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Activation of expression of genes coding for extracellular matrix proteins in Tat-producing glioblastoma cells

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ABSTRACT **The Tat protein of human immunodeficiency virus type 1 has been increasingly implicated in directly contributing to the disease AIDS by altering the expression of strategic cellular genes. In this study we demonstrate that the presence of the human immunodeficiency virus type 1 regulatory protein Tat is associated with a significant induction in the expression of certain protein components of the extracellular matrix in glial-derived cells. Northern blot analysis reveals that in cells expressing Tat there is a marked elevation in the steady-state RNA levels for fibronectin and types I and III collagen. Metabolic labeling of the Tat-producing cells demonstrates that this induction is also reflected at the level of protein synthesis. Transient transfection experiments indicate that the presence of Tat results in increased transcription of fibronectin and αI type I collagen promoters. Possible mechanisms for this phenomenon and their significance with regard to AIDS are discussed.**

The human immunodeficiency virus type 1 (HIV-1) is the etiologic agent of AIDS and has been directly implicated in some of the clinical manifestations such as acquisition of an immunodeficient state, neurological disorders, and the development of malignancies (1–3). There is evidence for direct infection by HIV-1 of a wide variety of cells including T and B lymphocytes, cells of the monocyte/macrophage lineage, neurons of the central nervous system (CNS), oligodendrocytes, astrocytes, microglia, and endothelial cells (4–7). Further, mounting evidence suggests that uninfected cells can be influenced at a distance by factors, both viral and cellular, released from infected cells (1, 4).

In addition to the classic retroviral structural genes *gag*, *pol*, and *env*, the HIV-1 genome encodes at least six regulatory genes (8). Among these, the regulator of expression of virion protein (*rev*) and the transactivator (*tat*) genes are essential to viral replication, and many studies have been directed at understanding the function of the encoded viral proteins and how they contribute to AIDS. The Tat protein is a potent activator of HIV-1 gene expression, by enhancing the rate of transcription from the viral long terminal repeat (LTR) (9). Since production of Tat is augmented upon activation, it serves to establish a strong positive feedback loop that results in a massive induction of HIV-1 gene expression and, consequently, further propagation of the infection (9).

In addition to causing a direct cytopathic effect in infected cells, HIV-1 infection results in the altered expression of strategic cellular genes (10, 11). Importantly, a number of immunoregulatory cytokines are released from infected cells *in vitro* and their serum levels are elevated in AIDS patients (1, 4). The altered expression of cellular factors is believed to be an integral part of the disease AIDS, facilitating the spread of the infection and contributing to immunodeficiency and the formation of neoplasms. Considerable evidence has accumulated suggesting that Tat-mediated activation is responsible for some of the altered cellular gene expression seen in HIV-1 infection. Tat induces the expression of tumor necrosis factor β and transforming growth factor β (refs. 12 and 13 and C.C., unpublished data). Substantial quantities of Tat are released from HIV-1-infected cells, and this Tat can be taken up by neighboring uninfected cells and thus exerts its functions in these cells (14, 15). Tat specifically inhibits antigen-induced proliferation of uninfected peripheral blood lymphocytes (16). Of great importance is the observation that Tat imparts a growth stimulation to cell lines derived from Kaposi sarcoma lesions *in vitro* (17). Further, transgenic mice expressing Tat develop dermal lesions similar to AIDS Kaposi sarcoma (18). These studies suggest a role for Tat in the pathogenesis of this particular common lesion in AIDS patients.

Our interest lies in the regulation of gene expression within the CNS. Noting that pathology within the CNS is a major component of HIV-1-associated disorders, we examined the influence of the Tat protein on cellular gene expression in a glial-derived cell line. We present detailed analysis of the effect of Tat on the expression of components of the extracellular matrix.

MATERIALS AND METHODS

Cells, Plasmids, and Transfections. Cells (U87-MG from American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal bovine serum and plated in 60- or 100-mm dishes for 24 h prior to transfection. Plasmids pTat, pAct-Tat, and pSV_L-Tat contain the *tat* gene of HIV-1 under the control of HIV-1 LTR, actin, and simian virus 40 late promoter regions, respectively. Plasmid pCD15-CAT containing U_3 and R regions of the HIV-1 LTR in front of the chloramphenicol acetyltransferase (CAT) gene was kindly provided by Suresh Arya (National Cancer Institute/National Institutes of Health, Bethesda, MD). Plasmids pAC-171 (kindly provided by Anne Olson, Lawrence Livermore Laboratories, Livermore, CA) and p0.6NF-CAT (kindly provided by Suzanne Bourgeois, Salk Institute) contain 804- and 510-base-pair fragments of human α1 type I collagen and human fibronectin upstream regulatory regions, respectively, cloned in front of the CAT gene. Plasmid pNeo is the neomycin-resistance (neo^r) gene downstream from the bovine papillomavirus promoter. Cells were transfected by the calcium phosphate coprecipitation method (19). Concentration of the transfected plasmid DNA was kept constant at 15 μg per 60-mm dish and 30 μg per 100-mm dish with test plasmid plus

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Abbreviations: HIV-1, human immunodeficiency virus type 1; LTR, long terminal repeat; CNS, central nervous system; CAT, chloramphenicol acetyltransferase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; neo^r, neomycin resistance.

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pUC19 plasmid DNA. Cell lines 3-8 and 3-10 express Tat under control of the HIV-1 LTR. Cell lines 4-1, 4-4, 4-9, 5-9, and 5-10 express Tat under control of the simian virus 40 late promoter.

CAT Assay. All extracts were made 36–48 h after transfection and CAT enzyme assays were performed as described (20).

RNA Preparation and Analysis. Total cellular RNA from two 100-mm plates was prepared by the hot acid/phenol procedure (21). RNA (20 μg) was electrophoresed in formaldehyde/agarose gels, transferred to nitrocellulose, and hybridized with cloned cDNA and DNA fragments (22, 23) derived from α_1 type III and α_1 type I collagen, fibronectin, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) labeled with [^{32}P]dCTP by nick-translation.

Protein Analysis. U87-MG and its derivatives were plated in 35-mm dishes to subconfluency. Cellular proteins were labeled with [^{14}C]proline at 5 μCi/ml for 48 h (1 Ci = 37 GBq). Media were adjusted to final concentrations of the following components: 5 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 5 mM *N*-ethylmaleimide, and 1 mM *p*-aminobenzamidine hydrochloride. The cell layers were detached and sonicated in an ice-cold solution of 0.15 M NaCl/50 mM Tris·HCl, pH 7.4/protease inhibitors. The labeled proteins in the medium and cell layers were analyzed by PAGE under reducing conditions. For collagen analyses, aliquots of pooled media and cells were processed by limited digestion with pepsin to remove the terminal propeptides of procollagen molecules as described (24) and then resolved on SDS/PAGE.

Immunofluorescence. Clonal U87-MG cells grown in 35-mm dishes were rinsed, dried, and fixed with acetone. The cells were incubated with anti-Tat monoclonal antibody (1269-1.2 obtained from Patricia D'Souza, AIDS Program, National Institute of Allergy and Infectious Diseases, National Institutes of Health), stained with fluorescein isothiocyanate-conjugated second antibody, and observed by fluorescence microscopy.

RESULTS

Generation of Tat-Producing Glial Cell Lines. To examine the role of the HIV-1 Tat protein in altering the pattern of cellular gene expression, we developed a panel of glial cells constitutively expressing Tat. Two expression systems were employed to create a spectrum of cell lines that produce different amounts of Tat. ptat expresses the 72-amino acid form of Tat under the control of the HIV-1 LTR and is expected to produce high levels of Tat as a result of positive feedback. pSV$_L$-tat expresses the 72-amino acid form of Tat under the control of the simian virus 40 late promoter and is expected to produce lower levels of Tat. Each of these constructs was cotransfected with a plasmid expressing the neo^r gene (pNeo, under control of the bovine papillomavirus promoter) into the glial cell line U87-MG and G418-resistant colonies were selected. Expression of the *tat* gene was examined in single-cell clones by direct visualization of the protein by indirect immunofluorescence using an anti-Tat monoclonal antibody (Fig. 1*A*). Substantial accumulation of Tat is observed and its nuclear localization is evident. Differences in intensity of staining suggest that different amounts of Tat protein are produced in different clones. The function of the stably expressed Tat protein was examined by transiently transfecting the newly established cell lines with an HIV-1 LTR–CAT reporter plasmid PCD15-CAT. HIV-1 LTR transcriptional activity is increased in Tat$^+$ cells, and the level of induction roughly correlates with the intensity of immunofluorescence staining, indicating that these cells are producing biologically active Tat (Fig. 1*B*).

RNA Expression of Genes Encoding Extracellular Matrix Proteins. Several intriguing features of the Tat-producing cell lines were observed, in particular, a slower rate of growth, and the ability to attach and grow in uncoated plastic dishes. To understand these phenomena, experiments were designed to examine the expression phenotype of these cell lines.

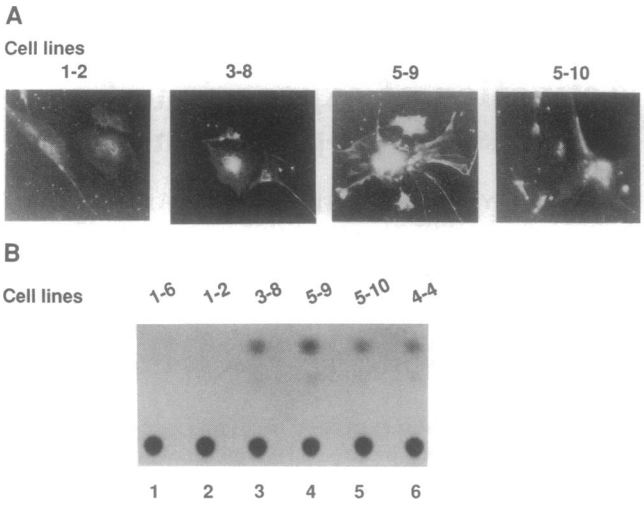

FIG. 1. Expression of HIV-1 Tat in transfected U87-MG glial cells. (*A*) Visualization of Tat in neo^r Tat$^+$ cells by indirect immunofluorescence. Cells were plated at low density in 35-mm dish and after 16 h were prepared for indirect immunofluorescence with an anti-Tat monoclonal antibody. (*B*) Biological activity of Tat produced in glial cells. Cells were plated in 60-mm dish and 16 h later were transfected with 0.5 μg of pCD15-CAT by the calcium phosphate method (19). The final amount of DNA was brought to 15 μg with pUC DNA. Extracts were prepared 48 h after transfection and analyzed for CAT enzyme activity during a 60-min reaction.

First, we examined the expression of the fibronectin gene by Northern blot hybridization (Fig. 2). Total RNA from control 1-6 cells (Fig. 2*A*, lane 1) and five selected Tat-producing clones [3-8 (lane 2), 3-10 (lane 3), 4-1 (lane 4), 4-4 (lane 5), and 4-9 (lane 6)] were analyzed on formaldehyde/agarose gels. Simultaneous hybridization with a fibronectin cDNA and a cDNA for GAPDH revealed a substantial increase in fibronectin expression in the Tat-producing cells. The extent of the induction of mRNA for fibronectin varied among the Tat$^+$ clones, whereas the levels of control GAPDH transcripts remained constant in the Tat$^+$ and Tat$^-$ clones (Fig. 2).

Clone 3-8 demonstrated very strong Tat activity, showed the greatest induction in fibronectin expression, and was selected for further analysis. The results shown in Fig. 3 indicate significant induction in the steady-state levels of RNA for both α1 type I and α1 type III collagen when compared to 1-2 (another G418-selected Tat$^-$ control cell line). This experiment also demonstrates the increase in fibronectin expression relative to an additional Tat$^-$ cell line.

Extracellular Matrix Protein Biosynthesis in Tat-Expressing Cells. To directly examine collagen protein synthesis in the Tat-producing cell lines, two neo^r Tat$^+$ clones (3-8 and 4-4) and two neo^r Tat$^-$ clones (1-2 and 1-6) were metabolically labeled for 48 h with [^{14}C]proline. Total cellular proteins and conditioned media containing total secreted proteins were analyzed by SDS/PAGE and fluorography. Comparison of [^{14}C]proline-incorporated proteins from these cells reveals enhancement of a major high molecular weight band comigrating with fibronectin and two additional bands that migrate in positions consistent with α1 type I and α2 type I procollagen chains (Fig. 4*A*). To confirm the identity of the proteins enhanced in Tat$^+$ cells, the [^{14}C]proline-labeled proteins from cell line 3-8 were subjected to limited pepsin digestion and the products were examined by SDS/PAGE (Fig. 4*B*). This result demonstrates the complete conversion of the procollagen molecules to collagen α1 and α2 chains after limited pepsin treatment and the marked increase in the amounts of these chains in the Tat$^+$ cell lines.

To directly investigate the level of fibronectin synthesis, ^{14}C-labeled proteins from the medium and cell layers from

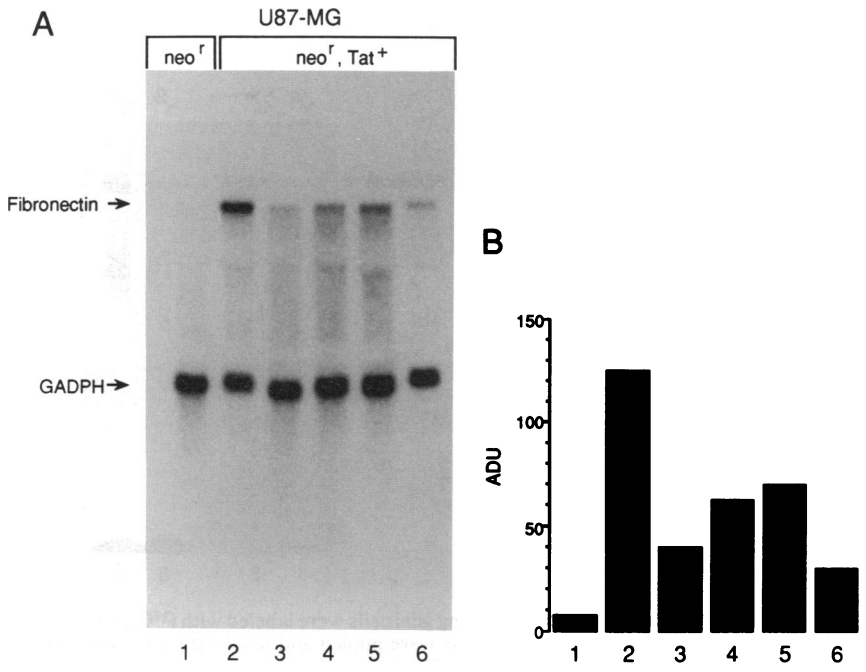

FIG. 2. Levels of fibronectin gene expression in Tat-producing glioblastoma cells. (*A*) Total RNA (20 μg) from control neo^r (lane 1) and five Tat-producing (lanes 2–6) clones was fractionated by electrophoresis in a 1.0% agarose gel and transferred to a nitrocellulose membrane. The blot was hybridized to ^{32}P-labeled cDNA fragments derived from fibronectin cDNA and GAPDH. (*B*) Densitometric analysis of bands corresponding to fibronectin in control neo^r (bar 1) and five neo^r Tat$^+$ cells (bars 2–6). The intensity of the bands corresponding to fibronectin RNA in various cell lines was normalized to GAPDH and rated arbitrarily on a scale of 0–150 adsorption units (ADU). Bars: 2, 3-8 cells; 3, 3-10 cells; 4, 4-1 cells; 5, 4-4 cells, 6, 4-9 cells.

Tat$^-$ (1-2) and Tat$^+$ (3-8) cell lines were examined by SDS/PAGE. The results reveal that the level of fibronectin is increased in the Tat-producing cells (Fig. 4*C*). The identity of fibronectin band was confirmed by immunoprecipitation using human fibronectin antibody (results not shown).

Transcription of α_1 (I) Procollagen and Fibronectin Genes. Comparison of the Tat$^-$ and Tat$^+$ cell lines clearly demonstrates that the protein and mRNA levels of specific components of the extracellular matrix are increased in the cell lines producing Tat. As each of these Tat$^+$ cell lines represents an isolated clone, one cannot rule out the possibility that matrix-enriched clones were selected by chance. However, this seems unlikely since several control Tat$^-$ clones were used for comparison (1-2 and 1-6 cells). Further, the observed increases in collagen and fibronectin expression parallel the observed levels of Tat activity. However, to directly address this question, we examined the transcriptional activity of the collagen and fibronectin promoters in the parental cell line U87-MG and evaluated the effect of Tat on transcription of these promoters. pAC-171 is a plasmid expressing the CAT reporter gene under control of 804 nucleotides of 5′ flanking sequences from the α1 type I collagen gene. This plasmid was transfected alone or cotransfected with a plasmid expressing Tat into U87-MG cells, and the levels of CAT activity were measured. The results demonstrate a dose-dependent increase in expression of this collagen promoter in response to Tat (Fig. 5*B*). Similarly, the reporter plasmid p0.6FN-CAT containing 510 nucleotides of the 5′ flanking sequences of the fibronectin gene was examined for a transcriptional response to Tat. The results demonstrate that fibronectin gene transcription is also increased in a dose-dependent manner in response to Tat (Fig. 5*A*). These results demonstrate that in the presence of Tat the transcriptional activity of these promoters is greatly induced; explaining, in part, the increased expression of these genes in Tat-producing cells.

DISCUSSION

In this report we have shown that the Tat protein of HIV-1 causes a significant increase in the expression of the extracellular matrix proteins fibronectin and types I and III collagen when present in a glial-derived cell line. Further, the results indicate that enhanced expression is mediated, at least in part, by transcriptional activation. It should be noted that the substantial enhancement seen in transcription of fibronectin and collagen is reflected by an equal increase in steady-state RNA levels; however, the levels of the corresponding proteins are elevated to a lesser extent. This suggests that posttranscriptional events, perhaps at the level

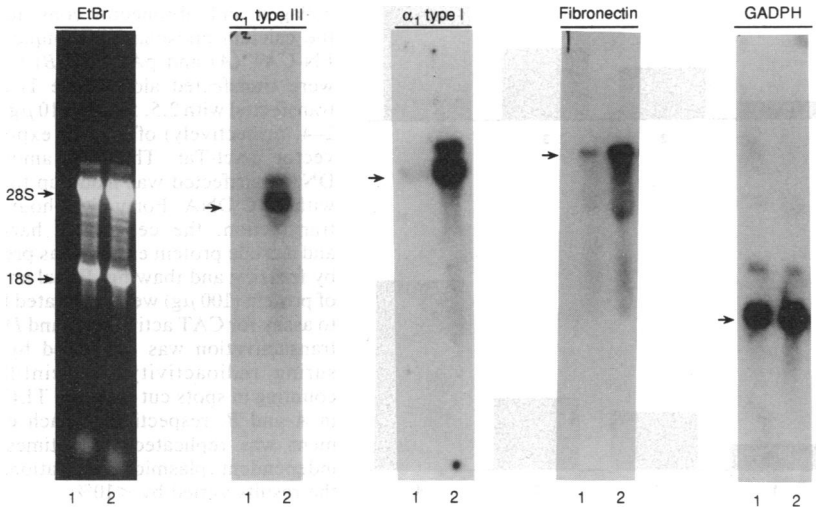

FIG. 3. Analysis of matrix-associated mRNAs in Tat-producing glial cells. RNAs from 1-2 (lanes 1) and 3-8 (lanes 2) cells were fractionated in formaldehyde/agarose gels, transferred to nitrocellulose, and hybridized to four cDNA probes [α_1 type III, α_1 type I, fibronectin, and control (GAPDH)]. Lanes: 1, neo^r 1-2 cells; 2, neo^r Tat$^+$ 3-8 clone.

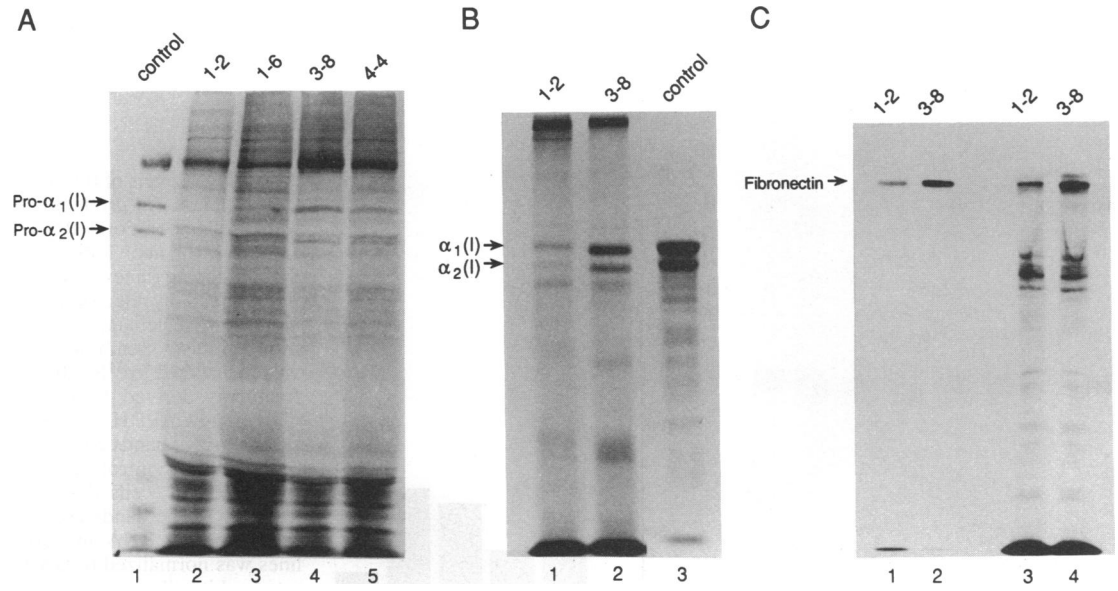

FIG. 4. Production of collagen and fibronectin. Subconfluent glial cells and Tat-producing glial cells were labeled with [^{14}C]proline at 5 μCi/ml for 48 h and medium and cell layers were harvested. (*A*) Equal aliquots of media and cells were pooled and resolved by SDS/PAGE (7% gel) under reducing conditions. Lane 1 is a control reference obtained from normal human lung fibroblasts, illustrating the position of pro α_1 (1) and pro α_2 (1) collagens and a major high molecular weight protein fibronectin that migrates above the collagens; lanes 2–5 are clones 1-2, 1-6, 3-8, and 4-4, respectively. (*B*) Samples of pooled media and cells containing equal cpm were digested with pepsin as described (24) and resolved by SDS/PAGE (7% gel) under reducing conditions. Lanes 1 and 2 are clones 1-2 and 3-8, respectively. Lane 3 is a control from normal human fibroblasts. (*C*) Clonal cell lines were labeled and extracted with 1 M urea and dialyzed. Aliquots of media (lanes 1 and 2) and cells (lanes 3 and 4) were separately sampled by SDS/PAGE (7% gel).

of translation or protein stability, may influence the net output of the Tat-induced cellular genes.

It is unclear whether Tat functions directly at the promoters of the genes for fibronectin and the α1 chains of type I and type III collagen or whether the observed induction represents a less-direct pathway. Within the HIV-1 LTR a sequence termed the TAR (trans-activation responsive) element has been identified as a primary target for Tat (25). Recently, it has been shown that additional sequences within the viral promoter are involved in mediating transactivation by Tat. Specifically, the G+C-rich sequences capable of binding transcription factor Sp1 mediate Tat transactivation in the HIV-1 promoter and may be responsible for the Tat responsiveness of certain non-HIV-1 promoters (refs. 26 and 27 and M.C., unpublished data). In addition, it has been demonstrated that the cis-response element that binds the transcription factor NF-κB is capable of mediating Tat transactivation in the appropriate cell type or after the appropriate stimulation (28, 29). The promoters of the genes for fibronectin and the α_1 chains of type I and type III collagen contain consensus binding sites for the transcription factor Sp1 (30, 31). Possibly, Tat induces transcription of these genes directly through a Tat responsive element in their promoters. It seems more likely, however, that enhanced expression of these matrix genes represents an indirect effect. Our results are consistent with a model in which Tat alters the expression of

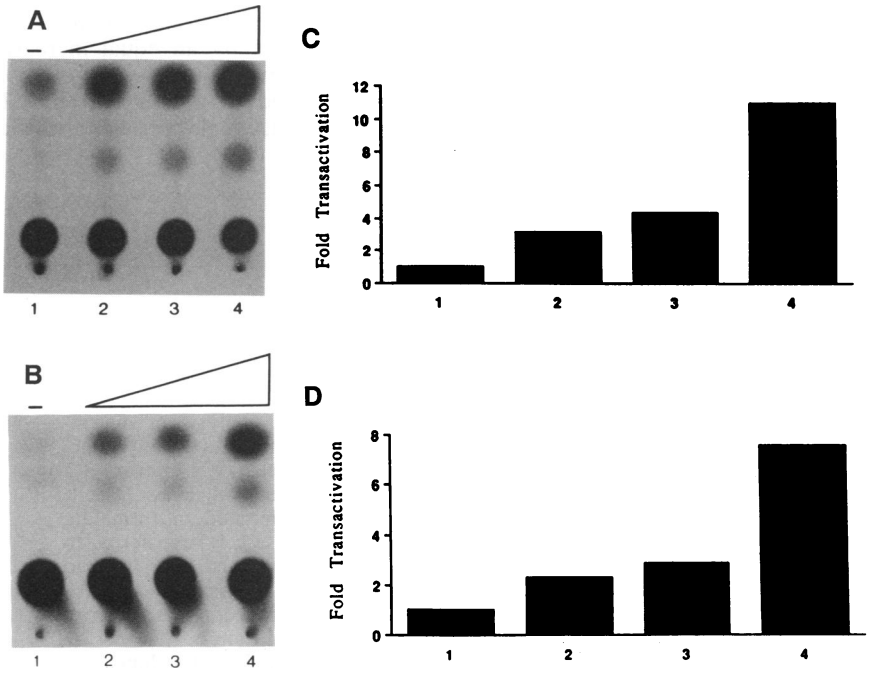

FIG. 5. Transient transfection with collagen and fibronectin constructs by the calcium phosphate technique. p0.6-FN-CAT (*A*) and pAC-171 (*B*) (2.5 μg) were transfected alone (lane 1) or co-transfected with 2.5, 5.0, and 10 μg (lanes 2–4, respectively) of the Tat expression vector pAct-Tat. The total amount of DNA transfected was made up to 15 μg with pUC DNA. Forty-eight hours after transfection, the cells were harvested and a crude protein extract was prepared by freezing and thawing. Equal amounts of protein (100 μg) were incubated for 1 h to assay for CAT activity. (*C* and *D*) Fold transactivation was quantified by measuring radioactivity by scintillation counting in spots cut from the TLC plate in *A* and *B*, respectively. Each experiment was replicated three times with independent plasmid preparations and the results varied by <10%.

a limited number of cellular regulatory factors that in turn mediate the altered physiology of the cells. We (C.C., K.K., and S.A., unpublished data) and others (13) have demonstrated the activation of transforming growth factor β expression by Tat. Because transforming growth factor β is a potent activator of matrix gene expression (32, 33), we speculate that this factor mediates the activation of fibronectin and collagen expression. Interestingly, a transgenic mouse model of HIV-associated nephropathy was recently reported in which an important feature was accumulation of extracellular matrix components (34). Although the authors could not attribute their observations to a particular viral factor, the *tat* gene was among those introduced into the mice.

The clinical course of AIDS is frequently complicated by multiple CNS disorders (2, 35). There is a significant correlation between the level of HIV-1 expression in the brain and clinical severity of the neurological complications (36–38). This strongly suggests that direct infection by HIV-1 or interaction with a viral product is responsible for the CNS pathology.

Within the CNS, which is a large reservoir for HIV-1, microglial cells appear to carry the greatest viral load (2, 4, 36). It has been suggested that migration of microglial cells, which are derived from the monocyte/macrophage lineage, from peripheral blood to the brain is responsible for introduction of HIV-1 to the CNS. Perhaps the presence of Tat in certain tissues, such as the CNS, results in the production of an extracellular matrix substrate that mediates the extravascular dissemination of HIV-1.

The marked stimulation of expression of genes encoding fibronectin and types I and III collagens in Tat-producing cells raises the possibility that a similar mechanism may be involved in the pathogenesis of certain idiopathic or autoimmune diseases characterized by pathologic fibrogenesis such as systemic sclerosis or idiopathic pulmonary fibrosis. This remains an attractive possibility, although no apparent transactivator similar to Tat has been identified in those fibrotic diseases.

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