

## Interaction of Retroviral Tax Oncoproteins With Tristetraprolin and Regulation of Tumor Necrosis Factor- $\alpha$ Expression

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**Background:** The Tax oncoproteins are transcriptional regulators of viral expression involved in pathogenesis induced by complex leukemogenic retroviruses (or delta-retroviruses, i.e., primate T-cell leukemia viruses and bovine leukemia virus). To better understand the molecular pathways leading to cell transformation, we aimed to identify cellular proteins interacting with Tax. **Methods:** We used a yeast two-hybrid system to identify interacting cellular proteins. Interactions between Tax and candidate interacting cellular proteins were confirmed by glutathione S-transferase (GST) pulldown assays, co-immunoprecipitation, and confocal microscopy. Functional interactions between Tax and one interacting protein, tristetraprolin (TTP), were assessed by analyzing the expression of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), which is regulated by TTP, in mammalian cells (HeLa, D17, HEK 293, and RAW 264.7) transiently transfected with combinations of intact and mutant Tax and TTP. **Results:** We obtained seven interacting cellular proteins, of which one, TTP, was further characterized. Tax and TTP were found to interact specifically through their respective carboxyl-terminal domains. The proteins colocalized in the cytoplasm in a region surrounding the nucleus of HeLa cells. Furthermore, coexpression of Tax was associated with nuclear accumulation of TTP. TTP is an immediate-early protein that inhibits expression of TNF- $\alpha$  at the post-transcriptional level. Expression of Tax reverted this inhibition, both in transient transfection experiments and in stably transfected macrophage cell lines.

**Conclusion:** Tax, through its interactions with the TTP repressor, indirectly increases TNF- $\alpha$  expression. This observation is of importance for the cell transformation process induced by leukemogenic retroviruses, because TNF- $\alpha$  overexpression plays a central role in pathogenesis. [J Natl Cancer Inst 2003;95:1846–59]

The primate T-cell lymphotropic viruses (PTLV-1, -2, and -3) and bovine leukemia virus (BLV) are members of the *Delta-retrovirus* genus of the *Retroviridae* family (1,2). These viruses infect either T or B lymphocytes and can lead to hematologic or

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neurologic disorders (3,4). The prototype virus, human T-lymphotropic virus type 1 (HTLV-1), propagates in CD4 or CD8 T cells and, after extended latency periods of 30–50 years, causes a rapidly fatal leukemia called adult T-cell leukemia/lymphoma (ATL). Infection with HTLV-1 is also associated with a degenerative neuromuscular disease referred to as tropical spastic paraparesis or HTLV-1-associated myelopathy (5). Infection of cattle with BLV leads to diverse lymphoproliferative manifestations, including tumors (lymphoma and lymphosarcoma) and/or persistent lymphocytosis, which is characterized by a stable increase in the number of circulating B cells. Another member of this group of viruses, HTLV-2, does not appear to be pathogenic, at least in non-immunosuppressed individuals. Infection with BLV or HTLV-1 results in the coordinate expression of several cellular genes, including those for various cytokines, such as interleukin 2 (IL-2) (6–8), IL-6 (9,10), granulocyte-macrophage colony-stimulating factor (11), and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (12–14). This aberrant expression of cytokines is thought to affect viral spread and disease progression. For instance, TNF- $\alpha$  plays a critical role in the physiology of HTLV-1-associated myelopathy by impairing the ability of astrocytes to take up extracellular glutamate. This deregulation of glutamate levels may affect the functions and survival of neurons and oligodendrocytes (15).

The mechanisms that underlie the process of leukemogenesis caused by complex retroviruses are still not clearly understood. Increasing evidence, however, supports an essential role for the tax gene. Tax protein is a transcriptional activator of cellular gene expression and is required for viral replication *in vivo*. Tax positively regulates transcription of viral genes and also activates transcription of several cellular genes, including IL-2 and its receptor, Bcl-xL, proliferating cell nuclear antigen (PCNA), vimentin, TNF- $\alpha$ , Krox 24, interferon  $\gamma$ , and transforming growth factor (TGF)- $\beta$  [reviewed in (1)]. The cellular metabolic pathways involved in gene regulation by Tax include cyclic adenosine monophosphate (cAMP) response element binding protein (CREB), nuclear factor  $\kappa$ B (NF- $\kappa$ B), and the serum responsive factor (SRF) transcription factors. Tax binds physically to members of the CREB/activation transcription factor family, thereby increasing their affinity for the cAMP response site [reviewed in (1)]. Tax also modulates the general transcription machinery via the p300/CREB binding protein (CBP) and p300/CBP-associated factor acetyltransferases (16–22). Activation of NF- $\kappa$ B-responsive genes by Tax is mediated by the interaction of Tax with the inhibitor of NF- $\kappa$ B (I $\kappa$ B) kinase (23–26), phosphorylation-dependent degradation of the I $\kappa$ B inhibitor, and subsequent release of NF- $\kappa$ B into the nucleus. Conversely, when transiently overexpressed, Tax also acts as a transcriptional repressor of bax (27), p53 (28), cyclins A and D3 (29), and  $\alpha$ - and  $\beta$ -polymerases (30), possibly interfering with apoptosis and gene stability. Besides these activities, Tax is able to promote and accelerate entry into the cell cycle by activating cyclin-dependent kinases via its interaction with cyclin D3 (31–34). Tax modulation of the cell cycle also occurs through at least two other mechanisms: 1) Tax activates the promoter of the E2F transcription factor and 2) Tax inhibits the p16<sup>INK</sup> cyclin-dependent kinase repressor (35,36). Overexpression of Tax in cells leads to various chromosomal abnormalities resulting from DNA damage (37–39), inhibition of nucleotide excision repair (40), and abrogation of the mitotic spindle assembly checkpoint (41), all of which are thought to be essential steps in the

leukemogenic process. Finally, the oncogenic capacity of Tax has been revealed by a series of transformation assays using primary fibroblasts (42–44), rodent cell lines, blood lymphocytes (45–47), and transgenic mice (48–51).

Together, these results describe a complex interconnection of pathways potentially regulated by Tax and support a key role of this viral protein during the leukemogenic process. To further dissect the mechanisms associated with transformation, we aimed to identify cellular proteins interacting with Tax. We report here the results of a genetic screen allowing the identification of the interacting protein tristetrapirolin (TTP), a repressor of TNF- $\alpha$  expression.

## MATERIALS AND METHODS

### Vector Constructs

Plasmid pBDTax was constructed by polymerase chain reaction (PCR) amplification of the BLV tax gene using primers 5'-TTTGTGCGACTAATGGCAAGTGTGTTGGTGG-3' and 5'-TTTCTGCAGTCAAAAAGGCCGGGAGAGCC-3'. The tax amplicon was cloned into vector pBDGal4 (Stratagene, Amsterdam, The Netherlands), which contains the DNA binding domain of the yeast transactivator Gal4. Used as controls, plasmids p53 and pLaminC (Stratagene) express the DNA binding domain of Gal4 fused to amino acids 72–390 of murine p53 and amino acids 67–230 of human Lamin C, respectively. Plasmid pSV40, which encodes a fusion protein of the Gal4-activating domain and region 84–708 of the SV40 large T antigen, was used as positive control. Plasmid pGexTax was obtained by PCR amplification of the BLV tax gene with the primers 5'-TTTGGATCCATGGCAAGTGTGTTGGTGG-3' and 5'-TTTCTGCAGTCAAAAAGGCCGGGAGAGCC-3'; the amplicon was subsequently inserted into pGex-2T downstream of the glutathione S-transferase (GST) sequences (Amersham Biosciences, Roosendaal, The Netherlands). Plasmid pGexTax1 (provided by F. Bex, Université Libre de Bruxelles, Brussels, Belgium) encodes the HTLV Tax-1 protein fused to GST.

The ovine TTP cDNA was obtained by reverse transcription of total RNA isolated from sheep peripheral blood mononuclear cells by using TRIzol and the SuperScript Preamplification System protocol (Invitrogen, Merelbeke, Belgium). TTP cDNA was amplified by 36 cycles of PCR using the Pwo enzyme (Roche, Brussels, Belgium) and the primers 5'-ACATCCTTCGGCCATCTGC-3' and 5'-AGCTGATCTGTACTAGGCAG-3'. Vectors pSGTTP and pEGFPTTP were constructed by subcloning the TTP amplicon into pSG5 (Stratagene) and pEGFP-C1 (Clontech, Erembodegem, Belgium), respectively. Plasmids hTTP(1–230), hTTP(1–206), and hTTP(1–173) expressing influenza virus hemagglutinin-tagged mutants of human TTP were derived from the wild-type hTTP vector by 3' end deletion of the TTP open reading frame at codons 230, 206, and 173, respectively. Plasmids pSGTax (43) and pTax1/pCMVTax/pIEXTax (provided by F. Bex, E. Wattel [Centre Léon Bérard, Lyon, France], and K. T. Jeang [National Institutes of Health, Bethesda, MD]) are mammalian expression vectors for BLV Tax and HTLV Tax-1 proteins, respectively. A series of plasmids containing BLV tax mutants and variants were used: pSGTax2XAsp, pSGTax106+293, pSGSer76, pSGSer103, pSGSer110, pSGSer264, pSGTyr1–2 (52), pDSPLOR, pDSPLORBglI, pDSPLOR $\Delta$ Ball-NdeI, pDSPLOR $\Delta$ SmaIRI, pD-

SPLORStuI (53), pSGTax395, pSGRex (54), pSGTaxM1–5 (55), pSG2.1 expressing both Tax and Rex, pSGTaxPT (which corresponds to exons 1 and 3 of the tax cDNA cloned into pSG5), and pSGTax(1–228) (which derives from pSGTax after digestion of the tax open reading frame with *EcoRI* and ligation). Vectors coding for HTLV Tax-1 mutants were pTax2–58, pTax3–6, pTax3–8, pTax105–114, pTax94–105, pTax94–114, pTax284–353, pTax337–353 (these plasmids encode Tax1 mutants harboring deletions between indicated residues); pTax313 (in which threonine codon 313 of the tax gene was replaced by an alanine; provided by K. T. Jeang); pTax300–301 (expressing a Tax protein mutated in serines 300 and 301, the two major phosphorylation sites); pG148V (obtained from F. Bex); pM22; and pM47 (encoding Tax proteins that are selectively able to activate gene expression via the CREB/ATF and NF- $\kappa$ B pathways, respectively; provided by E. Wattel). pSGTax1(1–244), which expresses a COOH-truncated mutant, was made by deletion of the tax1 cDNA region encoding residues between 244 and 353. Reporter plasmids pLTRCAT (43), pLTR1CAT, pRSVCAT, and p3XTRECAT (provided by D. Derse, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD) harbor, respectively, the BLV long terminal repeat (LTR), the HTLV-1 LTR, the RSV (Rous Sarcoma Virus) promoter, and three repeats of the 12-*O*-tetradecanoylphorbol-13-acetate (TPA) response element sequences cloned upstream of the chloramphenicol acetyltransferase (CAT) gene. Vector pEGFPMSX contains the human Msx2 cDNA cloned into the pEGFP-C1 plasmid (Clontech). In plasmid CMV3'TNF, the CAT gene is driven by the cytomegalovirus (CMV) promoter and is controlled by the 3' untranslated region (UTR) of the TNF- $\alpha$  mRNA. Plasmid CMV3'TNFUA– is the corresponding control plasmid lacking the AU-rich element of the UTR (56).

### Yeast Two-Hybrid System

This technique is based on the coexpression of two hybrid proteins, one containing Tax fused to the yeast Gal4 binding domain and the other corresponding to a cellular protein linked to the Gal4 activation domain. Interaction between Tax and a cellular protein creates a functional Gal4 transcription factor whose activity can be selected and quantified.

A human placenta cDNA library was inserted into vector pACT2 (Clontech) downstream of the yeast Gal4 activation domain and transformed using the LiAc/polyethylene glycol protocol into yeast PJ696 MAT $\alpha$  (genotypes *trp1*–901, *leu2*–3, 112, *ura3*–52, *his3*–200, *gal4* $\Delta$ , *gal80* $\Delta$ , *GAL2*-ADE2, *LYS2::GAL1-HIS3*, and *met2::GAL7-lac*; details on the strain are available from Clontech and Stratagene). Yeast cells were plated onto synthetic dropout medium lacking leucine, scraped, counted, and stored at  $-70^{\circ}\text{C}$ . PJ696 MAT $\alpha$  yeast, which are PJ696 MAT $\alpha$  yeast containing the URA3 YE $\rho$ 50 vector (Stratagene), were transfected with plasmid pBDTax and plated onto synthetic dropout medium lacking tryptophan and uracil to select for the bait plasmid and the mating phenotype. PJ696 MAT $\alpha$  cells transformed with the cDNA library were mated with PJ696 MAT $\alpha$  yeast containing pBDTax in YPAD medium (1% bacto-yeast extract, 2% bacto-peptone, 2% glucose, and 0.6% adenine) for 4 hours. Cells were transferred to synthetic dropout solid medium lacking uracil, leucine, tryptophan, and histidine and supplemented with 2 mM 3-amino-1,2,4-triazole (Sigma, Bornem,

Belgium) to reduce background of histidine selection. Five days after mating, colonies were assayed for  $\beta$ -galactosidase expression. To this end, colonies were replicated on Whatman filters, grown overnight in YPAD medium, lysed in liquid nitrogen, and incubated in Z buffer (60 mM Na<sub>2</sub>HPO<sub>4</sub>, 40 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM KCl, 1 mM Mg<sub>2</sub>SO<sub>4</sub>) containing 0.33 mg/mL of the  $\beta$ -galactosidase substrate X-gal (Eurogentec, Seraing, Belgium). Positive colonies were cultivated in synthetic dropout liquid medium lacking uracil, leucine, tryptophan, and histidine, and plasmid DNA was isolated and transformed by electroporation into *Escherichia coli* HB101. Bacteria were then plated into M9 minimal medium containing dropout nutrients but lacking leucine (57). The plasmids were extracted by using Qiagen columns (Qiagen Westburg, Leusden, The Netherlands), and the inserts were sequenced.

### Quantification of $\beta$ -Galactosidase Activity

Cultures were grown overnight in synthetic dropout medium lacking uracil, leucine, tryptophan, and histidine, diluted fivefold in YPAD medium, and incubated until the optical density at 600 nm (OD<sub>600</sub>) reached 0.5–0.8. After one wash in Z buffer, the yeast cells were lysed by three freeze–thaw cycles using liquid nitrogen. Cell lysates were then resuspended in 0.7 mL of Z buffer containing 0.27%  $\beta$ -mercaptoethanol and 160  $\mu\text{L}$  of ortho-nitrophenyl- $\beta$ -D-galactopyranoside at 4 mg/mL in Z buffer, as a substrate of  $\beta$ -galactosidase. After a 1-hour incubation at 30  $^{\circ}\text{C}$ , the reactions were stopped by the addition of 400  $\mu\text{L}$  of 1 M Na<sub>2</sub>CO<sub>3</sub>, and the OD<sub>420</sub> was determined.

### GST Pulldown Assay

The prokaryotic expression plasmids pGexTax, pGexTax1, and pGex-2T were transformed into the HB101 strain of *E. coli*. Overnight cultures were diluted sixfold in fresh NZY medium (Invitrogen) containing ampicillin at 100  $\mu\text{g}/\text{mL}$  and incubated until the OD<sub>600</sub> reached 0.7. After induction with 1 mM isopropylthiogalactoside, bacteria were allowed to grow for an additional 3 hours. Bacteria were then harvested, washed with phosphate-buffered saline (PBS), and lysed by sonication and by incubation for 3 hours at 4  $^{\circ}\text{C}$  in the presence of 1% Triton X-100. After centrifugation at 10 000g for 15 minutes at 4  $^{\circ}\text{C}$ , the supernatant was mixed with glutathione-Sepharose beads (Amersham Biosciences) for 1 hour at 4  $^{\circ}\text{C}$ . Finally, the beads were washed four times with PBS and stored at 4  $^{\circ}\text{C}$  as a 15% suspension in the presence of a mixture of protease inhibitors (Complete; Roche). The amount of GSTTax, GSTTax1, or GST polypeptides bound to the beads was quantified using the Bio-Rad assay (Bio-Rad, Nazareth, Belgium). For the GST pulldown assay, equal amounts of fusion proteins were added to 5  $\mu\text{L}$  of rabbit reticulocyte lysates (*in vitro* transcription–translation; Promega, Leiden, The Netherlands) programmed with pS-GTTP plasmid in the presence of a mixture of <sup>35</sup>S-methionine and <sup>35</sup>[S]-cysteine (Promix; Amersham Biosciences). After gentle shaking for 3 hours at 4  $^{\circ}\text{C}$  in NETN binding buffer (200 mM NaCl, 20 mM Tris–HCl [pH 8], 1 mM EDTA) and 0.5% Nonidet P-40 [NP40]), the beads were washed four times in binding buffer. Bound proteins were eluted in sodium dodecyl sulfate (SDS) sample buffer, resolved on a 12.5% SDS–polyacrylamide gel, soaked in amplifying solution (Enlightening; PerkinElmer Life Sciences, Schwadorf, Austria), and visualized by autoradiography.

## Co-immunoprecipitation

HeLa–Tat cells (provided by V. Ciminale, University of Padua, Padua, Italy) were cultivated at 37 °C in a 5% CO<sub>2</sub>–air humidified atmosphere in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum, penicillin at 100 U/mL, amphotericin B at 0.25 µg/mL, streptomycin at 100 µg/mL, and 1 mM sodium pyruvate. One day before transfection, the cells were divided and seeded at a density of 3.5 × 10<sup>5</sup> to 5 × 10<sup>5</sup> cells per 10–30 cm<sup>2</sup>. The cells were next transfected with 2–3 µg of plasmid DNA by using Lipofectamine Reagent (Invitrogen) or GeneJammer (Stratagene) according to the manufacturer’s instructions.

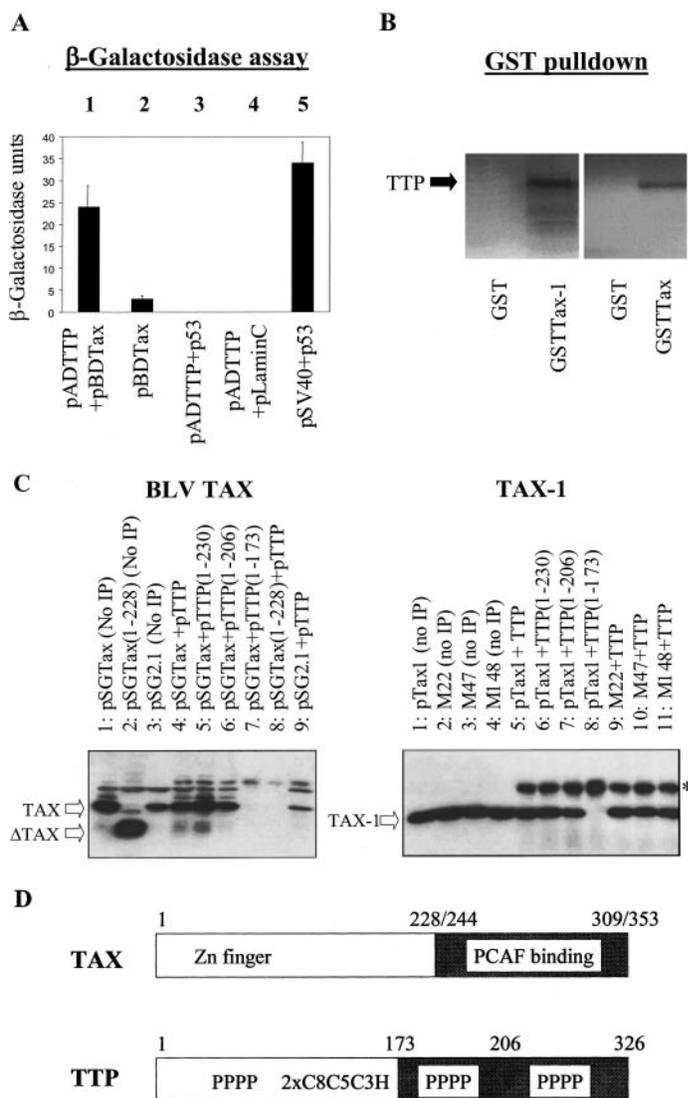
Thirty hours after transfection, the cells were washed in PBS, scraped from the dish, and lysed in NET buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris–HCl [pH 8], 0.5% NP40, and 0.25% sodium deoxycholate) containing proteolytic inhibitors (Complete; Roche). The lysates were then immunoprecipitated with rabbit anti-hemagglutinin antibody (final concentration = 0.01 µg/µL; Santa Cruz Biotechnology, Santa Cruz, CA) and with Protein A–Sepharose beads (Roche). The immunoprecipitates were washed three times in NET buffer and once in TNE (10 mM Tris–HCl [pH 8.3], 150 mM NaCl, and 1 mM EDTA) and subjected to electrophoresis on a denaturing polyacrylamide gel. After transfer of the proteins to polyvinylidene difluoride membranes, the filters were saturated with 1% Blocking Reagent (Roche), incubated overnight with anti-Tax antibodies (500-fold dilution), washed in TBST (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, and 0.1% Tween 20), and antibody binding was revealed by chemiluminescence using horseradish peroxidase–conjugated anti-mouse immunoglobulin antibody (1000-fold dilution), according to the manufacturer’s instructions (Roche).

## Confocal Microscopy

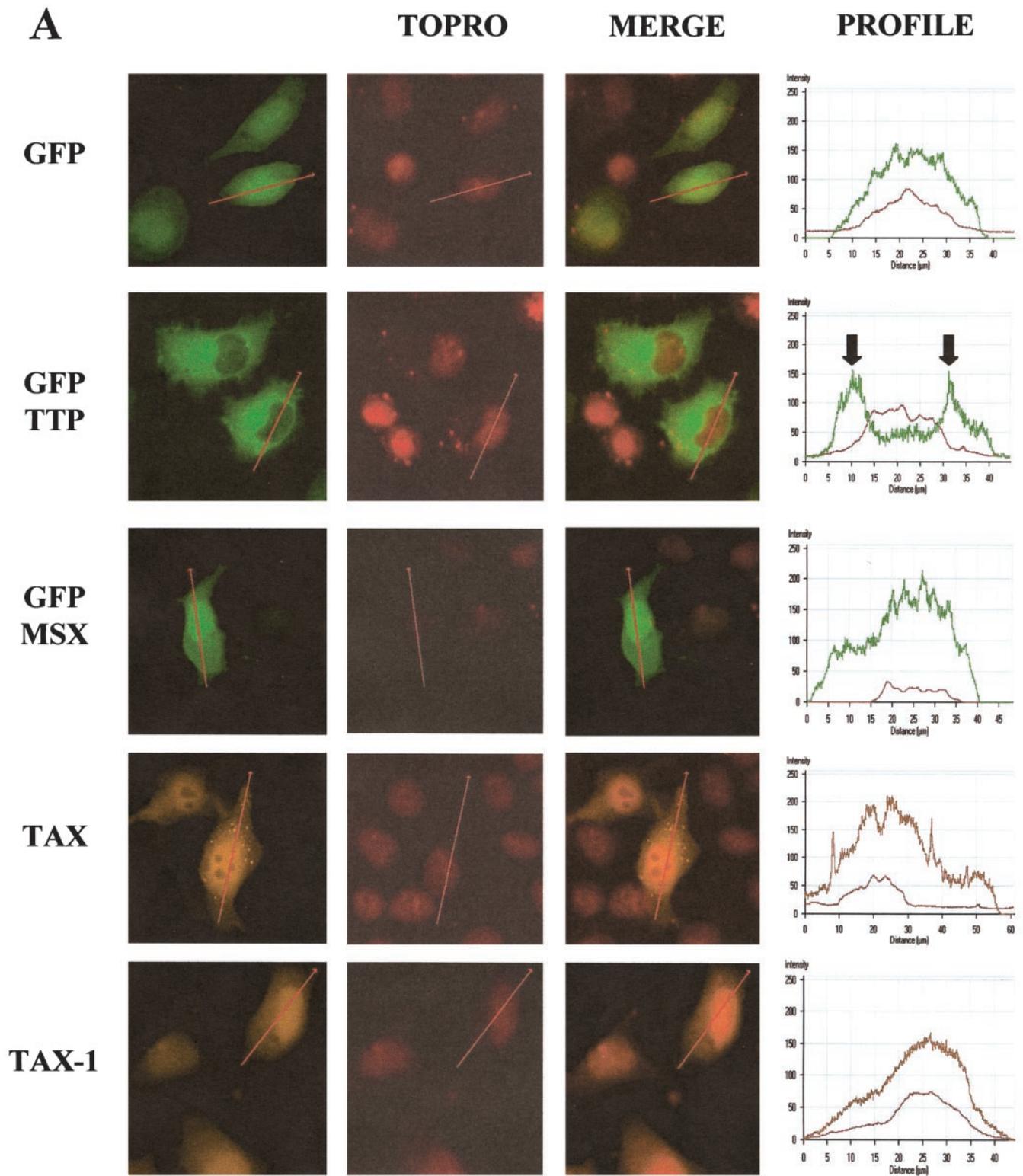
Twenty hours after transfection, 3.5 × 10<sup>5</sup> to 5 × 10<sup>5</sup> HeLa–Tat cells per 10–30 cm<sup>2</sup> were fixed in 3.7% formaldehyde (20 minutes at 4 °C), permeabilized with 0.1% NP40 (10 minutes), and incubated with anti-Tax antibodies (200-fold-diluted Tax monoKI for HTLV-1 and 5A5 monoclonal antibodies and L75 rabbit anti-Tax COOH-peptide for BLV, 1000-fold dilution) and then with Alexa 546–coupled anti-mouse or anti-rabbit immunoglobulin conjugates (1000-fold dilution; Molecular Probes, Leiden, The Netherlands) for 1 hour at 37 °C. After nuclear fixing with TOPRO-3 (a DNA intercalating fluorescent dye; Molecular Probes) and fixing with mounting medium (Prolong Antifade kit; Molecular Probes), the cells were analyzed using a Zeiss fluorescence confocal microscope (Axiovert 200 with LSM 510; Carl Zeiss Microscopy, Jena, Germany).

## CAT Assays

Two micrograms of reporter plasmids (pLTRCAT, pLTR1CAT, pRSVCAT, p3XTRECAT, CMV3’TNF, and CMV3’TNFUA–) and different amounts of effector vectors (pSGTax, pTax1, and pSGTTP) were transfected into 3 × 10<sup>5</sup> HeLa–Tat, D17, or HEK 293 cells by means of Lipofectamine Reagent. Forty-eight hours after transfection, cells were washed three times in PBS and lysed by three freeze–thaw cycles; CAT activities were then determined as described previously (55). <sup>14</sup>C–acetylated chloramphenicol migrating into the Econofluor (PerkinElmer Life Sciences) scintillation medium was counted at regular intervals by a Packard analyzer (Packard BioScience, Meriden, CT).



**Fig. 1.** Tristetraprolin (TTP) interacts with human T-lymphotropic virus 1 (HTLV-1) and bovine leukemia virus (BLV) Tax proteins in yeast and *in vitro*. **A**) PJ696 yeast cells transformed with different vectors (pADTTP, pBDTax, p53, pLaminC, and/or pSV40, as indicated) were cultivated in YPAD medium, and β-galactosidase activity in cell lysates was determined using ortho-nitrophenyl-β-D-galactopyranoside as a substrate. Data represent the mean values of three independent experiments. Error bars indicate 95% confidence intervals. **B**) TTP protein was synthesized using rabbit reticulocyte lysates programmed with pSGTTP vector in the presence of <sup>35</sup>S-labeled methionine and cysteine. Lysate was mixed with equal amounts of glutathione S-transferase (GST), GSTTax-1, or GSTTax proteins bound to glutathione-Sepharose beads. After incubation, the mixtures were subjected to electrophoresis on a 12.5% sodium dodecyl sulfate–polyacrylamide gel, and bands were revealed by autoradiography. A representative experiment of three is shown. **C**) HeLa cells were transfected with Tax and TTP expression vectors, as indicated. Twenty-four hours after transfection, cells were lysed and the TTP protein was immunoprecipitated with an influenza virus hemagglutinin–specific antibody. Protein complexes were then analyzed by western blot using anti-Tax antibodies. In lanes labeled “no IP,” the immunoprecipitation step was omitted. \* indicates nonspecific binding. ΔTax indicates the carboxyl terminal–truncated mutant of Tax. **D**) Schematic representation of the Tax and TTP domains involved in binding. PPPP are the tetraproline repeats in TTP and 2xC8C5C3H are the zinc finger–like structures in TTP. The carboxyl-terminal end of Tax has been involved in p300/CBP-associated factor (PCAF) interaction (transactivation domain). Amino acids 228/244 and 309/353 correspond to BLV/HTLV-1 Tax residues, respectively.



**Fig. 2.** Tristetraprolin (TTP) and Tax colocalize in mammalian cells, as shown by confocal microscopy. **A**) HeLa-Tat cells were transfected with pEGFPC1 (encoding green fluorescent protein; GFP), pEGFPTTP (expressing a fusion polypeptide between GFP and TTP; GFP/TTP), pEGFPMSX (Msx is another protein binding to Tax), pTax1, or pSGTax (coding for human T-lymphotropic virus 1 [HTLV-1] Tax-1 or bovine leukemia virus [BLV] Tax, respectively), as indicated. Twenty hours after transfection, cells were fixed, permeabilized, and labeled with Tax-specific antibodies and an Alexa 546-conjugated secondary antiserum. Finally, labeled cells were stained with TOPRO-3 and analyzed with

a confocal microscope (Zeiss LSM510 coupled with an Axiovert 200 microscope). Merge corresponds to the simultaneous acquisition of all three fluorochromes. **B**) The fluorescence intensities (graphs) were assessed along the **red line segments** (drawn on the photographs on left). The **green, orange, and red lines** in the profiles correspond to the relative intensities of GFP, Alexa 546, and TOPRO-3, respectively. **White arrows** indicate colocalization between Tax (BLV Tax [**top**] and HTLV-1 Tax [Tax-1; **bottom**]) and TTP. Redistribution of TTP from the cytoplasm to the nucleus in the presence of Tax is indicated by a **black arrow**. (Continued on facing page).

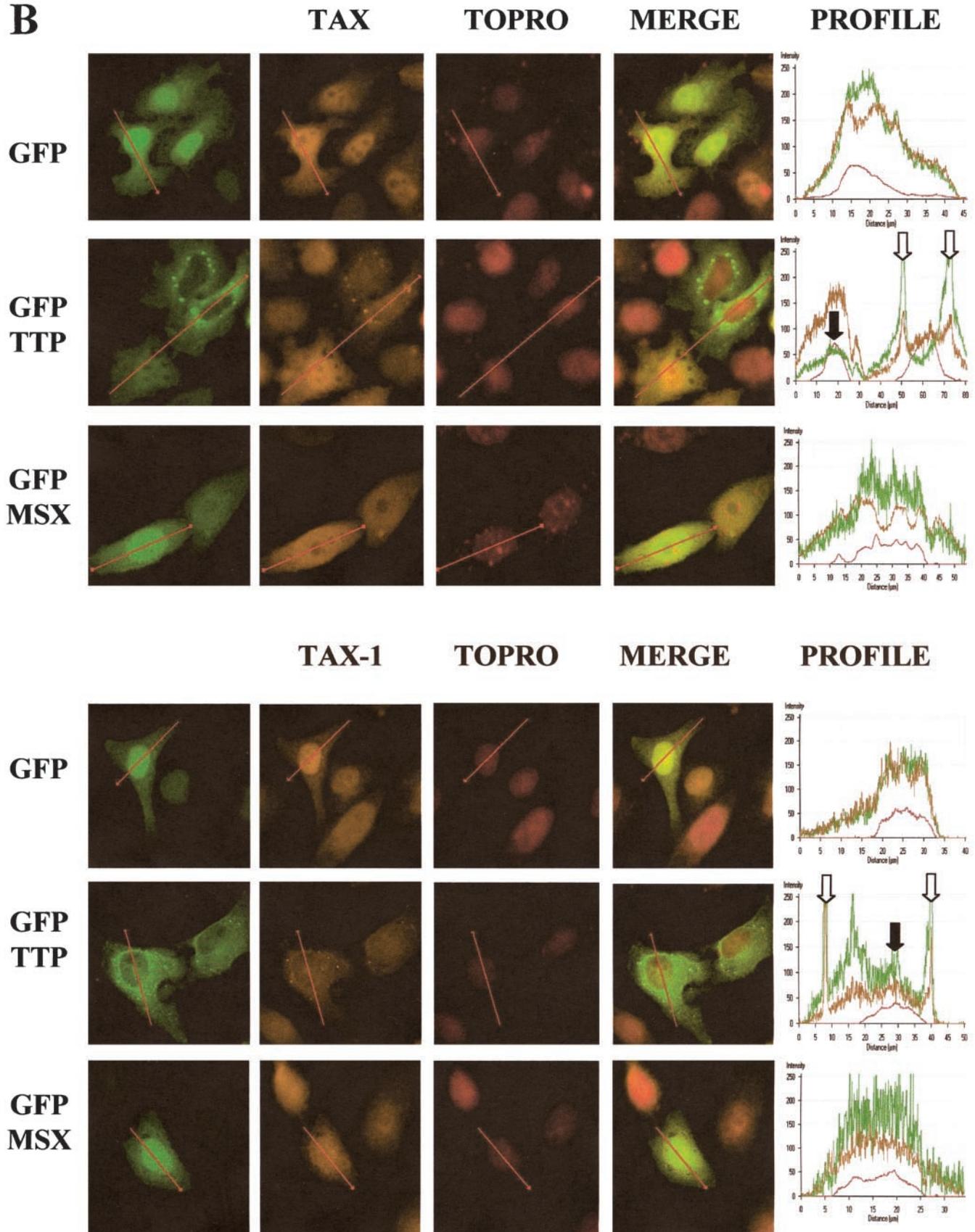
**B**

Fig. 2. (Continued from facing page).

## Titration of TNF- $\alpha$ in Macrophage Cell Lines

RAW-Tax, RAW-Tax1, RAW-TTP, RAW-Tax+TTP, RAW-Tax1+TTP, and RAW-pSG5c were derived from RAW 264.7 macrophages after cotransfection with plasmid pRc/CMV+pSGTax, pTax1, pSGTTP, pSGTax+pSGTTP, pTax1+pSGTTP or the empty vector pSG5c, respectively. Cells were transfected by means of Lipofectamine and cultured in Dulbecco's modified Eagle medium supplemented with 5% fetal calf serum, 1 mM sodium pyruvate, penicillin at 100 U/mL, and streptomycin at 100  $\mu$ g/mL. After 48 hours at 37 °C, cells were selected with 1 mg/mL G418 sulfate for 1 week, and pools of clones were further maintained in the presence of reduced concentrations of the antibiotic (0.4 mg/mL of G418 sulfate). Cells were plated at a density of  $8 \times 10^5$  cells in six-well dishes and stimulated with lipopolysaccharide (LPS at 100 ng/mL). TNF- $\alpha$  production in the culture supernatants was determined 6 hours after stimulation with an enzyme-linked immunosorbent assay (Biosource International, Nivelles, Belgium) according to the manufacturer's instructions.

## RESULTS

### Cellular Proteins Interacting With Tax

To identify cellular factors interacting with Tax, we performed a genetic screen based on the two-hybrid methodology. We used Tax as a bait (vector pBDTax) to screen a human placenta cDNA library containing cDNAs fused to the Gal4 transcriptional activation domain. After two rounds of selection and titration of  $\beta$ -galactosidase activity, Tax-binding clones ( $n = 7$ ) were isolated and sequenced. They were found to encode Tax interaction protein 1 (GenBank accession number AF028823), Msx2 (BAA06549), transducin beta chain 2 (XP 005013), human glucose transporter (K03195), pyrroline-5-carboxylate dehydrogenase (U24266), an unknown protein (KIAA0184), and human TTP (M92844). Here, we present the characterization of one of these clones, TTP, a protein already known to be involved in TNF- $\alpha$  regulation (58).

First, the strength and specificity of the interaction between Tax and TTP were estimated by titration of  $\beta$ -galactosidase activity (Fig. 1, A). Substantial activity was obtained by the coordinate action of pADTTP and pBDTax (lane 1) but not with the empty vectors alone or in combination with nonspecific sequences (lanes 2, 3, and 4). Only a weak signal was measured with pBDTax alone (lane 2), indicating that Tax contains an activation domain, as described previously (59,60). The  $\beta$ -galactosidase activities resulting from coexpression of pADTTP and pBDTax approached the  $\beta$ -galactosidase activity of the positive control (pSV40+p53, lane 5), supporting the notion that the interaction between Tax and TTP was strong and specific. Under similar conditions, a TTP-related protein, TIS11b/Berg-36/ERF1 (X99404), did not exhibit substantial binding to Tax (data not shown).

To further confirm the specificity of the interaction between Tax and TTP, GST pull-down assays were performed. TTP was pulled down by Tax proteins from HTLV-1 (GSTTax-1) and BLV (GST-Tax) but not by GST alone (Fig. 1, B). Similar results were obtained from co-immunoprecipitation assays using lysates from cells transfected with Tax and TTP expression

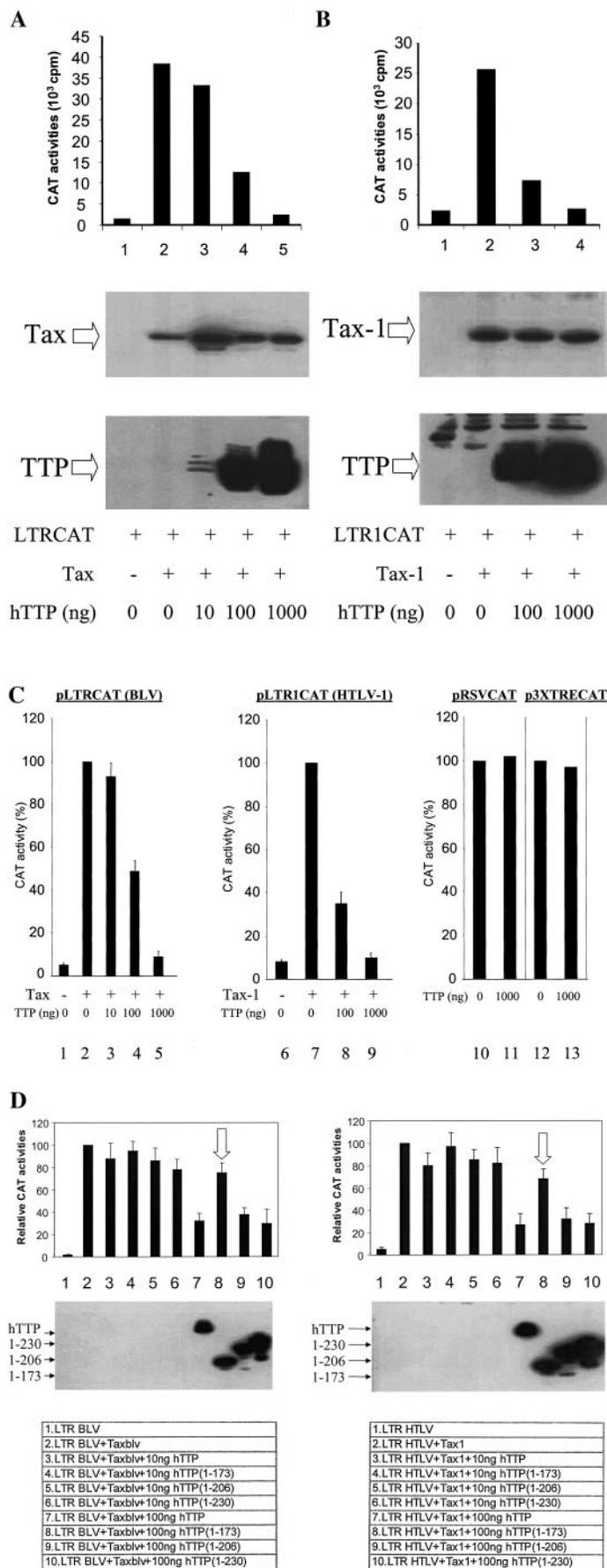
vectors (Fig. 1, C, lanes 1 and 4 for BLV Tax and lanes 1 and 5 for HTLV Tax-1, respectively).

To identify the domains of Tax required for binding, a series of Tax mutants ( $n = 22$  and  $n = 14$  for BLV Tax and HTLV Tax-1, respectively; see "Materials and Methods" section) were tested under similar conditions. Among these, the majority of Tax mutants or variants retained the ability to interact with TTP (Fig. 1, C, lane 9 for BLV Tax and lanes 9–11 for HTLV Tax-1; and data not shown). Only a large carboxyl-terminal truncation (plasmid pSGTax(1–228); Fig. 1, C, lane 8) abrogated binding, indicating that residues 228–309 of BLV Tax belong to the interaction domain. The corresponding deletion in HTLV Tax-1 (at position 244) also abolished the interaction between Tax1 and TTP (data not shown). Interestingly, the region at positions 244–353 has been identified as an activation-specific region and a p300/CBP-associated factor binding domain (18) (Fig. 1, D). A similar approach with TTP mutants identified residues 173–326 as being required for Tax binding (Fig. 1, C, lanes 5–7 for BLV Tax and lanes 6–8 for HTLV Tax-1). It thus appears that both the Tax and TTP carboxyl-terminal domains are involved in their mutual binding (Fig. 1, D). These findings suggest that the Tax proteins bind specifically to TTP *in vitro*, further demonstrating the specificity of their interaction.

### Colocalization of Tax and TTP in Mammalian Cells

We next performed confocal microscopy to assess the subcellular localizations of Tax and TTP in mammalian cells. For this purpose, HeLa-Tat cells, previously shown to be particularly convenient for microscopic examination, were transfected with expression vectors for HTLV Tax-1 (pTax1), BLV Tax (pSGTax), TTP tagged with green fluorescent protein (pEGFP-TTP) and, as controls, GFP alone (pEGFP-C1) or GFPMSX (a hybrid between GFP and Msx2, another protein interacting with Tax). Both BLV Tax and HTLV Tax-1 were localized in the nucleus (stained by TOPRO-3) and in the cytoplasm of the transfected cells, as revealed by specific Tax antibodies and Alexa 546 conjugates (Fig. 2, A: TAX and TAX-1). Under these experimental conditions, substantial amounts of Tax were thus found in the cytoplasm, suggesting a possible role in different subcellular compartments, as indicated by other studies (61,62). In contrast, the GFPTTP protein yielded mainly a cytoplasmic pattern, as previously reported in cycling cells (63), whereas GFP alone did not localize to a defined organelle (Fig. 2, A: GFPTTP and GFP). Finally, Msx2 was nuclear, but considerable staining was also observed in the cytoplasm (GFPMSX).

Coexpression of Tax and TTP provoked a dramatic change in the localization of TTP, with two patterns being observed. First, TTP was more concentrated in the nucleus of the cells in the presence of Tax than in its absence, but not in their nucleolus (Fig. 2, B, black arrows in the fluorescence intensity profiles, compare with GFPTTP in Fig. 2, A). It thus appears that coexpression with Tax directs TTP into the nuclear compartment. Second, Tax and TTP colocalized in the cytoplasm in a region surrounding the nucleus, as revealed by the perfect match of the green (TTP) and orange (Tax) fluorochromes (Fig. 2, B, white arrows). In contrast, no colocalization occurred between Tax and GFP alone, and a different profile was generated by Tax and Msx2 coexpression (Fig. 2, B: GFP and GFPMSX). Based on



confocal microscopy, we thus conclude that Tax and TTP colocalize in cells, further supporting the specificity of their interaction.

### Tax Transactivation in the Presence of TTP

To address the biologic significance of the interaction between Tax and TTP, we first analyzed the ability of Tax to activate viral transcription in the presence of TTP. To this end, expression vectors encoding BLV or HTLV Tax-1 proteins were cotransfected into HeLa cells together with reporter plasmids (pLTRCAT or pLTR1CAT) harboring the viral promoter sequences cloned upstream of the CAT gene. Forty-eight hours after transfection, HeLa cells were harvested and CAT activities were determined in the lysates. In the presence of Tax, an increase in the levels of CAT activity revealed the activation of LTR-directed gene expression, as expected (Fig. 3, compare lanes 1 and 2 on panels A and B). In the presence of TTP, the ability of Tax to transactivate the LTR promoters was inhibited in a dose-dependent manner (Fig. 3, A, lanes 3–5 and 3–4 for BLV and HTLV-1, respectively). In fact, it was possible to nearly completely abolish Tax transactivation in the presence of high doses (1000 ng) of TTP vector (panel A, lane 5, and panel B, lane 4). As shown by western blot, inhibition of transactivation was not due to a decrease in the levels of Tax but rather was linked to TTP expression (Fig. 3, A and B, lower panels). Similar results were obtained with different cell lines: canine D17 cells (Fig. 3, C, lanes 1–9) and HEK 293 cells (data not shown). As a control for specificity, equivalent amounts of TTP

**Fig. 3.** Tristetraprolin (TTP) inhibits Tax transactivation of the viral long terminal repeat (LTR) promoter during transient transfection experiments. **A**) The pLTRCAT reporter construct (2  $\mu$ g), the effector plasmid pSGTax (encoding bovine leukemia virus [BLV] Tax; 10 ng), and different amounts of hTTP (coding for human TTP; as indicated in nanograms) were transfected into HeLa cells. Chloramphenicol acetyltransferase (CAT) activities were determined from the lysates 48 hours after transfection. An aliquot from these lysates was separated on a 12.5% sodium dodecyl sulfate (SDS)–polyacrylamide gel, and a western blot was performed using anti-BLV Tax or anti-influenza virus hemagglutinin (HA) antibodies. **White arrows** indicate the positions of Tax and TTP proteins. **B**) The pLTR1CAT reporter construct (2  $\mu$ g), the effector plasmid pTax1 (encoding HTLV Tax-1; 10 ng), and different amounts (in nanograms) of hTTP (coding for human TTP) were transfected into HeLa cells. CAT activities were determined from the lysates 48 hours after transfection. An aliquot from these lysates was separated on a 12.5% SDS–polyacrylamide gel, and a western blot was performed using anti-HTLV Tax-1 or anti-HA antibodies. **White arrows** indicate the positions of Tax1 and TTP proteins. **C**) Two micrograms of reporter constructs (pLTRCAT, pLTR1CAT, pRSVCAT, and p3XTRECAT) and 10 ng of effector plasmids (pSGTax or pHTLVTax1 coding for BLV Tax [lanes 1–5] and HTLV Tax-1 [lanes 6–9], respectively) were transfected into D17 cells with different amounts (in nanograms) of pSGTTP vector (for TTP). CAT activities were determined 48 hours after transfection. As a control for specificity, equivalent amounts of TTP plasmid did not affect the promoter of Rous Sarcoma Virus (pRSVCAT) or the p3XTRECAT reporter containing three repeats of 12-*O*-TPA response elements (lanes 10–13). **D**) Two micrograms of reporter constructs (pLTRCAT, pLTR1CAT) and 10 ng of effector plasmids (pSGTax or pHTLVTax1 coding for BLV Tax and HTLV Tax-1, respectively) were transfected into HeLa cells with different amounts (in nanograms) of hTTP vector (for TTP) or carboxyl terminal–truncated mutants hTTP(1–173), hTTP(1–206), and hTTP(1–230). CAT activities were determined 48 hours after transfection. An aliquot from these lysates was separated onto a 12.5% SDS–polyacrylamide gel, and a western blot was performed using anti-HA antibodies (for HA-tagged TTP proteins). **White arrows** indicate that mutant hTTP(1–173) does not inhibit Tax-dependent transactivation.

plasmid did not affect the promoter of RSV (pRSVCAT) or the p3xTRECAT reporter gene containing three repeats of TPA response elements (Fig. 3, C, lanes 10–13). To identify the TTP domain involved in inhibition of Tax transactivation, vectors expressing truncated versions of TTP were analyzed (Fig. 3, D). Deletion of the carboxyl-terminal end abrogated the function (plasmid pTTP(1–173); lanes 8, white arrows). The absence of TTP-associated inhibition was not the consequence of a lack of protein expression (western blots in Fig. 3, D). We conclude that TTP specifically impedes Tax-dependent transactivation of both BLV and HTLV-1 LTRs but does not alter gene expression controlled by two other promoters (RSV and TRE).

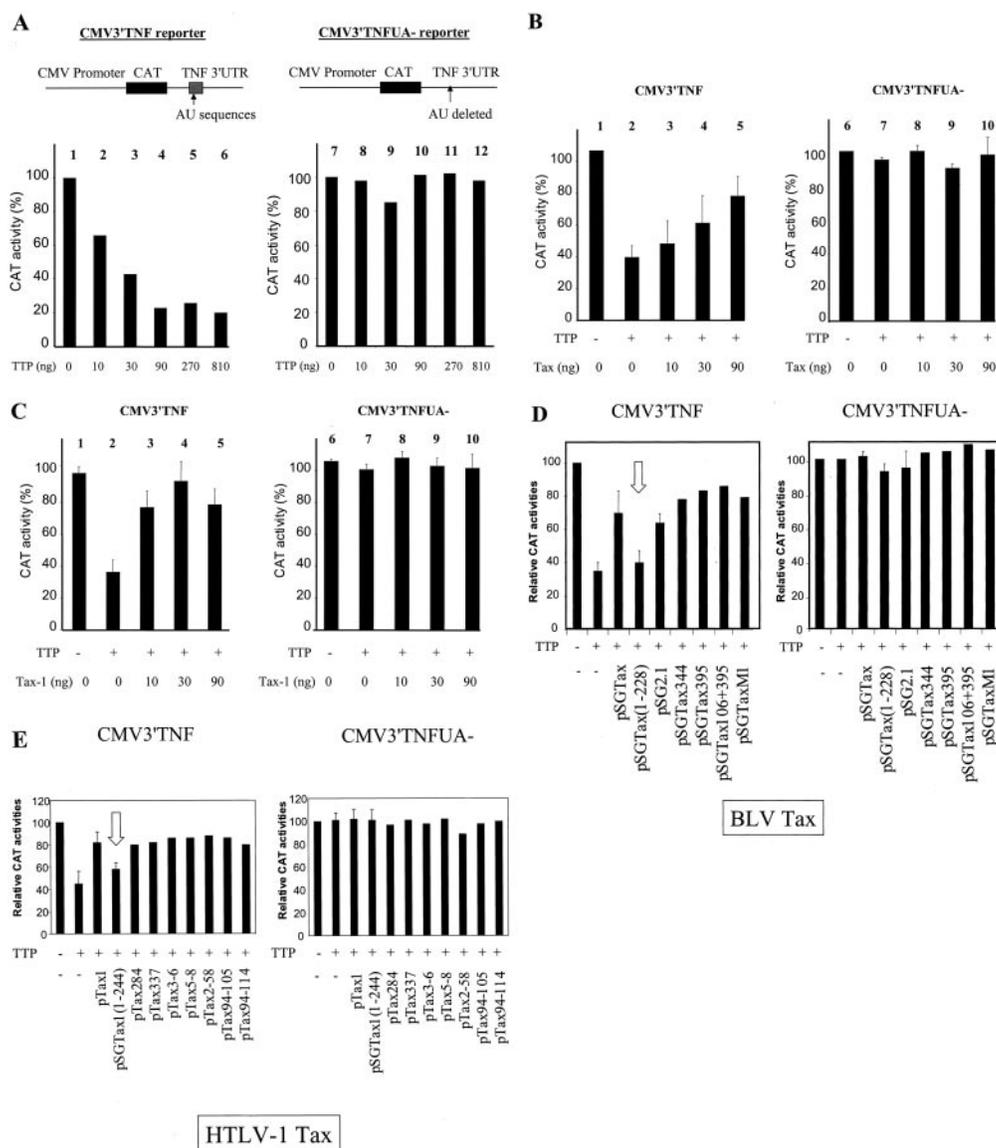
### TTP Function in the Presence of Tax

The major function of TTP is to interact with the AU-rich element present in the 3' UTR of TNF- $\alpha$  mRNA (58). This interaction is associated with a concomitant post-transcriptional regulation of TNF- $\alpha$ , leading to a decrease in TNF- $\alpha$  mRNA stability (64–66). Post-transcriptional regulation of TNF- $\alpha$  was evaluated in transient transfection experiments using a reporter construct harboring the CAT gene placed under control of the

CMV promoter and 3' UTR sequences of TNF- $\alpha$  (plasmid CMV3'TNF) (56,67) (Fig. 4, A). Transfection of this reporter into HeLa cells led to the expression of CAT activity that was arbitrarily normalized to 100% (lane 1). Increasing doses of TTP vector (10–810 ng) induced a gradual reduction in the levels of CAT enzyme (lanes 2–6), revealing the ability of TTP to inhibit 3' UTR-dependent expression. As a control, no inhibition of CAT activity was observed when TTP was coexpressed with the CMV3'TNFUA- reporter lacking the AU-rich element of the TNF- $\alpha$  mRNA (Fig. 4, A, lanes 7–12).

To assess the ability of the BLV Tax and HTLV Tax-1 proteins to interfere with TTP function, increasing doses of the pSGTax and pTax1 vectors were included in the transfection mixtures. Inhibition of CMV3'TNF induced by TTP (Fig. 4, B, compare lanes 1 and 2) was reverted in a dose-dependent manner by coexpression of BLV Tax (lanes 3–5), with levels reaching 80% of those seen in the absence of TTP. HTLV Tax-1 was even more efficient in restoring CAT activity driven by the CMV3'TNF reporter (Fig. 4, C, lanes 3–5). As controls, cotransfection of pSGTax or pTax1 did not affect the expression of the CMV3'TNFUA- reporter, ex-

**Fig. 4.** Tax reverts tristetraprolin (TTP) inhibition in transient transfection assays. **A)** HeLa-Tat cells were transfected with 2  $\mu$ g of either CMV3'TNF or CMV3'TNFUA- reporter plasmids and increasing amounts (in nanograms) of TTP expression vector (pSGTTP). Forty-eight hours after transfection, chloramphenicol acetyltransferase (CAT) activities were determined. Data are the mean values of relative CAT activities of two independent experiments. **B)** pSGTTP (30 ng) was cotransfected into HeLa-Tat cells with 2  $\mu$ g of reporter plasmids (CMV3'TNF or CMV3'TNFUA-) and different quantities of bovine leukemia virus (BLV) Tax vector (pSGTax), as indicated. Data are the means of three independent experiments. Error bars indicate 95% confidence intervals (CIs). **C)** pSGTTP (30 ng) was cotransfected into HeLa-Tat cells with 2  $\mu$ g of reporter plasmids (CMV3'TNF or CMV3'TNFUA-) and different quantities of HTLV-1 Tax vector (pTax1 for Tax-1), as indicated. Data are the means of three independent experiments. Error bars indicate 95% CIs. **D)** pSGTTP (30 ng) was cotransfected into HeLa-Tat cells with 2  $\mu$ g of reporter plasmids (CMV3'TNF or CMV3'TNFUA-) and 90 ng of vectors coding for wild-type BLV Tax and mutants, as indicated. Data are the means of two or three independent experiments. Error bars indicate 95% CIs (no error bars appear if only two experiments were performed). **E)** pSGTTP (30 ng) was cotransfected into HeLa-Tat cells with 2  $\mu$ g of reporter plasmids (CMV3'TNF or CMV3'TNFUA-) and 90 ng of vectors coding for wild-type HTLV-1 Tax (pTax1) and mutants, as indicated. Data are the means of two or three independent experiments. Error bars indicate 95% CIs (no error bars appear if only two experiments were performed).



cluding a direct activation of the CMV promoter by Tax (Fig. 4, B and C, lanes 6–10).

To identify the Tax domain required for TTP repression, a series of Tax mutants were tested under similar conditions (the number of mutants used for BLV Tax and HTLV Tax-1 were 22 and 14, respectively). Among these, only the carboxyl terminal-truncated mutants, BLV pSGTax(1–228) (Fig. 4, D, white arrow) and HTLV pSGTax1(1–244) (Fig. 4, E, white arrow) had lost their ability to revert TTP inhibition. Thus, the potential inhibitory effect exerted by Tax resides in the domain that interacts with TTP (*see* Fig. 1, C). Together, our results demonstrate that, in transient transfection experiments, Tax reverts TNF- $\alpha$  3' UTR-dependent inhibition induced by TTP and that this function requires the carboxyl-terminal domain of Tax.

### Modulation of TNF- $\alpha$ Expression by Tax and TTP

To further assess the functional interplay between TTP and Tax, expression of TNF- $\alpha$  was directly evaluated in macrophages in the presence of both proteins. For this purpose, RAW 264.7 macrophages were stably transfected with various combinations of expression vectors for TTP and Tax (pSGTax for BLV or pTax1 for HTLV-1), as indicated in Fig. 5. In the absence of LPS (Fig. 5, A, unstimulated), basal levels of TNF- $\alpha$  were low, except in the presence of Tax (RAW-Tax and RAW-Tax1 cells). In cells expressing both Tax and TTP (RAW-Tax+TTP and RAW-Tax1+TTP), secretion of TNF- $\alpha$  was completely abolished. Under these conditions, TTP thus counteracted the activation of TNF- $\alpha$  induced by Tax. When RAW 264.7 macrophages were stimulated with LPS (Fig. 5, B), TNF- $\alpha$  synthesis was stimulated, reaching up to 5000 pg/mL of supernatant (RAW or RAW-pSG5 stably transfected with an empty vector). LPS stimulation of TNF- $\alpha$  production was only

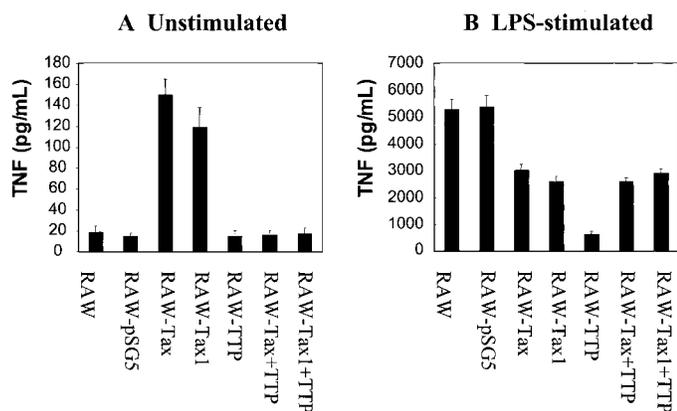
slightly reduced in the presence of Tax (RAW-Tax and RAW-Tax1) but was strongly inhibited in cells expressing TTP (RAW-TTP). Coexpression of Tax reverted TTP inhibition and restored high levels of TNF- $\alpha$  (RAW-Tax+TTP and RAW-Tax1+TTP).

Together, these experiments illustrate a complex interplay between Tax and TTP in macrophages. Under unstimulated conditions, TTP reduces TNF- $\alpha$  expression activated by Tax, whereas in the presence of LPS Tax reverts the inhibition induced by TTP.

### DISCUSSION

In this article, we demonstrated that TTP interacts directly with the oncoviral Tax transactivator proteins. TTP, which is also known as Nup475 or TIS11, is encoded by an immediate-early response gene that is induced by a variety of mitogens and growth factors, including serum, phorbol esters (phorbol myristate acetate [PMA]), insulin, and platelet-derived growth factor (PDGF) (68–73). The TTP protein is widely distributed, with particularly high levels of expression in lymph nodes, thymus, and spleen (70). In addition, TTP transcription occurs in freshly isolated BLV-infected lymphocytes and in HTLV-1 cell lines (data not shown). TTP is predominantly localized in the nucleus of quiescent fibroblasts and macrophages but translocates rapidly to the cytoplasm upon cell stimulation (58,63). Deficiency of TTP results in a complex inflammatory syndrome in mice that is characterized by an excess of circulating TNF- $\alpha$  that can be prevented by repeated injections of antibodies to TNF- $\alpha$  (58). Macrophages derived from these knockout mice exhibit enhanced TNF- $\alpha$  mRNA stability and subsequent increase of the corresponding protein (58).

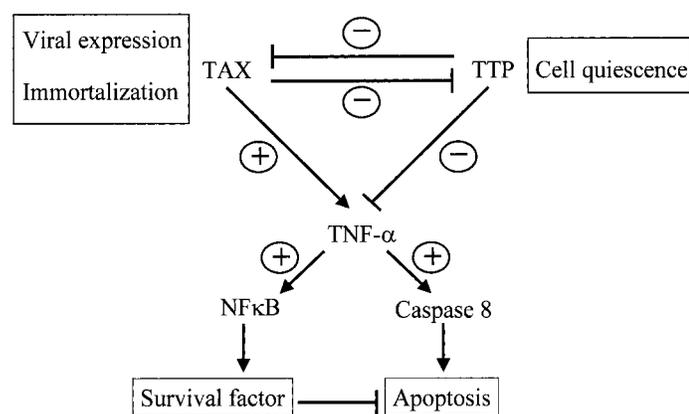
At the molecular level, TTP binds to AU-rich elements located in the 3' UTR of the TNF- $\alpha$  mRNA and provokes its destabilization (65,66). Interaction between TTP and RNA requires the integrity of unusual CX8CX5CX3H zinc fingers located at residues 109–128 and 147–166 in TTP (65,66,74). These structures are not required for Tax binding because we mapped the region involved in the interaction at the carboxyl-terminal end of the TTP protein (Fig. 1, D). It thus appears that the Tax protein probably does not compete directly with TTP for RNA binding. The dissociation between the RNA- and Tax-interaction domains of TTP instead supports an indirect mechanism of inhibition of TTP function. Alternatively, Tax may interfere with TTP/RNA complex formation by directly disrupting intra-molecular conformational changes of the TTP protein. An indirect mechanism of TTP inhibition is, however, also supported by our confocal microscopy experiments. Coexpression of Tax and TTP was indeed associated with a delocalization of the TTP protein and its partial retention into the nucleus (Fig. 2), suggesting that inhibition of TTP function could result from a modification of its subcellular targeting. Furthermore, redistribution of TTP was accompanied by an almost perfect colocalization with Tax proteins in a region essentially concentrated around the nucleus but detectable throughout the cell. In fact, this phenotype is dependent on the cell type because HEK 293 fibroblasts did not exhibit the same pattern (data not shown). In addition, the Tax proteins neither co-immunoprecipitated with TTP in these cells nor inhibited TTP function, suggesting the possible involvement of other factors required for complex formation (75).



**Fig. 5.** Expression of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) induced by Tax in macrophages. **A**) RAW 264.7 cells (RAW) stably transfected with pSG5 (RAW-pSG5), pSGTax (RAW-Tax), pTax1 (RAW-Tax1), pSGTTP (RAW-tristetraprolin [TTP]), or combinations of them (RAW-Tax+TTP and RAW-Tax1+TTP) were cultivated for 6 hours, and TNF- $\alpha$  expression was determined from the culture supernatants by using an enzyme-linked immunosorbent assay (ELISA). Results (in picograms per milliliter) are the means of two experiments done in triplicate. Under these unstimulated conditions, TTP reduces TNF- $\alpha$  expression induced by Tax. **B**) RAW stably transfected with RAW-pSG5, RAW-Tax, RAW-Tax1, RAW-TTP, RAW-Tax+TTP, and RAW-Tax1+TTP were cultivated for 6 hours in the presence of lipopolysaccharide. TNF- $\alpha$  expression was determined from the culture supernatants by using an ELISA. Results (in picograms per milliliter) are the means of two experiments done in triplicate. In the presence of lipopolysaccharide (LPS-stimulated), Tax reverts the inhibition induced by TTP.

Functional interplay between Tax and TTP has been observed not only in HeLa cells but also in RAW 264.7 macrophages and in primary rat embryo fibroblasts. In these latter cells, Tax induces immortalization and, in cooperation with activated Ha-Ras, yields complete transformation (42,43). Interestingly, co-expression of TTP inhibited oncogenesis initiated by Tax and Ha-Ras (data not shown), indicating that TTP may participate in a process of cellular protection against cell transformation. Similarly, because TTP is predominantly localized in the nucleus of quiescent fibroblasts (58,63), Tax-induced redistribution of TTP could result in exit from the cell cycle and entry into a resting stage. In other words, TTP could be part of a cellular mechanism that interferes with proliferation of the infected cells and therefore could belong to a feedback loop process that inhibits Tax (Fig. 6). In this context, Gao et al. (76) indicated that CCH-type zinc finger proteins might have antiretroviral activities.

The fact that TTP function as well as both activities of Tax (i.e., immortalization of primary rat embryo fibroblasts and transactivation of viral expression) were concomitantly affected by their interactions is noteworthy. Although a general quenching effect on gene expression by TTP could account for inhibition of Tax transactivation, control promoters (RSV and 3XTRE) were not modulated under the same conditions, supporting a specificity of this effect (Fig. 3). Furthermore, the immortalization and transactivation functions of Tax, both of which are affected by TTP, are independent (55). Because Tax interacts with specific subunits of the proteasome (77–80), a straightforward interpretation is that the Tax/TTP complex is targeted to this organelle and is subject to simultaneous proteolytic degradation. Our preliminary results indeed indicate that the stabilities of Tax and TTP are reduced when both proteins are coexpressed (data not shown). In this context, variations in the stoichiometry between the Tax proteins and TTP or their respective kinetics could differentially affect the function of one protein over the other. Evidence for antagonistic effects resulting from TTP/Tax interactions was obtained by titrating TNF- $\alpha$  synthesized by macrophages containing both proteins (Fig. 5).



**Fig. 6.** Model summarizing the Tax–tristetraprolin (TTP) interplay. Positive (+, arrows) and negative (–, ⊥) activities between retroviral Tax oncoproteins, TTP, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), nuclear factor  $\kappa$ B (NF- $\kappa$ B), and caspase 8 are presented. TTP inhibits both Tax functions, i.e., immortalization of primary cells and transcriptional activation of viral expression. Tax interaction leads to translocation of TTP into the nuclear compartment, potentially leading to cell quiescence. Tax activates TNF- $\alpha$  directly via the promoter and indirectly via TTP inhibition. TNF- $\alpha$  activates two conflicting pathways, one leading to caspase 8-dependent apoptosis and the other to NF- $\kappa$ B-triggered survival.

Under LPS-stimulated conditions, TTP-dependent inhibition of TNF- $\alpha$  expression was reverted by ectopic expression of Tax (Fig. 5, B). Of note, the relative induction of TNF- $\alpha$  in the presence of Tax appeared to be lower than in its absence, possibly due to its potential toxicity (compare panels A and B: from 20 pg/mL to 5000 pg/mL for RAW-pSG5 versus 120–150 pg/mL to only 2500–3000 pg/mL for RAW-Tax). In contrast, Tax stimulation of TNF- $\alpha$  was abrogated by TTP (Fig. 5, A). Together, our results reveal a remarkable bipartite interplay between the functions of the Tax oncoproteins and TTP (Fig. 6).

The inhibition of TTP by Tax resulted in the stabilization of AU-rich element-containing mRNAs (Fig. 4) and an increase in the TNF- $\alpha$  protein levels in macrophages (Fig. 5). It thus appears that TNF- $\alpha$  is regulated at two different levels: post-transcriptional (this study) and initiation of mRNA transcription (81). It should be mentioned that, under our experimental conditions, we could not detect any significant transcriptional regulation using a reporter construct containing the TNF- $\alpha$  promoter in RAW 264.7 cells (data not shown). The finding that TNF- $\alpha$  expression is dually regulated by the Tax proteins underlines the importance of this cytokine in the pathogenic mechanisms associated with HTLV-1 and BLV. And indeed, TNF- $\alpha$  protein levels are increased in species infected by these viruses (9,10,13,14,82–85). TNF- $\alpha$  is an inflammatory cytokine whose downstream signaling is characterized by opposing effects [reviewed, for instance, in (86–88)]. The TNF- $\alpha$  pathways bifurcate at the type I and II receptors, leading either to caspase 8-dependent apoptosis or, alternatively, to NF- $\kappa$ B activation and rescue from cell death (Fig. 6).

Although the role of TNF- $\alpha$  during pathogenesis is not fully understood, our data cast light on the mechanisms by which two retroviruses (i.e., HTLV-1 and BLV) infecting different cell types (T or B lymphocytes) are able to modulate TNF- $\alpha$  expression. This observation is of importance for the cell transformation process induced by leukemogenic retroviruses because TNF- $\alpha$  overexpression plays a central role in pathogenesis. Importantly, other cytokines, like granulocyte-macrophage colony-stimulating factor and IL-3, are also subject to TTP-dependent regulation (65,89–92) and are involved in oncoviral pathogenesis (11,82,84,93–98). Modulation of TTP activity by the Tax proteins could thus initiate a much broader range of effects on various cytokines, leading ultimately to pathogenesis. Stabilization of short-lived mRNAs coding for a series of cytokines thus appears to be an important aspect of the pleiotropic effects associated with the Tax proteins.

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## NOTES

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