Human Immunodeficiency Virus Type 1 Tat Does Not Transactivate Mature *trans*-Acting Responsive Region RNA Species in the Nucleus or Cytoplasm of Primate Cells

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Human immunodeficiency virus (HIV)-encoded transactivator Tat is essential for viral gene expression and replication. By interacting with a nascent RNA stem-loop called the *trans*-acting responsive region (TAR), Tat increases rates of initiation and/or elongation of HIV transcription. Several reports have also suggested that Tat has additional effects on mature HIV RNA species including modification of primary transcripts in the nucleus and their increased translation in the cytoplasm. These posttranscriptional effects are most pronounced in the *Xenopus* oocyte. To investigate directly whether Tat has similar effects on viral transcripts in cells that are permissive for HIV replication, we cotransfected and microinjected human and monkey cells with Tat and TAR in the form of DNA or RNA. Whereas Tat transactivated TAR DNA targets, it did not transactivate TAR RNA targets in the nucleus of microinjected cells or in the cytoplasm of transfected cells. We conclude that in cells permissive for viral replication, Tat exerts its effect primarily at the level of HIV transcription.

Human immunodeficiency virus (HIV) is the etiological agent of AIDS (15, 21). Early in infection, multiply spliced viral RNA species are produced (8, 44). These transcripts code for regulatory proteins Tat, Rev, and Nef (8, 31). Tat and Rev are positive transactivators that are essential for viral gene expression and replication (11, 16, 35). Localized in the nucleus, Tat is a 15-kDa protein that interacts with a nascent RNA stem-loop called the *trans*-acting responsive region (TAR) to increase rates of HIV transcription (24, 41).

Tat might also increase the expression of HIV genes at a posttranscriptional step (8). In one study, levels of steady-state RNA were not altered by the addition of Tat, and it was considered that Tat affects the translation of viral transcripts (36). Although in most studies Tat increased HIV transcription (29, 32, 33), levels of TAR RNA could not always account for disproportionately higher amounts of viral or reporter proteins (7, 46). To reconcile these differences, a bimodal mechanism for Tat action was proposed in which Tat affected transcriptional as well as posttranscriptional steps (7). Since Tat interacts with an RNA stem-loop that is found in nascent and all mature HIV RNA species, Tat might have more than one mechanism of action (13, 37, 45).

Recently, positive effects of Tat on TAR RNA were observed in the *Xenopus* oocyte (3, 4). Here, TAR RNA was not translated in the absence of Tat. However, when coinjected with Tat into the nucleus, TAR RNA could be efficiently translated. Since little or no transcriptional effect could be demonstrated, these posttranscriptional effects could account for the transactivation by Tat in the *Xenopus* oocyte (3, 4). However, this mechanism of Tat action might be a peculiarity of the *Xenopus* oocyte. To investigate whether Tat has similar posttranscriptional effects in cells that are permissive for HIV replication, we transfected and microinjected plasmids and RNA containing TAR and Tat sequences into HeLa, CV1, and COS cells.

MATERIALS AND METHODS

Recombinant DNA. Plasmids pHIVCAT, pSVTAT, and pSVTATZX have been previously described (25, 32, 38). TAR, chloramphenicol acetyltransferase (CAT), and Tat sequences were also subcloned into plasmid vectors that facilitated transcription of sense (coding) RNA by using Sp6 polymerase (26). Hybrid TAR-CAT (TARCAT) sequences were placed into pGEM-2 (Promega Biotec, Madison, Wis.) by first digesting pHIVCAT with *Bgl*III and *Bam*HI, followed by digestion of pGEM2 with *Eco*RI and *Bam*HI and ligating the TARCAT fragment with two complementary bridging oligonucleotides which contain the Sp6 promoter and the 5' end of TAR (Fig. 1A) (pSP6TARCAT). CAT was placed into pSCSP6 (26) by using *Hind*III and *Bam*HI (pSP6CAT). A Tat fragment with *Bgl*III-to-*Bam*HI ends was placed into pSP64T (26), which contains the untranslated leader sequence of β -globin 5' to and a poly(A) tract 3' to the polylinker sequence (pSP6gloTAT). For in vitro transcription with Sp6 polymerase, these plasmids were linearized with *Bam*HI, *Sma*I, and *Bam*HI, respectively (Fig. 1). 7-methyl-GpppG was added to 5' ends of these transcripts following the manufacturer's instructions (Promega Biotec, Madison, Wis.). In some RNA preparations, poly(A) tails were added by the poly(A) polymerase. After RNA synthesis, DNA was digested with DNase I, followed by extractions with phenol-chloroform and with chloroform and by ethanol precipitation. Pellets were dried in a vacuum and resuspended in doubly distilled, diethyl pyrocarbonate-treated H₂O. RNA concentrations were checked by UV spectroscopy (28).

Cell culture and transient transfection assays. COS and CV1 cells were grown in 10-cm petri dishes with Dulbecco modified Eagle medium supplemented with 10% fetal calf serum and antibiotics. Before transient transfection assays, HeLa cells were 50 to 75% confluent. The medium was aspirated, and the cells were washed two times with serum-free medium including 50 mM Tris-HCl (pH 7.5). Serum-free medium (3 ml) was added to each plate together with 2 ml of DEAE-dextran at 1 mg/ml. After the addition of 50 μ l of 5 mg

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FIG. 1. Plasmid constructions for the synthesis of RNA. (A) As described in Materials and Methods, TARCAT sequences from pHIVCAT were placed downstream from the Sp6 promoter by using two synthetic oligonucleotides from the *Eco*RI site in the polylinker to the *Sac*I site in TAR. Transcription by Sp6 polymerase initiated at the HIV-1 cap site, denoted by an arrow before the TAR box and above the sequence of the synthetic oligonucleotides. *Bam*HI denotes the site where this plasmid was linearized before transcription by Sp6 polymerase. The Sp6 promoter is depicted as a closed circle, whereas TAR (positions +1 to +180) and CAT sequences are shown as open boxes. (B) CAT sequences were placed 3' to the Sp6 promoter as described in Materials and Methods. *Sma*I denotes the site where this plasmid was linearized before transcription by Sp6 polymerase. (C) Tat sequences were placed 3' to the Sp6 promoter and the β -globin (β -glo) leader. *Bam*HI denotes the site where this plasmid was linearized before transcription by Sp6 polymerase. "An" represents the polyadenylation site of simian virus 40, which was placed 3' of Tat coding sequences. Tat sequences are depicted as an open box.

of chloroquine per ml in serum-free medium, 5 μ l of RNA and/or DNA was added to each tissue culture plate. Cells were incubated with the nucleic acid for 2 to 3 h at 37°C, after which the medium was aspirated and the cells were washed two times with 5 ml of medium containing 10% fetal calf serum. Finally, 10 ml of medium containing 10% fetal calf serum was added, and the cells were incubated for 24 to 48 h before CAT assays were performed as described previously (25, 32, 38, 39).

Microinjections. COS and CV1 cells were cultured on 25-mm glass coverslips 1 to 2 days before microinjections. The microinjection into nuclei of COS and CV1 cells was controlled by a pneumatic controller and micromanipulator (Narishige, Tokyo, Japan). Injections were performed at 37°C on a heated chamber at 60 to 88 $\times$ magnification with a 40 $\times$ objective lens and a 2.2 $\times$ zoom lens attached to the camera port of an inverted microscope (Nikon, Osaka, Japan). All injections were monitored through a video camera with a zoom lens, displayed on a video monitor, and recorded. Solutions containing RNA were heat denatured at 50°C and either passed through a 0.2- μ m-pore-size filter or spun for 30 min at 12,000 $\times$ g before microinjection. RNA samples were backloaded with a loading pipette placed

against the shoulder of the injection needles, which were pulled from capillary tubing (1-mm outer diameter, 0.7-mm inner diameter) to a tip diameter of 0.15 to 0.25 μ m. A volume of 20×10^{-15} to 40×10^{-15} liter of nucleic acid, at 1 μ g/ μ l for RNA and 0.25 μ g/ μ l for DNA, was injected into each cell nucleus. A plasmid containing the firefly luciferase reporter gene under the control of the Rous sarcoma virus promoter (pRSVLUC) was also used as a coinjection marker to normalize expression levels of injected cells (12).

Approximately 100 cells were injected per sample, and most of these cells were counted after processing. Forty-eight hours after injection, cells were fixed with 4% (vol/vol) freshly diluted formaldehyde in phosphate-buffered saline (PBS), quenched, permeabilized with 0.2% Nonidet P-40 in PBS (NPBS), and processed for indirect immunofluorescence with a monoclonal antibody specific for CAT (a generous gift of C. Gorman) and a polyclonal antibody raised against firefly luciferase (used at 1:800 dilution) (D. Chin). Cells were incubated for 30 min each with appropriate secondary antibody (Texas red-labeled goat anti-mouse and fluorescein isothiocyanate-labeled sheep anti-rabbit antibodies, both from Accurate Chemicals, Westbury, N.Y.) and washed with NPBS. After the final wash in NPBS, cells were stained with 2 μ g of Hoechst 33528 dye per ml and washed in distilled water. The coverslip was mounted on a glass slide with 0.1% (wt/vol) phenylenediamine in 90% glycerol–20 mM Tris-HCl (pH 9.5) and stored for up to 7 days at –20°C. Approximately 20% of DNA- or RNA-injected cells expressed luciferase and CAT.

Image analysis and densitometry. Fluorescent cells were imaged with a silicon-intensified target camera (Dage, Michigan City, Ind.), and 33 to 45 frames were digitized with a frame grabber (Perceptics, Knoxville, Tenn.) in a 512 by 512 pixel format at 40 $\times$ or 60 $\times$ (1.6 numerical aperture [NA]) magnification. Cells were visualized by epi-illumination with a mercury excitation beam through a set of neutral density filters, spanning 0.005 to 100% transmission, to maintain fluorescent signals within the calibrated linear range of the camera. Texas red and fluorescein dichroic mirrors and filter sets were obtained from Omega Optical. All images were temporarily stored on a hard disk and archived to an erasable optical disk. Images were corrected for background and camera shading with TCL-Image (Multihouse, Delft, The Netherlands) and Image (W. Rasband, National Institutes of Health, Bethesda, Md.) software as described previously (5). Briefly, the Texas red (CAT) and fluorescein isothiocyanate (luciferase) fluorescence intensity of each injected cell and two to three adjacent, noninjected cells was determined from background and shading-corrected images. The noninjected cells typically had less than 5 to 10% of the signal of the injected cells, and luciferase expression varied less than threefold among all injected cells. The Texas red and fluorescein fluorescence of injected cells were corrected for the background fluorescence of noninjected cells. Finally, the total CAT fluorescence of injected cells was normalized for variations in luciferase expression and neutral density filter settings (23).

To aid in the alignment of luciferase and CAT images, outlines of nuclei (determined by segmentation analysis of the Hoechst image) and cell borders (determined from the phase image when possible) were superimposed upon several of the fluorescence images. To improve visual signal detection, images were inverted to show black objects on a white background. Final images were displayed on a 256-gray scale monitor and photographed with Technical Pan

FIG. 2. Cotransfections of TAR targets and Tat effectors as DNA or RNA. (A) Cotransfections of TAR DNA and RNA targets with the nonfunctional Tat plasmid (pSVTATZX). Basal level of expression from pHiVCAT is given as 100%. This corresponds to a CAT enzymatic activity of 0.13%. Basal levels of expression of TARCAT and CAT RNA species were 8 and 24% of those observed with pHiVCAT, respectively. Filled bar depicts cotransfections with pHiVCAT, i.e., the TAR DNA target, whereas striped bars represent cotransfections with TARCAT and CAT, i.e., TAR RNA target and CAT RNA, respectively. Standard errors of the mean are given above the bar graphs. (B) Cotransfections of TAR DNA and RNA targets with the functional Tat plasmid (pSVTAT) and TAT RNA. Fold transactivation (FOLD-TA) of pHiVCAT by pSVTAT is given as 100%, which corresponds to 131-fold of basal levels (filled bar). Fold transactivation observed with Tat in the form of RNA is given in the adjoining striped bar. Fold transactivation observed with TARCAT and CAT RNA targets by Tat DNA and RNA effectors is depicted to the right of cotransfections with the TAR DNA target. Combinations of targets and effectors are given below the bar graphs. Pluses and minuses indicate the presence and absence of a particular plasmid or RNA in the cotransfection, respectively.

film (Eastman Kodak Co., Rochester, N.Y.) and developed with fresh HC110 developer (Kodak) at a 1:25 dilution.

RESULTS

Effects of Tat on TAR DNA and RNA targets in the cytoplasm of HeLa cells. To determine whether Tat can transactivate TAR RNA targets, we cotransfected RNA species containing HIV long terminal repeat and Tat sequences (Fig. 1) into HeLa cells. As controls, TAR DNA targets were cotransfected with Tat DNA and RNA effectors. TAR and Tat RNA targets and effectors are called TARCAT and TAT RNA (Fig. 1). TAR and Tat DNA targets and effectors are called pHiVCAT and pSVTAT (25). All RNA species were capped, and cotransfections and microinjections were performed with polyadenylated and nonpolyadenylated transcripts. However, polyadenylation by commercial poly(A) polymerase did not affect our results (data not shown). Thus, all subsequent experiments were done with capped nonpolyadenylated RNA species.

To measure basal levels of expression, we also cotransfected TAR targets with a plasmid that encodes a nonfunctional Tat protein (pSVTATZX) (25). When TAR DNA target was cotransfected with a functional Tat effector, levels of transactivation varied between 100- and 150-fold. As observed previously, levels of TARCAT RNA and CAT protein as measured by primer extension (32) and CAT enzymatic assays (Fig. 2) (32) increased proportionately in these transfections.

TAR DNA target was also cotransfected with the TAT RNA effector synthesized by Sp6 polymerase (Fig. 3). Tat protein synthesized by this TAT RNA could be measured by immunofluorescence (data not shown). In cotransfections in which the TAR target was in the form of DNA and the Tat effector was in the form of RNA, even greater levels of

transactivation were observed than when both targets and effectors were in the form of DNA (Fig. 2 and 3). Furthermore, TAT RNA transactivated pHiVCAT in a dose-dependent fashion over a concentration gradient from 5 to 0.05 μ g (Fig. 3). Higher levels of transactivation observed with TAT RNA compared with pSVTAT could represent earlier and more rapid translation of the transfected TAT RNA. This result also confirms that RNA species were stable in our transient transfection assay. In sharp contrast, when TARCAT RNA was cotransfected with TAT RNA, no increase in CAT activity was observed. Thus, Tat did not transactivate TAR RNA targets. However, this TARCAT RNA was able to direct the synthesis of CAT protein detected by the CAT enzymatic assay (Fig. 2 and 3) and by immunofluorescence (data not shown).

To determine the effect of TAR RNA sequences on CAT RNA translation, we also transfected CAT RNA lacking TAR sequences into HeLa cells (Fig. 2). Threefold-higher levels of CAT activity were observed with CAT RNA compared with TARCAT RNA irrespective of the presence or absence of Tat. This is in agreement with previous reports which demonstrated that TAR RNA is translated less efficiently than other RNA species because of the secondary structure of TAR RNA (29, 30), the inhibition of translation by double-stranded RNA-dependent kinase (14, 40), or both.

Effects of Tat on TAR DNA and RNA targets in the nuclei of COS cells. To determine whether Tat can transactivate TAR RNA targets, we microinjected identical plasmid constructions and RNA species into the nuclei of COS and CV1 cells. Our RNA preparations were divided for analysis by both cotransfection and microinjection assays. Since amounts of nucleic acid delivered were measured, up to 2,500 DNA and 10,000 RNA molecules were delivered into each nucleus in the experiments presented. This contrasts with far fewer RNA molecules that could enter the cytoplasm of cells by

FIG. 3. Cotransfections of pHIVCAT with the Tat effector in the form of DNA or RNA. (A) Fold transactivation of the TAR DNA target (pHIVCAT) in the presence of functional Tat over nonfunctional Tat is given. Filled bars represent cotransfections with plasmids (pHIVCAT and pSVTAT), whereas striped bars represent cotransfections of the TAR DNA target (pHIVCAT) with decreasing amounts of TAT RNA (from 5 to 0.05 μ g per transfection). Standard errors of the mean are given above the bar graphs. (B) Results are presented as percent fold transactivation (% FOLD-TA) of CAT activities in panel A. Fold transactivation of pHIVCAT by pSVTAT is given as 100%, which corresponds to 113-fold basal levels. As in panel A, striped bars represent cotransfections of pHIVCAT with decreasing amounts of TAT RNA (from 5 to 0.05 μ g per transfection). Standard errors of the mean are given above the bar graphs. Combinations of targets and effectors are given below the bar graphs as in Fig. 3.

cotransfection (17). Levels of targets and effectors were varied over a 100-fold range of concentrations, which did not change our findings.

When targets and effectors were introduced as DNA, efficient transactivation was observed by indirect immunofluorescence (Fig. 4). However, when TARCAT RNA was used, no transactivation by Tat RNA was observed (Fig. 5).

Twofold-higher levels of CAT activity were observed after nuclear injection of CAT RNA versus TARCAT RNA (Fig. 5C), which is consistent with the inhibitory effect of TAR RNA sequences found in cotransfections of the same RNA species (see above). Finally, RNA species were stable in both assays as verified by translation of Tat from TAT RNA and CAT from TARCAT and CAT RNA species. Thus, Tat

FIG. 4. Coinjection of pHIVCAT and pSVTAT into COS cells. (A) COS cells were microinjected with pHIVCAT, pRSVLUC, and pSVTATZX. Luciferase (LUC) immunofluorescence (fluorescein isothiocyanate) is shown in the panel on the left, and CAT immunofluorescence (Texas red) is shown in the panel on the right. Outlines that represent nuclear and cellular borders were superimposed onto the luciferase and CAT fluorescence images. The faint nuclear autofluorescence of these cells is equivalent to that of adjacent noninjected cells. (B) COS cells were microinjected with pHIVCAT, pRSVLUC, and pSVTAT. Again, luciferase and CAT immunofluorescences are shown in the left and right panels, respectively. The small bar in the bottom right panel measures 5 μ m. (C) Densitometric analysis of the CAT immunofluorescence of COS cells injected with pHIVCAT and pSVTATZX (-Tat) or pSVTAT (+Tat). CAT immunofluorescence was corrected for the background fluorescence of noninjected cells and normalized for luciferase immunofluorescence as described in Materials and Methods. Values are given in arbitrary fluorescence units. Standard errors of the mean are indicated above the bars. Data are from 17 microinjected cells in this experiment and are representative of three independent experiments.

FIG. 5. Coinjection of TARCAT and TAT RNAs into COS cells. (A) COS cells were microinjected with TARCAT RNA and pRSVLUC. As in Fig. 4, luciferase (LUC) immunofluorescence (fluorescein isothiocyanate) is shown in panels on the left, and CAT immunofluorescence (Texas red) is shown in panels on the right. Outlines of nuclei were superimposed onto the luciferase immunofluorescence. The bar in the panel on the left measures 15 μ m. (B) COS cells were microinjected with TARCAT RNA, pRSVLUC, and TAT RNA. Two separate fields from two independent experiments are shown. In the upper left panel, the faint background fluorescence of a binucleated noninjected cell is seen, which is indicated by its outlined nuclei. Bars in panels on the left measure 20 μ m. (C) Densitometric analysis of the CAT immunofluorescence of COS cells injected with TARCAT, CAT, and pRSVLUC in the absence (-) or presence (+) of TAR RNA. The standard error of the mean is given above the bars. TARCAT and CAT RNA data are from 30 and 23 cells in this experiment, respectively, and are representative of three experiments with different preparations of RNA. All values were calculated as described in the legend to Fig. 4.

is unable to transactivate TAR-containing RNA in either the cytoplasm or nucleus of cells that are permissive for HIV replication. However, in both assays, Tat could transactivate TAR DNA targets, which implies that Tat affects HIV transcription.

DISCUSSION

In this study, we could not detect any effect of Tat on TAR RNA species in either the nucleus or cytoplasm of cells that are permissive for HIV replication. That cotransfected RNA remains in the cytoplasm is supported by previous transient transfection assays (3) and by studies of U1 small nuclear RNA in the *Xenopus* oocyte (19). When injected into the cytoplasm, U1 small nuclear RNA with a trimethyl cap is efficiently transported into the nucleus, whereas U1 small nuclear RNA with a monomethyl cap (7-methyl-GpppG) remains in the cytoplasm (19). All our cotransfected RNA species contained the monomethyl cap. Therefore, this study confirms and extends previous observations that Tat has only transcriptional effects in primate cells (20, 25, 27, 33).

Cotransfections and microinjections are complementary techniques. Aside from differences in targeting, there are qualitative and quantitative differences between them. In cotransfections, amounts of nucleic acid that enter the cytoplasm are unknown, but subsequent measurements represent quantitative assays of cell populations (17), whereas in microinjections, amounts of input nucleic acid are known but assays of reporter genes are indirect and semiquantitative (23). Nevertheless, microinjection assays likely yield a

more uniform response in individual cells than the variability of expression found in cotransfection assays (17). Although we varied conditions with both techniques, more nucleic acid was delivered by microinjection. This resulted in higher basal levels of expression of microinjected RNA species. However, in all cases, varying the concentrations of targets and effectors in either system did not change our results (for an example, see Fig. 3).

Note that we injected fourfold-lower amounts of TAR DNA than TAR RNA targets because TAR DNA could direct the synthesis of more TARCAT RNA over the 24 to 48 h of the assay. Furthermore, it was difficult to compare values from the CAT enzymatic assay with immunofluorescence, which might underestimate the fold transactivation due to background fluorescence. However, we normalized values for microinjections to luciferase expressed from coinjected pRSVLUC. Despite differences in our assays, Tat effects could easily be measured on TAR DNA targets, whereas no transactivation could be demonstrated with TAR RNA targets.

We did not analyze directly target RNA levels after cotransfection or microinjection for the following reasons. First, significant CAT activity was obtained from cotransfected TARCAT and CAT RNA and visible immunofluorescence could be documented with both RNA species in microinjected cells. Second, TAT RNA was more functional than Tat DNA, implying that degradation of that RNA was not significant even at low levels of input RNA. Third, in both cotransfection and microinjection assays, TARCAT compared with CAT RNA showed similar translational

suppression by TAR RNA as was previously observed in other in vitro and in vivo systems (14, 30, 40).

Previously, we observed the accumulation of short, non-polyadenylated transcripts corresponding to the stem-loop in TAR in the absence of Tat (25, 39). In the presence of Tat, mostly full-length, polyadenylated RNA species accumulated (25, 39). Since introduced RNA species were not rapidly degraded, and since levels of expression from TARCAT when compared with CAT RNA were constant between cotransfections and microinjections, our data also confirm that these short TAR RNA species do not represent degradation of mature TARCAT RNA.

In our cells, transactivation of TAR DNA targets by Tat extends the observation that Tat affects HIV transcription in primate cells that are permissive for HIV infection and replication (20, 25, 27, 33). Although this study cannot exclude effects of Tat on cotranscriptional events such as RNA processing by RNase H, release, and compartmentalization, these are unlikely. First, no effects of Tat on processing or release of HIV transcripts could be demonstrated in kinetic studies of HIV transcription in in vivo and in vitro systems (33, 43). Second, whereas specific compartmentalization was implied in the *Xenopus* oocyte by identical effects of Tat on TAR DNA and TARCAT RNA but not on cytomegalovirus-TAR DNA targets (4), no such effects on TARCAT RNA could be demonstrated on TARCAT RNA in our primate cells.

Our findings are in stark contrast with microinjections into the *Xenopus* oocyte, in which direct effects of Tat on TARCAT RNA were observed (3, 4). These investigators concluded that the HIV long terminal repeat or mature TARCAT RNA directs the TARCAT RNA into a compartment that makes these RNA species refractory to translation in the cytoplasm and that Tat somehow reverses this effect (4). Presumably, Tat does not have to interact with the TAR RNA for these effects because a Tat truncated from amino acids 1 to 48 could rescue translation of TARCAT in the *Xenopus* oocyte (3). In our cells, microinjection of Tat synthesized from amino acids 1 to 47 had no effect (data not shown). Furthermore, we demonstrated previously that Tat does not function in primate cells without its basic domain from amino acids 48 to 58 unless fused to another protein that can tether Tat to RNA near the HIV promoter (39). We conclude that although Tat has a very profound effect on TAR RNA in the *Xenopus* oocyte, this effect is not observed in primate cells that are permissive for HIV infection and replication. Thus, from the standpoint of both target and effector, the *Xenopus* oocyte might represent a unique system of translational control, which cannot be extrapolated to differentiated eukaryotic cells. Perhaps one of the specialized mechanisms that suppresses translation of mRNA in the *Xenopus* oocyte (1, 6, 9, 10, 18, 22, 34, 42) also blocks translation of TAR RNA. Thus, it would be of great interest to examine *Xenopus* embryos or later stages of frog development for similar effects of Tat on TAR RNA and to examine sequences of TAR RNA obtained from the *Xenopus* oocyte for modification of nucleotides.

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