TAR RNA decoys inhibit Tat-activated HIV-1 transcription after preinitiation complex formation

Paul R. Bohjanen1,2, Yi Liu1,3 and Mariano A. Garcia-Blanco1,2,3,*

1Department of Pharmacology and Cancer Biology, 2Division of Infectious Diseases, Department of Medicine and 3Department of Microbiology, Levine Science Research Center, Duke University Medical Center, Durham, NC 27710, USA

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ABSTRACT

The ability of the HIV-1 Tat protein to trans-activate HIV-1 transcription in vitro is specifically inhibited by a circular TAR RNA decoy. This inhibition is not overcome by adding an excess of Tat to the reaction but is partially overcome by adding Tat in combination with nuclear extract, suggesting that TAR RNA might function by interacting with a complex containing Tat and cellular factor(s). A cell-free transcription system involving immobilized DNA templates was used to further define the factor(s) that interact with TAR RNA. Preinitiation complexes formed in the presence or absence of Tat were purified on immobilized templates containing the HIV-1 promoter. After washing, nucleotides and radiolabelled UTP were added and transcription was measured. The presence of Tat during preinitiation complex formation resulted in an increase in the level of full-length HIV-1 transcripts. This Tat-activated increase in HIV-1 transcription was not inhibited by circular TAR decoys added during preinitiation complex formation, but was inhibited by circular TAR decoys subsequently added during the transcription reaction. These results suggest that TAR decoys inhibit Tat-activated HIV-1 transcription after preinitiation complex formation, perhaps by interacting with components of transcription complexes.

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) uses the host cell’s transcription machinery to produce the RNA transcripts necessary for viral replication. Although the cellular transcription machinery can sustain a low basal level of HIV-1 transcription, the viral regulatory protein Tat is required for high level HIV-1 transcription and can activate HIV-1 transcription by >100-fold (1–3). In the absence of Tat most of the RNA transcripts produced from the HIV-1 promoter are short transcripts resulting from early transcription termination, whereas in the presence of Tat the number of long full-length HIV-1 transcripts increases (4–7). Several cellular proteins have been shown to bind to Tat in vitro, including several components of the transcriptional machinery (8–12). Tat has also been shown to associate with the RNA polymerase II holoenzyme (13,14). The functional significance of these interactions is not clear, but it is possible that transcription complexes that contain Tat are modified such that they are capable of elongation competence. Perhaps Tat increases the elongation competence of transcription complexes by regulating phosphorylation of the C-terminal domain (CTD) of the RNA polymerase II large subunit (10,15).

Activation of HIV-1 transcription by Tat depends on the presence of an intact TAR RNA element. TAR is a 59 nt RNA stem–loop structure that forms the 5′-end of all HIV-1 transcripts. TAR consists of two stems, a 4 nt bulge and a 6 nt loop (16–20; see Fig. 1). Tat binds to the TAR bulge region in vitro (21–23) and may bind this same region in vivo, since mutations in the TAR bulge that inhibit Tat-binding in vitro also abolish Tat-mediated transcriptional activation in vivo (3,24,25). Interestingly, mutations in the TAR loop that have little or no effect on Tat binding in vitro abolish Tat-activated HIV-1 transcription in vivo (21,26–28), suggesting that a cellular factor that recognizes the TAR loop is required for Tat function.

Experiments involving TAR RNA decoys provide further evidence that a cellular loop binding factor is necessary for Tat-activated HIV-1 transcription. Overexpression of TAR RNA decoys in a human T cell line rendered these cells resistant to HIV-1 replication (29). In addition to inhibiting HIV-1 replication, TAR decoys also inhibited HIV-1 RNA expression as assessed by Northern blot (30). In contrast, TAR decoys containing a mutation in the TAR loop that allowed efficient Tat-binding in vitro did not inhibit HIV-1 RNA expression and did not inhibit HIV-1 replication (30). These experiments suggest that TAR decoys do not function simply by binding to and sequestering Tat, but rather interact with a cellular factor that recognizes the TAR loop.

An in vitro system that utilizes transcription-competent HeLa nuclear extracts has been used to study the role of TAR RNA in regulating Tat-activated HIV-1 transcription. In this system, as in in vivo transfection systems, Tat induced an increase in transcription from the HIV-1 promoter and this increase in HIV-1 transcription depended on the presence of a functional TAR sequence (31,32). TAR decoys specifically inhibited Tat-activated HIV-1 transcription in this system, whereas TAR decoys containing a mutation in the TAR loop did not inhibit Tat-activated transcription.
HIV-1 transcription (32,33). This result is consistent with the results in vivo (30) and provides further evidence that a cellular factor interacts with the TAR loop. TAR decoys containing a mutation in the TAR bulge also failed to inhibit Tat-activated HIV-1 transcription (33). Thus the cellular factor does not recognize the TAR loop in isolation, suggesting either that this factor recognizes a structure dependent on bulge as well as loop sequences or that this factor requires Tat for binding.

In the present work TAR decoys were used as biochemical tools to further study the role of TAR RNA in regulating Tat-activated HIV-1 transcription in vitro. Although inhibition of Tat-activated HIV-1 transcription by TAR decoys was not reversed by addition of excess Tat (33), this inhibition was partially reversed by additional nuclear extract plus Tat, suggesting that TAR RNA might interact with Tat as well as with cellular factor(s). TAR decoys were also found to specifically inhibit Tat-activated transcription from purified preinitiation complexes. The results suggest that TAR decoys function after preinitiation complex formation and likely interact with Tat and cellular components of the transcription machinery.

MATERIALS AND METHODS

Synthesis of circular TAR RNA decoys

The plasmids pTC, pTC-31/34 and pTC-BL (33) were used as templates for synthesis by T7 RNA polymerase of TAR circle RNA, 31/34 circle RNA and bulgeless circle RNA respectively. RNA synthesis and circularization were carried out using the self-splicing activity of a group I permuted intron–exon as described previously (33). Circular RNA was gel purified and quantified by measuring the optical density at 260 nm. The circular TAR RNA, 31/34 RNA and bulgeless RNA molecules are depicted in Figure 1.

In vitro transcription in HeLa nuclear extracts

HeLa nuclear extracts were prepared as previously described (34). In vitro transcription reactions were carried out for 30 min at 30°C in a 25 µl volume containing 8–16 µl nuclear extract, 14 mM HEPES, pH 7.9, 14% glycerol, 68 mM potassium chloride, 15 mM sodium chloride, 7 mM magnesium chloride, 4 mM sodium citrate, 250 µM poly(I)-poly(C), 300 ng poly(di)poly(dC), 1 mM DTT, 10 mM creatine phosphate, 0.1 mM EDTA, 0.05 M each ATP, CTP and GTP, 40 µM UTP and 0.1 µCi [α-32P]UTP (3000 Ci/mmol; New England Nuclear). Each reaction also contained 100 ng BamHI-cut plasmid pBC12/HIV/SEAP (35), which served as template for transcription from the HIV-1 promoter, and 250 ng AatII-cut plasmid pFLBH (36), which served as template for transcription from the adenovirus major late (AdML) promoter. Some reactions contained 50–200 ng Tat protein (37) and/or 2–100 pmol circular TAR RNA or mutant TAR RNA. The circular RNA was the last component added to the reactions. The reactions were stopped and newly transcribed RNA was isolated and separated by electrophoresis on 6% urea–polyacrylamide gels as previously described (37,38). Bands on the gel were visualized by autoradiography and quantified using a Molecular Dynamics phosphorimager.

Transcription from purified preinitiation complexes

Plasmid pBC12/HIV/SEAP (35) was amplified from positions –466 to +1125 relative to the start of HIV-1 transcription using PCR. The PCR product was digested with EcoRI at position –466 and then biotinylated by filling in the recessed ends with biotin-14-dATP using the Klenow fragment of DNA polymerase I. To immobilize the DNA template, 250 ng biotinylated DNA was suspended in 25 µl buffer containing PBS supplemented with 0.1% BSA and 0.1% (v/v) Tween 20 and was then added to each well of a streptavidin-coated panel (Labsystems). The panels were rotated on an orbital shaker for 1 h at room temperature and then each well was washed three times with 75 µl 0.1 mg/ml PMSF and 0.1% (v/v) Nonidet P-40. Transcription reactions were then performed by adding to each well 25 µl pre-mixed sample containing 10 µl HeLa nuclear extract in buffer D supplemented with 4 mM sodium citrate. Some reactions contained 400 ng purified recombinant Tat protein and/or 50 pmol circular TAR decoys or mutant TAR decoys. The reactions were incubated at 30°C for 20 min and then the wells were washed three times with 75 µl buffer D supplemented with 0.1 mg/ml PMSF and 0.1% (v/v) Nonidet P-40. Transcription reactions were then performed by adding to each well 25 µl solution containing 14 mM HEPES, pH 7.9, 14% glycerol, 68 mM potassium chloride, 15 mM sodium chloride, 7 mM magnesium chloride, 4 mM sodium citrate, 250 µM poly(I)-poly(C), 300 ng poly(di)poly(dC), 1 mM DTT, 10 mM creatine phosphate, 0.1 mM EDTA, 625 µM each ATP, CTP and GTP, 40 µM UTP and 10 µCi [α-32P]UTP (3000 Ci/mmol; New England Nuclear). At this point, 50 pmol circular TAR decoy or mutant circular TAR decoy was added to some reactions. After a 9 min incubation at 30°C the reactions were stopped and radiolabelled RNA was isolated and separated by electrophoresis on 6% urea–polyacrylamide gels. Bands on the gel were visualized by autoradiography and quantified using a Molecular Dynamics phosphorimager or a Molecular Dynamics densitometer.
RESULTS AND DISCUSSION

TAR decoys inhibit Tat-activated HIV-1 transcription in an in vitro system involving HeLa nuclear extracts (32,33). In this system exogenous Tat induced an increase in HIV-1 transcription but had no effect on transcription from the adenovirus major late (AdML) promoter, which was used as an internal control (Fig. 2, lanes 1 and 2). When an HIV-1 promoter containing the ΔTAR mutation (32) was used as an internal control the same result was seen; Tat induced an increase in transcription from the wild-type but not the mutant HIV-1 promoter (data not shown). This Tat-dependent increase in HIV-1 transcription was inhibited by circular TAR decoys (Fig. 2, lanes 3–5) but not by circular TAR decoys containing the 31/34 loop mutation (Fig. 2, lanes 6–8). The inhibitory activity of circular TAR decoys was specific, because TAR decoys did not inhibit transcription from the AdML promoter (Fig. 2) or from an HIV-1 promoter containing the ΔTAR mutation (data not shown). Circular rather than linear TAR decoys were used for these experiments because circular TAR decoys were found to be very stable in HeLa nuclear extracts, whereas linear TAR decoys were relatively unstable (33).

Experiments were performed to determine if TAR decoys inhibit Tat-activated HIV-1 transcription by interacting with cellular proteins, as has been previously suggested (29,32,33). We have previously shown that inhibition of Tat-activated HIV-1 transcription by TAR decoys could not be reversed by adding an excess of Tat protein to the transcription reaction (33), suggesting that TAR decoys do not function simply by binding to and sequestering Tat. The amount of HeLa nuclear extract added to the transcription reactions was titrated to determine if the inhibitory activity of the TAR decoy could be reversed by additional nuclear extract (Fig. 3a). In the absence of Tat, as the amount of HeLa nuclear extract was increased an increase in the level of HIV-1 and AdML transcription was observed (lanes 1–4). Addition of 100 ng Tat protein induced an increase in HIV-1 transcription but no increase in AdML transcription (lanes 5–8). Further addition of 3 pmol circular TAR decoy inhibited this Tat-activated increase in HIV-1 transcription (lanes 9–12). Quantification of this experiment indicated that the amount of HeLa nuclear extract was increased the degree of inhibition of HIV-1 transcription by the TAR decoy was relatively unchanged (Fig. 3b). This result suggests that additional nuclear extract was unable to reverse the inhibitory activity of TAR decoys. If Tat binds to TAR as a preformed complex with cellular proteins, as has been suggested (39), then inhibition of Tat-activated HIV-1 transcription by TAR decoys might be reversed by additional cellular proteins plus Tat. As seen in Figure 3c (left), circular TAR decoys inhibited Tat-activated HIV-1 transcription in a concentration-dependent manner. When additional nuclear extract as well as additional Tat was used (Fig. 3c, right) the magnitude of inhibition for a given amount of TAR was less (Fig. 3d). Thus the combination of additional nuclear extract plus additional Tat was capable of partially reversing the inhibition of Tat-activated HIV-1 transcription. These data suggest that TAR decoys interact with at least one cellular factor and that this cellular factor(s) may require Tat in order to bind to TAR. This supports previous results suggesting that TAR RNA interacts with Tat and at least one cellular factor (29,32,33,39,40) and is consistent with a previous genetic analysis of the Tat–TAR interaction that suggested that Tat binds to TAR as a preformed complex with at least one cellular factor in vivo (39). Since Tat has been shown to interact with various components of the cellular transcription machinery (8–10,12), including RNA polymerase II transcription complexes (11,13,14) and RNA polymerase II holoenzyme (13,14), it is possible that TAR RNA actually interacts with a preformed complex that contains Tat and components of transcription complexes.
Experiments were carried out to determine if TAR decoys interact with complexes that assemble on a DNA template containing the HIV-1 promoter. A DNA template containing the HIV-1 promoter was biotinylated at its 5'-end and was immobilized onto streptavidin-coated wells. Preinitiation complexes were formed by incubating HeLa nuclear extract with the immobilized template in the presence or absence of Tat. After washing, cold nucleotides and radiolabelled UTP were added and newly transcribed RNA was analysed on 6% urea–polyacrylamide gels. The presence of Tat during preinitiation complex formation resulted in an increase in the level of full-length HIV-1 transcripts (Fig. 4; Liu et al., manuscript submitted). The effect of TAR decoys on this Tat-dependent increase in HIV-1 transcription was studied. To determine if TAR decoys could inhibit Tat-activated HIV-1 transcription after preinitiation complex formation, preinitiation complexes were formed in the presence of Tat, template-bound complexes were washed and TAR decoys were added during the transcription reaction. Under these conditions the Tat-activated increase in HIV-1 transcription was specifically inhibited by circular TAR decoys but not by TAR decoys that contained either bulge or loop mutations (Fig. 4a and c). These data suggest that TAR decoys specifically inhibit Tat-activated HIV-1 transcription after preinitiation complex formation. To determine if TAR decoys could function prior to initiation of transcription, circular TAR decoys were added along with Tat during preinitiation complex formation, the template-bound complexes were washed and then transcription was measured. Under these conditions TAR decoys had no effect on Tat-activated HIV-1 transcription (Fig. 4b and d).
Figure 4. TAR decoys inhibit Tat-activated HIV-1 transcription after preinitiation complex formation. (a) Preinitiation complexes were formed by incubating immobilized DNA templates that contained the HIV-1 promoter with 10 µl HeLa nuclear extract in the absence (–) or presence (+) of 400 ng Tat protein for 20 min at 30°C. After the immobilized templates were washed three times, nucleotides and radiolabelled UTP were added to each reaction. The indicated reactions also contained 50 pmol circular TAR decoy (TAR), circular TAR decoy that contained the 31/34 loop mutation (31/34) or circular TAR decoy that contained the bulgeless mutation (BL). The reactions were incubated at 30°C for 9 min and radiolabelled transcripts were isolated and separated by electrophoresis. The position of migration of the 1125 nt run-off HIV-1 transcript is indicated by an arrow. The positions of migration of single-stranded DNA markers are shown to the left of the figure as the number of deoxynucleotides. (b) Preinitiation complexes were formed by incubating the immobilized HIV-1 template with 10 µl HeLa nuclear extract in the absence (–) or presence (+) of 400 ng Tat protein. The reactions were incubated at 30°C for 20 min. After the immobilized templates were washed three times, nucleotides and radiolabelled UTP were added to each reaction. The reactions were incubated at 30°C for 9 min and radiolabelled transcripts were isolated and separated by electrophoresis. (c) The level of full-length HIV-1 transcription was quantified for the experiment in (a) and two other independent experiments. For the experiments shown in this figure trans-activation was calculated by dividing the level of full-length HIV-1 transcription for each lane by the level of full-length HIV-1 transcription in the absence of Tat. The data is expressed as the mean ± SEM from the three experiments. (d) The level of full-length HIV-1 transcription was quantified for the experiment in (b) and two other independent experiments. Trans-activation was calculated as described in (c). The data is expressed as the mean ± SEM from the three experiments.

This lack of inhibition by TAR decoys was not due to degradation of the decoys prior to the transcription reaction, because inhibition was observed if the washing step after preinitiation complex formation was eliminated (data not shown). Exogenous ATP was added along with Tat and TAR decoys during preinitiation complex formation, the template-bound complexes were washed and transcription was measured. No inhibition of Tat-activated HIV-1 transcription was seen under these conditions (data not shown). Taken together, these results suggest that TAR decoys function after preinitiation complex formation and not prior to this. Perhaps the TAR binding site does not assemble or assume the proper conformation until after transcription begins.

Although the mechanism by which TAR decoys inhibit Tat-activated HIV-1 transcription has not yet been elucidated, it appears that TAR decoys function by interacting with a preformed complex consisting of Tat and cellular factors. Since Tat has been shown to associate with RNA polymerase II transcription complexes as well as with RNA polymerase II holoenzyme in the absence of TAR RNA (13,14,41), it is possible that TAR RNA interacts with preformed Tat-associated transcription complexes. This contention is supported by the observation that TAR RNA interacted with RNA polymerase II in the presence of a nuclear extract fraction that contained essential cellular factors (42,43). Perhaps binding of endogenous TAR RNA to Tat-associated RNA polymerase II transcription complexes activates these complexes such that they become more elongation competent. The finding that TAR decoys inhibit Tat-activated HIV-1 transcription after preinitiation complex formation is consistent with the hypothesis that TAR decoys bind to and inhibit Tat-associated transcription complexes. It is likely that TAR decoys function by competing with endogenous TAR for this binding. Although several cellular proteins have been identified that bind to TAR RNA in vitro (31,40,44–46), the role...
of these proteins in regulation of HIV-1 transcription has not been defined. The results presented here predict that functional TAR binding factors are components of complexes that assemble on HIV-1 templates. Identification of the components of the transcription machinery that interact with TAR RNA will lead to a better understanding of the mechanism by which TAR RNA regulates HIV-1 transcription.

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