Journal of General Virology (1999), **80**, 777–782. Printed in Great Britain

Translational effects of peptide antagonists of Tat protein of human immunodeficiency virus type 1

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The Tat (*trans*-activator of transcription) regulatory protein of human immunodeficiency virus (HIV-1) acts by interacting with the TAR RNA domain of nascent viral transcripts and with cellular proteins to increase viral transcription. In Jurkat-derived HCLE-D36 cells, which are stably transfected with the chloramphenicol acetyltransferase (CAT) reporter gene expressed from the TAR-encoding long terminal repeat (LTR) of HIV-1, CAT protein expression is dependent on Tat. The Tat9-K-biotin peptide antagonist of Tat binds specifically to TAR RNA and competes with Tat for binding. In the cellular expression system, Tat9-K-biotin reduces Tat-dependent CAT expression. However, while the Tat antagonist greatly reduces CAT protein production and polysome association of CAT mRNA, it has little effect on CAT mRNA levels, suggesting that the antagonist works at the post-transcriptional level.

Introduction

Human immunodeficiency virus (HIV-1) gene expression from its integrated proviral form is activated by the virally encoded Tat (*trans*-activator of transcription) protein, which interacts with a specific RNA domain, denoted TAR (Tat-responsive element), consisting of the first 57 nucleotides at the 5′ end of all viral transcripts (Cullen, 1992; Gaynor, 1995; Jones & Peterlin, 1994). Tat binding to TAR RNA is mediated by a basic RNA-binding domain (Dingwall *et al.*, 1989; Roy *et al.*, 1990; Selby *et al.*, 1989) in this 86–102 amino acid nuclear protein, which is encoded by multiply spliced viral transcripts. TAR forms a secondary structure comprising a partially base-paired stem, a six nucleotide unpaired loop and a three pyrimidine bulge 5′ to the loop. Tat binds through its basic domain to the bulge and two adjacent base pairs of TAR (Dingwall *et al.*, 1989; Roy *et al.*, 1990; Selby *et al.*, 1989). Binding of Tat to TAR induces conformational changes in the RNA that may play an important role in the transactivation process (Zacharias & Hagerman, 1995). Transactivation by Tat appears to occur by a transcriptional mechanism, with TAR functioning essentially as an enhancer (Berkhout & Jeang, 1992; Kao *et al.*, 1987; Kato *et al.*, 1992; Laspia *et al.*, 1989; Marciniak & Sharp, 1991), whereby TAR affinity for Tat mediates an increased local concentration of Tat near the transcription-initiation complex (Southgate & Green, 1991). Tat functions mainly by stimulating elongation of transcripts by RNA polymerase II, relieving the stalling of transcription near the initiation site seen in the absence of Tat (Keen *et al.*, 1996). Tat acts on transcription complexes in combination with RNA polymerase II, transcription factors and host cellular factors, including a specific cellular kinase (Jones, 1997), some of which are recruited by the Tat–TAR complex (Mavankal *et al.*, 1996; Parada & Roeder, 1996; Southgate *et al.*, 1990; Wu-Baer *et al.*, 1995; Zhou & Sharp, 1995). It has also been suggested that Tat associates tightly with polymerase paused near TAR, and recruits cellular kinases that phosphorylate the carboxy-terminal domain repeats of RNA polymerase II (Jones, 1997). During transcription, Tat appears to remain associated with the elongation complex, and is removed from the TAR RNA which functioned in its recruitment (Keen *et al.*, 1997). Transcriptional effects of Tat may also be mediated by its interaction with the Sp1 transcriptional regulator, which binds to sites in the HIV-1 enhancer region (Jeang *et al.*, 1993). In

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addition to this transcriptional mechanism, Tat has been suggested to act at the translational level (Braddock *et al*., 1989; Cullen, 1986; Huang *et al*., 1994; Rosen *et al*., 1986), and TAR-independent effects of Tat on host cell gene expression have been reported (Yang *et al*., 1997).

We have previously reported (Choudhury *et al*., 1998) a Tat-antagonistic compound, *N*-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Cys(S-biotin)-NH_2 (Tat10-biotin or Tat9-C-biotin), which contains the nine amino acid sequence of the TAR-binding basic domain of Tat protein. Tat9-C-biotin avidly competes with Tat for binding to TAR (Wang *et al*., 1995), and inhibits Tat-dependent gene expression of a stably transfected chloramphenicol acetyltransferase (CAT) gene expressed from the HIV-1 long terminal repeat (LTR) in cultured Jurkat cells (Choudhury *et al*., 1998). This Tat antagonist also inhibits acute infection of MT2 cells by HIV-1, as assayed by syncytium induction, cytotoxicity and production of reverse transcriptase (Choudhury *et al*., 1998). Despite the ability of Tat9-C-biotin to markedly inhibit Tat-dependent expression of CAT protein from the LTR–CAT construct, Northern blot analysis did not reveal any significant reduction in CAT mRNA levels in response to this antagonist, or the related Tat9-K-biotin antagonist [in which Cys(S-biotin) is replaced by Lys(ε-biotin)]. This suggested a post-transcriptional effect, which we now report to be the effect of Tat-9-K-biotin on polysome association of CAT mRNA produced from the Tat-dependent LTR–CAT construct.

Methods

■ **Peptide synthesis.** Tat9-K-biotin was synthesized manually on PAL resin by Fmoc chemistry with reagents from PerSeptive Biosystems. Fmoc-Lys (ε-biotin) was from Bachem. After purification by reverse-phase high-performance liquid chromatography, the structure was confirmed by mass spectrometry for the molecular ion.

■ **Binding assay.** As described previously (Choudhury *et al*., 1998; Wang *et al*., 1995), Tat9-K-biotin was tested for its ability to compete with Tat–PEG (a Tat-RNA-binding domain peptide with an appended 5 kDa polyethylene glycol tail) for binding to a 27-mer TAR RNA fragment, as assayed by a competitive gel shift assay in the presence and absence of excess yeast tRNA.

■ **Cell viability and CAT assays.** Jurkat-derived HLCE-D36 cells (Choudhury *et al*., 1998), stably transfected with a recombinant plasmid derived from the pRep10 EBV episomal vector (Invitrogen) containing the HIV-1 LTR linked to the CAT reporter gene, were grown in RPMI 1640 medium supplemented with HEPES (10 mM), glutamine (4 mM), foetal bovine serum (10%; GIBCO BRL), penicillin (50 U/ml), streptomycin (50 U/ml) and hygromycin B (0·3 mg/ml; Boehringer Mannheim). Expression of Tat protein in these cells was initiated by transfection using DEAE-dextran with pAR(Tat), which contains the HIV-1 LTR and HIV-1 sequences mapping between 5·7 and 6·3 kb, encoding the 72 amino acid first exon of Tat. CAT protein levels were determined (Choudhury *et al*., 1998) using an ELISA kit (Boehringer Mannheim), and cell viability was determined using 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma).

Transient expression of pSV2-CAT (Gorman *et al*., 1982), in which CAT expression is driven from an SV40 promoter, was studied in Jurkat cells transfected by using the cationic liposome reagent DMRIE-C (Life Technologies). This transfection method was required to obtain CAT expression in Jurkat cells. CAT expression and cell viability were assayed as in the stably transfected cell line HLCE-D36.

■ **Preparation of polysomes.** All procedures were performed at 0–4 °C, unless otherwise indicated. Polysomes were prepared as described by Katze *et al*. (1986) from cells collected by centrifugation at 700 ***g*** for 10 min and were washed twice with PBS containing cycloheximide (100 µg/ml). The cell pellet was suspended in buffer containing 10 mM Tris–HCl (pH 7·5), 10 mM NaCl, 1·5 mM $MgCl_2$, cycloheximide (100 µg/ml) and RNase inhibitor (40 U/ml; Promega), and then Triton X-100 (0·5%) was immediately added. After 5 min on ice, Tween 20 (1%) and sodium deoxycholate (0·5%) were added, and the cells were disrupted by homogenization in a Dounce homogenizer. Cell debris and nuclei were pelleted by centrifugation for 10 min at 12000 ***g***, and the resulting cytoplasmic extract was layered on a linear sucrose gradient (10–50%) in 10 mM Tris–HCl (pH 7·5), 5 mM magnesium acetate and 100 mM KCl. The sucrose gradient was centrifuged for 3 h at 40000 r.p.m., at 4 °C in a Beckman SW41Ti rotor. Gradient fractions (1 ml) were collected with continuous monitoring at 254 nm using an ISCO UA-5 UV-detector. The gradient fractions were treated with proteinase K (200 µg/ml; Boehringer Mannheim), extracted with phenol–chloroform, and RNA was collected by ethanol precipitation.

■ **Northern blot hybridization.** Cells were collected and RNA was extracted by using the RNeasy kit (Qiagen). RNA in sample buffer (5·4% glycerol, 3·5 M formamide, 50% formaldehyde, 1 × MOPS, bromphenol blue and 0·1 µg/ml ethidium bromide) was denatured and fractionated by formaldehyde–agarose gel electrophoresis (1% agarose in 1 × MOPS buffer, 0·66 M formaldehyde) at 85 V for 4 h. A 0·24–9·5 kb RNA ladder (GIBCO BRL) was used as a marker. After electrophoresis, the gel was washed in water at room temperature for 1 h and for 30 min in 10 × SSC (1 × SSC = 300 mM NaCl, 30 mM sodium citrate, pH 7·0). RNA was then transferred to a Hybond-N membrane (Amersham) by capillary elution with 10 × SSC overnight. RNA was cross-linked to the membrane using the 'UV Stratalinker 2400' (Stratagene) at auto cross-link setting. The membrane was washed at 63 °C for 15 min in 2 × SSC, 0·1% SDS and then was prehybridized at 44 °C in hybridization solution [5 × SSPE (1 × SSPE = 150 mM sodium chloride, 10 mM sodium phosphate, 1 mM sodium EDTA), 2 × Denhardt's solution, formamide (50%), dextran sulfate (5%), SDS (1%)], and denatured sonicated salmon sperm DNA (200 µg/ml) in a roller bottle in a hybridization oven for 4 h and hybridized for 16–24 h. The DNA probe for CAT, a 1·6 kb *Hin*dIII–*Bam*HI fragment of pSV2CAT, was labelled with [α-^{32}P]dCTP (sp. act. 3000 Ci/mmol; NEN-Dupont), using a random priming labelling kit (Boehringer Mannheim). A control probe was made using a 0·585 kb *Kpn*I–*Eco*RI fragment of *β*-actin cDNA. Unincorporated radioactivity was removed with a G-25 Sephadex spin column (Boehringer Mannheim). The membrane was stripped after probing by immersion in a boiling solution of 0·1% SDS followed by cooling to room temperature and then reprobed. After hybridization the membrane was washed under stringent conditions (twice for 20 min at room temperature in 2 × SSC, 0·1% SDS, and then for 30 min at 55 °C in 0·1 × SSC, 0·1% SDS). After each probing, the membrane was exposed to X-ray film to obtain an autoradiographic image, and radioactivity was quantified by phosphorimager analysis (GS250, Bio-Rad).

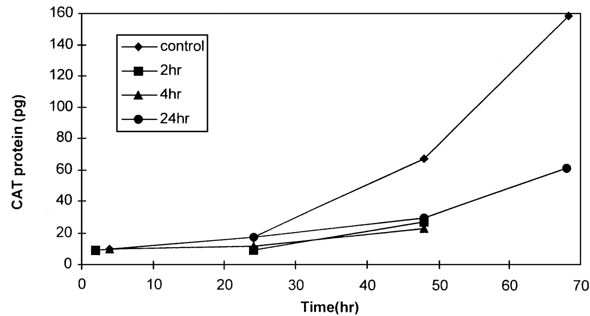

Fig. 1

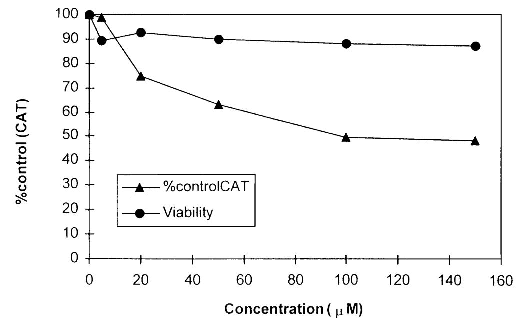

Fig. 2

Fig. 1. Time-course of Tat9-K-biotin inhibition of CAT protein expression induced by transfection with pAR(Tat). Tat9-K-biotin (50 μM) was added to HLCE-D36 cells 2, 4 or 24 h after transformation by pAR(Tat). Cells were harvested at 24, 48 and 68 h and were assayed for CAT protein levels. Each time-point was determined in duplicate, with the difference between duplicates ranging from 0·1 to 5·3 pg.

Fig. 2. Inhibition of Tat-induced CAT expression by Tat9-K-biotin. HLCE-D36 cells were transfected with pAR(Tat) and Tat9-K-biotin was added at the indicated concentrations 30 h later. CAT protein levels and cell viability were assayed at 48 h. Each measurement was done in triplicate; standard errors were below 4·8% for CAT and below 6·6% for viability determinations.

Results

Tat antagonist activity of Tat9-K-biotin

Jurkat-derived HLCE-D36 cells constitutively express a low level of CAT protein. As shown in Fig. 1 (control), transfection with Tat expression plasmid results in a marked increase in CAT protein. Presumably the induction of CAT by transfection with pAR(Tat) occurs at the transcriptional level, although we cannot rule out possible additional Tat effects on translation, which have been suggested previously (Braddock *et al.*, 1989; Cullen, 1986; Huang *et al.*, 1994; Rosen *et al.*, 1986). Tat9-C-biotin and Tat9-K-biotin bind TAR RNA with similar avidity ($k_d \sim 10$ nM) and with similar specificity in the presence of a 750-fold (by weight) excess of yeast tRNA. Tat9-K-biotin (50 μM) added 2, 4 or 24 h after transfection with the Tat expression plasmid nearly completely blocks the Tat-induced increase in CAT protein (Fig. 1). Tat9-K-biotin showed a dose-response relationship (Fig. 2) for reducing CAT protein production by HLCE-D36 cells after transfection with plasmid pAR(Tat), comparable to that reported previously for Tat9-C-biotin (Choudhury *et al.*, 1998). The CAT protein levels at 48 h are only reduced by 50% after Tat9-K-biotin addition at 30 h (Fig. 2) due to the production of CAT protein prior to peptide addition. The peptide nearly completely blocks accumulation of additional CAT protein. As previously reported for Tat9-C-biotin (Choudhury *et al.*, 1998), the inhibitory peptide did not reduce cell viability at any dose tested, despite the nearly complete inhibition of CAT synthesis. Similar results were obtained when the peptide was added at 44 h following transfection and CAT protein level was measured at 68 h.

When CAT was expressed from the SV40 promoter in Jurkat cells transfected by pSV2-CAT, addition of Tat9-C-biotin at 24 h resulted in only 26·7% inhibition of CAT levels measured at 48 h. In these experiments, untreated transfected cells produced 30 pg of CAT at 48 h. Although we do not have a Jurkat cell line expressing stably transfected CAT from a non-LTR promoter, this result indicates that the SV40 promoter is much less sensitive to inhibitory peptides than is the LTR promoter.

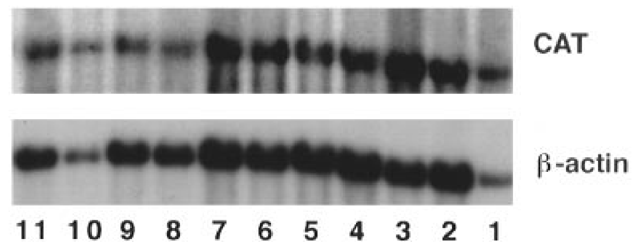

Fig. 3. Effect of Tat9-K-biotin on pAR(Tat)-induced CAT mRNA level. Levels of CAT and *β*-actin mRNA were determined by sequential Northern blot hybridization of the same membrane. RNA was extracted from HLCE-D36 cells transfected with pAR(Tat) and treated with Tat9-K-biotin (or untreated control cells); CAT protein from these cells was analysed in Fig. 2. Lanes 1–5 show RNA from control cells (without Tat9-K-biotin) at 2, 4, 24, 48 and 68 h after transfection, respectively. RNA in the other lanes include RNA from cells treated with peptide at 2 or 4 h and extracted at 24 h (lanes 6 and 7), from cells treated at 2 or 4 h and extracted at 48 h (lanes 8 and 9), and from cells treated at 24 h and extracted at 48 h and 68 h (lanes 10 and 11).

CAT mRNA was substantially increased following transfection with pAR(Tat) (data not shown). However, the CAT mRNA level, as shown in Northern blot analysis (Fig. 3), failed to show a major change in response to treatment with the peptide (relative to the levels of control *β*-actin mRNA), despite the marked decrease seen in protein levels. Densitometric analysis (Bio-Rad) of this image revealed the ratio of band intensity for CAT:*β*-actin hybridization to remain unchanged by treatment, with values in all lanes lying in the range 0·97–1·15, except for lanes 1 (2·9) and 10 (2·1). This suggests that the inhibitory effect might be mediated at the

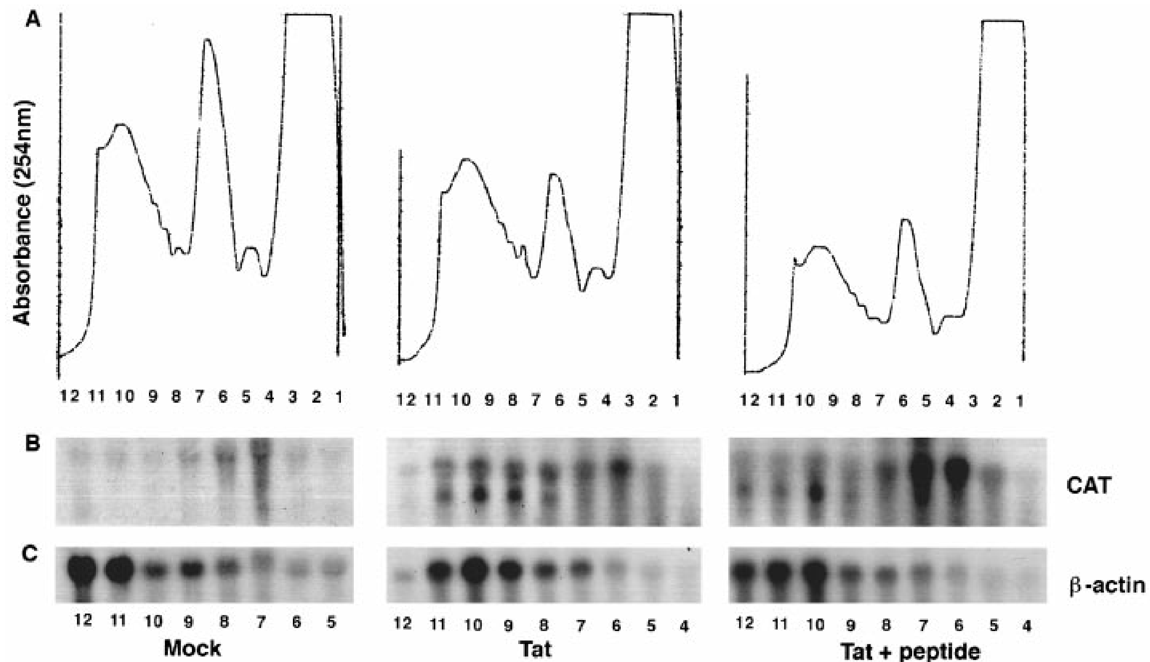

Fig. 4. Tat9-K-biotin reduces polysome-association of CAT mRNA. Cells without plasmid ('Mock'), transfected with pAR(Tat) or transfected and treated with Tat9-K-biotin peptide (50 μM peptide added 4 h after transfection and incubated for an additional 42 h) were lysed and extracts were analysed for polysome content as described in Methods. (A) Absorbance profiles (254 nm) of 10–50% sucrose gradients. The indicated fractions were collected from the top of each gradient with continuous monitoring of absorbance. (B) Northern blot analysis of RNA extracted from each gradient fraction and probed for CAT mRNA. No hybridization was detected in fractions 1–3 in any gradient. (C) Northern analysis of the same membrane reprobed for *β*-actin. In (B) and (C), the lane numbers correspond to the fractions indicated in (A). In all three gradients, the monosome peak was in fraction 7 and polysomes were collected in fractions 9–11.

level of RNA localization or translation rather than at the level of RNA synthesis or stability.

Effect of Tat9-K-biotin on polysome association of CAT mRNA

To study a possible translational effect at the level of ribosomal loading onto mRNA, cytoplasmic extracts prepared from the cells transfected with (control cells) and without (mock cells) Tat expression plasmid pAR(Tat) were subjected to sucrose density-gradient centrifugation (Fig. 4). As can be seen in Fig. 4, the general shape of the polysome profiles from mock cells (no transfection), transfected cells and Tat9-K-biotin-treated transfected cells are similar, although the ratio of polysomes to monosomes seems modestly increased in the two gradients from the transfected cells. Total RNA from each fraction was resolved by electrophoresis in 1% agarose–formaldehyde denaturing gels and was blotted into Hybond-N nylon membrane. The Northern blot was sequentially probed with labelled DNA specific for cellular mRNA encoding CAT and *β*-actin. The low level of cytoplasmic CAT mRNA produced in the absence of pAR(Tat) was found almost entirely in the monosome fraction. In contrast, the enhanced level of CAT mRNA produced in the presence of pAR(Tat) was detected in both the monosome (lanes 4–7) and polysome fractions (lanes 8–11). The *β*-actin mRNA was associated mostly with polysome fractions in both mock and control cells. The data demonstrated that Tat not only induces CAT mRNA but also specifically increases association of CAT mRNA with polysomes. The dependence on Tat of polysomal association and subsequent translation of CAT mRNA is consistent with the marked increase in CAT protein production in cells transfected with Tat expression plasmid pAR(Tat). Note that two bands of CAT mRNA are seen in these gels; the larger presumably represents the full-length polyadenylated transcript and the shorter form is that processed by partial deadenylation during translation.

In pAR(Tat) transfected cells, CAT mRNA was approximately equally distributed in monosome and polysome fractions. However, following Tat9-K-biotin exposure, most CAT mRNA was distributed in the monosomal fraction and a much smaller portion was in the polysomal fraction. In contrast, Tat9-K-biotin has no detectable effect on the sedimentation of *β*-actin mRNA (Fig. 4). Thus the effect of Tat9-K-biotin was to decrease the amount of CAT mRNA in polysomes while increasing the amount associated with monosomes. Tat9-K-biotin appears to reduce the fraction of CAT mRNA associated with polysomes much more dramatically than it affected total CAT mRNA levels. This is consistent with translation inhibition of CAT expression by Tat9-K-biotin in the absence of significant alteration in the CAT mRNA level.

Discussion

The data presented here indicate that Tat protein causes an increase in polysome association of Tat-dependent CAT mRNA, as well as a dramatic increase in transcription; the Tat9-K-biotin Tat antagonist reduces polysome association of this mRNA. The HIV-1-encoded nuclear phosphoprotein Rev has been widely studied as a potentiator of stability and transport into the cytoplasm of unspliced and partially spliced mRNAs. However, it has recently been reported (Arrigo & Chen, 1991; D'Agostino *et al*., 1992) that Rev is required for polysome association and translation of the *gag/pol* and *vpu/env* viral mRNAs of HIV-1, with the same effect seen on a *gag*-RRE (Rev responsive element) mRNA expressed from a Rev-responsive expression plasmid. This result leaves open the possibility that the regulatory proteins of HIV-1 might act at multiple levels of gene expression.

We have previously shown that Tat9-C-biotin competes with Tat protein for binding to TAR RNA *in vitro*, and that it reduces Tat-dependent CAT expression in the indicator cells used in this report. We have shown in this report that CAT expressed from the Tat-independent SV40 promoter is less sensitive to this inhibition. Tat9-C-biotin also inhibits HIV-1 infection of cells in culture (Choudhury *et al*., 1998). The activity of a 9-mer peptoid–peptide hybrid compound resembling the Tat RNA-binding domain (GGP64222) to similarly inhibit transactivation in a cellular assay has also been reported (Hamy *et al*., 1997). Both of these studies measured the effect of the inhibitors on indicator protein expression. We report that in the cellular assay used here, Tat-dependent expression of CAT protein is about as sensitive to inhibition by Tat9-K-biotin as by Tat9-C-biotin. The inhibitory peptide Tat-9-K-biotin resulted in reduced polysome association of CAT mRNA, consistent with inhibition of CAT expression occurring at a post-transcriptional level. Whether or not Tat itself exerts any effect at the translational level, as previously suggested (Braddock *et al*., 1989; Cullen, 1986; Huang *et al*., 1994; Rosen *et al*., 1986), Tat9-K-biotin inhibits Tat-dependent gene expression translationally. During transactivation, Tat has been shown to initially associate with TAR RNA, but once it interacts with other cellular factors and the RNA polymerase II complex, it is removed from TAR, and presumably migrates down the newly synthesized RNA along with the transcription elongation complex (Keen *et al*., 1997). This removal of Tat from TAR is believed to be dependent on protein–protein interactions between the non-RNA binding domains of Tat and other transcriptional complex proteins. Tat9-K-biotin must lack the domains required for these protein–protein interactions, so that even if transcription is not markedly inhibited, Tat9-K-biotin remains associated with the 5′ end of the transcripts and thus inhibits the process of translational initiation. Since polysome-associated messages are translationally active (Warner *et al*., 1963), Tat9-K-biotin appears to reduce gene expression of Tat-dependent genes by preventing mRNA from associating with polysomes, rather than by inhibiting the transcriptional effects of Tat. The relatively greater translational effect of Tat-inhibitory peptides on Tat-dependent gene expression may relate to the 30-fold greater localization of these peptides to the cytoplasm relative to the nucleus of treated Jurkat cells (Choudhury *et al*., 1998). This result suggests that a Tat antagonist peptide might inhibit HIV-1 at different steps in the virus replication cycle involving RNA function.

This work was supported by The New Jersey Commission on Science and Technology and the Gustavus and Louise Pfeiffer Research Foundation. We thank D. E. Georgopoulos for technical assistance.

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Received 2 September 1998; Accepted 9 November 1998