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Synthesis of Functional Human Immunodeficiency Virus *tat* Protein in Baculovirus as Determined by a Cell-Cell Fusion Assay

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The human immunodeficiency virus *tat* protein is a strong *trans*-activator of the expression of mRNAs originating from the viral long terminal repeat. We have expressed the first 72 amino acids (coding exon 1) of this protein in eucaryotic *Spodoptera frugiperda* SF9 cells by using a baculovirus vector, *Autographa californica* nuclear polyhedrosis virus. We show that the baculovirus vector stably produced the 72-amino-acid form of the *tat* protein but was unable to stably synthesize a larger 101-amino-acid full-length version of the same polypeptide. The 72-amino-acid *tat* protein, when introduced into mammalian fibroblasts by using a cell-cell fusion technique, functionally *trans*-activated the expression of the human immunodeficiency virus long terminal repeat.

Human immunodeficiency virus (HIV) is the etiological agent for acquired immunodeficiency syndrome (AIDS) (3, 4). The HIV retrovirus is approximately 10 kilobases (kb) in size and contains *gag*, *pol*, and *env* genes which are flanked by long terminal repeat (LTR) sequences. In addition, the virus also contains at least five other nonstructural genes (7, 8), of which one, *tat*, has been extensively studied.

The *trans*-activator protein *tat* of HIV is encoded by a doubly spliced mRNA generated through the joining of two separate coding exons (2, 26, 29) (Fig. 1). This mRNA codes for a protein of approximately 14 kilodaltons (kDa) (10) which functions as a potent *trans*-activator for the expression of genes under the control of the HIV LTR. Depending on the particular HIV isolate, the *tat* protein varies in exact size (e.g., 86 amino acids for the HXB2 isolate and 101 amino acids for the SF2 isolate). However, it has been demonstrated that only the initial 58 N-terminal amino acids (23) of the first *tat* coding exon (5, 9, 15, 26) are necessary for *trans*-activator activity.

Despite studies showing the necessity of *tat* function for HIV viability in T_4 lymphocytes (6, 8), the exact mechanism of *tat* action remains unclear. It has been suggested that *tat* can act at both a transcriptional and posttranscriptional level (5, 13, 15, 17, 19, 21, 29) to increase the quantity and utilization of mRNAs. The presence of regions of basic amino acid residues and a cluster of seven cysteines in the *tat* protein suggest a nucleic acid-binding domain (15, 17, 22). There is as yet no evidence for a direct interaction between HIV *tat* and either DNA or RNA.

One approach to elucidating the biological mechanism(s) of HIV *tat* function lies in the acquisition of useful amounts of functional protein. Consistent with this goal, we have transferred a DNA fragment containing the first coding exon, the biologically active N-terminal portion of the HIV *tat* protein, into baculovirus (Fig. 1). The amino acid sequence of this DNA is presented in Fig. 2A.

We verified, prior to the transfer of DNA into baculovirus, that this first coding exon independently retained intact HIV *trans*-activating function. A chimeric plasmid (pSVtat12) containing the same DNA fragment placed downstream of the simian virus 40 (SV40) early promoter was constructed and functionally compared with a full-length cDNA of HIV *tat* controlled by the same promoter (pSVtat [13]) (Fig. 2B). Both plasmids were separately introduced into a CV-1-derived cell line (cBENNCAT-3) which contained an integrated copy of an HIV LTR-*cat* gene (pBENNCAT [9]) (Fig. 2B, lanes 3 and 4). Alternatively, we cotransfected pSVtat12 or pSVtat with stoichiometric amounts of pBENNCAT plasmid into CV-1 cells (Fig. 2B, lanes 7 and 8). Within the limits of experimental variability and consistent with the results of previous investigators (5, 9, 15, 23, 26), we found that the first 72 amino acids was comparable to the full-length *tat* protein in activating the expression of an HIV LTR-linked *cat* gene. As a negative control, we detected no significant *trans*-activation in CV-1 cells of the HIV LTR by the HTLV-I $p40^x$ gene (Fig. 2B, lanes 2 and 6). The difference in acetylation observed in lanes 3 and 4 (Fig. 2B) represents an experimental variability in this single experiment. In other transfections, we detected minor differences between the levels of *trans*-activation of the integrated HIV LTR-*cat* by pSVtat12 compared with those by pSVtat.

An *EcoRI-KpnI* DNA fragment containing the HIV *tat* coding exon 1 (map positions 5401 to 5625 [19]) was placed into the polylinker cloning region of polyhedrin transfer vector pAc610 (Fig. 1C) (28), which was modified to contain an SV40 polyadenylation signal. The resulting plasmid, ptat, was transfected with DNA from a wild-type baculovirus, *Autographa californica* nuclear polyhedrosis virus, into insect SF9 cells. By virtue of in vivo recombination events, hybrid baculoviruses that have incorporated the HIV *tat* sequence into the polyhedrin gene were generated. Independent isolates of recombinant baculovirus were plaque purified. One isolate, vActat, was used in subsequent studies.

We determined the synthesis of HIV *tat* protein in SF9 cells infected with vActat virus. A rabbit antiserum directed against a synthetic peptide consisting of the first 20 amino acids of the *tat* protein (Fig. 2A) was prepared. SF9 cells were mock infected (Fig. 3A, lane 1), infected with vAcPx (an HTLV-I $p40^x$ recombinant baculovirus [11]) (Fig. 3A, lane 2), infected with vActat (Fig. 3A, lane 3), or infected

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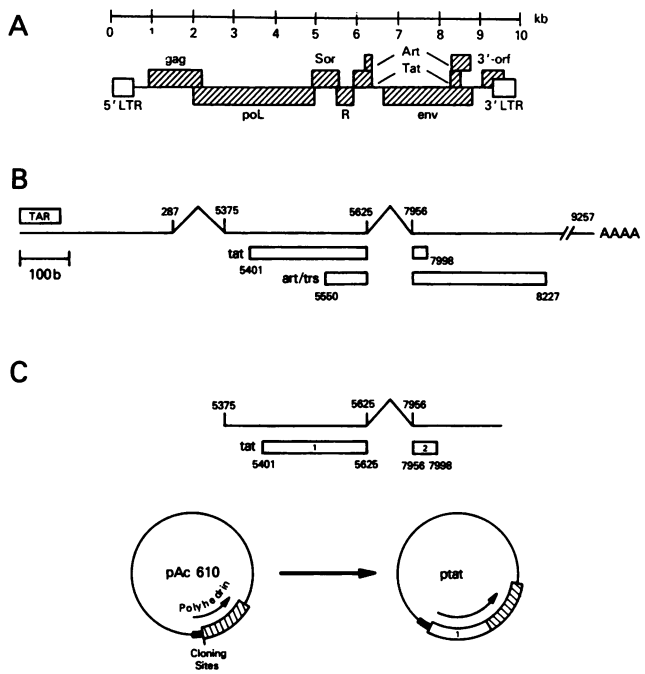

FIG. 1. Schematic representations of the HIV genome and construction of a baculovirus expression plasmid containing the HIV *tat* gene. (A) The HIV genome is presented to show the relative locations of the structural and nonstructural viral genes. (B) Putative splice donor and acceptor sites and map coordinates for the HIV *tat* and *art/trs* mRNAs. (C) Cloning of HIV *tat* coding exon 1 into baculovirus transfer vector pAc610 to generate plasmid ptat. In ptat, the HIV *tat* coding exon 1 is driven by the polyhedrin promoter. orf, Open reading frame; b, bases; TAR, *trans*-activation-responsive sequence. Nucleotide numbering is based on Ratner et al. (19).

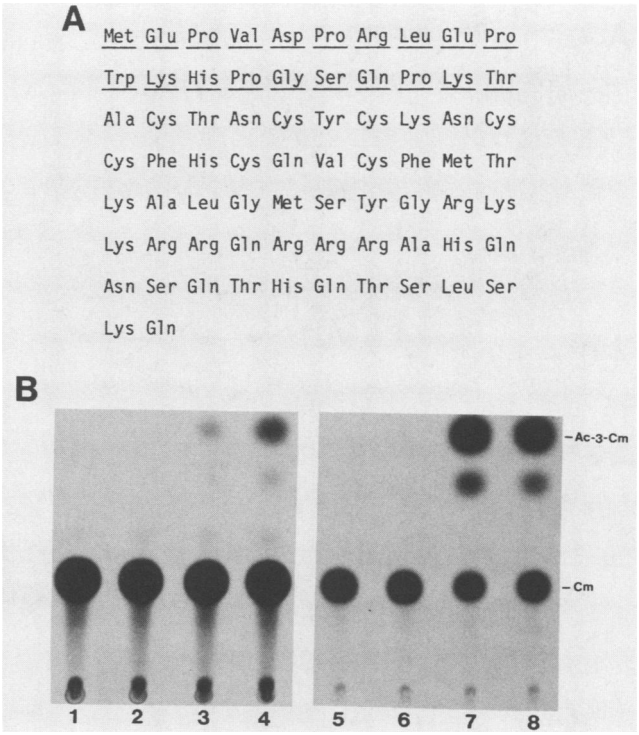

FIG. 2. (A) The 72 amino acids of the HIV *tat* first coding exon. A synthetic peptide of the first 20 amino acids (underlined) was used to immunize New Zealand White rabbits to generate specific antiserum. (B) *trans*-Activation capacity of the HIV *tat* coding exon 1. Coding exon 1 or a full-length cDNA of coding exons 1 and 2 was individually placed downstream of the SV40 early promoter to generate plasmids pSVtat12 and pSVtat, respectively. These two DNAs were separately transfected into cBENNCAT-3 cells, a CV-1 cell line which contains an integrated copy of an HIV LTR-*cat* gene, or cotransfected with an HIV LTR-*cat* plasmid, pBENNCAT (9), into CV-1 cells. CAT activities were assayed 48 h after transfection. Lanes: 1, cBENNCAT-3 cells alone; 2, cBENNCAT-3 cells transfected with 1 μg of an HTLV-I $p40^x$-producing plasmid (pHTLVpX [16]); 3, cBENNCAT-3 cells transfected with 1 μg of pSVtat12; 4, cBENNCAT3 cells transfected with 1 μg of pSVtat; 5, CV-1 cells alone; 6, CV-1 cells transfected with 1 μg of pBENNCAT and 1 μg of pHTLVpX; 7, CV-1 cells transfected with 1 μg of pBENNCAT and 1 μg of pSVtat12; 8, CV-1 cells transfected with 1 μg of pBENNCAT and 1 μg of pSVtat. Ac-3-Cm, Acetylated chloramphenicol; Cm, chloramphenicol.

with a plaque-purified stock of recombinant baculovirus containing a full-length *tat* cDNA of HIV SF2 isolate (Fig. 3A, lane 4) for 36 h prior to pulse labeling with 20 μCi of [^{35}S]cysteine per ml. We immunoprecipitated cell lysates with either preimmune or immune rabbit serum. In SF9 cells infected with vActat, a novel protein with an apparent mobility of 13 kDa (Fig. 3A, lane 3, right) was detected. This protein was antigenically confirmed in separate immunoprecipitations (data not shown) with a second rabbit antiserum (gift of B. Cullen) generated against an *Escherichia coli*-synthesized HIV *tat* protein. We believe that the 13-kDa *tat* protein exists as a homogeneous species. Preliminary labeling experiments with $^{32}P_i$ suggested that this polypeptide is devoid of phosphate groups (Jeang, unpublished observation).

We next assessed the stability of baculovirus-produced *tat* protein. vActat-infected cells were pulse-labeled for 3 h with [^{35}S]cysteine (Fig. 3B, lane 2) and then chased in unlabeled medium for 10 h (Fig. 3B, lane 3), 20 h (Fig. 3B, lane 4), or 30 h (Fig. 3B, lane 5). The cells were solubilized into sodium dodecyl sulfate (SDS) sample buffer (2% SDS, 0.1 M β-mercaptoethanol, 50 mM Tris hydrochloride [pH 7.5], 30% glycerol) and analyzed by polyacrylamide gel electrophoresis followed by autoradiography. A densitometric measurement of the HIV 13-kDa *tat* polypeptide band in the autoradiogram suggested that the half-life for this protein in SF9 cells is less than 10 h (Fig. 3B, compare lanes 2 and 3 and lanes 3 and 4). We detected very little radiolabeled *tat* protein after a 20-h chase period (Fig. 3B, lane 4).

The biological activity of baculovirus-synthesized *tat* protein was tested by using a cell-cell fusion protocol. We used polyethelene glycol (PEG) to fuse cBENNCAT-3 cells to mock-infected SF9 cells (Fig. 4A, lane 1), SF9 cells infected with wild-type baculovirus (Fig. 4A, lane 2), or SF9 cells infected with vActat virus (Fig. 4A, lane 3). Measurement of chloramphenicol acetyltransferase (CAT) activities indicated that the integrated HIV LTR-*cat* gene in cBENNCAT-3 cells was activated by vActat-infected (Fig. 4A, lane 3) but not by wild-type-virus-infected (Fig. 4A, lane 2) or mock-infected (Fig. 4A, lane 1) cells.

We verified that this *trans*-activation of the HIV LTR-*cat* gene was from exogenously introduced *tat* protein and not from the translation in cBENNCAT-3 cells of *tat* mRNA delivered from infected SF9 cells through the fusion event. In a second series of assays, we used untreated (Fig. 4B, lane 2) or vActat-infected cells treated with 100 μM anisomycin for 20 h (Fig. 4B, lane 3). This treatment with anisomycin inhibited de novo protein synthesis in SF9 cells by more than 99% (data not shown). Furthermore, from the

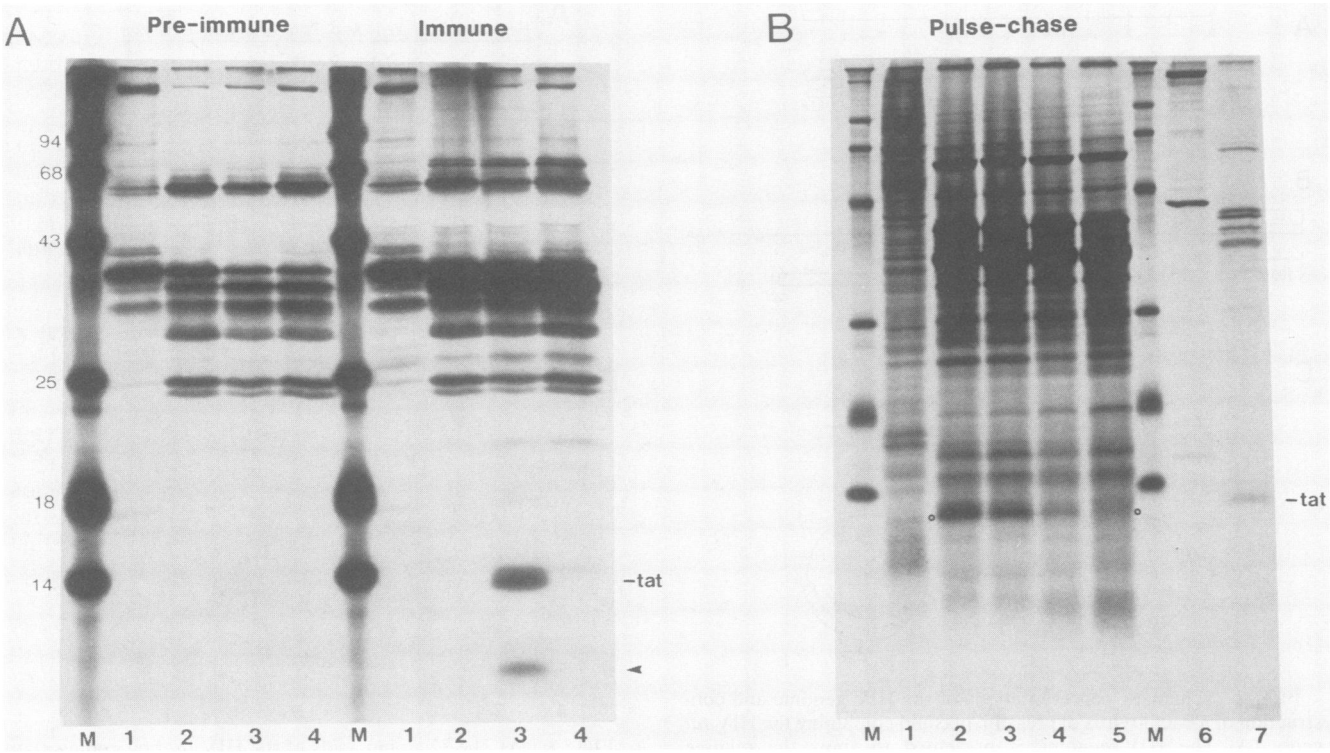

FIG. 3. (A) Immunoprecipitation of infected SF9 cells. SF9 cells were mock infected or infected with virus for 36 h prior to labeling with 20 μCi of [^{35}S]cysteine per ml for 3 h. Cells were lysed with RIPA buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS), and proteins were immunoprecipitated with preimmune or immune serum directed to the HIV *tat* protein. An autoradiogram of an SDS–10% polyacrylamide gel is shown. Lanes: M, molecular size markers (sizes in kilodaltons); 1, mock-infected SF9 cells; 2, SF9 cells infected with vAcPx (recombinant baculovirus containing HTLV-I p40^{x} gene [11]); 3, SF9 cells infected with vActat; 4, SF9 cells infected with a stock of recombinant baculovirus containing a full-length cDNA of the HIV *tat* gene. The position of a 13-kDa *tat* protein in lane 3 is indicated. Arrow points to a presumed proteolytic product of *tat* which varied in intensity from experiment to experiment. (B) Stability assessment of baculovirus-synthesized HIV *tat*. SF9 cells were infected with vActat for 36 h prior to a 3-h labeling period. After pulse-labeling, cells were chased in unlabeled medium for various times. Cellular proteins were directly solubilized into SDS sample buffer (lanes 1–5) or lysed with RIPA buffer and immunoprecipitated (lanes 6 and 7) with anti-*tat* serum. Lanes: M, molecular size markers; 1, mock-infected SF9 cells; 2, pulse-labeled vActat-infected SF9 cells; 3, vActat-infected SF9 cells pulse-chased for 10 h; 4, vActat-infected SF9 cells pulse-chased for 20 h; 5, vActat-infected SF9 cells pulse-chased for 30 h; 6, cell lysate from mock-infected SF9 cells immunoprecipitated with anti-*tat* serum; 7, cell lysate from vActat-infected SF9 cells immunoprecipitated with anti-*tat* serum. The position of the 13-kDa HIV *tat* protein is labeled and indicated by open circles.

results of the pulse-chase experiment (Fig. 3B), we reasoned that the duration (20 h) of metabolic inhibition should prevent a significant accumulation of *tat* protein synthesized prior to anisomycin addition. Indeed, when cell-cell fusions were carried out with anisomycin-treated vActat-infected SF9 cells (Fig. 4B, lane 3), no increase in CAT activity over the baseline (Fig. 4B, lane 1) was seen. A parallel fusion performed with untreated vActat-infected cells, as expected, demonstrated activation of the integrated HIV LTR-*cat* gene (Fig. 4B, lane 2). We do not believe that the difference in CAT activities was due to the transfer into cBENNCAT-3 cells of residual amounts of inhibitor molecules retained within the anisomycin-treated SF9 cells. Radiolabeling of cells after fusion showed no significant variation in total [^{35}S]cysteine incorporation between the three different samples (data not shown).

We also controlled for the possible perturbation by anisomycin of the steady-state levels of *tat* mRNAs. Northern (RNA) blot analysis of mRNAs isolated from vActat-infected cells, not treated (Fig. 4C, lane 2) or treated with anisomycin (Fig. 4C, lane 3), showed no major differences in the accumulated level of *tat* mRNAs. This result reaffirmed that the cell-cell fusion-mediated *trans*-activation was specific for *tat* protein and not *tat* mRNA.

In this study we have expressed the biologically active N-terminal 72 amino acids of the HIV *tat* protein in a baculovirus vector. A recombinant baculovirus, vActat, that contained the first HIV *tat* coding exon in place of the polyhedrin gene was plaque purified. This virus synthesized approximately 1 to 5 mg (quantitated by silver staining of immunoprecipitated protein; data not shown) of a 13-kDa *tat* protein per liter (2.5×10^8 cells) of infected SF9 cells. The level of HIV *tat* synthesis is roughly 100 times less than the expression of a counterpart HTLV-I protein (HTLV-I p40^{x}) from a similarly generated recombinant baculovirus (11). This may reflect some inherent instability of the HIV *tat* protein in SF9 cells. Consistent with this idea, we encountered significant difficulties in isolating a recombinant baculovirus that expressed a full-length cDNA (both coding exons 1 and 2) of the HIV *tat* gene. Stocks of this recombinant baculovirus carrying a full-length *tat* cDNA produced minimal amounts of intact protein (Fig. 3A, lane 4).

The synthesis of HIV *tat* protein in baculovirus presents some potential advantages over similar expression in *E. coli* (1, 10, 15). The baculovirus-SF9 cell system offers the ability to construct a variety of eucaryotic posttranscriptional modifications, including glycosylation (25), splicing (12), signal peptide cleavage and extracellular secretion (24, 25), and

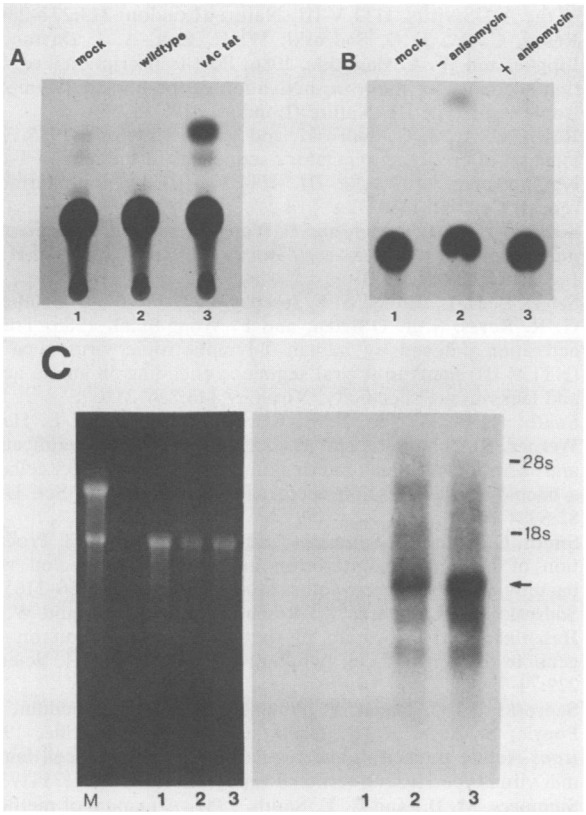

FIG. 4. Functional assay for baculovirus-synthesized HIV *tat*. (A) cBENNCAT-3 cells (10^6 cells) were trypsinized and suspended with equal numbers of mock-infected or infected (36 h after virus inoculation) SF9 cells in 2 ml of 100% (wt/vol) PEG 1000 in serum-free medium with 100 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.0) for 1 min at room temperature. Cells were pelleted at 2,000 rpm in a tabletop centrifuge, washed once with serum-free medium, and seeded into culture dishes in medium plus 10% fetal bovine serum. CAT activities were assayed 20 h after seeding. Lanes: 1, cBENNCAT-3 fused with mock-infected SF9 cells; 2, cBENNCAT fused with wild-type-infected SF9 cells; 3, cBENNCAT-3 cells fused with vActat-infected SF9 cells. Autoradiogram was exposed for 24 h. (B) cBENNCAT-3 cells were fused to SF9 cells infected with vActat virus for 36 h and then maintained in normal insect cell medium or medium plus 100 μM anisomycin for 20 h. SF9 cells treated with anisomycin were washed three times with phosphate-buffered saline prior to use in cell-cell fusions. Lanes: 1, cBENNCAT-3 fused with mock-infected SF9 cells; 2, cBENNCAT-3 fused with vActat-infected SF9 cells; 3, cBENNCAT-3 fused with vActat-infected cells treated with anisomycin. Autoradiogram was exposed for 4 h. (C) Northern blot analysis of *tat*-specific RNA in vActat-infected cells with or without anisomycin treatment. Left, Ethidium bromide-stained gel; right, autoradiogram of the same gel after transfer to nitrocellulose paper and probing with a ^{32}P-nick-translated HIV *tat* gene-specific DNA probe. Lanes: M, marker 28s and 18s rRNAs isolated from CV-1 cells; 1, RNA from SF9 cells infected with wild-type baculovirus; 2, RNA from SF9 cells infected with vActat; 3, same as lane 2 except that cells were incubated for 20 h in anisomycin prior to RNA isolation. Relative positions of the 28s and 18s rRNAs are indicated. Arrow points to *tat*-specific RNA. In infected SF9 cells, we routinely saw only the 18s rRNA after ethidium bromide staining.

phosphorylation (14). In addition, we have found that proteins overexpressed in baculovirus vectors generally do not suffer from the problems of solubility often encountered in procaryotic systems. It is therefore not unexpected that both the HIV (this study) and HTLV-I (11) *trans*-activator proteins are made in functional forms in the baculovirus-SF9 cell system. The availability of soluble HIV *tat* protein should permit a rapid assessment of the binding properties of this polypeptide to nucleic acids.

We have characterized the biological activity of baculovirus-synthesized HIV *tat* by optimizing a PEG fusion technique for use with SF9 cells. This fusion procedure represents a rapid and general means for the introduction of baculovirus-produced proteins into recipient indicator cells. We resorted to this approach because many different experiments indicated that HIV *tat*, unlike HTLV-I p40^x (11), was incapable of *trans*-activating an LTR-linked reporter gene in the SF9 cell background. This observation held true in both cotransfection (pBENNCAT and pSVtat transfected together into SF9 cells) and superinfection (transfection of pBENNCAT into SF9 cells followed by infection with vActat) experiments. One possible explanation for this finding may be that the HIV *tat* protein requires the cooperation of cellular factors that are absent in eucaryotic SF9 cells for the activation of responsive LTR sequences. We believe that the ability to deliver biologically active HIV *tat* protein (i.e., vActat-infected SF9 cells) through a simple procedure (i.e., PEG fusion) into responsive indicator cells (cBENNCAT-3) will permit an examination of the requirements for de novo protein synthesis in the mechanism of *tat*/HIV-LTR *trans*-activation.

This project was initiated under George Khoury's leadership. We are grateful for his scientific input and guidance and regret his passing. We thank Malcolm Martin for advice and for the use of various HIV recombinant plasmids, Paul Luciw for plasmid pSVtat, Bryan Cullen for antiserum to HIV *tat*, and Max Summers for introducing us to baculovirus expression technology.

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