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Interaction of the Second Coding Exon of Tat with Human EF-1δ Delineates a Mechanism for HIV-1-Mediated Shut-Off of Host mRNA Translation

Hua Xiao,[1] Christine Neuveut, Monsef Benkirane, and Kuan-Teh Jeang
Molecular Virology Section, Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892-0460

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HIV-1 Tat has pleiotropic functions. While its most studied role is to activate transcription from the retroviral long terminal repeat (LTR)-promoter, Tat also has functions as a secretable growth factor, a T-cell activator, and an inducer of cellular apoptosis, amongst others. For its transcriptional function, the first coding exon of Tat appears wholly sufficient; however, lentiviruses (HIVs and SIVs) maintain and conserve a second coding exon for Tat. While the function(s) of the second exon of Tat has remained largely unknown, its integrity in lentiviral genomes suggests biological importance, possibly a role in non-transcriptional activities. To understand better the biology of the second exon of Tat in HIV-1 infection of cells, we have searched for cellular proteins that bind specifically to this protein domain. Here, we report that the human translation elongation factor 1-delta (EF-1δ) binds to the second exon of HIV-1 Tat. Interaction between Tat and EF-1δ dramatically reduces the efficiency of the translation of cellular, but not viral, mRNAs. These findings suggest that a non-transcriptional activity of Tat modulates cellular protein synthesis, thereby affecting the metabolism of host cells.

HIV-1 Tat is a potent activator of transcription from the viral long terminal repeat (LTR). Tat, however, is also essential for viral replication (1, 2), can activate or repress the transcription of various cellular genes and function as a secretable growth factor for Kaposi-sarcoma-like cells (reviewed in ref. 3). *In vitro,* Tat can activate quiescent T-lymphocytes (4, 5) and, in some settings, induce their apoptosis (6) perhaps partly through a suppression of manganese-dependent superoxide dismutase (Mn-SOD; 7). Collectively, these findings and others (reviewed in ref. 3) have fueled the speculation that Tat has pleiotropic non-transcriptional functions in the life-cycle of HIV-1 (8, 9).

HIV-1 Tat is encoded by two exons. Exon 1 contains amino acids 1 to 72; and exon 2 contains amino acids 73 to 101 (10). Tat's transcriptional activity for its cognate LTR is primarily contained within the first coding exon; most transfection studies have found that deletion of exon 2 minimally perturbs the ability of Tat to transactivate efficiently a LTR-reporter plasmid (reviewed in ref. 11). Despite this, it remains amply evident that all lentiviruses maintain and conserve the amino acid sequences in the second coding exon of Tat (10) suggesting functional importance. If the second coding exon of Tat does not participate in transcription, then one might reason that it subserves some of the pleiotropic non-transcriptional functions which have been attributed to Tat. Indeed, experimentally, the second exon of Tat has been found necessary both for T-cell activation (5) and suppression of Mn-SOD (7).

To explore roles for the second exon during HIV-1 infection of cells, we searched using yeast two-hybrid technology (12) for cellular proteins that would bind this protein domain. In screening 3×10^6 independent clones from a human library, we isolated a cDNA which encodes a cellular protein that, upon independent verification, specifically binds the second exon of Tat. On further characterization, we noted that this cellular factor is human translation elongation factor-1 delta (EF-1δ). Here, we show that protein-protein interaction between Tat and EF-1δ represses the translation of host-cell, but not HIV-1, mRNAs. We discuss the implications of host protein synthesis shut-off for HIV-1 infection.

[1] Author to whom correspondence should be addressed at: Building 4, Room 306, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892-0460. Fax: (301) 402-0226. E-mail: hxiao@atlas.niaid.nih.gov.

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MATERIALS AND METHODS

Yeast two-hybrid cloning. The MATCHMAKER II two-hybrid system (Clontech) was used to screen a HeLa cell cDNA library. To construct a GAL4-Tat Exon 2 bait plasmid, HIV-1 sequence coding for amino acids 64-101 of Tat was PCR-amplified and fused to the GAL4 DNA binding domain in pHGX1 (13). A total of three million colonies were screened. One of the cDNAs that we isolated was identical to the C-terminal half (amino acids 144-280) of the human translation elongation factor 1-delta, EF-1δ (14).

Protein affinity chromatography. GST-fusion proteins containing either full length or N-terminal half of EF-1δ were constructed in pGEX-2T, and MBP-fusion proteins containing Tat1-72 or Tat1-101 were constructed in pMAL-c2 (New England Biolabs). The resulting GST- and MBP-fusion proteins were expressed in and purified from *E. coli.* Protein affinity chromatography (AC) was performed using purified GST-EF-1δ bound to glutathione sepharose beads. The beads were equilibrated with a mixture of MBP-Tat72 protein, MBP-Tat101 protein, and control MBP protein. After extensive washes with affinity chromatography buffer (ACB, 10 mM Hepes, pH7.5, 1 mM MgCl2, 0.1 mM DTT, 10% glycerol) containing 100 mM KCl, bound proteins were eluted and analyzed by Western blotting with anti-MBP antibody.

In vitrotranscription/translation. In vitro transcription and translation were performed using reagents purchased from Invitrogen. CAT transcript was generated from a plasmid containing CAT coding sequence under T7 promoter.

Transfection of recombinant HIV-1 viruses and immunoprecipitation. Recombinant HIV-1 derivatives (see Figure 3) were transfected into HeLa cells. An SV40 early promoter-driven CD4-expressing plasmid was co-transfected with various forms of HIV-1 molecular clones into HeLa cells. 15 hours after transfection, α-amanitin was added to the cells to halt mRNA transcription, and 3 hours later ^{35}S-methionine was added to the cells for three additional hours to label proteins. ^{35}S-labeled HIV-1 and CD4 proteins were immunoprecipitated with anti-HIV-1 or anti-CD4 antibodies and resolved by SDS-PAGE.

RNase protection assays. RNase protection assays (RPA) were carried out according to manufacturer's protocol (Ambion, Inc.). Total RNA was prepared from transfected HeLa cells that were incubated with 3μg/ml α-amanitin for three hours, and quantified against known amounts of nucleic acid standards either by ethidium bromide staining or by OD-readings at 260 nm. Each RPA contained 10 μg of total RNA hybridized to 10^5 cpm of single-stranded RNA probe. The DNA template for preparation of single-stranded actin RNA probe were purchased from Ambion, Inc. To prepare single-stranded CD4 RNA probe, a CD4 DNA fragment was PCR-amplified with a 3′-primer containing the SP6 promoter and a 5′-primer containing 20 unrelated nucleotides. The resulting DNA was used as template to generate an antisense probe using SP6 RNA polymerase. Upon hybridization to CD4 mRNA, the unrelated 20 nucleotides which has no hybridizing counterparts in the mRNA will be digested by RNase A/T1, thus protected probe after digestion can be distinguished easily from input probe. HIV-1 RNA probe protected HIV-1 sequences from position 4021 to 4401, and was synthesized from pGEM-4Z/POL which contains sequences from position 4021 to 4401 of HIV-1 under SP6 promoter in pGEM-4Z.

RESULTS AND DISCUSSION

To explore directly potential biological role(s) for the second exon of Tat we asked whether there are cellular factors that specifically recognize this protein domain. Thus, we screened a human cDNA library using a yeast two-hybrid protocol for Tat second-exon binding proteins. Using the second-exon as bait, a human cDNA fragment encoding amino acids 144-280 of translation elongation factor-1δ (14) was specifically recovered (Figure 1).

A positive result in the two-hybrid assay suggests protein-protein contact; however, it does not formally exclude the possibility that EF-1δ and Tat might have interacted in yeast through a bridging protein. To confirm independently that EF-1δ binds the second exon of Tat directly, we assessed this interaction using purified protein generated from GST- or MBP-fusion technology. Hence, we constructed, expressed, and purified a GST-EF-1δ fusion protein. GST-EF-1δ was bound to glutathione sepharose beads, and the resulting resin was used in protein affinity chromatography (AC). We equilibrated either GST- (Fig. 2A, lane 2) or GST-EF-1δ-resin (Fig. 2A, lane 3) with a solution containing equal amounts of three proteins, MBP, MBP-Tat1-72 fusion protein, and MBP-Tat1-101 fusion protein (Fig. 2A, input; lane 1). We then asked which (if any) of the 3 proteins would bind either GST- or GST-EF-1δ. Both sets of resin, after equilibration, were washed with 10 column volumes of affinity chromatography buffer (ACB, 10 mM Hepes, pH7.5, 1 mM $MgCl_2$, 0.1 mM DTT, 10% glycerol, 100 mM KCl); and proteins that remained bound on the resin were eluted with ACB containing 500mM KCl. Subsequently, the eluates (Fig. 2A, lanes 2 and 3) and the input (Fig. 2A, lane 1) were analyzed by Western blotting with anti-MBP antibody which recognizes all three proteins (Fig. 2A, lane 1). In Figure 2A, lane 3, we found that MBP-Tat1-101, which contains the full length two-exon Tat protein, was preferentially retained by GST-EF-1δ resin. The findings that neither MBP nor MBP-Tat1-72 bound strongly to GST-EF-1δ (Fig. 2A, lane 3) and that none of the three input proteins bound strongly to GST (Fig. 2A, lane 2) support a specific interaction of EF-1δ with the second exon of Tat.

Earlier studies had indeed suggested that Tat has a translational function (15-19). While such proposed activity has remained controversial, the current finding that Tat binds EF-1δ suggests that HIV-1 might modulate host protein synthesis by targeting this component of the cellular translational machinery. To assess this hypothesis functionally, we asked whether the addition of Tat to an active *in vitro* rabbit reticulocyte translation lysate would perturb the efficiency of protein synthesis. As shown in Figure 2B, titration of purified recombinant Tat protein into translation reactions progressively reduced the translatability of CAT mRNA. At 1.0 μg of MBP-Tat101, translation was reduced by more than 20-fold (Fig. 2B; compare lanes 1 and 5) when compared to basal activity (Fig. 2B, lane 1). By contrast, addition of 1.0 μg of MBP had very little effect on the efficiency of translation (Fig. 2B, compare lanes

hEF-1δ

1 MATNFLAHEK IWFDKFKYDD AERRFYEQMN GPVAGASRQE NGATVILRDI

51 ARARENIQKS LAGSSGPRGS SGTSGDHGEL VVRIASLEVE NQSLRGVVQE

101 LQQAISKLEA RLNVLEKSSP GHRARPTDPA RISMRQVEPP AKK*PATPAED*

151 *DEDDDIDLFG* ***SDNEEED****KEA AQLREERLRQ YAEKKAKKPA LVAKSSILLD*

201 *VKPWDDETDM AQLEACVRSI QLDGLVWGAS KLVPVGYGIR KLQIQCVVED*

251 *DKVGTDLLEE EITKFEEHVQ SVDIAAFNKI*

FIG. 1. Amino acid sequence of the human translation elongation factor 1-δ (14). The C-terminal half (*italics*) of EF-1δ was identified in our yeast two-hybrid screen. Major features of EF-1δ include a leucine-zipper (the regularly spaced leucines are in bold/underlined), which may be involved in protein/protein interactions, and a potential CK II kinase site (bold/*italics*).

1 and 3), and addition of MBP-Tat72 produced a much reduced interference with CAT mRNA translation (Fig. 2B, compare lanes 1 and 7). These results agree with the finding that Tat binds EF-1δ and support a functional activity in translation.

The ability of Tat to down-modulate host protein synthesis was assessed using a second approach. In place of cell-free lysate, we asked whether an intracellular translational effect could be documented. In this approach, we examined protein synthesis in cells transfected with various HIV-1 molecular clones which are otherwise isogenic except for differences in their Tat-encoding sequences (see Figure 3). Thus, for example, Tat(−) GV/4GSTm cannot express a functional Tat protein (due to premature stop codons at amino acids 11 and 12), while Tat72GV/GSTm and Tat101GV/4GSTm express respectively either a 1-exon or a two-exon Tat protein (Fig. 3). To ensure that these genomes all transcribe equivalently, the Tat-responsive site (TAR RNA)

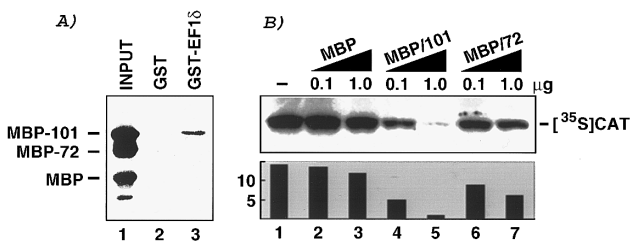

FIG. 2. Tat interacts with EF-1δ and inhibits translation *in vitro*. A) Tat interacts with EF-1δ. A mixture of equivalent amounts of maltose binding protein (MBP), MBP-Tat72 and MBP-Tat101 fusion proteins was equilibrated with glutathione sepharose beads prebound with either GST or GST-EF-1δ. After washes with 10 column volumes of ACB/100 KCl, bound proteins were eluted and analyzed by Western blotting with an anti-MBP antibody. Lane 1, input mixture of purified MBP, MBP-Tat72 and MBP-Tat101 proteins; lane 2, proteins retained on GST column; and lane 3, proteins retained on GST-EF-1δ column. B) Tat inhibits translation of a CAT transcript *in vitro*. Purified MBP or MBP-Tat fusion proteins were added to *in vitro* translation reactions as indicated. Lane 1, no protein added; lanes 2 and 3, addition of 0.1 and 1.0 μg of MBP, respectively; lanes 4 and 5, addition of 0.1 and 1.0 μg of MBP-Tat101, respectively; and lanes 6 and 7, addition of 0.1 and 1.0 μg of MBP-Tat72, respectively.

in the 5′ LTR (20, 21) was deliberately disabled, and each genome was engineered to express a GAL4-VP16 fusion protein which activates the *cis*-LTR through artificially inserted GAL4 binding sites. Co-transfection of the appropriate HIV-1 derivative with either a CAT reporter driven by the native HIV-1 LTR or a reporter driven by GAL4 sites confirmed that GAL4-VP16 was equivalently and functionally expressed from all genomes and that Tat was expressed from Tat72GV/4GSTm and Tat101GV/4GSTm (data not shown).

Each of these modified HIV-1 genomes was transfected individually into HeLa cells with a second plasmid expressing the full length CD4 cDNA under the control of the SV40 immediate-early promoter (note that CD4 protein is not endogeneously expressed in HeLa cells). 15 hours after transfection, α-amanitin was added to the cells to block transcription, and three hours hence ^{35}S-methionine was added to the cells for three additional hours to label proteins. ^{35}S-labeled viral and CD4 proteins were immunoprecipitated with anti-HIV-1 or anti-CD4 and resolved by SDS-PAGE. Figure 4A shows that co-transfection of CD4 with the HIV-1 genome expressing full length Tat (Tat101) reduced by 10-fold the amount of CD4 protein (compare lane 2 with lane 4). By contrast, transfection of a Tat(−) HIV-1 derivative produced no reduction in CD4 protein (Fig. 4A, compare lane 1 with lane 4), and transfection of the HIV-1 genome that expresses Tat72 also had a minimal effect on the amount of CD4 protein (Fig. 4A, compare lane 1 to lane 3). As a normalizing control for CD4, we examined the viral proteins gp120 and p55 expressed in the same transfected-cells. In contrast to the CD4 profiles, cell samples transfected with each of the three HIV-1 variants showed the same amounts of both gp120 and p55 (Fig. 4B and C). Thus, at this level of resolution, the inhibitory effect of Tat is preferentially on the translation of cellular, as contrasted with viral, proteins.

To rule out that reduction in CD4 protein occurs at the level of transcription instead of translation, we compared mRNA levels using an RNase protection assay. As shown in Figure 5, cells transfected with ei-

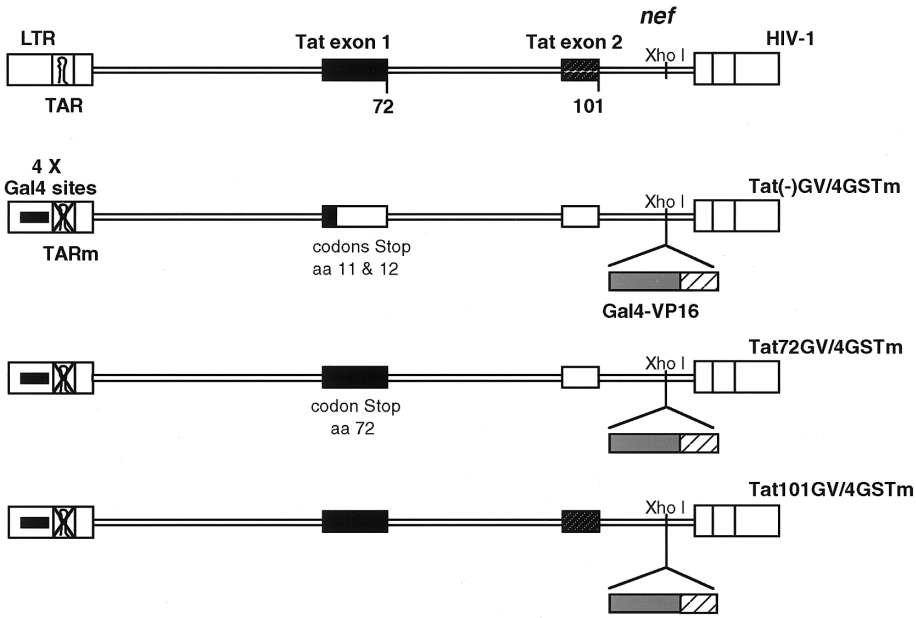

FIG. 3. Construction of HIV-1 derivatives. All derivatives were constructed from a laboratory HIV-1 molecular clone, pNL-43, using standard techniques (35). These HIV-1 derivatives contain four GAL4 binding sites (GS) in place of the natural upstream transcriptional control sequences and a GAL4-VP16 fusion inserted into the XhoI site in the *nef* gene. A mutation was introduced into the trans-activator response element (TAR) to eliminate the effect of Tat on transcription of these derivatives. In addition, stop codons were introduced at codon positions 11 and 12 to generate Tat(−)GV/4GS/Tm, at 72 to generate Tat72GV/4GS/Tm. The expression and functionality of Tat and GAL4-VP16 from these HIV-1 derivatives were confirmed by co-transfection of these derivatives with an HIV 1-CAT reporter driven either by the natural LTR sequence or by synthetic GAL4 binding sites.

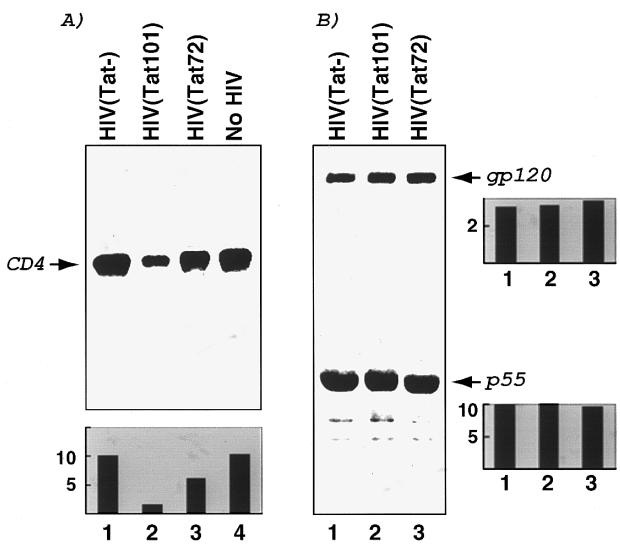

FIG. 4. Tat down-regulates translation of cellular but not HIV-1 proteins *in vivo*. Various forms of recombinant HIV-1 molecular clones (see Figure 3) were co-transfected with a CD4-expressing plasmid into HeLa cells. Proteins were labeled with ^{35}S-methinione, immunoprecipitated with either anti-CD4 or anti-HIV-1 antibodies, and analyzed by SDS-PAGE. A) CD4 proteins. B) HIV-1 viral proteins. Migration positions of gp120 and p55 are indicated. Protein signals are quantitated and graphed in relative units in the various insets.

ther Tat(−) HIV-1, Tat72 HIV-1 or with Tat101 HIV-1 expressed indistinguishable levels of CD4 (Fig. 5A) and viral mRNAs (Fig. 5B). These results support the idea that the reduction in CD4 protein is likely at the translational step, although we cannot formally exclude that Tat might affect CD4 stability.

During the course of this work, we noted that another group had reported that the HSV1 α regulatory protein ICP0 also binds EF-1δ (22). ICP0, like Tat, was previously believed to be exclusively a transcriptional transactivator. Yet Roizman and colleagues showed elegantly that this protein has a significant translation function both *in vitro* and intracellularly. Herpes viruses are large DNA viruses not at all related to human retroviruses. Thus, unless it is for a significant functional reason, Herpes simplex proteins are not expected to conserve fortuitously protein domains with HIV-1 polypeptide. The finding that ICP0 binds EF-1δ prompted an obvious challenge–does the binding portion of ICP0 share protein homology with the second exon of Tat. In figure 6, we show a protein alignment of Tat and ICP0 using the MacVector program. Interestingly, the second exon of Tat does show some similarities to the binding portion of ICP0 for EF-1δ. Considering that the viruses are not expected to be related, it is significant that over 30% of the amino acids in the second exon of Tat are similar to the relevant portion

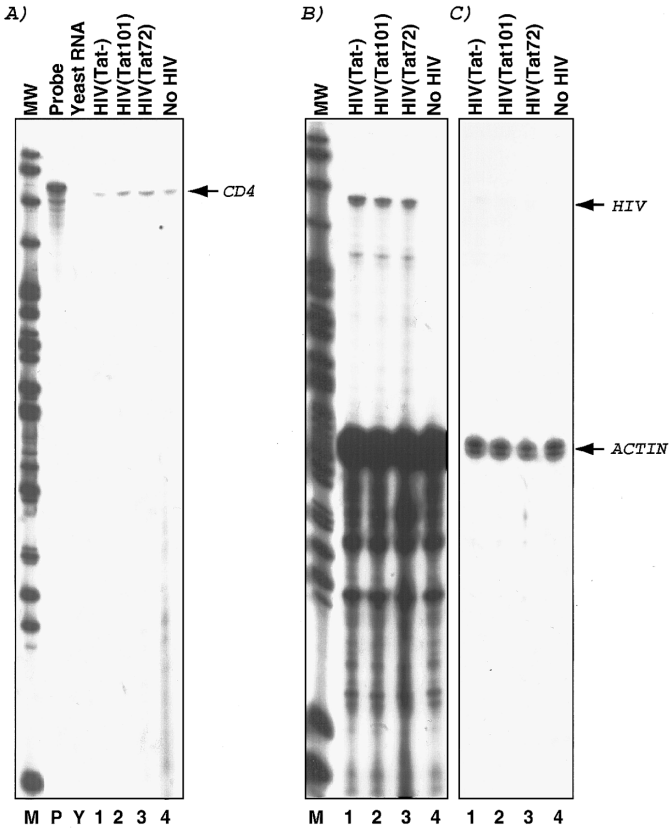

FIG. 5. RNase protection assays of HIV-1 and cellular mRNAs. Total RNAs from transfected cells were hybridized to a CD4 probe (A) or to a mixture of a 32p-labelled single-stranded HIV-1 and an actin probe (B). After digestion with RNase A/T1, the protected products were analyzed by PAGE. (C) is a shorter exposure of (B) to show more clearly the protected actin probe.

of ICP0 (Fig. 6). Although this level of similarity may not be statistically impressive, random comparisons of Tat with a number of proteins revealed that even this level of similarity occurs only between Tat and the EF-1δ binding portion of HSV1 ICP0 and corresponding portion of ICP0 proteins from related viruses. For example, Tat and the corresponding portion of HSV2 ICP0 share about 25% amino acid identities, which is a better conservation than between Tat and HSV1 ICP0 (Fig. 6)

Is Tat plausibly a translational regulator? This question can be considered on several levels. First, there clearly exists a large body of literature that supports a role of Tat in protein translation (for example see refs. 15-19). Second, although such studies have been discounted largely because Tat has been viewed to be a nuclear transcription factor, there could be a minor partition of Tat protein to the cytoplasm. We note that this protein (~15kDa in full-length size) is considerably smaller than 50kDa and may thus be expected to diffuse from the nucleus into the cytoplasm through nuclear pore complexes (23, 24). Hence, one would expect a subpopulation of Tat protein to be found in the cytoplasm where it could interact with EF-1δ. Third, the maintenance of the ORF in the second coding exon in Tat has long been enigmatic. It is well-understood that this coding exon is largely dispensable for transcriptional function (reviewed in ref. 11). The fact that HIV-1 RT has a high error rate (reviewed in ref. 25) and yet the second coding exon has remained "open" suggests that this region likely has a highly selected function. Interaction with EF-1δ could conceivably be that function. This seems all the more reasonable when one considers that two otherwise unrelated proteins, Tat and ICP0, share observations of binding EF-1δ and maintain similar protein motif(s) for such binding (Fig. 6).

Why would viruses effect a shut-off of host mRNA translation? Shut-off of host protein translation by viruses is not surprising and appears to be a common process through which many viruses "hijack" protein synthesis machinery to redirect it towards producing large amounts of viral proteins (reviewed in ref. 26). While a shut-off of protein synthesis can have many different effects, one well-known result of translational inhibition is that it leads to a block in cell cycle progression from G1 to S phase (27, 28). From several perspectives, a prolonged G1 phase might be particularly advantageous for HIV-1. For instance, HIV-1 can productively infect T-lymphocytes that have been blocked by drugs from entering the S phase of the cell-cycle (for example see ref. 29). This suggests that events in G1 and not events concomitant with DNA-synthesis and/or mitosis are of significant importance for this virus. Indeed, several reports have indicated that the completion of reverse transcription and competent prepara-

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ICP01  RAATHPPSDS GPGGRGQGPG TPLTSSAASA SSSSASSSSA PTPAGAAS
Tat1   RRQRRRAPQD SQTHQASLSK QPASQSRGDP TGPTESKKKV ERETETDP
ICP02  RAAPRRAPDS DSGDRGHGPL APASAGAAPP SASPSSQAAV AAASSSSA
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FIG. 6. The C-terminal half (including the second exon) of Tat shares sequence similarities with a segment of HSV ICP0 that binds EF-1δ. Sequences shown represent amino acids 52 to 99 of HIV-1 Tat/SF2 (middle), amino acids 529 to 577 of HSV-1 ICP0 (top), and amino acids 547 to 594 of HSV-2 ICP0 (bottom). Sequence comparisons were performed with the MacVector program. Amino acid identities and similarities are boxed.

tion for integration of HIV-1 genome into host chromosomes occur during G1 (30-32). Consistent with these ideas is the observation that HIV-1, unlike other retroviruses, can productively infect non-dividing macrophages. Thus should Tat-interaction with EF-1δ result in translational inhibition leading to prolonged G1, then this would appear to benefit certain aspects of the HIV-1 life-cycle. Such an idea would not be unprecedented since it has recently been proposed that HIV-1 uses the Vpr protein to prolong selectively the G2-phase of the cell-cycle in order to effect higher levels of viral gene expression (33, 34).

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