Nucleic Acids Research, 1996, Vol. 24, No. 19 3733–3738

A small circular TAR RNA decoy specifically inhibits Tat-activated HIV-1 transcription

Paul R. Bohjanen[1,2,3], Richard A. Colvin[3,5], M. Puttaraju[4], Michael D. Been[4] and Mariano A. Garcia-Blanco[1,2,3,5,*]

[1]Division of Infectious Diseases, [2]Department of Medicine,[3]Department of Molecular Cancer Biology, [4]Department of Biochemistry and [5]Department of Microbiology, Duke University Medical Center, Durham, NC 27710, USA

Received June 24, 1996; Revised and Accepted August 12, 1996

ABSTRACT

Linear TAR RNA has previously been used as a decoy to inhibit HIV-1 transcription *in vitro* and HIV-1 replication *in vivo*. A 48 nucleotide circular RNA containing the stem, bulge and loop of the HIV-1 TAR element was synthesized using the self-splicing activity of a group I permuted intron–exon and was tested for its ability to function as a TAR decoy *in vitro*. This small circular TAR molecule was exceptionally stable in HeLa nuclear extracts, whereas a similar linear TAR molecule was rapidly degraded. The TAR circle bound specifically to Tfr38, a peptide containing the TAR-binding region of Tat. The ability of Tat to *trans*-activate transcription from the HIV-1 promoter *in vitro* was efficiently inhibited by circular TAR RNA but not by TAR circles that contained either bulge or loop mutations. TAR circles did not inhibit *trans*-activation exclusively by binding to Tat since this inhibition was not reversed by adding excess Tat to the transcription reaction. Together, these data suggest that TAR circles act as decoys that inhibit *trans*-activation by binding to Tat and at least one cellular factor. These data also demonstrate the utility of small circular RNA molecules as tools for biochemical studies.

INTRODUCTION

RNA–protein interactions play important roles in a wide variety of biological processes including transcription, splicing, nucleo-cytoplasmic transport, RNA degradation and translation. The use of RNA molecules as biochemical tools for studying these processes may be limited by the rapid degradation of RNA by nucleases present in biological samples. Compared with linear RNA molecules, circular RNA molecules are often resistant to nucleases (1–3) and could potentially function as useful biochemical tools for studying RNA–protein interactions. For example, if a circular RNA molecule has a structure that resembles an endogenous linear RNA molecule, the circular RNA could be used as a decoy to probe RNA–protein interactions and to study the role of these interactions in functional assays.

The human immunodeficiency virus type 1 (HIV-1) *trans*-activation response element (TAR) is a 59 nucleotide (nt) RNA stem–loop structure that forms the 5′-end of HIV-1 transcripts (4–6). TAR consists of a stem, a 4 nt bulge and a 6 nt loop (7–11). The HIV-1 viral protein Tat, which induces *trans*-activation of HIV-1 transcription by >100-fold *in vivo* (12–14), binds specifically to the TAR bulge *in vitro* (15–17). Several experiments suggest that at least one cellular factor binds to the TAR loop (18–25). In fact, several cellular factors have been identified that bind to the TAR loop *in vitro* (23,24,26–28), but none of these factors have been clearly shown to regulate HIV-1 transcription.

In vitro transcription assays using HeLa nuclear extracts have been used to study HIV-1 transcription. In this system, as in *in vivo* transfection systems, Tat induces increased transcription from the HIV-1 promoter, and this increase in HIV-1 transcription depends on the presence of a functional TAR sequence (24,25). Linear TAR RNA decoys functioned to inhibit Tat-mediated *trans*-activation in this system (25). In contrast, a linear TAR RNA that contained a loop mutation did not inhibit HIV-1 transcription (25), suggesting that loop sequences are required for Tat-mediated *trans*-activation.

In this work, a 48 nt circular RNA molecule that contained stem, bulge and loop sequences from the HIV-1 TAR element was synthesized using the auto-catalytic splicing of a group I permuted intron–exon. This circular TAR RNA, which was extremely stable in HeLa nuclear extracts, functioned successfully as a TAR decoy in terms of its ability to bind specifically to a Tat peptide and in terms of its ability to specifically inhibit Tat-mediated *trans*-activation *in vitro*. This study demonstrates that stable circular RNA molecules designed to mimic known RNA structures can be useful tools for studying RNA–protein interactions.

MATERIALS AND METHODS

Plasmids

The plasmids pTC, pTC-31/34 and pTC-BL were used as templates for the synthesis of TAR circle RNA, 31/34 circle RNA and bulgeless circle RNA, respectively. These plasmids were constructed by inserting synthesized oligonucleotides containing the sequence 5′-CTAGCCAGATCTGAGCCTGGGAGCTCTC-TGG-3′ (pTC), 5′-CTAGCCAGATCTGAGCCCAAAAGCTC-TCTGG-3′ (pTC-31/34) or 5′-CTAGCCAGAGAGCCTGGG-AGCTCTCTGG-3′ (pTC-BL) into the *Nhe*I site of plasmid pPR120 (3). The correct sequence and orientation of inserts for each plasmid was confirmed by DNA sequencing using the

* To whom correspondence should be addressed at: Department of Molecular Cancer Biology, Box 3686, Duke University Nedical Center, Durham, NC 27710, USA

dideoxy method (Sequenase, US Biochemicals). The plasmid pT7-157 was used as a template for synthesis of linear TAR RNA. This plasmid was derived from pT7-TAR (27) but was modified such that a *Hin*dIII site was inserted at position +63 immediately downstream of the TAR stem sequence. The plasmids pFLBH (29) and pBC12/HIV/SEAP (30) were used as templates for transcription in HeLa nuclear extracts to produce the AdML and HIV-1 transcripts respectively. All plasmid DNA was isolated from overnight cultures by the alkaline lysis method and purified by equilibrium density centrifugation in cesium chloride containing ethidium bromide (31).

RNA synthesis using T7 RNA polymerase

RNA synthesis was carried out as described by Milligan and Uhlenbeck (32) with minor modifications. The plasmids pTC, pTC-31/34, pTC-BL and pT7-157 were digested with *Hin*dIII (New England Biolabs) to create linear templates for *in vitro* transcription using T7 RNA polymerase. T7 RNA polymerase was prepared as described by Grodberg and Dunn (33). Transcription reactions containing 40 mM Tris–HCl (pH 8.1), 26 mM magnesium chloride, 1 mM spermidine, 5 mM dithiothreitol, 1 mM ribonucleotide triphosphates, 0.1 mg/ml linear plasmid DNA, 10 µg/ml T7 RNA polymerase, 0.01% Triton X and 0.04% polyethylene glycol (average molecular weight 8000) were incubated for 4 h at 37°C. In order to increase the efficiency of auto-catalytic splicing of precursor RNA, the reactions were incubated at 42°C for an additional 2 h. Radiolabelled RNA was prepared by including 0.2 nM [α-^{32}P]UTP (3000 Ci/mmol; New England Nuclear) in the transcription reaction. The reaction mixtures were then separated by electrophoresis on 12% urea–polyacrylamide gels. Gel slices containing the RNA of interest were identified by UV shadowing and were cut out of the gel. This RNA was eluted from the gel slices overnight in a buffer containing 0.1% SDS, 0.5 M ammonium acetate and 10 mM magnesium acetate, recovered by ethanol precipitation, and quantitated by measuring the optical density at 260 nm and/or by measuring radioactivity with a scintillation counter.

Partial hydrolysis of RNA

Partial hydrolysis of RNA was accomplished by incubating 0.1 pmol of circular or linear TAR RNA in 50 mM sodium bicarbonate (pH 9.0) and 1 mM EDTA for 3 min at 90°C. The partially hydrolyzed RNA was separated by electrophoresis on a 24% urea–polyacrylamide gel and was visualized by autoradiography on Hyperfilm (Amersham).

RNA stability assay

Radiolabelled circular or linear TAR RNA (0.1 pmol) was incubated with 10 µl HeLa nuclear extract (150 µg protein) in 100 µl reactions containing 50 mM Tris–HCl (pH 7.5), 100 mM sodium chloride, 10 mM magnesium chloride and 0.1 mg/ml yeast tRNA for 0–12 h. The reactions were stopped by adding an equal volume of 25 mM EDTA and 80% formamide. Aliquots of the reaction mixtures were then separated by electrophoresis on a 6% urea–polyacrylamide gel. Bands on the gel were visualized by autoradiography and were quantified on a Molecular Dynamics PhosphorImager.

Electrophoretic mobility shift assay

Radiolabelled circular TAR RNA (0.5 pmol) was incubated at room temperature for 10 min with 1 pmol of Tfr38 peptide (17) in the presence or absence of varying amounts of cold competitor RNA in a buffer containing 20 mM HEPES (pH 7.9), 10 mM magnesium chloride, 100 mM potassium chloride, 5% glycerol and 0.5 mg/ml yeast tRNA in a volume of 20 µl. A 4 µl volume of loading buffer containing 50% glycerol, 0.25% bromophenol blue and 0.25% xylene cyanol was added to each reaction, and then the reactions were separated by electrophoresis in a non-denaturing 8% polyacrylamide gel (acrylamide to bis-acrylamide ratio of 62:1) using a running buffer containing 45 mM Tris-borate, 45 mM boric acid and 2 mM EDTA. The gel was dried and bands were visualized by autoradiography and quantified on a Molecular Dynamics PhosphorImager.

In vitro transcription assay

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 10 µl nuclear extract (150 µg protein), 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 ng poly I–poly C, 300 ng poly dI–poly dC, 1 mM DTT, 10 mM creatine phosphate, 0.1 µM EDTA, 625 µM each of ATP, CTP and GTP, 40 µM UTP, 10 µCi [α-^{32}P]UTP (3000 Ci/mmol; New England Nuclear), 100 ng HIV-1 template (pBC12/HIV/SEAP cut with *Bam*HI) and 250 ng AdML template (pFLBH cut with *Aat*II). Some reactions also contained 15–1500 ng Tat protein (35) and/or 1–10 pmol circular 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 (35,36). Bands on the gel were visualized by autoradiography and quantified using a Molecular Dynamics PhosphorImager.

RESULTS

Circular TAR RNA can be synthesized using the self-splicing activity of a group I permuted intron–exon

Group I permuted intron–exons, which undergo auto-catalytic splicing to generate circular products (3,37), were used to synthesize circular TAR RNA. The sequence from +18 to +44 of HIV-1 TAR was shown by mutational analysis to represent the minimal TAR sequence for *in vivo* function (4). The plasmid pPR120 (3), which contained a group I permuted intron–exon sequence, was modified by inserting this minimal TAR sequence into the exon sequence. After linearization with a restriction endonuclease, this plasmid was used as a template for transcription by T7 RNA polymerase. The linear precursor RNA was expected to undergo self-splicing at 42°C in the presence of magnesium and GTP to yield a circular RNA product and two linear products (Fig. 1a). The products of an incomplete splicing reaction are shown in Figure 1b (lane S). The bands were identified by comparison with auto-catalytic RNA splicing products produced from the parent plasmid (37). The identity of the circular RNA product was confirmed by partial alkaline hydrolysis (Fig. 1b). Random nicking of linear TAR RNA (TAR) produced multiple products that appeared as a ladder when separated by electrophoresis. In contrast, random nicking of circular TAR RNA (TAR

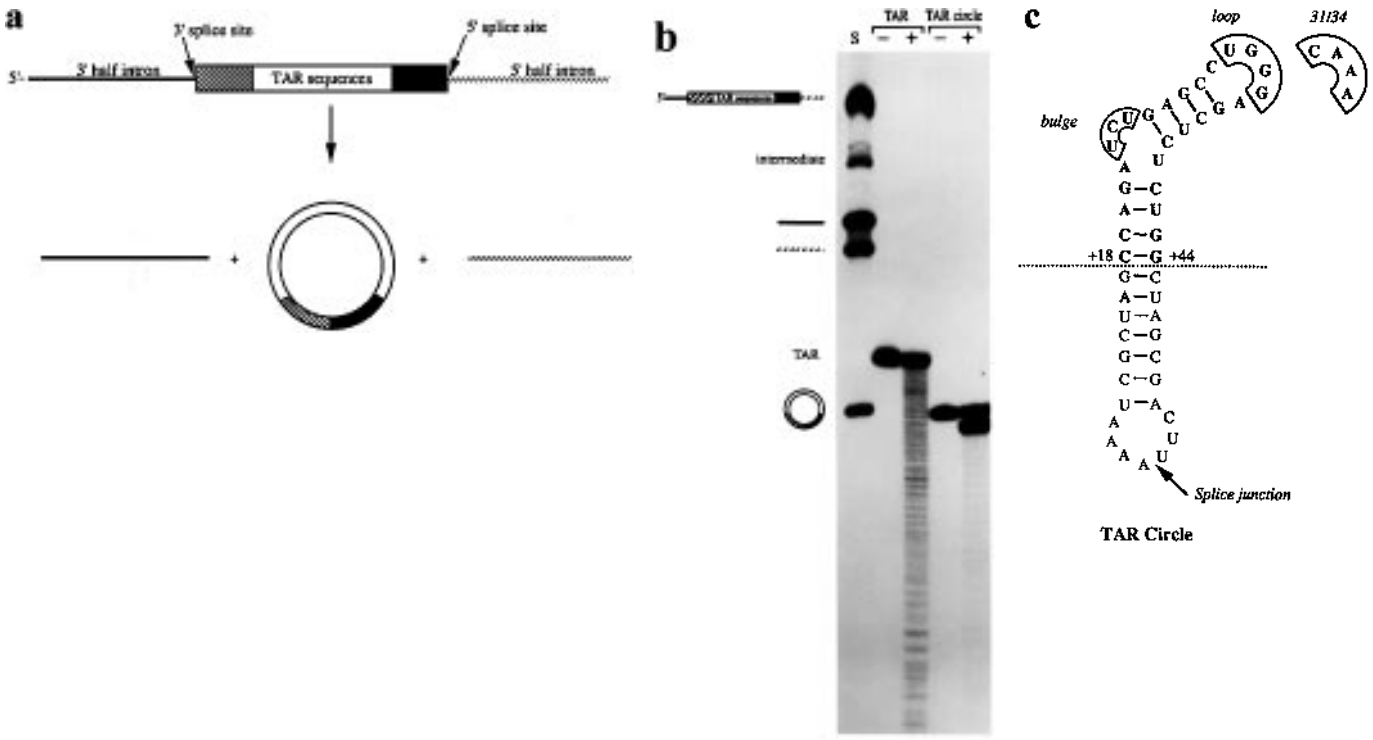

Figure 1. Synthesis of circular TAR RNA. (**a**) The plasmid pPR120, which contained a group I permuted intron–exon sequence, was modified by inserting sequences from the HIV-1 TAR element from +18 to +44 into the exon sequence. The linear transcript produced by T7 RNA polymerase (top) undergoes self-splicing at 42°C in the presence of magnesium and GTP to form a circular product and two linear products (bottom). (**b**) The radiolabelled products of an incomplete splicing reaction were separated by electrophoresis (lane S). The positions of migration of the parent linear transcript, the linear and circular products and a splicing intermediate are indicated to the left of the figure. Radiolabelled linear TAR RNA (TAR) or circular TAR RNA (TAR Circle) that was partially hydrolyzed by sodium bicarbonate (+) or was untreated (–) was also separated by electrophoresis on the same gel. The gel was dried, and the bands were visualized by autoradiography. (**c**) The predicted sequence and secondary structure of the TAR circle is shown. The sequences above the dotted line were derived from HIV-1 TAR RNA from +18 to +44. The sequences below the dotted line were derived from the parent exon. The position of the splice junction is shown with an arrow. The bulgeless circle was identical to the TAR circle except the indicated 3 nt from the bulge were deleted. The 31/34 circle contained the indicated substitutions in the 6 nt loop sequence.

Circle) produced a single band that displayed increased electrophoretic mobility. The expected sequence of the circular TAR RNA is shown in Figure 1c. The sequence of the splice junction has been previously determined by primer extension in circular RNAs produced from the parent plasmid (37).

Compared with linear TAR RNA, circular TAR RNA is exceptionally stable

Other circular RNA molecules have been shown to be relatively resistant to degradation by nucleases (1–3). If circular TAR RNA is also resistant to nucleases, it could be a very useful biochemical tool. Therefore, the stability of circular TAR RNA was compared with linear TAR RNA in HeLa nuclear extracts. Linear TAR RNA was rapidly degraded (Fig. 2) with a half life of only 20 min. In contrast, circular TAR RNA was very stable and was not appreciably degraded after 12 h. Circular TAR RNA is not completely resistant to nucleases, however, since it is degraded in the presence of serum (data not shown). The marked stability of circular RNA in nuclear extracts suggests that the TAR circle would be a very useful tool if it possessed functional properties of native TAR RNA.

A Tat peptide binds specifically to circular TAR RNA

Several studies have shown that the HIV-1 Tat protein binds through its basic domain to the TAR bulge *in vitro* (15,17,38–40). A Tat-derived peptide, Tfr38, consisting of the C-terminal 38 amino acids of Tat, was shown to bind to TAR with similar specificity as the full-length Tat protein (17). Circular TAR RNA was examined for its ability to interact with the Tfr38 peptide. As shown in Figure 3, the Tfr38 peptide bound specifically to radiolabelled circular TAR RNA (TAR circle) as detected using an electrophoretic mobility shift assay. Using this assay, the affinity of Tfr38 for circular TAR RNA was equivalent to the affinity for linear TAR RNA (data not shown). In the experiment shown in Figure 3, most of the binding by Tfr38 to radiolabelled TAR circle was competed with a 10-fold excess of unlabelled TAR circle (lanes 3–5). A 10-fold excess of a circular TAR RNA containing a mutation in the loop (31/34 circle) also competed for Tfr38 binding but to a slightly lesser extent (lanes 6–8). In contrast, a circular TAR RNA in which the bulge was deleted (BL circle) competed poorly for Tfr38 binding even at a 100-fold molar excess (lanes 9–11). Compared with the TAR circle, the affinities of the 31/34 circle and the BL circle for the Tfr38 peptide were 4-fold and 50-fold lower, respectively. This pattern

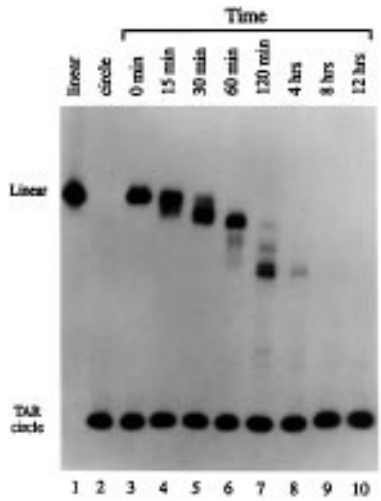

Figure 2. Circular TAR RNA is exceptionally stable in HeLa nuclear extracts. Radiolabelled linear and circular TAR RNA were incubated together with HeLa nuclear extracts at 37°C for the indicated times (lanes 3–10). The reactions were stopped by the addition of formamide and EDTA and were separated by electrophoresis. After the gel was dried, the bands were visualized by autoradiography and were quantified on a Molecular Dynamics PhosphorImager. The positions of migration of linear TAR RNA (lane 1, Linear) and circular TAR RNA (lane 2, TAR circle) are indicated.

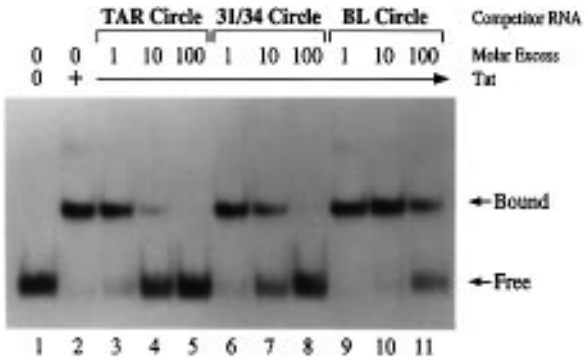

Figure 3. A Tat peptide binds specifically to circular TAR RNA. Radiolabelled TAR Circle (0.5 pmol) was incubated with 1 pmol of Tfr38 peptide in the absence or the presence of a 1-, 10- or 100-fold molar excess of unlabelled TAR circle, 31/34 circle, or bulgeless circle (BL Circle) competitor RNA. The reaction products were separated by electrophoresis under non-denaturing conditions. After the gel was dried, the bands were visualized and quantified.

of binding is consistent with previously reported binding of Tat to linear TAR in which bulge mutations abolished Tat binding while loop mutations had little or no affect (15,41–45). These data suggest that the TAR circle may have bulge and loop structures similar to native TAR RNA.

Circular TAR RNA inhibits Tat-mediated *trans*-activation *in vitro*

Linear TAR RNA decoys have been shown to inhibit Tat-mediated *trans*-activation in an *in vitro* system (25). In order to determine if circular TAR RNA could also inhibit Tat-mediated *trans*-activation, circular TAR RNA was added to *in vitro* transcription reactions containing HeLa nuclear extracts. As seen in Figure 4a, transcription from the HIV-1 promoter increased when exogenous Tat was added to the reaction while transcription from the adenovirus major late (AdML) promoter remained relatively unchanged (lanes 1 and 2). This specific increase in HIV-1 transcription was inhibited by the addition of TAR circle RNA to the reaction, and the amount of inhibition increased as the amount of TAR circle was increased from 1 to 10 pmol (lanes 3–5). In contrast, *trans*-activation was not inhibited by 31/34 circle RNA (lanes 6–8) or bulgeless circle RNA (lanes 9–11). Quantification of three independent transcription experiments revealed similar results (Fig. 4b). The TAR circle inhibited the Tat-mediated increase in HIV-1 transcription by 77.4%, whereas the 31/34 circle and the bulgeless circle had no significant effect. These data suggest that bulge and loop structures are both required for TAR to function as a decoy in this assay. Basal HIV-1 transcription in the absence of Tat was not affected by TAR circle, 31/34 circle, or bulgeless circle RNA (Fig. 5a and data not shown).

The amount of Tat added to transcription reactions was titrated over a 100-fold range to determine if the inhibition of *trans*-activation could be overcome by adding excess Tat (Fig. 5). As the amount of Tat was increased, the level of HIV-1 transcription increased to a peak at 150 ng (12 pmol) of Tat and then decreased (Fig. 5b). At high amounts of Tat (500–1500 ng; 40–120 pmol) the level of AdML transcription also appeared to decrease. Although the mechanism for this decrease is unknown, this phenomenon may be due to sequestration by Tat of limiting cellular factor(s) necessary for HIV-1 and AdML transcription. As was also seen in Figure 4, addition of 10 pmol of circular TAR RNA inhibited the increase in HIV-1 transcription induced by Tat, and the addition of excess Tat (up to 30-fold more Tat than was used in Fig. 4) did not overcome this inhibition. This result suggests that the TAR circle did not inhibit HIV-1 transcription simply by binding to Tat but also interacted with at least one cellular factor.

DISCUSSION

A 48 nt circular RNA containing the stem, bulge and loop of HIV-1 TAR RNA was synthesized and examined to determine its utility as a biochemical tool. This circular TAR RNA was found to be extremely stable in HeLa nuclear extracts while a similar linear TAR RNA molecule was rapidly degraded. Although the mechanism for this increased stability has not been defined, the circular RNA does not have ends that are accessible to exonucleases. It is also possible that the extensive secondary structure of the circular TAR RNA could contribute to its stability.

Circular TAR RNA functioned as a TAR decoy by binding specifically to the Tfr38 peptide. The binding of Tfr38 to the circular TAR RNA appeared to involve the bulge since deletion of the bulge reduced the relative binding affinity by 50-fold. The relative affinity of Tfr38 binding to a circular TAR RNA in which the loop sequence from +31 to +34 was altered (Fig. 1c) was also determined. This particular mutation in the TAR loop was chosen because the equivalent mutation *in vivo* nearly abolished Tat-mediated *trans*-activation (15,46,47). The 31/34 circle had a similar but slightly lower affinity (~4-fold) for Tfr38 binding compared with the TAR circle. This result differs slightly from the result of Roy *et al.* (15) who found that linear TAR RNA and linear 31/34 RNA have the same relative affinities for Tat. Roy *et al.*, however, examined only a single point rather using titrated amounts of cold competitor RNA, and they could have missed small differences in relative affinities. It is unclear whether the slight decrease in the relative affinity resulting from the 31/34 loop mutation seen in these *in vitro* binding experiments could

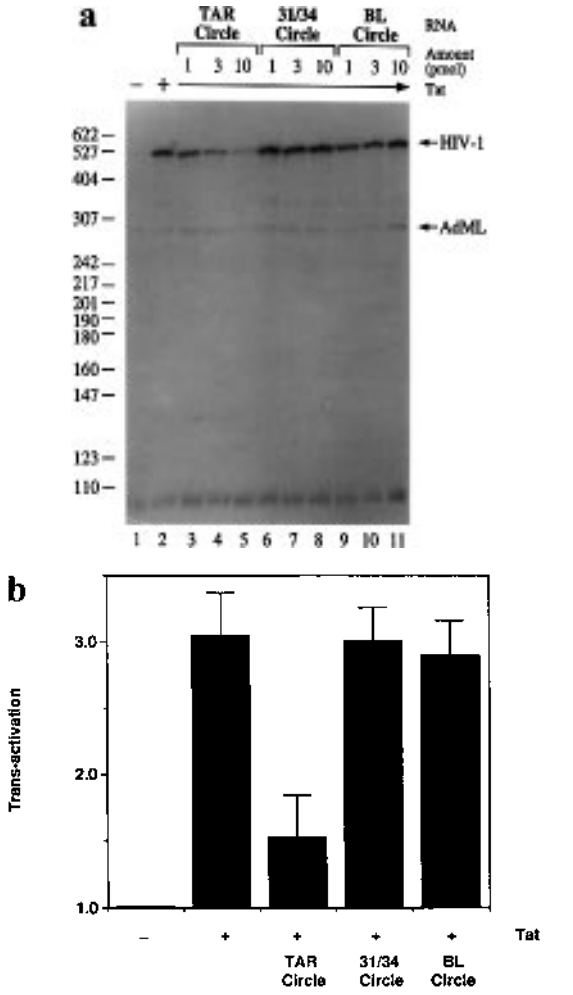

Figure 4. Inhibition of Tat-mediated *trans*-activation by circular TAR RNA. (**a**) *In vitro* transcription reactions containing HIV-1 and AdML templates were carried out using HeLa nuclear extracts in the presence (+) or absence (–) of 50 ng (4 pmol) of Tat protein. Titrated amounts (1–10 pmol) of TAR circle, 31/34 circle or bulgeless circle (BL Circle) RNA were added to the indicated reactions. The reactions were incubated for 30 min at 30°C, and radiolabelled transcripts were isolated and separated by electrophoresis on a denaturing polyacrylamide gel, and the bands were visualized by autoradiography. The positions of migration of the 552 nt HIV-1 transcript and the 290 nt AdML transcript are indicated with arrows. The positions of migration of single stranded DNA markers of varying lengths are shown to the left of the figure as the number of deoxynucleotides. (**b**) *In vitro* transcription reactions containing HIV-1 and AdML templates were carried out in the presence (+) or absence (–) of 50 ng (4 pmol) of Tat protein. Some reactions contained 10 pmol of TAR circle, 31/34 circle or bulgeless circle (BL Circle) RNA. Radiolabelled transcripts were isolated and separated by electrophoresis on denaturing gels. The bands were quantified on a Molecular Dynamics PhosphorImager, and the level of HIV-1 transcription was normalized for each lane by dividing the intensity of the HIV-1 band by the intensity of the AdML band. *Trans*-activation was calculated by dividing the normalized level of HIV-1 transcription for each lane by the normalized level of HIV-1 transcription in the absence of Tat. The data is expressed as the mean and standard error of the mean from three independent experiments.

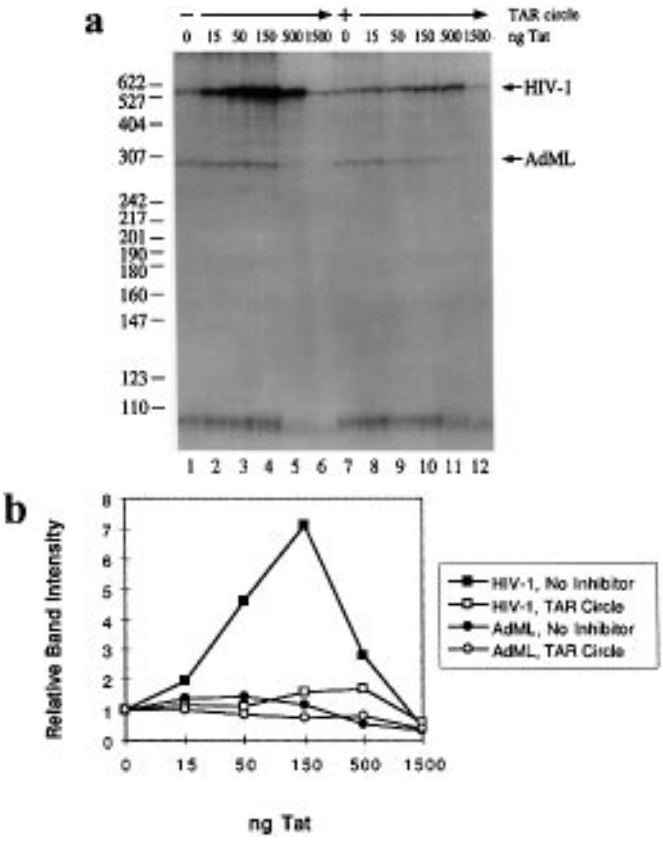

Figure 5. The inhibition of *trans*-activation by circular TAR RNA is not overcome by the addition of excess Tat. (**a**) *In vitro* transcription reactions containing HIV-1 and AdML templates and titrated amounts of Tat protein (0–1500 ng; 0–120 pmol) were carried out in the presence (+) or absence (–) of 10 pmol of TAR circle RNA. Radiolabelled transcripts were isolated and separated by electrophoresis on denaturing gels, and the bands were visualized by autoradiography. The positions of migration of the HIV-1 and AdML transcripts are indicated with arrows. The positions of migration of single-stranded DNA markers of varying lengths are shown to the left of the figure as the number of deoxynucleotides. (**b**) Bands from the gel in (a) were quantified using a Molecular Dynamics PhosphorImager. Relative band intensity was calculated by dividing the intensity of each band by the intensity of the corresponding band in the absence of Tat. The x-axis scale is non-linear to allow easier visual interpretation of the data.

explain the dramatic decrease in *trans*-activation caused by the 31/34 mutation *in vivo*. Overall, the results reported here are consistent with previous reports that mutation of the TAR bulge nearly abolished Tat binding while mutation of the TAR loop had little or no effect (15,17,42–45,48) and suggest that the structure of the bulge and loop in circular TAR RNA is similar to linear TAR RNA.

Previous studies have shown that linear TAR RNA functioned as a decoy to inhibit Tat-mediated *trans*-activation *in vitro* (25) and viral replication *in vivo* (49). In the experiments presented here, circular TAR RNA also functioned as a TAR decoy to inhibit Tat-mediated *trans*-activation *in vitro* (Figs 4 and 5). Circular TAR RNA was found to be superior to linear TAR RNA for this purpose because the results produced using circular TAR RNA were much more reproducible. This was possibly due to the increased stability of circular RNA.

Although the Tat peptide bound to the 31/34 circle *in vitro* (Fig. 3), the 31/34 circle did not inhibit Tat-mediated *trans*-activation even at concentrations 10-fold higher than those used in Figure 4 (data not shown). These data support the supposition that the TAR circle inhibits *trans*-activation by binding to Tat as well as to at least one cellular protein that recognizes loop sequences. The finding that the inhibition of Tat-mediated *trans*-activation by the TAR circle is not reversed by adding an excess of Tat to the

reaction also supports this. Whether or not this cellular factor is the same as one of the previously reported TAR-binding factors (23,24,26–28) is unknown.

The finding that the bulgeless circle did not inhibit Tat-mediated *trans*-activation suggests that the cellular factor cannot recognize loop sequences in isolation. Thus, it appears that bulge and loop sequences are both required for a circular TAR RNA to inhibit *trans*-activation. Potential explanations for these data include (i) the cellular factor cannot bind to the TAR loop in the absence of Tat, but binding by Tat to the bulge changes the structure of the loop allowing the cellular factor to bind, (ii) Tat and the cellular factor bind to TAR as a pre-formed complex, or (iii) the cellular factor recognizes a structure that is dependent on bulge as well as loop nucleotides. The first explanation is supported by biochemical structural studies that suggest that the structure of the loop changes upon binding of a Tat peptide to the bulge (7), and the second explanation is supported by previous genetic studies that suggest that Tat binds to TAR as a pre-formed complex *in vivo* (50). The finding presented here that high concentrations of Tat inhibit *trans*-activation *in vitro* (Fig. 5) suggests that Tat binds to and sequesters a cellular factor necessary for *trans*-activation. Previous studies in our laboratory have shown that HeLa nuclear extracts could be specifically depleted of *trans*-activation activity by passage through Tat affinity columns, providing further evidence that Tat interacts directly with cellular factors (35). One of these factors has been purified and cloned (Suñé *et al*., manuscript in preparation). Whether or not this factor is the same as the loop-binding factor remains to be determined.

The experiments presented in this manuscript show that circular TAR RNA displays structural and functional properties of its linear counterpart. The TAR circle binds specifically to a Tat peptide and appears to inhibit *trans*-activation by interacting with Tat and at least one cellular factor. The dramatically increased stability of the circular TAR RNA molecule makes it a very useful tool for the biochemical studies presented here as well as for future studies. Circularization may prevent RNA degradation in other systems and allow the use of RNA as a biochemical tool in a wider range of applications. Perhaps in the future, circular RNA decoys directed against intracellular targets could even be used as therapeutic agents.

ACKNOWLEDGEMENTS

We thank K. Weeks and D. Crothers for the kind gift of the Tfr38 peptide. We also thank C. Suñé, Z. Pasman and M. Velaz-Faircloth for their critical reading of this manuscript. The Keck Foundation is acknowledged for their generous support of the Levine Science Research Center at Duke University, where much of this work was performed. P.R.B. was supported by a Howard Hughes Medical Institute Postdoctoral Fellowship for Physicians. R.A.C. was a predoctoral fellow in the MSTP at Duke University. This work was supported by grants from the VA to M.A.G.-B. and from the NIH to M.D.B.

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