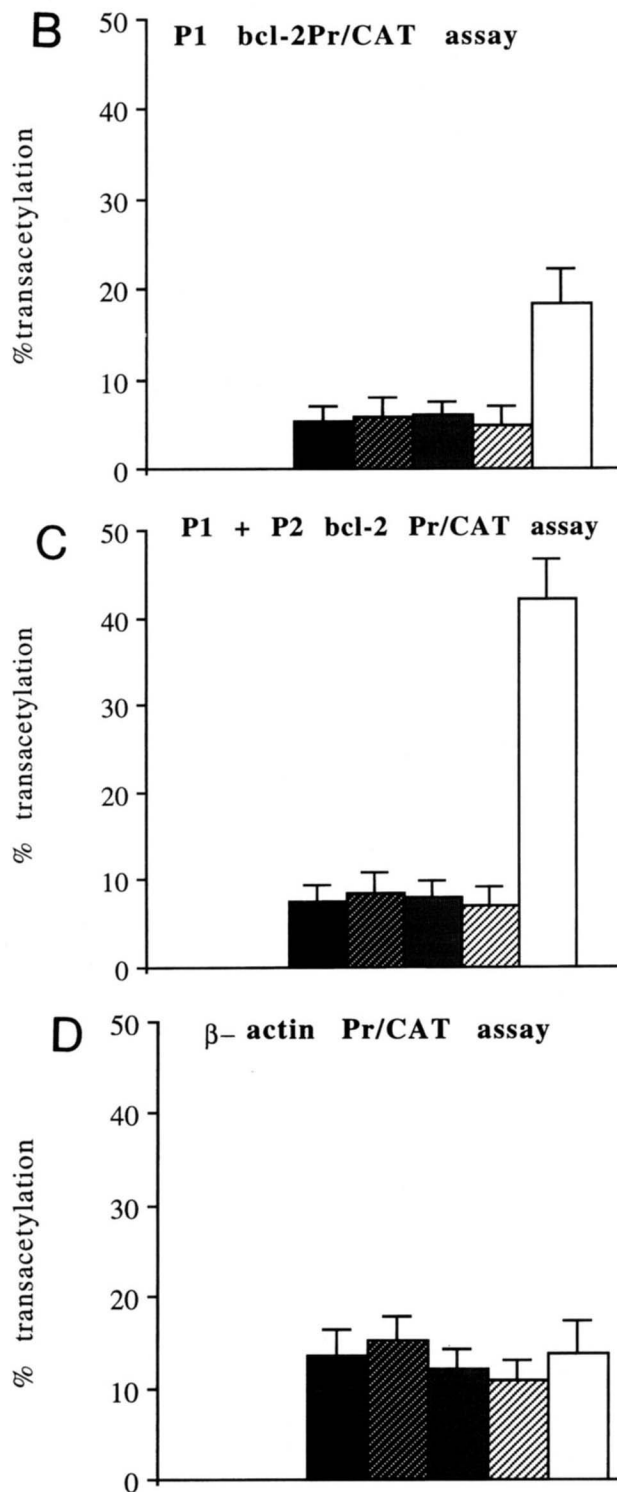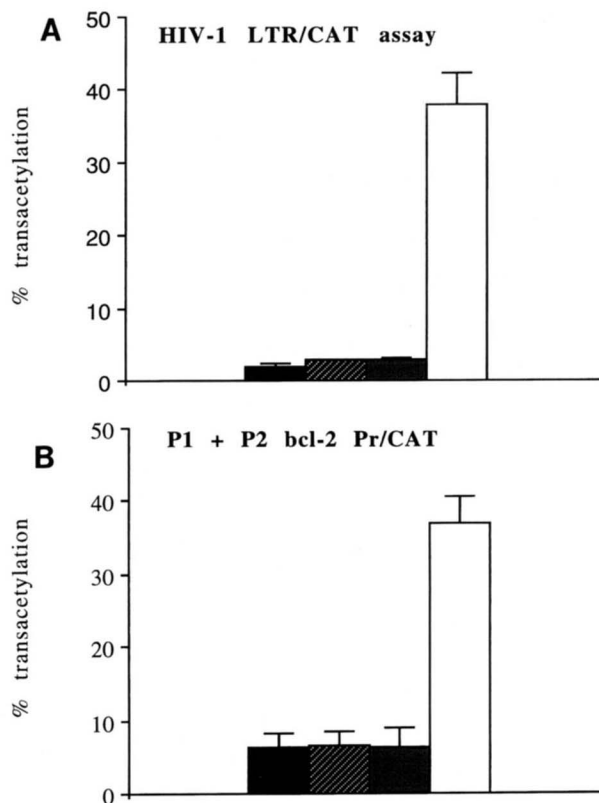

Fig 2. Upregulation of the Bcl-2 promoter activity in Jurkat T cells stably transfected with tat. Jurkat cell lines permanently transfected with tat/S or tat [22]/S or pRPneo-SL3 as well as parental Jurkat cells were cotransfected with (A) HIV-1 LTR/CAT or (B) P1+P2 Bcl-2 Pr/CAT plasmids. HIV-1 LTR and Bcl-2 promoter activities were measured as the percentage of transacetylation in a CAT assay. Data are reported as the mean ± standard deviation of three independent transfection experiments performed in duplicate. (■) Parental Jurkat; (▨) Jurkat + pRPneo-SL3; (▦) Jurkat + tat-22/S; (■) Jurkat + tat/S.

using a RNase protection kit (Boehringer Mannheim) according to the manufacturer's instructions. The specific Bcl-2 RNA probe was represented by a digoxigenin-labeled *Eco*RI-*Bam*HI fragment (563 bases) of the B4 cloned in PSPT18 vector.[30] The control RNA probe was a 255-bases probe specific for human β-actin provided by the manufacturer.

Hybrids were detected with alkaline phosphatase-conjugated anti-digoxigenin immune serum (Boehringer Mannheim) using a chemi-luminescent substrate, as previously described.[36] The results were recorded on Polaroid film or Kodak X-OMAT film (Eastman Kodak, Rochester, NY).

Analysis of Bcl-2 protein expression by Western blotting and indirect immunofluorescence staining shown by flow cytometry. Gel electrophoresis was performed as described by Laemmli.[37] Briefly, cell lysates were obtained by sonicating the cells for 2 minutes and boiling for 5 minutes in 62.5 mmol/L Tris buffer, pH 6.8, containing 2% SDS, 5% β-mercaptoethanol, and 10% glycerol. Samples derived from 2×10^6 viable PBMCs or Jurkat cells, containing approximately 100 to 150 μg of proteins, were migrated in 12% acrylamide gels and blotted onto nitrocellulose filters. Blotted filters were blocked for 30 minutes in a 3% suspension of dried skimmed milk in phosphate-buffered saline (PBS) and incubated overnight at 4°C with 1:100 dilution of anti–Bcl-2 MoAb (Dako, Glostrup, Denmark) or 1:500 dilution of antitubulin MoAb (Sigma). Filters were washed
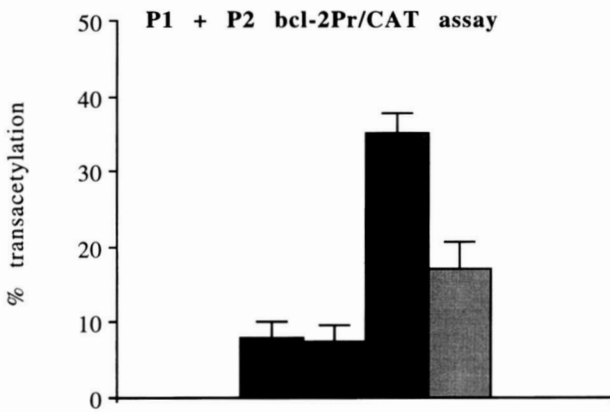

Fig 1. (Cont'd).

P1 + P2 bcl-2Pr/CAT assay

Fig 3. Selective blocking of the effect of Tat on the Bcl-2 promoter. Jurkat stably transfected with tat/S or with the pRPneo-SL3 control vector were cotransfected with the combination of an antisense RNA expression construct (tat/AS) and P1+P2 Bcl-2 Pr/CAT. Bcl-2 promoter activity was measured as the percentage of transacetylation in a CAT assay. Data are reported as the mean ± standard deviation of three independent transfection experiments performed in duplicate. (■) Jurkat-pRPneo-SL3; (■) Jurkat-pRPneo-SL3 + tat/AS; (■) Jurkat-tat/S; (□) Jurkat-tat/S + tat/AS.

and further incubated for 1 hour at room temperature with 1:1,500 peroxidase-conjugated antimouse IgG (Sigma) in 0.1% bovine serum albumin (BSA). Specific reactions were shown with the ECL Western blotting detection reagent (Amersham Corp, Arlington Heights, IL).

For the indirect immunofluorescence analysis, Jurkat cells and PBMCs were fixed in PBS 2% paraformaldehyde for 20 minutes at room temperature, washed twice with PBS containing 10 mmol/L

glycin, and permeabilized in PBS Triton X 1% for 5 minutes at 4°C. After two washings with PBS, cells were resuspended in PBS plus 10% normal goat serum for 10 minutes at room temperature before adding of anti–Bcl-2 MoAb (1:50; Dako) for 30 minutes at 37°C. After two washings with PBS, a polyclonal goat antimouse (1:100) antibody conjugated to fluorescein (GAM-FITC) was added to cells and incubated for 30 minutes at 37°C. The cells were finally washed and analyzed. The negative controls consisted of an isotype-matched unreactive antibody (1:50 dilution of anti-p66 human cytomegalovirus [HCV]; Du Pont Co, Wilmington, DE) followed by identical second-layer labeling as above. The expression of Bcl-2 protein was investigated by a FACScan flow cytometer (Becton Dickinson, San José, CA).

Detection of apoptosis. Apoptosis was evaluated combining flow cytometry, transmission electron microscopy and DNA gel electrophoresis techniques, as previously described.[17] For the flow cytometry procedure, 2×10^5 Jurkat cells or PBMCs were cultured for 1 to 4 days with low serum concentrations (IMDM plus 0.5% FCS) in the absence or presence of native or recombinant Tat. Cells were then harvested by centrifugation at 200g for 10 minutes at 4°C. The pellets were treated with 0.5 μg RNase (Type I-A; Sigma) and resuspended in PBS containing 50 μg/mL propidium iodide. Analysis was performed on a FACScan flow cytometer with the FL2 detector in logarithmic mode using Lysis II software (Becton Dickinson).

For DNA gel electrophoresis experiments, 2×10^6 cells were pelleted and resuspended in 20 mL of 10 mmol/L EDTA, 50 mmol/L Tris-HCl (pH 8) containing 0.5% (wt/vol) sarkosyl and 0.5 mg/mL proteinase K, and incubated at 50°C for 1 hour. Ten milliliters of 0.5 mg/mL RNase A was added to each sample and incubated at 50°C for 1 hour. Samples were heated at 70°C and 10 mL of 10 mmol/L EDTA (pH 8.0) containing 1% (wt/vol) low gelling temperature agarose, 0.25% (wt/vol) Bromophenol blue, and 40% (wt/vol) sucrose was mixed with each sample before loading into the dry wells of a 2% (wt/vol) agarose gel containing 0.1 μg/mL ethidium

A Bcl-2

B GAPDH

C Bcl-2

←protected fragment
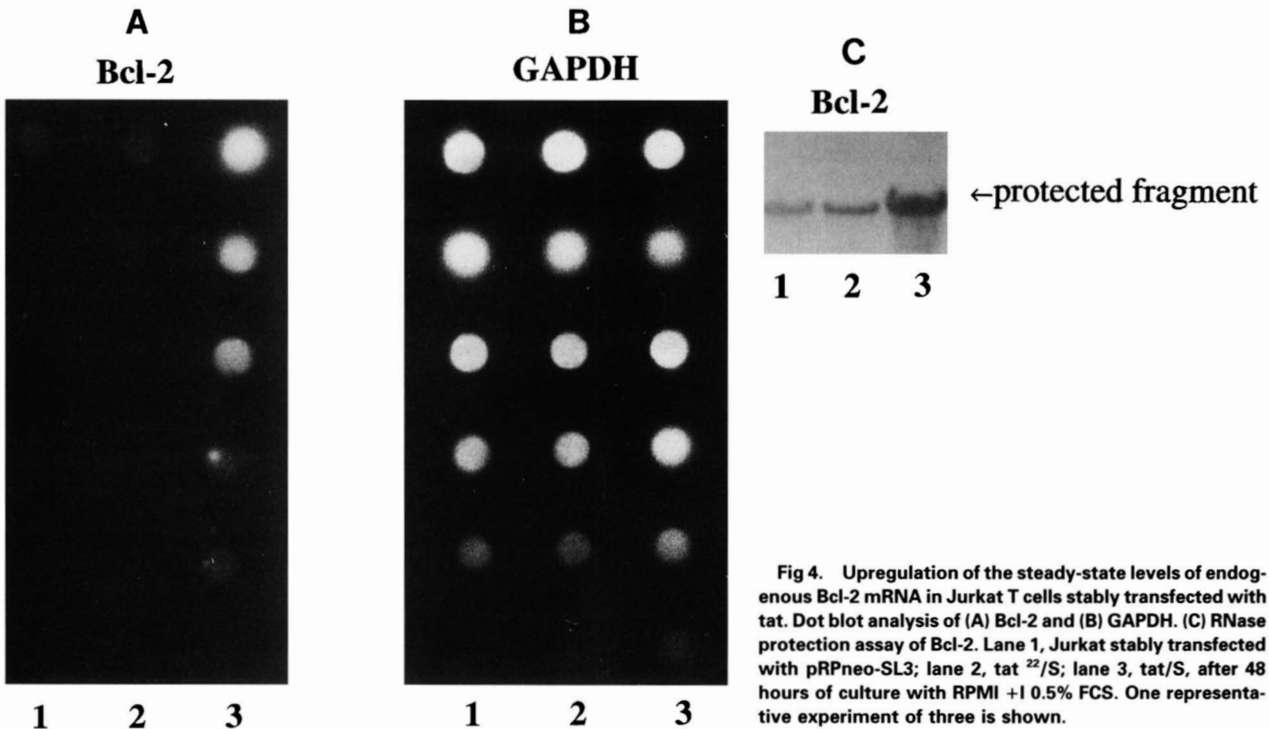
Fig 4. Upregulation of the steady-state levels of endogenous Bcl-2 mRNA in Jurkat T cells stably transfected with tat. Dot blot analysis of (A) Bcl-2 and (B) GAPDH. (C) RNase protection assay of Bcl-2. Lane 1, Jurkat stably transfected with pRPneo-SL3; lane 2, tat[22]/S; lane 3, tat/S, after 48 hours of culture with RPMI +I 0.5% FCS. One representative experiment of three is shown.

A **Bcl-2 protein**

←25kD

1 2 3

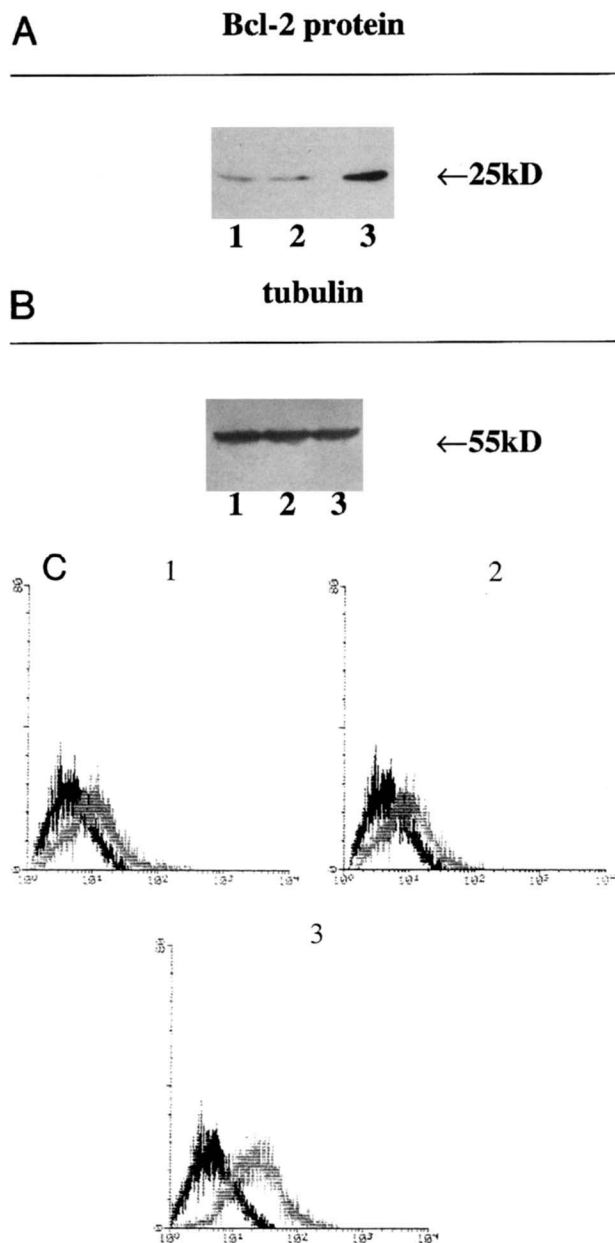## B **tubulin**

←55kD

1 2 3

C

Fig 5. Upregulation of endogenous Bcl-2 protein in Jurkat T cells stably transfected with tat. Western blot analysis of (A) Bcl-2 and (B) tubulin proteins performed on cell lysates from 2×10^6 cells. (C) Analysis of Bcl-2 protein by indirect (anti–Bcl-2 + GAM-FITC) immunofluorescence shown by flow cytometry. Wright areas represent cells treated with anti–Bcl-2 + GAM-FITC, whereas left areas are negative controls (cells treated with anti-p66 of HCV + GAM-FITC). Horizontal axis, relative Bcl-2 expression detected by fluorescence intensity (logarithmic scale). Vertical axis, relative number of cells. Lane 1, Jurkat stably transfected with pRPneo-SL3; lane 2, tat ^{22}/S; lane 3, tat/S, after 48 hours of culture with RPMI + 0.5% FCS. One representative experiment of three separate experiments is shown.

bromide. Electrophoresis was performed in 2 mmol/L EDTA, 800 mmol/L Tris-phosphate (pH 7.8) until the marker dye had migrated 3 to 4 cm.

For transmission electron microscopy, cells were fixed with 2.5% glutaraldehyde.

Statistical analysis. The results were expressed as the mean ± standard deviation (SD) of three or more experiments performed in duplicate. Statistical analysis was performed using the two-tailed Student's *t*-test.

RESULTS

Effect of transient expression of HIV-1 Tat on Bcl-2 promoter activity. The reporter constructs P1 Bcl-2Pr/CAT and P1+P2 Bcl-2Pr/CAT, where CAT gene expression is driven by P1 or P1+P2 Bcl-2 promoters (Fig 1A), were transiently cotransfected with tat/S, tat/AS, tat ^{22}/S, or pRPneo-SL3 backbone vectors in Jurkat cells (Fig 1B and C). After 48 hours of culture in RPMI plus 1% FCS, CAT activity was significantly ($P < .01$) higher in the presence of Tat expression (tat/S) as compared with transactivation values detected when the tat/AS, tat ^{22}/S, or pRPneo-SL3 vectors were used. Tat was significantly ($P < .01$) more efficient in transactivating the P1 + P2 Bcl-2Pr/CAT (5-fold) than P1 Bcl-2Pr/CAT (3-fold), thus indicating that both Bcl-2 promoters were required for optimal transactivation by Tat. Moreover, Tat transactivating effect on Bcl-2 promoter was specific, because Tat did not induce CAT expression when the reporter gene was under the transcriptional control of the β-actin promoter (β-actin Pr/CAT; Fig 1D). This first group of data suggested that, in transient assays, expression of Tat was able to transactivate the promoter of Bcl-2 gene in lymphoid Jurkat cells and that both P1 and P2 promoters are required for optimal activity. Based on these results, the P1+P2 Bcl-2 Pr/CAT plasmid was used in the following experiments.

Effect of stable expression of Tat on Bcl-2 promoter activity. To explore the effect of constitutive expression of Tat on Bcl-2 promoter activity, Jurkat cells were electroporated with tat/S, tat ^{22}/S, or the pRPneo-SL3 vectors and stable polyclonal cell cultures were selected in G418. Expression of tat/S-or tat ^{22}/S-specific mRNAs in these cultures was shown by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis (data not shown). The presence of Tat protein was then evaluated by monitoring its transactivating activity on HIV-1 LTR with the pU3RCAT reporter plasmid.[38] The results of these experiments showed transactivation of the HIV-1 LTR only in Jurkat cells stably transfected with tat/S, indicating endogenous production of functional Tat protein in this cell line (Fig 2A). The effect of constitutive expression of Tat on Bcl-2 gene promoter was then assayed by transient transfection of the cell cultures with the P1+P2 Bcl-2Pr/CAT. The results of these experiments (Fig 2B) were similar to those previously observed in transient cotransfection assays. CAT activity was fourfold to fivefold ($P < .01$) higher in Jurkat cells stably transfected with the tat/S expressing vector as compared with Jurkat cells containing tat ^{22}/S or pRPneo-SL3 vectors and as compared with the parental Jurkat cells.

To provide an independent proof that the increase of Bcl-2 promoter activity was specifically mediated by Tat, we selectively blocked endogenous tat gene expression using an antisense-tat expressing plasmid. Transient cotransfections of tat/AS and P1+P2 Bcl-2Pr/CAT vectors selectively reduced ($P < .01$) Bcl-2 promoter activity in Jurkat cells con-
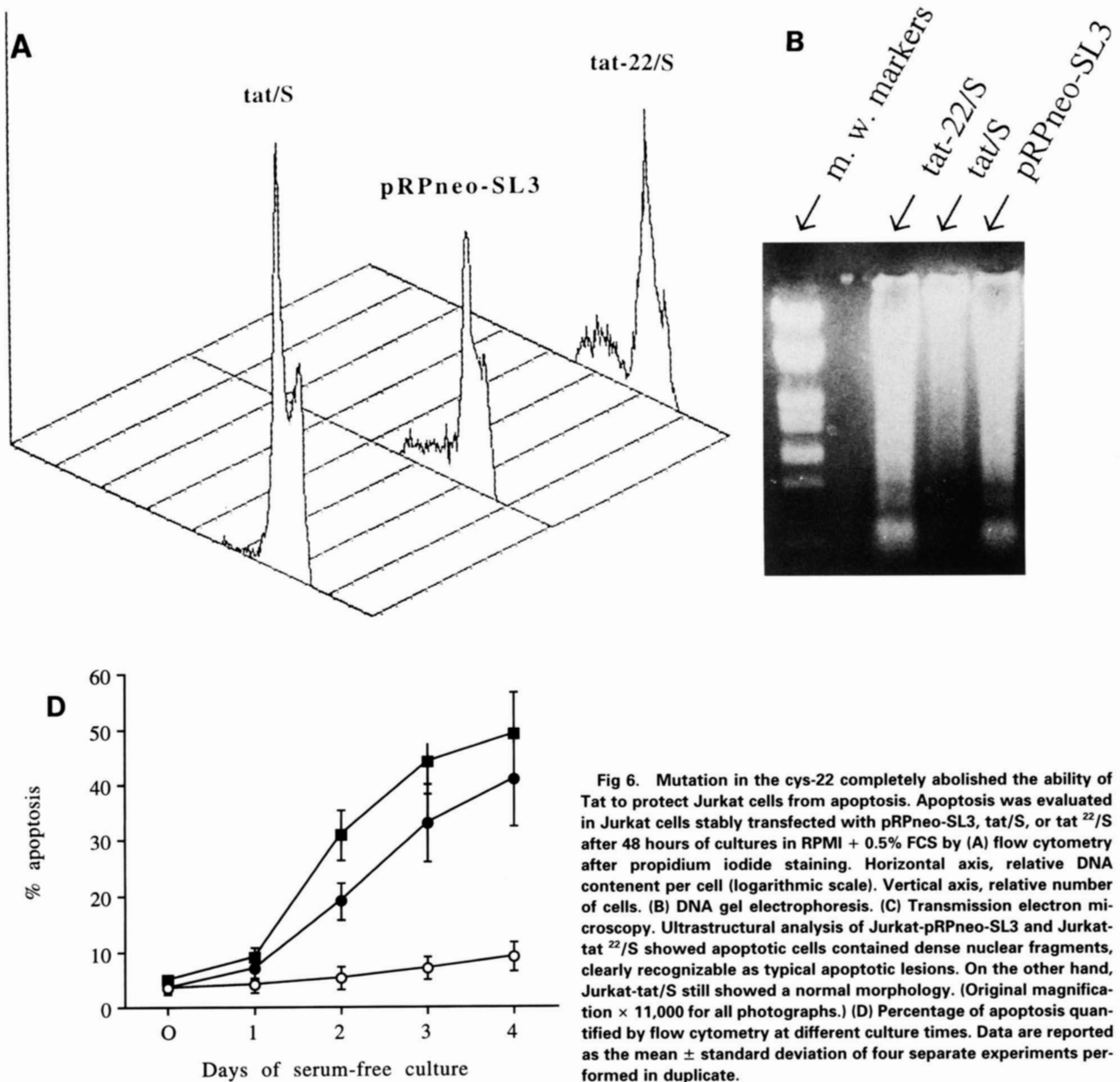
Fig 6. Mutation in the cys-22 completely abolished the ability of Tat to protect Jurkat cells from apoptosis. Apoptosis was evaluated in Jurkat cells stably transfected with pRPneo-SL3, tat/S, or tat ^{22}/S after 48 hours of cultures in RPMI + 0.5% FCS by (A) flow cytometry after propidium iodide staining. Horizontal axis, relative DNA contenent per cell (logarithmic scale). Vertical axis, relative number of cells. (B) DNA gel electrophoresis. (C) Transmission electron microscopy. Ultrastructural analysis of Jurkat-pRPneo-SL3 and Jurkat-tat ^{22}/S showed apoptotic cells contained dense nuclear fragments, clearly recognizable as typical apoptotic lesions. On the other hand, Jurkat-tat/S still showed a normal morphology. (Original magnification × 11,000 for all photographs.) (D) Percentage of apoptosis quantified by flow cytometry at different culture times. Data are reported as the mean ± standard deviation of four separate experiments performed in duplicate.

stitutively expressing tat/S but did not affect Bcl-2 promoter function in Jurkat cells stably transfected with the control vector (pRPneo-SL3) alone (Fig 3). These results also suggest that tat, constitutively expressed by the cells, specifically transactivates Bcl-2 promoter.

Effect of Tat on the endogenous Bcl-2 gene expression and apoptosis induced by serum withdrawal. An artificial Bcl-2 promoter-CAT fusion construct differs from the endogenous Bcl-2 gene with respect to the flanking DNA sequences, the RNA transcript, and possibly the chromatin structure. Therefore, in this group of experiments the effect of Tat on the steady-state levels of endogenous Bcl-2 mRNA (Fig 4A through C) and protein (Fig 5A through C) was evaluated after 48 hours of culture in RPMI plus 0.5% FCS. A combination of techniques was used for this purpose: dot

blot (Fig 4A and B) and RNase protection (Fig 4C) for the study of mRNA; Western blot (Fig 5A and B) and indirect immunofluorescence staining shown by flow cytometry (Fig 5C) for the study of Bcl-2 protein. Both endogenous Bcl-2 mRNA and protein levels were constantly higher in Jurkat tat/S than in Jurkat tat ^{22}/S or Jurkat transfected with the pRPneo-SL3 control vector.

The upregulation of Bcl-2 gene expression was reflected by the inhibition of apoptosis in cells cultured in RPMI plus 0.5% FCS for several days (Fig 6A through D). Jurkat cells stably transfected with tat/S showed a low level of apoptosis also after 4 days of culture (<10%). Conversely, cells transfected with tat ^{22}/S or with the control vector showed a progressive increase of apoptosis. These results suggest that the Tat-induced upregulation of endogenous Bcl-2 gene ex-
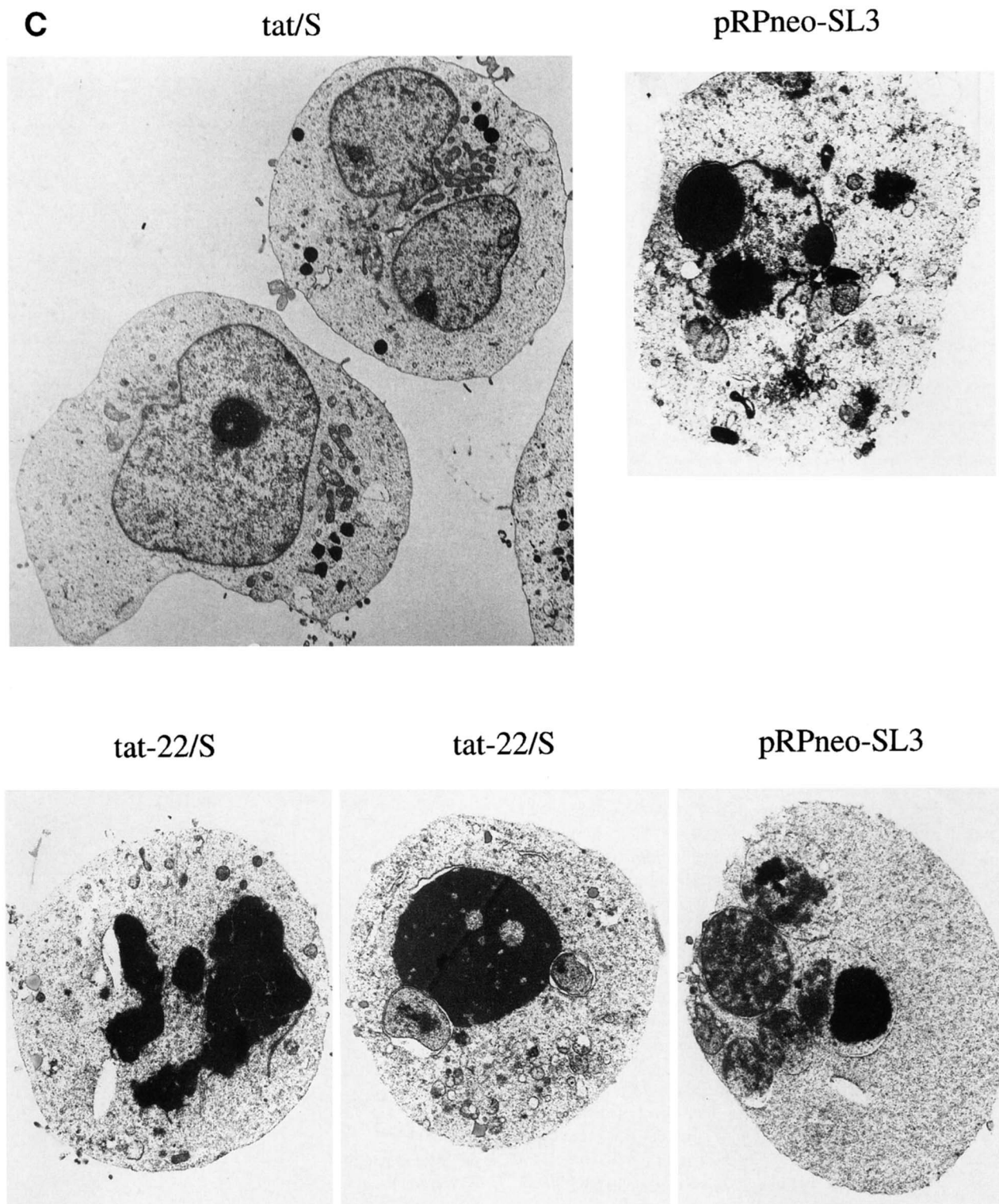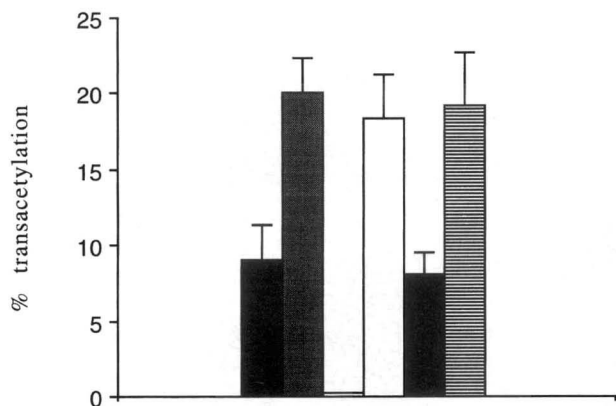
Fig 6. (Cont'd).

Fig 7. Upregulation of the Bcl-2 promoter activity by extracellular Tat. Jurkat were cultured in the presence of picomolar (5 ng/mL) concentrations of recombinant Tat or 50% serum-free PC12-tat culture medium (CM). The stimulating effect of native Tat was specifically blocked by the addition in culture of 5 μg of anti-Tat MoAb but not of 5 μg anti-p24 control antibody. Bcl-2 promoter activity was measured as the percentage of transacetylation in a CAT assay. Data are reported as the mean ± standard deviation of three independent transfection experiments performed in duplicate. (■) Jurkat; (■) Jurkat + recombinant Tat (5 ng/mL); (□) Jurkat + PC12-tat CM; (■) Jurkat + PC12-tat CM + anti-Tat MoAb; (▨) Jurkat + PC12-tat CM + anti-p24 MoAb.

pression protects the cells from apoptotic death. Moreover, it appears that the mutation in the 22cys-22gly (tat [22]/S) inactivates Tat transactivation of Bcl-2 gene expression, abolishing its ability to protect Jurkat cells from apoptosis.

Effect of extracellular Tat on the Bcl-2 promoter. It has been shown that Tat secreted by acutely infected cells or tat-transfected cells[10-12] can be taken up by neighbouring cells and transactivate HIV-1 LTR[39-41] or heterologous cellular genes[25] in bystander cells. To test the effect of extracellular Tat on Bcl-2 promoter activity, we added either native or recombinant Tat protein to Jurkat cells transiently transfected with P1+P2 Bcl-2Pr/CAT (Fig 7). It has been shown that tat-transfected cells are able to secrete a number of cytokines in culture.[22-25] To minimize the possible interference of human cytokines on the transactivation activity of the Bcl-2 promoter, as a source of native Tat protein we used the supernatant of stably tat-transfected rat PC12 cells.[12] Picomolar concentrations (5 to 10 ng/mL) of both native and recombinant Tat protein significantly increased P1+P2 Bcl-2Pr/CAT activity in Jurkat cells, although to a lower extent (2-fold) than endogenous Tat (5-fold, Fig 1C). Pretreatment of PC12-tat supernatant with optimal (5 μg/mL) concentrations of monoclonal anti-Tat antibody raised against an N-terminal peptide of Tat protein neutralized Tat transactivating activity on Bcl-2 promoters (Fig 7). The effect was specific because an MoAb to p24 did not inhibit Tat function. These results indicate that also extracellular Tat upregulates Bcl-2 gene promoters.
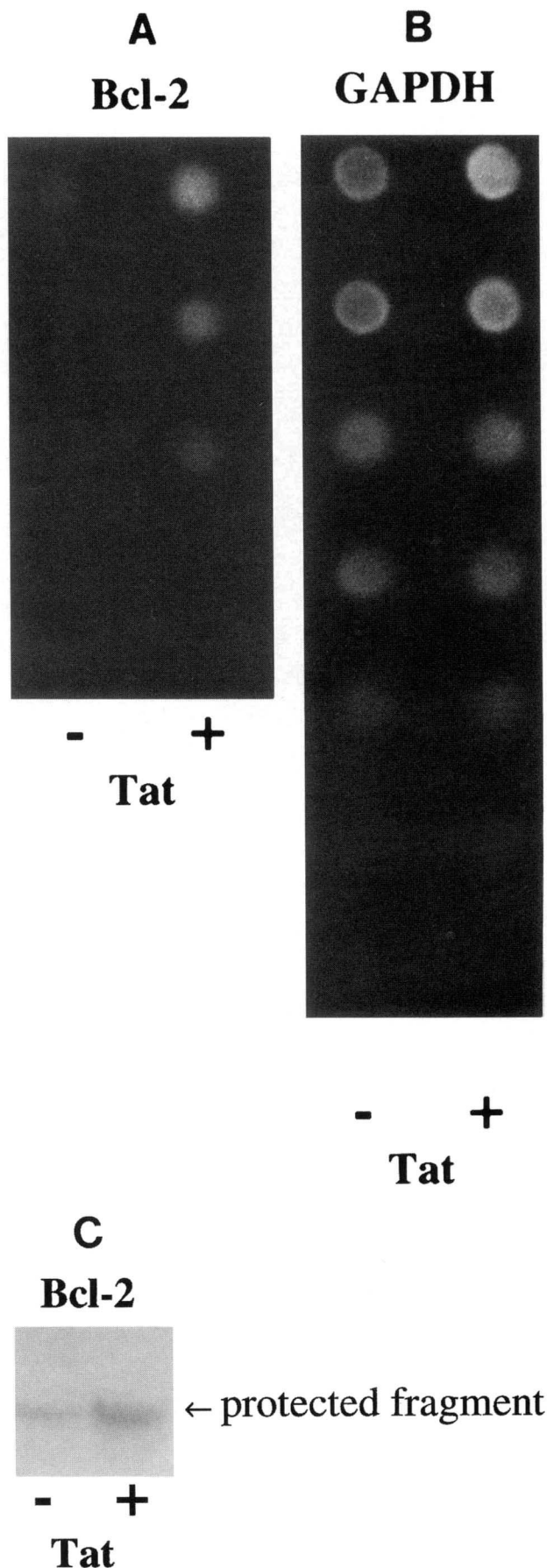
Fig 8. Upregulation of Bcl-2 RNA in PBMCs cultured in the presence of 5 ng/mL of recombinant Tat. Dot blot analysis of (A) Bcl-2 and (B) GAPDH and RNase protection assay (C) of Bcl-2 were performed after 48 hours of culture in RPMI plus 0.5% FCS ± Tat. One representative experiment of three separate experiments is shown.
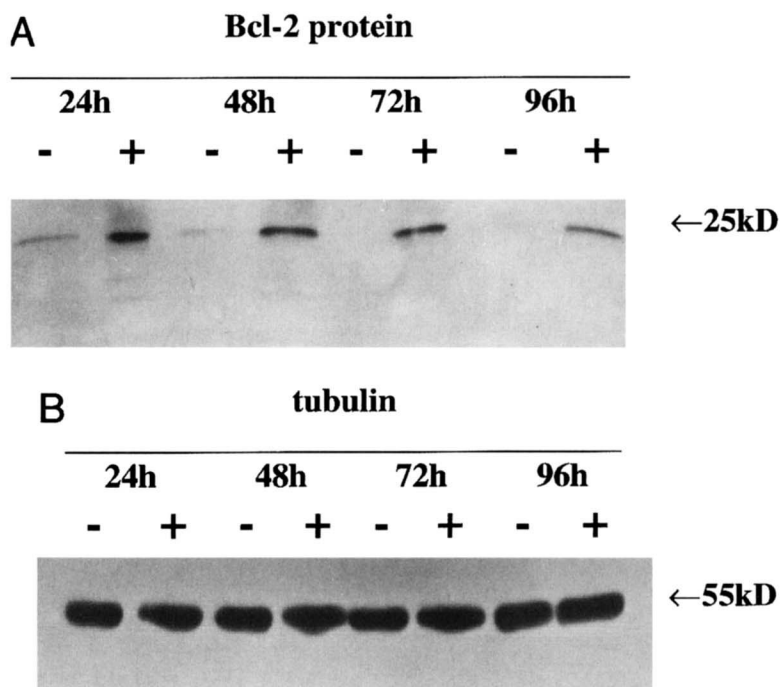

← protected fragment

Fig 9. Upregulation of Bcl-2 protein in PBMCs cultured in the presence of 5 ng/mL of recombinant Tat was accompanied by a significant protection from apoptosis. Western blot analysis of (A) Bcl-2 and (B) tubulin proteins, performed after 24 to 96 hours of culture in RPMI plus 0.5% FCS ± Tat. (C) Analysis of Bcl-2 protein performed by indirect (anti–Bcl-2 + GAM-FITC) immunofluorescence and shown by flow cytometry after 48 hours of culture in RPMI plus 0.5% FCS ± Tat. Horizontal axis, relative Bcl-2 expression detected by fluorescence intensity (logarithmic scale). Vertical axis, relative number of cells. Wright areas represent cells treated with anti–Bcl-2 + GAM-FITC, whereas left areas are negative controls (cells treated with anti-p66 of HCV + GAM-FITC). (D) Percentage of apoptosis quantified by flow cytometry at different culture times. Data are reported as the mean ± standard deviation of four separate experiments performed in duplicate.

Effect of extracellular Tat on the endogenous Bcl-2 gene expression in PBMCs. Normal T cells and malignant Jurkat T cells differ in growth control and requirement for growth factors. Therefore, the expression of endogenous Bcl-2 gene was also evaluated in PBMCs cultured in low serum (0.5% FCS) in the absence or presence of 5 ng/mL recombinant Tat. We chose to investigate the effect of Tat on Bcl-2 gene expression in unfractionated PBMCs rather than in purified lymphocytes to mimic more closely the physiologic in vivo situation in which lymphocytes interact with antigen-presenting cells and Tat exerts its effects in a complex contest of cell-to-cell interactions and network of cytokines.

Bcl-2 mRNA (Fig 8A through C) and protein (Fig 9A through C) were examined at different culture times (24 to 96 hours or 1 to 4 days). Whereas Bcl-2 expression was progressively downregulated in PBMCs cultured in in RPMI + 0.5% FCS, the addition of 5 ng/mL of Tat induced a sustained expression of Bcl-2 RNA and protein that was clearly detectable also after 4 days of culture. The prolonged expression of Bcl-2 protein in PBMCs cultured in the presence of Tat was reflected by a significantly ($P < .01$) lower level of apoptosis from day 2 of culture onwards (Fig 9D). Similar results were obtained when 5 ng/mL of Tat was added to Jurkat cells (data not shown). From these data we concluded that extracellular Tat affects Bcl-2 gene expression also in primary PBMC cultures, resulting in inhibition of programmed cell death.

DISCUSSION

Bcl-2 oncoprotein was first identified as a result of its involvement in the t(14;18) translocation that is frequently associated with human follicular B-cell lymphoma.[26] Characterization of its genomic organization showed that Bcl-2 consists of three exons,[30,31] whereas two promoters are responsible for the initiation of Bcl-2 transcription. Bcl-2 expression is tissue-specific[42] and developmentally regulated,[43] appearing at sites characterized by a high rate of apoptotic cell death. In the lymphoid compartment, Bcl-2 is upregulated in resting mature T and B cells, whereas it is downregulated in the majority of proliferating B cells of the germinal centers[42] and in a significant fraction of circulating T cells.[44] The relatively long half life of Bcl-2 mRNA (3 to 4 hours) and protein (>10 hours)[43] accounts for the quite long period required for a complete downregulation of Bcl-2 expression in lymphoid cells. Bcl-2 belongs to a family of proteins that can form homodimers and heterodimers and participate as either repressors or inducers of programmed cell death.[45-48] Thus, regulation of apoptosis in lymphocytes is complex and modulated by the interaction of several gene products.

The data presented here show that the transactivator Tat protein of HIV-1 upregulates Bcl-2 gene expression in both transformed Jurkat T cells and primary PBMCs. The effects of Tat on promoter activity, mRNA expression, and protein synthesis suggest that Bcl-2 upregulation was primarily caused at the transcriptional level. No significant differences were observed between the constitutive and transient effects of Tat on T cells. Of note, a single mutation in the cys[22] that is critical for the formation of intramolecular disulphide bonds[4] completely abolished the transactivating activity of Tat on either HIV-1 LTR or the heterologous Bcl-2 promoter, showing that the structural integrity of the activating domain of Tat is absolutely required for both functions.

The molecular mechanism of Tat function on Bcl-2 expression remains to be fully elucidated. The structure of Bcl-2 5′ untranslated region contains two distinct promoters. The predominant GC-rich promoter, P1, displays multiple start
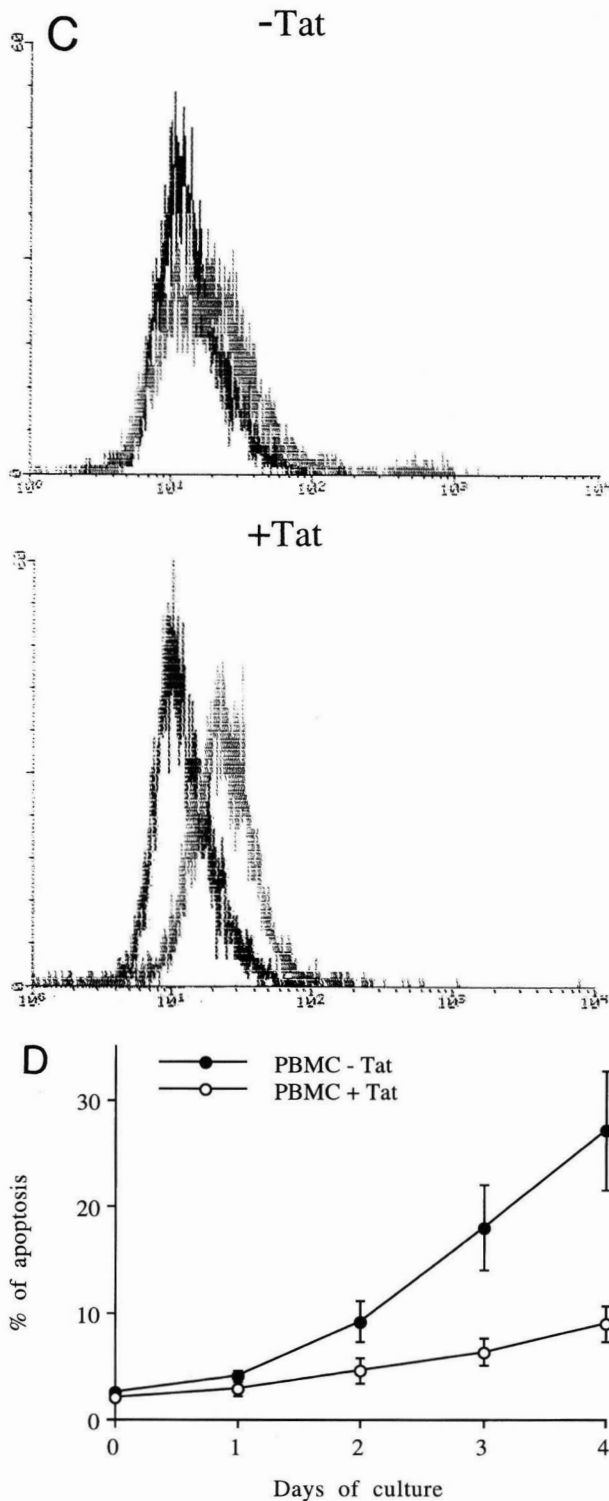
Fig 9. (Cont'd).

discrete initiation sites and an octamer motif.[30,31] Because it has been shown that Tat can directly bind to SP1[49] and to the TATA binding protein TFIID,[50] the Tat-mediated upregulation of Bcl-2 gene expression might result from a specific interaction of Tat with these two basal transcription factors. However, it is also possible that Tat upregulates Bcl-2 expression indirectly through the release of stimulatory cytokines, such as IL-2 or IL-6.[24,25] However, the findings that the human Bcl-2Pr/CAT was upregulated (G. Zauli, unpublished observations) also when transfected in a rat pheochromocytoma PC12 cell line stably expressing tat[12] renders this last hypothesis unlikely.

The endogenous expression of Tat in transfected Jurkat T cells mimicks the condition of acutely HIV-1–infected cells. Nevertheless, this might not correctly represent the pathologic situation in vivo in which extracellular Tat could play a more relevant role in promoting cell survival of bystander uninfected cells. In this respect, exogenous recombinant or native Tat was also able to upregulate Bcl-2 expression, probably as a consequence of cellular uptake and direct targeting of Tat to the nucleus.[8,9] However, the transactivation effect of extracellular Tat on Bcl-2 promoter may also depend on specific membrane interactions of Tat with integrin receptors.[18,19,51,52] Combinations of these two mechanisms are also possible. It is conceivable that extracellular Tat is concentrated in lymphoid tissues at sites at which HIV-1 replication is active also during the clinically latent period of HIV-1 infection.[53] A close contact of cells at such sites might induce uptake of Tat by surrounding cells that are particularly efficient. In this respect, it is noteworthy that the upregulation of Bcl-2 expression in PBMCs was achieved with very low (picomolar) concentrations of recombinant Tat. Similar concentrations of Tat have been detected in the supernatant of HIV-1–infected or tat-transfected cells.[10-12]

A number of reports have shown that nanomolar concentrations (1 to 10 μg/mL) of recombinant Tat inhibit the antigen-dependent proliferation of isolated T lymphocytes,[13-15] possibly through an inappropriate interaction of the basic region of Tat with the CD26 surface antigen[15] or through the release of inhibitory cytokines.[22,23] Of note, these inhibitory effects on T-cell proliferation are not detectable in in the presence of monocytes.[54] While this report was in press, Li et al[55] reported that 60 to 300 nmol/L of recombinant Tat or endogenously expressed Tat may induce apoptosis in both primary PBMC and Jurkat T-cell lines. However, these findings are unlikely to have a physiologic significance in vivo because extremely high concentrations of recombinant Tat were used. Moreover, it is difficult to reconciliate these data with the original work of Caputo et al[56] showing that the same Jurkat-tat cell lines used in Li et al's study had a growth advantage as well as an increased resistence to apoptosis[17] with respect to either parental Jurkat cells or Jurkat cells transfected with the control vector. Altogether, these data suggest that Tat influences lymphoid cell survival and proliferation in a complex way, mainly depending on the concentrations of Tat reached intracellularly and/or extracellularly and the presence or absence of accessory cells.

Finally, it is tempting to speculate that upregulation of

sites and includes seven consensus binding sites for the SP1 transcription factor. The second promoter, P2, is located approximately 1.3 kb downstream from the first. It includes both a CCAAT box and a TATA element and displays two

Bcl-2 expression achieved in primary PBMCs at picomolar concentrations of extracellular Tat might be relevant also to the pathogenesis of the frequent neoplastic disorders present in HIV-1–seropositive individuals. For instance, a subset of Epstein-Barr virus (EBV)-negative large-cell lymphomas in acquired immunodeficiency syndrome (AIDS) patients are polyclonal, and an orderly evolution of the neoplasm from a polyclonal lymphoproliferation to a monoclonal process has been appreciated.[57] These polyclonal lymphomas often contain HIV-1–infected macrophages or macrophage-like cells, which are believed to play a key role in tumorigenesis. Thus, Tat protein could participate to the polyclonal lympho-proliferation that anticipates monoclonal transformation in several ways. Endogenous Tat might contribute to the im-mortalization of HIV-1–infected macrophages upregulating Bcl-2 expression and stimulating the release of inflammatory cytokines[22-25] by HIV-1–infected cells. In addition, actively released extracellular Tat might directly interfere with the survival of neighbouring B and T uninfected lymphocytes in the germinal centers, which are normally characterized by extensive apoptotic cell death.[42]

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