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Release, Uptake, and Effects of Extracellular Human Immunodeficiency Virus Type 1 Tat Protein on Cell Growth and Viral Transactivation

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During acute human immunodeficiency virus type 1 (HIV-1) infection or after transfection of the *tat* gene, Tat protein is released into the cell culture supernatant. In this extracellular form, Tat stimulates both HIV-1 gene expression and the growth of cells derived from Kaposi's sarcoma (KS) lesions of HIV-1-infected individuals (AIDS-KS cells). Tat protein and its biological activities appear in the cell supernatants at the peak of Tat expression, when the rate of cell death is low (infection) or cell death is undetectable (transfection) and increased levels of cytoplasmic Tat are present. Tat-containing supernatants stimulate maximal AIDS-KS cell growth but only low to moderate levels of HIV-1 gene expression. This is due to the different concentrations of exogenous Tat required for the two effects. The cell growth-promoting effects of Tat peak at between 0.1 and 1 ng of purified recombinant protein per ml in the cell growth medium and do not increase with concentration. In contrast, both the detection of nuclear-localized Tat taken up by cells and the induction of HIV-1 gene expression or replication require higher Tat concentrations (≥100 ng/ml), and all increase linearly with increasing amounts of the exogenous protein. These data suggest that Tat can be released by a mechanism(s) other than cell death and that the cell growth-promoting activity and the virus-transactivating effect of extracellular Tat are mediated by different pathways.

Human immunodeficiency virus type 1 (HIV-1) is the causal agent of AIDS (5, 22, 32, 39). The infection is characterized by immune dysfunction, opportunistic infections, and frequent occurrences of proliferative diseases, particularly Kaposi's sarcoma (KS) (21, 30). HIV-1 possesses several regulatory genes, among which *tat* encodes an early protein (Tat) that transactivates HIV-1 gene expression through interaction with the Tat activation response (TAR) element of the HIV-1 long terminal repeat (LTR). This is essential for viral replication (1, 6, 10, 17, 18, 27, 28, 40, 41, 43, 50). We and others have previously shown that Tat possesses other activities. During acute infection of T cells by HIV-1, Tat is released into the cell culture supernatant (14). In this extracellular form, Tat stimulates the growth of spindle cells of vascular origin derived from KS lesions of patients with AIDS (AIDS-KS cells) and that of normal vascular cells which have been exposed to inflammatory cytokines (3, 14, 36). These results, and data showing that *tat*-transgenic mice develop dermal lesions resembling KS (49), have suggested that Tat plays a role in the pathogenesis of AIDS-associated KS (13). Previous data also indicated that extracellular Tat protein can be taken up by cultured cell lines, can be localized into the nuclei, and can transactivate HIV-1 LTR-directed gene expression (3, 6, 19, 20, 23). Transcellular transactivation by Tat has also been observed by coculturing cells containing the HIV-1 promoter with a Tat-expressing cell line (29).

Similar activities have recently been reported for the human T-cell leukemia-lymphoma virus type I (HTLV-I) transactivator protein, Tax (33, 35, 47), indicating that transactivator proteins of human retroviruses may mediate both viral and cellular effects. However, the events associated with the release, the uptake, and the biological effects of extracellular Tat are not yet well understood.

In this study, we investigated whether during acute infection of T cells by HIV-1, Tat is released at concentrations sufficient to stimulate HIV-1 gene expression in a paracrine fashion and the events associated with the release of biologically active protein by cells transfected with the *tat* gene. As the Tat-containing supernatants from both HIV-1-infected and Tat-transfected cells stimulate maximal AIDS-KS cell growth but only low levels of HIV-1 gene expression, we analyzed the kinetics of dose-response for these activities. This analysis was done by using recombinant purified Tat protein in assays of AIDS-KS cell growth, LTR-directed gene expression, and viral replication. Since the nuclear uptake of Tat is a prerequisite for transactivation, we also determined the concentration of exogenous protein necessary to detect nuclear-localized Tat with AIDS-KS cells. The results from these studies suggest that different pathways mediate the cellular and viral effects of extracellular Tat protein.

MATERIALS AND METHODS

Cell cultures. The T-cell lines H9 and Jurkat, the epithelial cell line COS-1, and HeLa-CD4$^+$ cells containing a Tat-defective HIV-1 provirus (HLM1 cell line) have all been previously described (14, 44). Primary cell cultures derived from KS lesions of patients with AIDS were established and cultured as previously described (14, 16, 37, 38, 46).

HIV-1 infection. H9 or Jurkat cells (10^6/ml) were infected by a cell-free method (14) with HIV-1 (IIIB isolate), as

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described in the legend to Fig. 1 and in Table 1, footnote *a*. During infection, cell death was continuously monitored by trypan blue dye exclusion. Conditioned media (CM) from infected or uninfected cells were prepared every 3 to 4 days (from days 4 to 24) as described previously (14) and utilized for HIV-1 transactivation assays (see below). For the coculture experiments, infected and uninfected cells were cocultured with COS-1 cells transfected with the HIV-1 LTR-chloramphenicol acetyltransferase (CAT) plasmids, as described in footnote *a* of Table 1.

Plasmid DNA and transfection into COS-1 cells. Wild-type *tat*-expressing vectors (CMV-TAT and PCV-TAT), the construct CMV-M-TAT containing a mutation in the basic region of Tat (Arg-52 to Leu), and the control vector DNAs (CMV-0 and PCV-0) have all been previously described (14, 45). The HIV-1 LTR-CAT construct (CD23-CAT) and its mutant (ΔBS-CAT), with a deletion of 4 nucleotides in the TAR region, have also been previously described (15). Transfection of these plasmids into COS-1 cells was performed by an electroporation procedure which was modified for COS-1 cells to minimize the cell death often observed with this technique (3, 8, 14). COS-1 cells were cultured in RPMI 1640 in a 175-cm^3 flask. When cell confluence was ~90%, cells were cold trypsinized, washed in phosphate-buffered saline (PBS) containing no Ca^{2+} or Mg^{2+}, and resuspended in 1.6 ml of electroporation buffer (272 mM sucrose, 7 mM potassium phosphate [pH 7.4], 1 mM magnesium chloride). An 800-μl volume of the cell suspension was gently mixed with 30 μg of total plasmid DNA and transferred to ice for 8 to 10 min. After being electroshocked (25 μF, 0.28 kV; Bio-Rad apparatus), cells were incubated on ice for 8 to 10 min and transferred very gently to a 75-cm^3 flask containing growth medium at 37°C. By this technique, more than 99% of the cells were alive and attached to the flasks 10 to 14 h after transfection. Cell death was also monitored (24, 48, and 72 h after transfection) by measuring the lactic dehydrogenase (LDH) content (with a commercially available kit [Sigma]) (42) of the cell supernatants. Growth media and supernatants from cells containing 1, 2, and 5% lysed cells (100% being the percentage of cells used for the transfection) were used as negative and positive controls, respectively (42).

CM from cells transfected with Tat-expressing plasmids or control vectors were prepared as described previously (14).

Tat protein and anti-Tat antibodies. The Tat protein used in these studies was expressed in *Escherichia coli* and isolated by successive rounds of high-pressure liquid chromatography and ion-exchange chromatography. Analysis of purified protein was by silver staining (Bio-Rad) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and indicated the protein to be >95% pure (6). The purified protein (containing 0.1 mM dithiothreitol) was stored lyophilized at −20°C. Because air oxidation of Tat leads to inactivation and the loss of biological activities (14, 48; also data not shown), Tat was resuspended in degassed buffer (PBS containing bovine serum albumin [BSA] [0.1%]) immediately prior to being used. For each procedure involving the use of Tat or Tat-containing supernatants, the plastic ware was previously rinsed in PBS-BSA or medium containing 10% fetal calf serum, respectively (3, 6, 14).

Rabbit polyclonal and mouse monoclonal anti-Tat antibodies were both raised against recombinant purified Tat protein expressed in *E. coli*, affinity purified with immobilized purified Tat protein, and analyzed by Western blotting (immunoblotting) and radioimmunoprecipitation analysis (RIPA) on Tat-transfected cells and Tat-expressing cell lines (3, 6, 14).

RIPA of Tat. HIV-1-infected and uninfected H9 cells (10^6/ml) and COS-1-transfected cells were metabolically labeled for 24 and 6 to 8 h, respectively, with both [^{35}S]methionine and cysteine (150 μCi/ml each). Supernatants (3 to 4 ml) and cell extracts (500 μl) were prepared and used for immunoprecipitation analysis with the polyclonal anti-Tat antibodies (1:100) as previously described (14).

Immunostaining of Tat. Immunocytochemical analysis of infected and transfected cells was performed with cytospin preparations, either by double indirect immunoperoxidase staining or by the alkaline phosphatase–anti-alkaline phosphatase method. Briefly, slides were fixed in cold acetone or acetone and chloroform (1:1), washed in Tris-buffered saline (TBS), and incubated (1 h at room temperature or overnight at 4°C) with the anti-Tat monoclonal antibodies (dilutions of 1:250, 1:500, and 1:1,000). Slides were then rinsed in TBS and secondary reagents were applied. The reaction of the alkaline phosphatase–anti-alkaline phosphatase was developed with the Alkaline Phosphatase Kit I (Vector, Burlingame, Calif.), and the peroxidase reaction was developed with diaminobenzidine and hydrogen peroxide. Slides were counterstained with Mayers (Merck) or Harris hematoxylin. The percentages of Tat-positive cells in duplicate samples for each experiment and in more than five fields per slide were evaluated. The controls utilized were (i) uninfected cells, (ii) cells transfected with control vectors, (iii) PBS, and (iv) unrelated antibodies.

HIV-1 LTR-directed gene expression after direct addition or scrape-loading of Tat or Tat-containing supernatants into COS-1 cells transfected with HIV-1 LTR or ΔBS-CAT constructs. COS-1 cells were transfected with the HIV-1 LTR-CAT plasmid (CD23-CAT) or its TAR deletion mutant (ΔBS-CAT) as described above and elsewhere (3). At 48 h posttransfection (cell confluence of 70 to 90%), cells were washed twice in PBS containing Ca^{2+} and Mg^{2+}. Then Tat or its buffer (in 10 ml of medium), Tat-containing supernatants from infected or *tat*-transfected cells, or the control supernatants from uninfected cells or cells transfected with the control vectors (10 ml) were added to the cells. Cells were directly incubated or immediately scraped with a rubber policeman (Costar), dispersed by pipetting, and then incubated at 37°C (19, 20, 23). Twelve to 16 h later, cell extracts were prepared and used for CAT assays by overnight incubation (3, 15, 26). Each experiment was performed with duplicate samples, and the resulting CAT activities were normalized to the protein content of the cell extracts.

Cell growth assays and blocking experiments with anti-Tat antibodies. AIDS-KS cell growth experiments were performed by cell counting and/or by [^{3}H]thymidine incorporation assays as described previously (3, 14, 37, 38, 46). For the cell-counting method, confluent cells were plated (5×10^3 or 7.5×10^3 per well in 12-well plates) and incubated overnight with medium alone with no growth supplements and then for 4 h in serum-free medium. The proteins or the CM (diluted in medium) were added to the cells in duplicate wells (1 ml per well) and replaced with fresh preparations 2 to 3 days later. Cells were counted at 4 to 6 days by trypan blue staining (in triplicate for each well). For the thymidine incorporation assays, AIDS-KS cells were plated at 5×10^2 to 9×10^2 per well in 96-well plates (Costar), and the proteins or the test CM (250 μl) and 1 μCi of [^{3}H]thymidine (6.7 Ci/mmol; NEN Research Products) per well were added simultaneously to each well (4 to 5 replicates per sample). The cells were harvested after 48 h, and the counts per

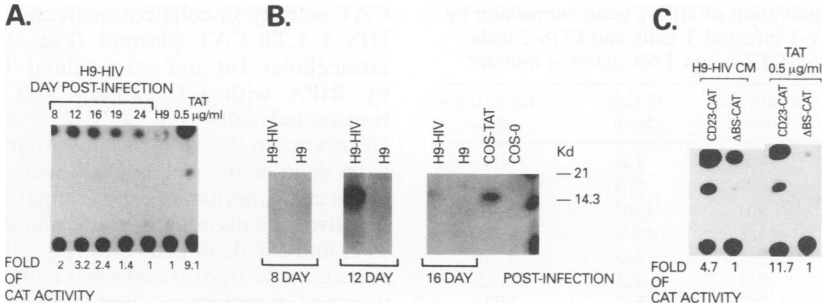

FIG. 1. (A and B) Activation of HIV-1 gene expression by CM from H9-infected cells and detection of extracellular Tat. (A) Activation of HIV-1 LTR-directed gene expression by scrape loading the CM from H9 cells at several time points after acute HIV-1 infection (H9-HIV) (100,000 cpm of reverse transcriptase per 2×10^7 cells) or from uninfected cells (H9) into COS cells transfected with the HIV-1 LTR-CAT plasmid. Transactivation values are relative to the values obtained with CM from uninfected cells (H9, onefold). Recombinant purified Tat (0.5 μg/ml) was used as the positive control. (B) RIPA of Tat in supernatants of metabolically labeled HIV-1-infected H9 cells (H9-HIV) and uninfected cells (H9). Supernatants from COS-1 cells transfected with the *tat*-expressing plasmid PCV-TAT (COS-TAT) or the vector DNA PCV-0 (COS-0) were used as controls (14). Molecular size markers are in kilodaltons. Both experiments were performed twice under the same conditions. (C) Activation of HIV-1 gene expression by CM from acutely HIV-1-infected H9 cells is due to Tat. HIV-1 LTR-directed CAT expression was evaluated after CM from HIV-1-infected H9 cells (600,000 cpm of reverse transcriptase per 2×10^7 cells) was scrape loaded into COS cells transfected with CD23-CAT or ΔBS-CAT plasmid. Shown are the highest levels of transactivation activity during infection. CAT activity is expressed as the ratio of CD23-CAT to ΔBS-CAT. For CM from uninfected cells or Tat buffer, the ratio of CD23-CAT to ΔBS-CAT was 1. The experiment was repeated twice (in duplicate samples) under the same conditions.

minute of incorporated thymidine was determined with a β-counter (1250 Beta Plate; LKB/Pharmacia). The results are expressed either as fold increases of cell proliferation after the addition of the proteins and test CM (calculated on the basis of the number of cells plated [onefold]) or as counts per minute. CM derived from HTLV-II-infected T cells (HTLV-II CM) and basic fibroblast growth factor (bFGF) were used as positive controls (3, 14, 16, 37, 38, 46). Tat buffer, medium alone, and the control CM were used as negative controls.

The inhibition of Tat-induced cell growth was performed by preincubating Tat or Tat-containing supernatants at 4°C for 6 to 12 h with serial dilutions of the anti-Tat polyclonal antibodies (14). Cell proliferation was then measured by cell counting and/or thymidine incorporation. Controls for specificity and toxicity were performed by adding the antibodies to Tat buffer, control CM, or the known growth inducers of AIDS-KS cells (HTLV-II CM or bFGF) as described previously (3, 14).

Cellular uptake of Tat. AIDS-KS cells were plated on glass slides (8 wells per slide). After 24 to 48 h, cells were washed and incubated for 1 h in serum-free medium. Serial dilutions of Tat (0.01, 0.1, 1, or 10 μg/ml) were then added to the cells, which were incubated either at 37 or at 4°C for 15, 30, 60, and 180 min. Cells were then washed with PBS, fixed in acetone and chloroform (1:1), and stained with the anti-Tat monoclonal antibodies as described above. Blocking of Tat uptake was performed by preincubating the protein for 1 to 2 h (4°C) with antibody prior to adding it to the cells. Each experiment was performed in duplicate. The procedure for the negative control was the same, except that Tat buffer was used.

Rescue of Tat-defective provirus by Tat. The HeLa-CD4-expressing cell line containing an integrated, nonrevertant, Tat-defective HIV-1 provirus (HLM1 cell line) (44) was used for rescue assays. The assay consists of complementing the lack of expression of Tat by supplying Tat protein and monitoring viral replication by measuring the viral core antigen (p24) released into the cell supernatants. Cells were seeded in 6-well plates (Costar) at 2×10^5 cells per well. After 24 h, cells were washed in PBS containing Ca^{2+} and Mg^{2+}, and Tat or its buffer was added or scrape loaded into the cells. After 24, 48, and 72 h of incubation at 37°C, p24 was monitored in the cell supernatants by an antigen capture assay (DuPont and Cellular Products). Each experiment was performed with duplicate samples.

RESULTS

Transcellular activation of HIV-1 gene expression by Tat released from acutely HIV-1-infected T cells. Previous studies have shown that acutely HIV-1-infected T cells release Tat into the culture medium (14) and that exogenous Tat protein transactivates HIV-1 gene expression (3, 6, 19, 20, 23). In order to determine whether during acute HIV-1 infection released Tat was able to transactivate HIV-1 gene expression in a paracrine fashion, CM from H9-infected T cells (H9-HIV CM) or from uninfected cells (H9 CM) from sequential time points postinfection were assayed for transactivating activity by scrape loading into COS-1 cells transfected with the HIV-1 LTR-CAT construct (3, 6). H9-HIV CM stimulated low levels of HIV-1 LTR-directed gene expression compared with those by purified Tat protein used as a positive control and with those by CM from uninfected control cells (onefold). Transactivation was transient and concordant with the presence of immunoreactive Tat in the cell supernatants (Fig. 1A and B). To verify whether the activation of HIV gene expression by CM was due to the presence of Tat in the CM, H9-HIV CM was scrape loaded into cells transfected with a deletion mutant of the Tat-responsive region (TAR) of the HIV-1 LTR (ΔBS-CAT) (15) (Fig. 1C). This mutant is not responsive to Tat but is still responsive to TAR-independent inducers of the HIV-1 LTR (3, 15). The transactivating effect of CM was markedly reduced (Fig. 1C), confirming that it was largely mediated by Tat. Similar results were obtained with Jurkat cells (data not shown).

In these experiments, however, HIV-1 transactivation was observed by utilizing the scrape-loading procedure. This allows the entry of exogenous proteins into cells by the avoidance of cell membrane-protein interactions (20, 23). To

TABLE 1. Transcellular activation of HIV-1 gene expression by coculture of acutely HIV-1-infected T cells and COS-1 cells transfected with the HIV-1 LTR or its TAR deletion mutant[a]

Cell type	Day postinfection	CD23/ΔBS ratio[b]	% Cell death	% Tat-positive cells
H9	3	1.0	1.8	ND
	6	1.9	5.0	2–4
	9	3.0	15.0	5–10
	12	1.1	45.0	1–2
Jurkat	3	1.5	2.0	3–5
	6	5.3	2.2	10–15
	10	2.7	45.0	ND
Control				
0.5 μg		2.5		
1.0 μg		5.1		

[a] H9 or Jurkat cells were acutely infected with HIV-1 (for H9 cells, 0.6 × 10^6 cpm of reverse transcriptase per 20 × 10^6 cells without preincubation of the cells with Polybrene; for Jurkat cells, 1.2 × 10^6 cpm of reverse transcriptase per 20 × 10^6 cells preincubated for 1 h with Polybrene). Every 3 to 4 days, infected and uninfected cells were monitored for cell death (by trypan blue staining) and expression of Tat (by immunohistochemistry) and cocultured in duplicate (3 × 10^6 to 6 × 10^6 in 10 ml of growth media) with COS-1 cells transfected 24 h earlier with the HIV-1 LTR-CAT plasmid (CD23-CAT) or its TAR deletion mutant (ΔBS-CAT). After 24 h, 5 ml of fresh media was added to the flasks, and incubation continued for an additional 24 h. Cell extracts were then assayed for CAT activity. Tat protein directly added at 0.5 and 1.0 μg was used as a positive control. The results are the averages of two independent experiments for each cell type (each in duplicate samples) under the same conditions.

[b] CAT activity (fold) is expressed as the value of the CD23-CAT/ΔBS-CAT ratio in infected versus that in uninfected cells or in recombinant purified Tat versus that in its buffer directly added to transfected COS cells.

verify whether during HIV-1 infection transactivation could also result from the cellular uptake of Tat (transcellular transactivation), H9 or Jurkat infected cells were cocultured every 3 to 4 days postinfection with COS cells transfected with the CD23-CAT or ΔBS-CAT plasmid (Table 1). At the same time that cells were cocultured, they were monitored for cell death and for the expression of Tat by immunohistochemistry. Tat protein was used as the positive control, but this time Tat was added directly and not scrape loaded into the cells. During infection, transcellular transactivation (expressed as the value of the CD23-CAT/ΔBS-CAT ratio in infected versus that in uninfected cells) was low to moderate, and for both cell types, it correlated with the percentage of Tat-expressing cells (Table 1). Transactivation was transient and peaked early during infection, at the moment of maximal Tat expression and before significant rates of death of infected cells (Table 1). Although transactivation was modest, in this case it was observed during coculturing of infected and target cells, suggesting a possible pathway for transcellular transactivation in vivo.

Release of Tat in supernatants from Tat-transfected COS-1 cells is not due to cell death. The previous results indicate that during acute HIV-1 infection, transactivation by extracellular Tat occurs before significant rates of death of infected cells. In order to assess the contribution of cell death to the release of Tat, COS-1 cells were transfected with the *tat*-expressing plasmid (PCV-TAT) or the control vector DNA (PCV-0) (14) by an electroporation method as described in Materials and Methods (3, 8, 14). Under these conditions, more than 99% of the cells are attached to the flasks 10 to 14 h posttransfection, and the highest expression of Tat is observed 34 to 38 h later (48 h posttransfection). This was monitored by immunostaining (5 to 10% of the transfected cells express Tat) (Fig. 2A) and by measuring HIV-1 LTR-CAT activity in cells cotransfected with PCV-TAT and the HIV-1 LTR-CAT plasmid (Fig. 2B). At this time, both intracellular Tat and extracellular Tat were easily detected by RIPA with cell extracts and supernatants from the transfected cells (Fig. 2C). Cell death was undetectable. However, to exclude the possibility that Tat was released from dead or damaged cells, levels of LDH were measured in the cell supernatants and compared with the levels present in growth medium and in supernatants from cells containing 1, 2, and 5% dead cells (42) (Fig. 2D). The level of LDH in supernatants from transfected cells was very similar to that detected in growth medium (≤40 U/liter) and lower than the levels detected in supernatants from cells containing 1% dead cells (~90 U/liter). These results confirm that Tat is released in the absence of cell death.

Tat released by transfected COS-1 cells induces maximal AIDS-KS cell growth but only low levels of HIV-1 gene expression. In previous studies, we have shown that Tat released during acute HIV-1 infection stimulates the growth of AIDS-KS cells (14). To correlate this observation with the HIV-1 transactivation ability of extracellular Tat, CM from cells transfected with *tat* (PCV-TAT) or the control vector (PCV-0) were utilized in cell growth experiments with AIDS-KS cells and in transactivation assays by the scrape-loading procedure (Fig. 3). The Tat-containing CM induced maximal AIDS-KS cell proliferation when compared with that by known inducers of AIDS-KS cell growth (CM from HTLV-II-infected cells and bFGF) (3, 14, 16, 37, 38, 46) (Fig. 3A, left panel). Cell growth was inhibited by preincubating the CM with anti-Tat antibodies, while the antibodies had no effect on the cell growth induced by the HTLV-II CM (Fig. 3A, right panel) (14). In contrast, only modest levels of HIV-1 LTR-directed gene expression were observed after the Tat-containing CM were scrape loaded into COS-LTR-transfected cells, compared with the levels with CM from cells transfected with the vector DNA (PCV-0) (Fig. 3B). Transactivation was reduced after the Tat-containing CM were scrape loaded into cells transfected with the ΔBS-CAT construct (Fig. 3C). This confirms that the transactivation is due to Tat. These results indicate that after transfection into COS cells, released Tat is biologically active and is capable of inducing maximal AIDS-KS cell growth; however, only modest levels of HIV-1 transactivation are observed.

Release of biologically active Tat by transfected cells depends upon the levels of expression and subcellular localization of the protein. In the previous experiments, we observed that the release of Tat required maximal expression of the gene. To clarify the relationships between expression, intracellular accumulation, and activity of Tat with its release from the cells, COS-1 cells were transfected with wild-type *tat* constructs expressed under two different promoters (PCV-TAT and CMV-TAT) (14, 45) and with a *tat* construct (CMV-M-TAT) expressing a protein mutated in the basic region (Arg to Leu at position 52). The CMV-M-TAT construct was chosen because the basic domain of Tat is necessary for the nuclear-nucleolar localization of the protein, which is required for viral transactivation (28, 43, 45). In fact, CMV-M-TAT protein does not properly accumulate in the nucleus, and it is less active in transactivation (45). Several parameters were simultaneously monitored 48 h after transfection of these constructs: (i) Tat expression and subcellular (nuclear and cytoplasmic) localization of the protein (by immunohistochemistry), (ii) intracellular and extracellular Tat content (by RIPA), (iii) intracellular Tat activity (by CAT assays with extracts from cells cotransfected with the HIV-1 LTR-CAT plasmid), and (iv) extracellular Tat activity (by

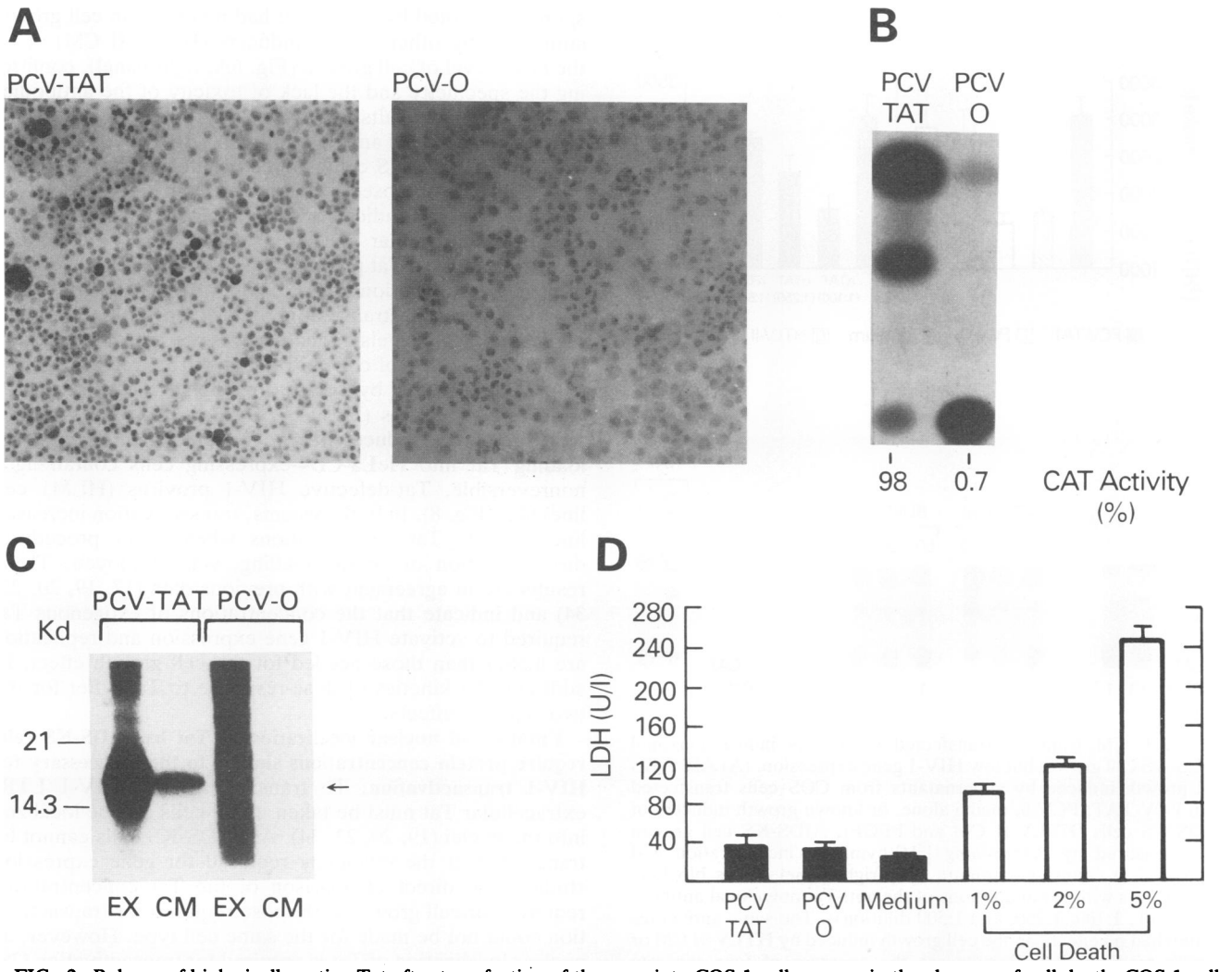

FIG. 2. Release of biologically active Tat after transfection of the gene into COS-1 cells occurs in the absence of cell death. COS-1 cells were transfected with PCV-TAT or PCV-0 as described in Materials and Methods. After 48 h, the cells were monitored for Tat expression by immunostaining with the anti-Tat monoclonal antibodies (A), by HIV-1 LTR transactivation assays with cell extracts from cells cotransfected with the CD23-CAT plasmid (1/10 of the cell extract normalized to the protein content and incubated for 2 h) (B), by RIPA of Tat in cell extracts and supernatants from metabolically labeled cells (C), and at the same time that LDH (units per liter) was measured in supernatants from COS-1 cells, growth medium, and supernatants from cells containing 1, 2, or 5% dead cells. The experiments were repeated six times under the same conditions.

AIDS-KS cell growth assays with CM from transfected cells). The results from these experiments are summarized in Table 2, and some examples are shown in Fig. 4 and 5.

The percentage of Tat-positive cells and total Tat content (intracellular and extracellular) were higher in PCV-TAT-transfected cells and lower in cells transfected with the CMV-M-TAT construct than in CMV-TAT-transfected cells (Fig. 4 and 5; Table 2). In all cases, the protein was detected in both the nuclei and the cytoplasm of the cells. However, cytoplasmic Tat was present in a larger fraction of cells transfected with PCV-TAT and was detected predominantly in the cytoplasm of CMV-M-TAT-transfected cells (Fig. 4; Table 2). The fraction of Tat released by the cells correlated with the level of total Tat content (higher in PCV-TAT-transfected cells) and with the levels of cytoplasmic Tat (prevalent in PCV-TAT- and CMV-M-TAT-transfected cells) (Fig. 5; Table 2). Interestingly, cells transfected with CMV-M-TAT released levels of Tat similar to those released by cells transfected with CMV-TAT, although the total Tat content and the percentage of Tat-positive cells were lower (Fig. 5B). This results in a proportionally higher fraction of protein released into the cell supernatants (Table 2). In agreement with these data, intracellular Tat activity was lower for the CMV-M-TAT construct than for its wild-type plasmid (CMV-TAT), while extracellular Tat activity was about the same for CMV-M-TAT and CMV-TAT (Table 2). These results suggest that the cellular release of Tat depends upon the presence of cytoplasmic protein, which, in turn, is increased under conditions of high-level gene expression or by mutations in *tat* which direct cytoplasmic localization.

Different concentrations of exogenous Tat are required for cell growth and viral gene expression or replication. In the experiments described above, we observed that Tat-containing supernatants induced maximal AIDS-KS cell growth but

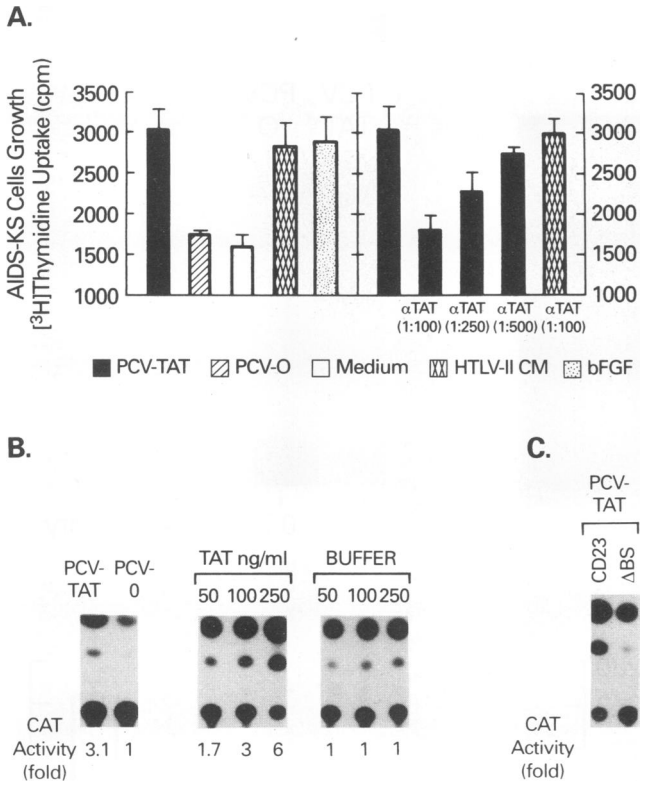

FIG. 3. CM from Tat-transfected COS cells induce maximal AIDS-KS cell growth but low HIV-1 gene expression. (A) AIDS-KS cell growth induced by supernatants from COS cells transfected with PCV-TAT, PCV-0, media alone, or known growth inducers of AIDS-KS cells (HTLV-II CM and bFGF). AIDS-KS cell growth was measured by determining [^{3}H]thymidine incorporation and expressed in counts per minute. The right panel shows blocking experiments with serial dilutions of the anti-Tat polyclonal antibodies (αTAT, 1:100, 1:250, and 1:500 dilutions). The same antibodies (1:100) had no effects on the cell growth induced by HTLV-II CM or growth medium (14). Results are the averages of four separate experiments. (B and C) Activation of HIV-1 LTR-directed gene expression by CM from COS cells transfected with PCV-TAT or PCV-0 or recombinant Tat and its buffer. CAT activities are relative to the CAT activity induced after CM from cells transfected with PCV-0 was scrape loaded into CD23-CAT-transfected cells (onefold) (B) or after Tat-containing CM was scrape loaded into ΔBS-CAT-transfected cells (onefold) (C). Recombinant purified Tat and its buffer (50, 100, or 250 ng/ml) were used as positive controls. Each experiment was repeated four or five times.

only low to moderate levels of HIV-1 transactivation. This suggested that cell growth requires a lower concentration of extracellular Tat than the transactivating effect. To verify this, serial dilutions of recombinant-purified Tat protein were added to AIDS-KS cells, and cell growth was monitored by both cell counting (Fig. 6A, left panel) and [^{3}H]thymidine incorporation (Fig. 6B). Tat stimulated cell growth at concentrations of 0.05 to 50 ng/ml, with peak activity at between 0.1 and 1 ng/ml (Fig. 6). No growth stimulation was observed with concentrations of Tat at 100 ng/ml or higher (Fig. 6). The cell proliferation stimulated by Tat was at the maximal inducible level compared with that by known growth simulators of AIDS-KS cells, such as HTLV-II CM and bFGF (Fig. 6) (3, 13, 14, 16, 36–38, 46). Preincubation of Tat with the anti-Tat antibodies abolished the growth response promoted by Tat, yet it had no effect on cell growth stimulated by other growth inducers (HTLV-II CM) or on the basal level of cell growth (Fig. 6A, right panel), confirming the specificity and the lack of toxicity of the antibodies (3, 14). Similar results were recently obtained with normal vascular endothelial and smooth-muscle cells, potential progenitors of AIDS-KS cells, which proliferate in response to Tat after being exposed to cytokines from activated T cells (3). These results indicate that only picomolar amounts, or less, of extracellular Tat are required to stimulate cell growth. The same Tat preparations were then used in assays of viral transactivation and replication (Fig. 7 and 8).

With COS cells transiently transfected with the HIV-1 LTR, detectable levels of gene expression were obtained by the direct addition of concentrations of Tat greater than 500 ng/ml (Fig. 7A) or by scrape loading of the protein (100 ng/ml) into the cells (Fig. 7B). Similar Tat concentrations were required to induce viral replication by adding or scrape loading Tat into HeLa-CD4-expressing cells containing a nonreversible, Tat-defective HIV-1 provirus (HLM1 cell line) (44) (Fig. 8). In both systems, transactivation increased linearly with Tat concentrations when either procedure, direct addition or scrape loading, was employed. These results are in agreement with previous data (17, 19, 20, 23, 34) and indicate that the concentrations of exogenous Tat required to activate HIV-1 gene expression and replication are higher than those needed for the cell growth effect. In addition, the kinetics of dose-response to Tat differ for the two types of effects.

Uptake and nuclear localization of Tat by AIDS-KS cells require protein concentrations similar to those necessary for HIV-1 transactivation. To transactivate the HIV-1 LTR, extracellular Tat must be taken up by cells and be localized into the nuclei (19, 20, 23, 34). As AIDS-KS cells cannot be transfected at the efficiency required for gene expression studies, the direct comparison of the Tat concentrations required for cell growth with those required for transactivation could not be made for the same cell type. However, as nuclear localization of Tat is required for transactivation (28, 43, 45), this was monitored after the addition of exogenous Tat to AIDS-KS cells. Cells were incubated with serial dilutions of Tat (from 0.01 to 10 μg/ml) for different periods (from 15 min to 3 h) and then stained with the affinity-purified anti-Tat monoclonal antibodies by the double indirect immunoperoxidase procedure (Fig. 9). Uptake and nuclear localization of Tat were clearly and consistently detectable only by incubating the cells with 100 ng of the protein per ml or more. These (particularly the nucleolar localization) increased by increasing the concentration of exogenous Tat or the time of incubation of the cells with the protein. An example of the nuclear uptake of Tat after incubation of the cells with 1 μg or 100 ng of the protein per ml is shown in Fig. 9. Staining was abolished when Tat was preincubated with the anti-Tat antibodies (Fig. 9B). Similar to the results of previous experiments (34), the cellular uptake of Tat was reduced (low-level and diffuse cytoplasmic staining only) by incubations being performed at 4°C instead of at 37°C (data not shown). The results indicate that the concentrations of extracellular Tat required for nuclear-nucleolar localization of the protein are higher than needed for the cell growth effect and are much closer to those necessary for viral transactivation or replication. As for transactivation, the nuclear uptake of Tat is concentration and time dependent, increasing linearly with both, in agreement with previous data (19, 20, 23, 34).

TABLE 2. Expression, subcellular localization, release, and activity of Tat from COS-1 cells transfected with PCV-TAT, CMV-TAT, and a *tat* gene mutated in the basic region (CMV-M-TAT)[a]

Vector(s)	Tat expression			Tat content[b]			Tat activity	
	Positive cells (%)	Nucleus[c]	Cytoplasm[c]	Total	Intracellular (%)	Extracellular (%)	Intracellular[d] (fold)	Extracellular[e] (cpm)
PCV-TAT	5–10	++	++	25	63.5	36.5	50	2,478
CMV-TAT	3–5	++	+	14.6	92.2	7.8	72	2,254
CMV-M-TAT	2–4	±	++	10	82.8	17.2	52	2,198
Vector DNAs	0	−	−	0	0	0	1	1,400

[a] COS-1 cells were transfected with plasmids as described in Materials and Methods. At 48 h after transfection, Tat expression was monitored by immunohistochemistry with the anti-Tat monoclonal antibodies (expressed as average values of percentages of positive cells from five fields per slide; duplicate samples were used for each experiment) and by determining nuclear versus cytoplasmic Tat localization. Intracellular and extracellular Tat was analyzed by RIPA of Tat with cell extracts (500 μl) and supernatants (4 ml) from metabolically labeled cells and measured by densitometric analysis (Gelscan XL; Pharmacia) of the immunoprecipitated Tat protein band (average values of short exposures for intracellular Tat and long exposures for extracellular Tat). Intracellular Tat activity was measured in cell extracts from cells cotransfected with the *tat* plasmids and the HIV-1 LTR-CAT plasmid (CD23-CAT); extracellular Tat activity was monitored in CM (dilutions, 1:2 and 1:4) by growth assays with AIDS-KS cells (by determining [^{3}H]thymidine incorporation). After transfection, cell death was not detectable (LDH measurements). Results are the averages of five independent experiments.

[b] Densitometry of the Tat protein band detected by RIPA. Values are expressed on an arbitrary scale, with a value of 10 representing the minimal total (intracellular plus extracellular) detected Tat signal. Intracellular and extracellular Tat contents are expressed as percentages of the total signal.

[c] −, negative; ±, not consistently detected; +, <50% of the Tat-positive cells; ++, 50 to 100% of the Tat-positive cells.

[d] CAT activity after 20 min of incubation relative to that with control vectors (onefold).

[e] AIDS-KS cell growth measured by [^{3}H]thymidine incorporation assay (standard deviation [SD] ≤ ±12%). CM from cells transfected with the control vector DNAs gave an average thymidine uptake of 1,400 cpm (SD ≤ ±11.5%). HTLV-II CM (positive control) gave an average value of 2,400 cpm (SD, ±10%).

DISCUSSION

We have shown that during acute HIV-1 infection or after transfection of the gene, Tat is released into the cell culture medium and can stimulate maximal AIDS-KS cell growth and low to moderate levels of HIV-1 gene expression. Release of Tat occurs when the rate of cell death is still low (during infection) or cell death is absent (after transfection) and when Tat expression is high. Expression of Tat, in fact, is an early event, necessary for optimal viral replication and productive infection. The release of Tat appears to be dependent on high expression of the gene and on the presence of cytoplasmic protein. The transfection method utilized in our studies is characterized by high expression of Tat and may mimic this early phase of HIV-1 replication, when high Tat expression results in accumulation in the cytoplasm and release into the medium. We failed to detect Tat in the culture supernatants from chronically infected cells or cell lines permanently expressing Tat (H9 and U937) (14a). This may be due to the lower expression of Tat observed with these cell types compared with that observed with transiently transfected or acutely infected cells or to the sensitivities of our assays, or other mechanisms may be operating in these other systems.

When the amount of the CMV-M-TAT product, mutated in the basic region, is compared with that expressed by the wild-type vector (CMV-TAT), the following observation can be made. There are (i) lower levels of intracellular Tat content, (ii) increased levels of cytoplasmic Tat, and (iii) similar levels of extracellular Tat release and biological activity. This suggests that the increased release of Tat is due to the levels of cytoplasmic localization of the protein. It has been proposed previously that cytoplasmic Tat has a

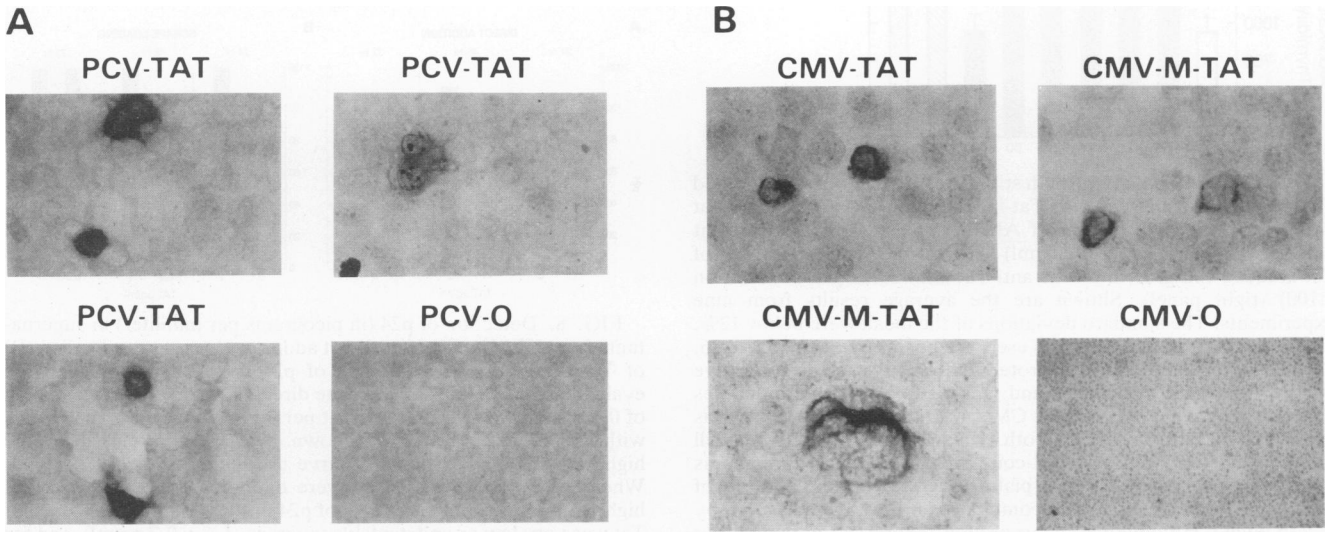

FIG. 4. Subcellular localization of Tat in COS-1 cells transfected with PCV-TAT and PCV-0 (A) and CMV-TAT, CMV-M-TAT, and CMV-0 (B). Immunostaining was performed by the double indirect immunoperoxidase method. For details, see footnote *a* in Table 2 and Materials and Methods.

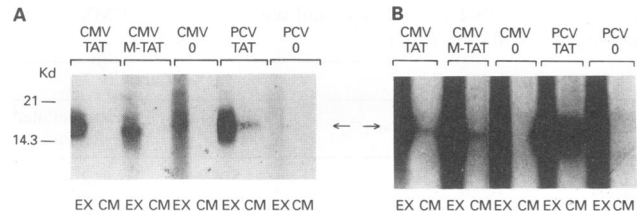

FIG. 5. RIPA of Tat with cell extracts (EX) and supernatants (CM) from metabolically labeled COS-1 cells after transfection with the *tat*-expressing vectors shown in Fig. 4. Exposure was for 12 h (A) or 7 days (B). For details, see footnote *a* in Table 2 and Materials and Methods.

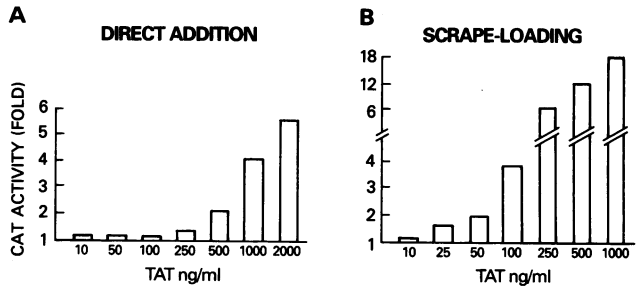

FIG. 7. Activation of HIV-1 LTR-directed gene expression by the direct addition (A) or scrape loading (B) of recombinant Tat into COS cells transiently transfected with the HIV-1 LTR-CAT plasmid. The activity values indicated are relative to that obtained with buffer alone (onefold) and are the averages of four to eight separate experiments. Standard deviation, ≤10%.

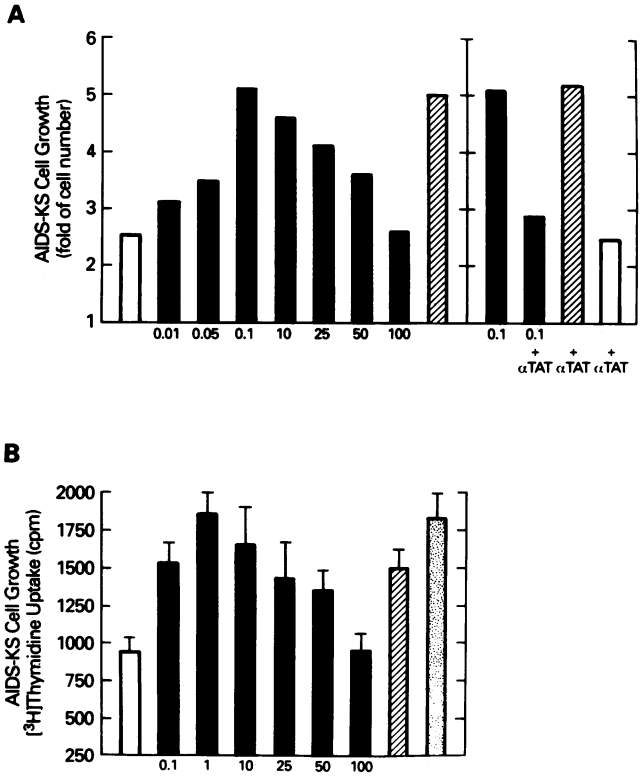

FIG. 6. AIDS-KS cell growth stimulated by recombinant purified Tat protein and inhibition of Tat-induced cell growth by anti-Tat antibodies. (A) Proliferation of AIDS-KS cells with recombinant purified Tat (0.01 to 100 ng/ml) (left panel) and inhibition of Tat-induced cell growth by the anti-Tat antibodies (αTAT, dilution 1:100) (right panel). Shown are the average results from nine experiments. The standard deviations of the mean were below 12%. HTLV-II CM (dilution, 1:5) was used as a positive control (3, 14, 16, 37, 38), and medium alone or protein buffer was used as a negative control. To verify specificity and lack of toxicity, the antibodies were also added to HTLV-II CM or to the medium alone, as previously described (14). In both types of assays, AIDS-KS cell growth was monitored by a cell-counting method and expressed as a multiple of the number of cells plated (onefold). (B) Proliferation of AIDS-KS-derived cells with recombinant purified Tat measured by determining [^{3}H]thymidine incorporation (expressed in counts per minute). The average results of five assays are shown. bFGF (50 ng/ml) and HTLV-II CM (1:5) were used as positive controls (3, 14, 16, 37, 38). □, medium; ■, TAT; ▨, HTLV-II CM; ▩, bFGF.

shorter half-life (27). Our results suggest that this may be due to the fact that a larger fraction of the cytoplasmic protein is released by the cells. There are several examples of proteins which are released in the absence of a secretory signal and which are found in the extracellular fluid in biologically active forms, such as bFGF and acidic FGF, interleukin-1, and platelet-derived endothelial cell growth factor (2, 9, 11, 16, 24, 25, 42). In particular, our data and preliminary results from experiments similar to those performed for interleukin-1 (42) suggest that a mechanism(s) other than cell death is responsible for the release of Tat.

The Tat-containing supernatants induce maximal AIDS-KS cell growth but modest levels of HIV-1 transactivation. Although viral transactivation by extracellular Tat can occur in a situation of close cell proximity or contact (Table 1) (29), these results suggest that the cell growth-promoting activity may be more biologically relevant than the virus-transactivating effect of extracellular Tat.

The experiments with purified Tat protein show that maximal cell growth is obtained with Tat concentrations much lower than those required to induce HIV transactiva-

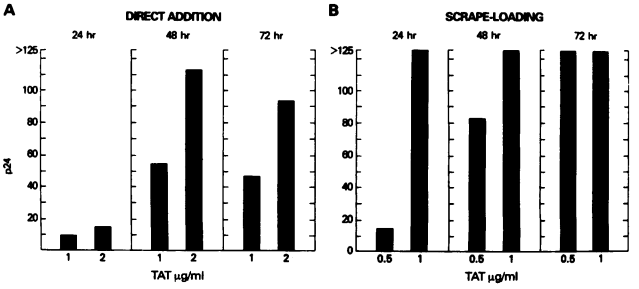

FIG. 8. Detection of p24 (in picograms per milliliter) in supernatants from HLM1 cells after direct addition (A) or scrape loading (B) of Tat into the cells. Amounts of p24 in the supernatants were evaluated 24, 48, and 72 h after the direct addition or scrape loading of 0.1, 0.25, 0.5, 1, or 2 μg of Tat per ml into the cells. The results with 0.5, 1, and 2 μg of Tat are shown. p24 concentrations above the highest point of the standard curve are indicated as >125 pg/ml. When some of these samples were diluted further, p24 was still higher than 375 pg/ml. The level of p24 production in the absence of Tat was very low or undetectable (average, 1.4 ± 2.7 pg/ml), and for each experiment it was subtracted from the values obtained in the presence of Tat. Results are the averages of 2 to 10 experiments for each Tat concentration. Standard deviation, ≤10%.

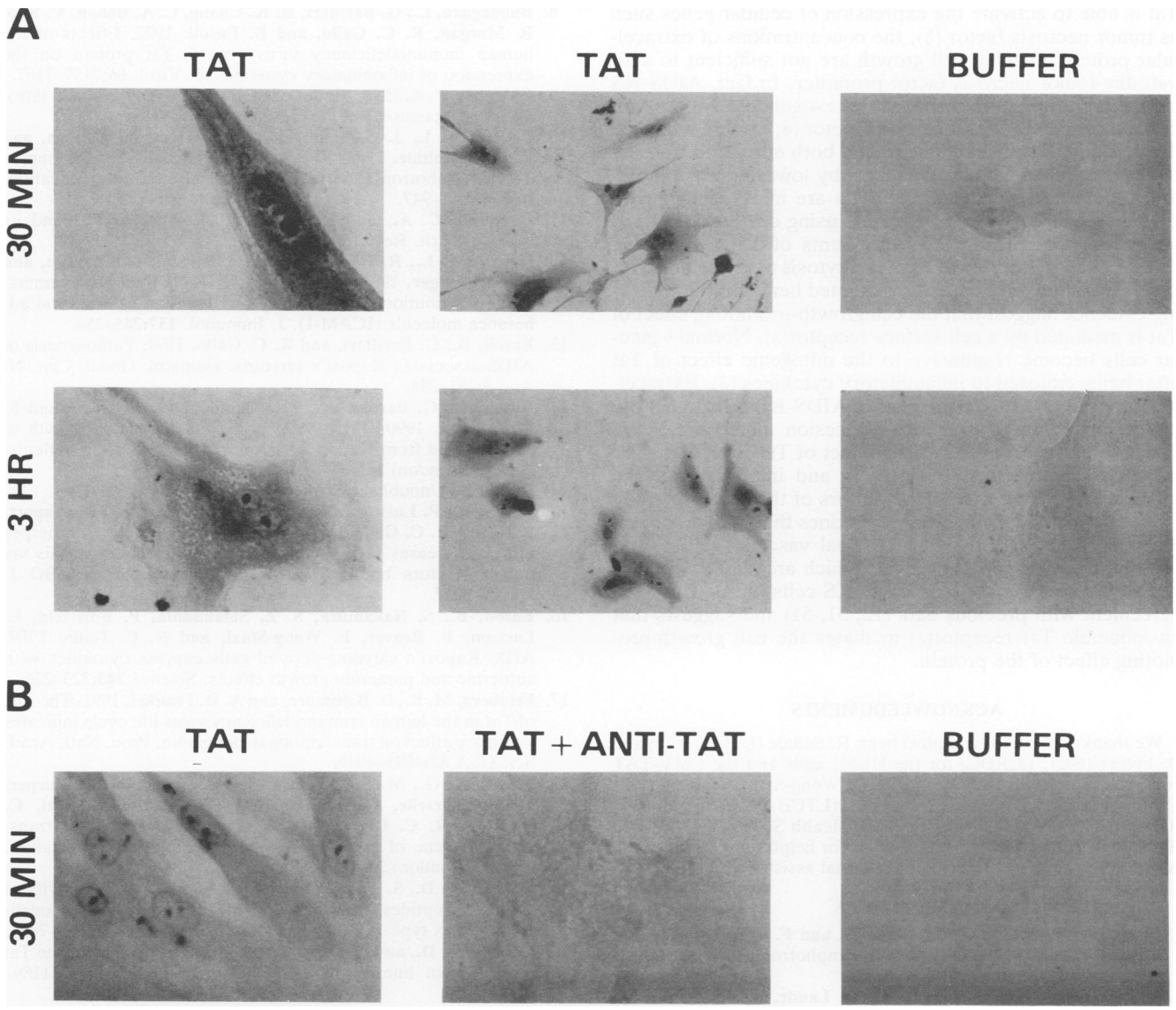

FIG. 9. Uptake of recombinant Tat by AIDS-KS cells and absence of staining by preincubating protein with anti-Tat antibodies. (A) Results with AIDS-KS cells incubated for 30 min or 3 h with 1 μg of Tat or control buffer per ml, respectively, at two different magnifications. (B) Tat (100 ng/ml) or buffer control was preincubated (1 to 2 h, on ice) in the absence or in the presence of anti-Tat antibodies (TAT + ANTI-TAT), and then the complex was added to the cells. After incubation, cells were stained with the anti-Tat monoclonal antibodies by the double immunoperoxidase method. The experiments were repeated four to eight times with Tat concentrations of 0.1 to 10 μg/ml and two to three times by incubating the cells at 4°C instead of at 37°C, as described in Materials and Methods. The figure shows the most representative results.

tion and viral replication, particularly after the direct addition of the protein (≥500 ng/ml). Viral transactivation by extracellular Tat increases linearly with increasing amounts of exogenous protein (19, 20, 23, 34), while the cell growth effect peaks at between 0.1 and 1 ng of Tat per ml and is not observed at concentrations higher than 50 to 100 ng/ml.

As AIDS-KS cells cannot be transfected at the efficiency required for transactivation studies, HIV-1 gene expression and cell growth could not be monitored by using the same cell type. However, uptake and nuclear localization of Tat are required for transactivation; thus, we studied this requirement by incubating AIDS-KS cells with concentrations of Tat that induced cell growth or viral transactivation (Fig. 9). Nuclear localization of Tat was observed at Tat concentrations similar to those needed for transactivating effects, confirming the previous data of others (34). As with transactivation, uptake and nuclear localization of Tat are dose dependent, increasing with concentration, and are dependent on the time of incubation of the cells with the protein. These data confirm that cell growth requires a lower concentration of extracellular Tat than does the transactivating effect.

The data presented here suggest that different pathways mediate the cell growth-promoting effect and the uptake-transactivation effect of extracellular Tat. Also in support of this are recent results indicating that, although extracellular

Tat is able to activate the expression of cellular genes such as tumor necrosis factor (8), the concentrations of extracellular protein inducing cell growth are not sufficient to activate the tumor necrosis factor promoter. In fact, AIDS-KS cells proliferating in response to exogenous Tat do not contain or secrete tumor necrosis factor (8; also unpublished data). Ongoing studies indicate that both entry and transactivation activity of Tat are reduced by lowering the temperature (data not shown). These data are in agreement with recent studies by other researchers using cell lines (29, 34), suggesting that the uptake of amounts of Tat resulting in transactivation is mediated by endocytosis of the protein (19, 20, 34). Conversely, the data presented here and the following evidence suggest that the cell growth-promoting effect of Tat is mediated by a cell surface receptor(s). Normal vascular cells become responsive to the mitogenic effect of Tat after being exposed to inflammatory cytokines (3). Extracellular Tat induces the attachment of AIDS-KS cells to culture plates, mimicking the effect of adhesion molecules of the extracellular matrix (4, 7). This effect of Tat is mediated by the RGD sequence of the protein and implies a specific interaction with cell surface receptors of the integrin family (4, 7). The same inflammatory cytokines that induce the cell responsiveness to Tat induce normal vascular cells to express integrin receptors at levels which are similar or identical to those expressed by AIDS-KS cells (3, 4). This is in agreement with previous data (12, 31, 51) and suggests that an inducible Tat receptor(s) mediates the cell growth-promoting effect of the protein.

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