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DOI
10.1093/nar/gkw167

Publication date
2016

Document Version
Final published version

Published in
Nucleic Acids Research

Citation for published version (APA):
Tat-dependent production of an HIV-1 TAR-encoded miRNA-like small RNA

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Received January 23, 2015; Revised March 02, 2016; Accepted March 03, 2016

ABSTRACT

Evidence is accumulating that retroviruses can produce microRNAs (miRNAs). To prevent cleavage of their RNA genome, retroviruses have to use an alternative RNA source as miRNA precursor. The trans-acting responsive (TAR) hairpin structure in HIV-1 RNA has been suggested as source for miRNAs, but how these small RNAs are produced without impeding virus replication remained unclear. We used deep sequencing analysis of AGO2-bound HIV-1 RNAs to demonstrate that the 3′ side of the TAR hairpin is processed into a miRNA-like small RNA. This ∼21 nt RNA product is able to repress the expression of mRNAs bearing a complementary target sequence. Analysis of the small RNAs produced by wild-type and mutant HIV-1 variants revealed that non-processive transcription from the HIV-1 LTR promoter results in the production of short TAR RNAs that serve as precursor. These TAR RNAs are cleaved by Dicer and processing is stimulated by the viral Tat protein. This biogenesis pathway differs from the canonical miRNA pathway and allows HIV-1 to produce the TAR-encoded miRNA-like molecule without cleavage of the RNA genome.

INTRODUCTION

MicroRNAs (miRNAs) are small RNAs (∼21 nt in size) that have important post-transcriptional regulatory roles by targeting messenger RNAs (mRNAs) for cleavage or translational repression (1). In the canonical pathway, miRNAs are derived from stem-loop RNA structures within long primary miRNA transcripts (pri-miRNAs). Recognition and cleavage of the pri-miRNA into a precursor miRNA (pre-miRNA) hairpin is mediated by the nuclear microprocessor complex that consists of at least two sub-units: the ribonuclease (RNAse) III Drosha and the double-stranded RNA (dsRNA)-binding protein DGCR8 (DiGeorge syndrome critical region protein 8) (2,3). The stem-loop structure in the pri-miRNA is recognized by DGCR8 and Drosha cleaves both strands at ∼11 base pairs (bp) from the base of the stem (4). The pre-miRNA is exported to the cytoplasm and further processed by the RNase III Dicer (5,6). Dicer cleaves both strands of the pre-miRNA at ∼2 helical turns (∼21 nt) from the Drosha cleavage sites (1,7). The guide strand of the double-stranded miRNA is loaded into the Argonaute2 (AGO2) protein, which is part of the RNA-induced silencing complex (RISC) to target a complementary mRNA, while the passenger strand is degraded (8,9).

Several DNA viruses encode miRNAs, including viruses of the Herpesviridae, Polyomaviridae and Adenoviridae (10). For retroviruses like human immunodeficiency virus type-1 (HIV-1), it has remained controversial whether they encode miRNAs. Retroviruses are RNA viruses with a DNA replication intermediate that is transcribed by RNA polymerase II (RNAP II) in the nucleus. Microprocessor and subsequently Dicer will thus be able to access and process the viral RNAs. Canonical production of an avian leukemia virus subgroup J (ALV-J) encoded miRNA has indeed been suggested (11). However, such canonical miRNA processing is expected to lead to RNA genome cleavage and inhibition of retrovirus replication (12). Recent studies revealed that bovine leukemia virus (BLV) (13,14), bovine foamy virus (BVF) (15) and simian foamy virus (SFV) (16) use a non-canonical pathway to produce miRNAs. These retroviruses encode sub-genomic RNA polymerase III (RNAP III) transcripts that serve as pre-miRNAs, thus avoiding degradation of their RNA genome. Several studies described miRNAs derived from the HIV-1 transacting...
responsive (TAR) RNA domain (17–21). These TAR miRNAs may prevent apoptosis of the infected cell for the purpose of increased virus production (22,23) or induce chromatin remodelling to induce latency (18,21). The relevance of these findings was however disputed because it was not clear how these miRNAs could be produced without impeding virus production (24–26).

The 57-nt TAR element is present at the 5′ and 3′ end of all HIV-1 RNA transcripts and can fold a stable hairpin structure (Figure 1). The best-studied function of the 5′ TAR element is its essential role in the activation of transcription from the long terminal repeat (LTR) promoter in the proviral genome (27). In the absence of the viral Tat transactivator protein, RNAP II initiates transcription but then as an TAR structure causes pausing and premature termination of transcription (28). This non-processive transcription results in the synthesis of short TAR transcripts (29–31) (Figure 1A). Upon binding of Tat and the cellular positivetranscription elongation factor b (P-TEFb) complex to TAR RNA, RNAP II is phosphorylated, which activates processive transcription and production of full-length viral RNAs (29).

MiRNAs are loaded in the AGO2/RISC complex to exert their regulatory function. We used this important characteristic to identify HIV-1 encoded miRNAs by deep sequencing of AGO2-bound viral RNAs. This analysis revealed that the TAR element encodes a miRNA-like small RNA that originates from the 3′ side of the hairpin. We used wild-type (wt) HIV-1 and TAR and Tat-mutated virus variants to investigate how this small RNA molecule is produced and the role of the Tat protein.

MATERIALS AND METHODS

Cell culture, transfection and infection

Human Embryonic Kidney 293T, HCT-116 and HCT-116-Ex5 cells (32) were cultured as monolayer in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37°C and 5% CO2. Cells (5 × 106 cells) were cultured in 25 cm2 flasks and transfected with 5 g HIV plasmid DNA using lipofectamin 2000 (Life technologies). When indicated, cells were transfected with 1 µg/ml doxycycline (dox; Sigma D-9891). At 2 days after transfection, virus production in culture medium samples was measured using CA-p24 ELISA (33). CD4-enriched human peripheral blood mononuclear cells (PBMC) were isolated from four donors, pooled, cultured and activated with IL-2 and phytohemagglutinin for 3 days, as described previously (35). 10 × 106 cells in 5 ml culture medium were infected with 293T-produced wt HIV-1 virus (corresponding to 40 ng CA-p24). After culturing the cells for 48 h, small intracellular RNA was isolated and analysed by northern blotting.

Plasmid constructs

The HIV-1 molecular clone pLAI (34) and the dox-dependent derivatives HIV-rtTA-Tatwt (35), HIV-rtTA-TARMut-Tatstop (35) and HIV-rtTA-ΔTAR5+3 (ER2-TatY26A variant in (36)) were previously described.

HIV-rtTA-ΔTAR5 was constructed by replacing the 3′ BamH1 – BglII fragment of HIV-rtTA-ΔTAR5+3 with the corresponding fragment of HIV-rtTA-TatY26A. HIV-rtTA-ΔTAR3 was constructed by replacing the 3′ BamHI – BglII fragment of HIV-rtTA-TatY26A with the corresponding fragment of HIV-rtTA-ΔTAR5+3. For mutation of the Sp1 sites in the 3′ LTR, this region was PCR-amplified with primers mutSp1 (GAAA GTCGAC AGAAATTCCG TGGCCTGTTCGAGGAATTCCG TGGCCTGTTCGAGGAATTCCG) and mutSp2 (GAAA GTCGAC AGAAATTCCG TGGCCTGTTCGAGGAATTCCG TGGCCTGTTCGAGGAATTCCG)
TCAGATGCTGCA; mutated nucleotides underlined; Sali site in italics and bold) and C(N1) (antisense primer annealing to the U5 region) (37), and with pBlue3LTERext-ΔU3-rtTA_F86Y/A209T-2Δteto, which includes Env, rtTA and 3′ LTR sequences of the HIV-rtTA genome (38), as template. The PCR product was digested with Sali and HindIII, and used to replace the corresponding 3′ LTR fragment in pBlue3LTERext-ΔU3-rtTA_F86Y/A209T-2Δteto. The BamHI-BglII fragment of the resulting shuttle plasmid pBlue3LTERext-ΔU3-rtTA_F86Y/A209T-2Δteto-mutSp1 was used to replace the corresponding Env-rtTA-3′ LTR sequences in HIV-rtTA-ΔTAR3′, which resulted in plasmid HIV-rtTA-ΔTAR3′-mutSp1.

HIV-Tatstop was constructed by replacing the NcoI-BamHI fragment of HIV-1 pLAI with the NcoI-BamHI site in the resulting shuttle plasmid pBlue3 (Promega), 0–1250 ng HIV-1, HIV-rtTA-Tatwt (35) or pBlueScript plasmid (Stratagene), and cultured for 48 h. HIV-rtTA transfected cells were transfected with 1 μg/ml dox (Sigma; D-9891).AGO2-bound small RNAs were isolated by immunoprecipitation using anti-FLAG M2 affinity gel (Sigma; A2220) as previously described (41). Five microgram RNA was loaded on a denaturing 15% PAGE gel for size fractionation and the 15–55 nt RNA fragments were isolated using a spin column (Ambion). The quality of the RNA was assayed on a Bioanalyzer 2100 (Agilent) using a small RNA chip. The SOLiD Small RNA Library Preparation protocol (Applied biosystems) was used to prepare an RNA library that was subsequently analysed using the SOLiD Wildfire system (Applied biosystems).

Bioinformatics

The SOLiD cospacereads obtained for HIV-1 and HIV-rtTA samples were analysed with LifeScope Genomic Analysis Software version 2.5 (Applied biosystems) using the small RNA pipeline and the highest quality value, allowing no mismatches. First, the libraries were mapped against the human genome filter-sequences (supplied with LifeScope) and mirBase (version 21; http://www.mirbase.org) to identify reads generated from cellular or irrelevant sources. The remaining reads were mapped to the reference sequence of HIV-1 LAI or HIV-rtTA. Reads that were also observed with the pBlueScript-transfected 293T cells were excluded.

Northern blot analysis

293T and HCT-116 (wt or Ex5) cells cultured in 25 cm² flaskswere transfected with 5 μg HIV-1, HIV-rtTA or pLTR-2Δteto-luceff plasmid. When indicated, cells were cotransfected with 50 or 500 ng Tat expressing plasmid (pTAT; (35)) or 1 μg/ml dox was added to the culture medium. Cells were harvested after 48 h and small intracellular RNA was isolated with the mirVana miRNA isolation kit (Ambion). Isolated RNA (2 μg HIV-rtTA or 4 μg LAI RNA sample) was analysed by northern blotting as previously described (20). To check for equal sample loading, the 5S rRNA and tRNA bands were visualized by ethidium bromide staining. The LNA oligonucleotide 5′-AAGCAGGTGGTTCTCCTAGTTAG-3′ (LNA-positions underlined) complementary to the 3′ end of the TAR hairpin was used as probe to detect miR-TAR-3p and TAR-containing RNAs.

Luciferase assay

293T cells were cultured in 0.5 ml medium in 1-cm² wells to 50% confluence and transfected with 50 ng pGL3-miR-TAR-3p WT or pGL3-TAR-3p WT (42), 0.5 ng pRL-CMV (Promega), 0–1250 ng HIV-1, HIV-Tatstop, HIV-TARΔbulge or Tatstop or shRNA-RT5 expression plasmid (42) and 0–1250 ng empty pcDNA3 vector (to complete the DNA to 1300 ng in total) using lipofectamin2000 (Life technologies). After culturing the cells for 48 h, the cells were lysed in 75 μl
Passive Lysis Buffer (Promega) and the firefly and renilla luciferase activities were measured with the Dual-Luciferase Reporter Assay System (Promega). The firefly luciferase activity was normalized to the renilla luciferase activity to correct for experimental variation.

Characterization of Dicer bound RNAs

293T cells (5 × 10^6 cells per 25 cm² flask) were transfected with 4 μg HIV-Tat^{top} plasmid and 1 μg FLAG-tagged Dicer plasmid (Addgene plasmid 19881 [43]) or 1 μg FLAG-tagged hepatitis C virus (HCV) E2 plasmid (44). At 48 h post-transfection, Dicer and E2 bound small RNAs were isolated by immunoprecipitation using anti-FLAG M2 affinity gel (Sigma; A2220) and analysed in a similar way as previously described for AGO2-binding RNAs (45). Briefly, RNAs were polyadenylated using the Poly(A) Polymerase Tailing Kit (Epicentre Biotechnologies) and reverse transcribed into cDNA using an oligo-dT30-adaptor primer. The cDNA products were PCR amplified with the forward primer (TGCGGATCCCCGTCCTCTCTGTTAGACCACA; TAR +1/+21 sequence underlined) or (TGGGATGAGGTAGTAGGTGGTATA; Let-7a-1 +1/+19 sequence underlined) and a reverse adaptor primer, and subsequently purified by agarose gel electrophoresis, ligated into a TA cloning vector (TOPO-TA cloning kit, Life Technologies) and sequenced.

RESULTS

Analysis of small HIV-1 RNAs bound by AGO2/RISC

In this study, we analyse miRNA production by wt HIV-1 and by the doxycycline (dox)-dependent HIV-rtTA variant (Figure 1A). In HIV-1, binding of the viral Tat protein to the TAR RNA hairpin at the 5' end of nascent transcripts activates processive transcription from the LTR promoter and allows production of full-length viral RNAs. In HIV-rtTA, this Tat-TAR transcription activation mechanism is functionally replaced by the dox-inducible Tet-On system (46,47). For this, (i) the Tat-TAR mechanism was inactivated through mutations in the TAR bulge and loop sequences that prevent Tat binding and trans-activation of transcription (TAR^{mut}; Figure 1B), (ii) the gene encoding the dox-dependent transcriptional activator protein rtTA was inserted at the site of the accessory nef gene that is not required for gene expression and virus replication in T cell lines and (iii) tet operator (tetO) sites to which the rtTA-dox complex can bind are inserted in the viral LTR promoter. Only in the presence of dox, rtTA will bind to the tetO-LTR promoter and activate processive transcription, which will result in the production of full-length RNAs. Because HIV-rtTA does not require the Tat-TAR mechanism for the activation of transcription, this virus allows deletion of TAR without blocking viral gene expression (36).

To characterize AGO2-binding HIV-encoded small RNAs, 293T cells were transfected with plasmids expressing FLAG-tagged AGO2 (40) and either the wt HIV-1 or HIV-rtTA proviral DNA genome. HIV-rtTA transfected cells were cultured with dox to activate viral gene expression. As a control, the pBluescript vector was co-transfected instead of an HIV plasmid. 293T cells are efficiently transfected and support a high level of virus production. These cells do not express the CD4 receptor needed for HIV-1 entry, which prevents reinfection and subsequent rounds of virus replication. Two days after transfection, AGO2-bound RNAs were isolated by immune precipitation. Small RNAs (~15–55 nt) were purified and analysed by SOLiD deep sequencing (Figure 1C). To identify the virus-derived reads, the data set was first filtered for sequences from cellular sources (Table 1). Non-viral reads that were also observed for pBluescript-transfected control cells were subsequently removed.

Approximately 3.5% (25 599 of 737 185) and 22.8% (62 772 of 275 420) of the mapped sequence reads corresponded to the HIV-1 and HIV-rtTA genome, respectively (Table 1). Alignment of these reads to the viral reference sequences revealed a prominent peak in the TAR region (Figure 2A and B). Whereas 54% of the HIV-1 reads (13 945 of 25 599 reads) corresponded to the TAR region, a significant larger number of TAR reads, 92%, were observed for HIV-rtTA (57 721 of 62 772 reads) (Table 1). Some other small peaks were observed in both alignments, but these peaks were much smaller than the peak in the TAR region. These reads did not correspond to a structured RNA region nor to any of the previously suggested HIV-1 miRNAs (17,48) and were not further investigated.

Nearly all TAR reads corresponded to the 3' half of the TAR region (Figure 2C). Analysis of the size distribution of these TAR reads demonstrated that their size varied slightly, with a major peak at 21 nt, which is in agreement with the general miRNA size (Figure 2D) (1,49). These results indicate that the 3' side of TAR is processed into a miRNA-like molecule that is loaded into AGO2/RISC. We further refer to this small RNA as miR-TAR-3p. No reads were observed corresponding to the 5' side of the TAR stem in the HIV-1 and HIV-rtTA samples.

Alignment of all TAR reads demonstrates that not a unique miR-TAR-3p species is produced, but rather a small RNA population with slightly variable 5' and 3' ends (Figure 2E). A similar heterogeneity in length is frequently ob-

<table>
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Total 737 185 275 420

Table 1. SOLiD deep sequencing analysis of AGO2-bound RNAs in HIV-1 and HIV-rtTA expressing cells

Analysis of 18 × 10^6 and 50 × 10^6 reads obtained for the HIV-1 and HIV-rtTA samples, respectively, with LifeScope Genomic Analysis Software using the small RNA pipeline and the highest quality value, allowing no mismatches, resulted in the mapping of 737 185 and 275 420 unambiguous reads. The number of reads with a viral or cellular origin is shown.
Figure 2. Deep sequencing analysis of small AGO2-bound HIV RNAs. (A–B) AGO2-bound RNAs isolated from 293T cells expressing HIV-1 (A) or HIV-rtTA (B) and FLAG-tagged AGO2 were analysed by SOLiD deep sequencing. The virus-encoded reads were aligned with the HIV-1 (A) and HIV-rtTA (B) RNA genome. The number of reads per nucleotide is shown. The reads corresponding to the repeat (R) region (including TAR) are shown at their 5' position. (C) Alignment of the HIV-1 and HIV-rtTA derived RNAs to the TAR sequence. The wt sequence is shown with the bulge and loop nucleotides mutated in TARmut boxed in grey. (D) The size distribution of the TAR-derived reads is shown for the HIV-1 (TARwt) and HIV-rtTA (TARmut) samples. The total number of TAR reads (13 945 TARwt reads; 57 721 TARmut reads) was set at 100% for each sample and the percentage of reads is shown for the 15–27 nt size window. (E) The TARwt and TARmut structure is shown with the position of the prevailing 5' (open arrowhead) and 3' (closed arrowhead) ends of the TAR-derived reads indicated. The percentage of TAR-derived reads that start or end at the indicated position is shown (cut off set at 5%). The vertical lines indicate the five most frequent miR-TAR-3p RNAs with their frequency.
Figure 3. TAR and miR-TAR-3p RNAs are produced by non-processive transcription. (A–B) 293T cells were transfected with the HIV-1 and HIV-rtTA constructs and cultured for 48 h without dox (HIV-1 and HIV-rtTA) or with dox (HIV-rtTA). (A) Intracellular small RNAs were isolated and analysed by northern blotting. An LNA probe complementary to the miR-TAR-3p sequence was used to identify the position of miR-TAR-3p and TAR RNAs. The position of the size markers is shown on the left. Ethidium bromide staining of small 5S rRNA (5S) and tRNAs is shown as loading control below the blot. (B) The CA-p24 level in the culture supernatant was measured to quantify gene expression. The mean ± SD for three experiments is shown. (C) Expected TAR-containing transcripts produced by the HIV-rtTA variants with the TARmut element in both the 5′ and 3′ LTR, only the 5′ or 3′ LTR, or no TAR element (Δ, TAR deletion). In the 3′TAR+3′mutSp1 variant, the TAR element in the 5′ LTR was deleted and the Sp1 binding sequences in the 3′ LTR region were mutated. This set of variants is based on the HIV-rtTA-TatY26A variant (47) and all constructs carry the TatY26A mutation (in addition to the TARmut mutations) to disable the Tat-TAR transcription activation mechanism. (D) 293T cells were transfected with the HIV-rtTA constructs shown in panel C and cultured for 48 h with dox. Small RNAs were isolated and analysed by northern blotting using the miR-TAR-3p probe, as described for panel A. (E) CA-p24 production was analysed as described for panel B. The mean ± SD for three experiments is shown. (F) CD4-enriched human PBMC were infected with 293T-produced wt HIV-1 virus and cultured for 48 h. Small RNAs were isolated and northern blot analysis was performed as described for Figure 3A. Mock, control uninfected PBMC. The RNA size marker is shown at the right (a short phosphorimager exposure is shown for this lane because of the high radio activity of the 32P-labeled marker fragments).
in agreement with the dox-control of viral processive transcription and full-length RNA production (Figure 3B). Production of the short TAR and miR-TAR-3p RNAs did however not depend on dox administration (Figure 3A; compare lanes 3 and 4), which confirms that non-processive transcription is sufficient for the production of the TAR RNAs and miR-TAR-3p.

TAR is present at both the 5′ and 3′ end of the full-length HIV-1 and HIV-rtTA transcripts (Figure 1A). To exclude the possibility that the TAR and miR-TAR-3p RNAs are produced from the TAR region at the 3′ end of full length RNAs, we analysed the small RNAs produced by HIV-rtTA variants with only the 5′ TAR, only the 3′ TAR or no TAR element (Figure 3C). As expected, deletion of both TAR elements abolished TAR and miR-TAR-3p RNA production (Figure 3D; lane 5). Deletion of the 3′ TAR did not affect TAR and miR-TAR-3p production (lane 4), which is in agreement with their 5′ TAR origin. Surprisingly, deletion of the 5′ TAR element did also not significantly reduce TAR and miR-TAR-3p RNA production (lane 2). Because transcriptional promoter activity of the 3′ LTR region may also result in the production of short TAR transcripts, we constructed an HIV-rtTA-3′TAR+3′mutSp1 variant in which the binding sites for the Sp1 transcription factor in the 3′ LTR were mutated (Figure 3C). These Sp1 mutations completely inactivate 3′ LTR promoter activity (Supplementary Figure S1). As a result, TAR sequences can only be transcribed as 3′ portion of extended HIV-rtTA-3′TAR+3′mutSp1 transcripts that initiate at the 5′ LTR and not as short TAR transcripts that initiate at the 3′ LTR. In contrast to the 3′ TAR construct, this 3′TAR+3′mutSp1 variant did not produce TAR and miR-TAR-3p RNAs (Figure 3D; compare lanes 2 and 3). These results demonstrate that not only the 5′ LTR but also the 3′ LTR in the HIV-rtTA plasmid is transcriptionally active and both LTR regions drive the production of short TAR transcripts. Surprisingly, deletion of either the 5′ or 3′ TAR element did not halve the observed TAR RNA level, but this was not further investigated. In agreement with previous observations (36), deletion of the 5′ TAR element did not reduce viral CA-p24 production (Figure 3E) and deletion of the 3′ TAR element or mutation of the 3′ Sp1 sites reduced the CA-p24 level only ~2-fold, which demonstrates that these mutations do not block full-length RNA production. Taken together, these results indicate that the TAR and miR-TAR-3p RNAs cannot be produced from the 3′ TAR region present in the full-length transcripts that are produced upon processive transcription initiating at the 5′ LTR promoter, but are exclusively derived from short transcripts resulting from non-processive transcription.

Northern blot analysis of the small RNAs isolated from CD4-enriched peripheral blood mononuclear cells (PBMC) infected with wt HIV-1 confirmed the production of the TAR precursor and miR-TAR-3p during virus replication in primary human CD4+ T cells (Figure 3F).

Tat is required for miR-TAR-3p production

The experiments with dox-controlled HIV-rtTA variants demonstrated that non-processive transcription from the LTR promoter results in production of short TAR RNAs and miR-TAR-3p. The bulge and loop sequence of the TAR element in these viruses had been mutated (TARmut) to prevent Tat binding. Because these mutations may influence TAR and miR-TAR-3p production, we next tested whether non-processive transcription from the wt HIV-1 LTR promoter, with a wt TAR sequence (TARwt), is also sufficient for production of both RNA species. For this, we analysed TAR and miR-TAR-3p production of a Tat-negative HIV-1 variant (HIV-Tatstop) that was constructed by introducing two translation stop codons in the Tat open reading frame (35). As expected, HIV-Tatstop gene expression is low and we measured a reduced level of CA-p24 production upon transfection of the construct in 293T cells (Figure 4A). The level of transcription and CA-p24 production could be restored by addition of Tat in trans through co-transfection of a Tat-expressing plasmid (pTat). Northern blot analysis of the intracellular small RNAs revealed an increase in TAR RNA upon Tat inactivation, but no miR-TAR-3p was detectable (Figure 4B; lane 2). Trans-complementation with Tat reduced TAR RNA and increased miR-TAR-3p production to the wt levels. These results demonstrate that the TAR RNAs are produced by Tat-independent, non-processive transcription from the HIV-1 LTR promoter. Furthermore, these data indicate that Tat does not only activate processive transcription, which results in elongated viral RNAs and viral protein production, but also stimulates processing of the TARwt RNAs into miR-TAR-3p. However, these experiments do not exclude that these processes are coupled and that elongated transcripts are required for miR-TAR-3p production.

To demonstrate that the elongated TAR-containing transcripts produced upon processive transcription are not the precursor for the miR-TAR-3p, we next analysed TAR and miR-TAR-3p production from an HIV-rtTA variant with wt TAR sequences and inactivated Tat gene (HIV-rtTA-Tatstop-TARwt). Processive transcription of this TARwt-Tatstop virus can be activated by the administration of dox (via dox-rtTA/tetO axis) or Tat (via Tat-TAR axis). Without dox and Tat, transcription is non-processive and we did indeed measure a low level of CA-p24 production in transfected cells (Figure 4C). Addition of dox or Tat activated processive transcription and increased CA-p24 production, and highest CA-p24 production was measured when both dox and Tat were present (Figure 4C). Analysis of the small RNAs revealed that, without dox and Tat, this virus produced a high level of TAR RNA but no miR-TAR-3p RNA (Figure 4D; lane 1). Dox administration did not increase the miR-TAR-3p level (Figure 4D; lane 3), which demonstrates that the elongated transcripts produced upon processive transcription are not sufficient for miR-TAR-3p production. In contrast, Tat administration reduced the TAR RNA level and increased the miR-TAR-3p level (Figure 4D; lane 2). These results confirm that non-processive transcription is sufficient for the production of TAR RNA and that Tat activates processing of TAR RNA into miR-TAR-3p.

Changes in the TAR RNA structure activate processing into miR-TAR-3p

Previous studies demonstrated that the 3-nt bulge causes a bend in the α-helical structure of the TARwt RNA hair-
Figure 4. Tat activates miR-TAR-3p production. (A) 293T cells were transfected with the HIV-1 plasmid or with the Tat-deficient HIV-Tatstop construct and a varying amount of Tat plasmid (0, 50 or 500 ng). The CA-p24 level in the culture supernatant was measured after 48 h. The mean ± SD for three experiments is shown. (B) Small RNAs were isolated and analysed by northern blotting using the miR-TAR-3p probe, as described for Figure 3A. (C) 293T cells were transfected with the Tat-deficient HIV-rtTA-Tatstop construct with a wild-type TAR element (TARwt). When indicated, cells were co-transfected with 500 ng pTat and cultured in the presence of dox. The CA-p24 level in the culture supernatant was measured after 48 h. The mean ± SD for three experiments is shown. (D) Small RNAs isolated from the transfected cells were analysed by northern blotting using the miR-TAR-3p probe, as described for Figure 3A.

Figure 5. Bulge deletion activates TAR RNA processing. (A) In the HIV-1 TARΔbulge mutant, the 3-nt TAR bulge (nt +22 to +24) was deleted. (B) 293T cells were transfected with wt HIV-1 and HIV-Tatstop variants with either a wt or bulge-deleted TAR element (TARwt and TARΔbulge, respectively). Small RNAs were isolated after 48 h and analysed by northern blotting using the miR-TAR-3p probe as described for Figure 3A. (C) A luciferase reporter construct containing the miRTAR-3p target sequence in the 3′ UTR was transfected into 293T cells together with 0–1250 ng HIV-1, HIV-Tatstop, HIV-Tatstop-TARΔbulge or a plasmid expressing a non-related shRNA-RT5 (42). Luciferase production was measured 48 h after transfection. The mean values ± SD for four experiments is shown. Statistical analysis using independent samples T-test analysis was used to compare the luciferase activities obtained without and with co-transfection of the HIV-1 or shRNA-RT5 constructs (*, P < 0.01).
in the bulge region of TAR$^{\text{mut}}$ affected the RNA structure and allowed partial cleavage.

The miR-TAR-3p can silence target RNAs

MiRNAs can target mRNAs with complementary sequences for cleavage or translational repression. To test the capacity of the miR-TAR-3p to knock-down gene expression, we constructed a luciferase reporter with the sequence complementary to miR-TAR-3p in the 3′ untranslated RNA region. Upon transfection of this reporter into 293T cells, a high luciferase level was measured after 48 hr (Figure 5C). This luciferase level gradually decreased when the cells were co-transfected with an increasing amount of the miR-TAR-3p expressing wt HIV-1 and HIV-Tat$^{\text{stop}}$, TAR$^{\text{Δbulge}}$ constructs. The latter was a more effective inhibitor, which is in agreement with increased miR-TAR-3p production (as demonstrated in Figure 5B). Co-transfection with the HIV-Tat$^{\text{stop}}$ construct that does not produce miR-TAR-3p did not reduce the luciferase level. Similarly, luciferase expression was not influenced by co-transfection of a control plasmid expressing a non-related short hairpin RNA (shRNA-RT5; (42)) with no sequence homology to the miR-TAR-3p target sequence. As an additional control experiment, a luciferase reporter construct with the shRNA-RT5 target sequence in the 3′ untranslated RNA region was transfected into 293T cells together with the different HIV-1 variants and the shRNA-RT5 expressing plasmid (Supplementary Figure S3). Expression of this luciferase reporter was significantly reduced by the shRNA-RT5 plasmid but not by the miR-TAR-3p producing HIV-1 and HIV-Tat$^{\text{stop}}$-TAR$^{\text{Δbulge}}$ constructs, which demonstrates the sequence specificity of miR-TAR-3p. Taken together, these results show that miR-TAR-3p is able to knock-down the expression of target RNAs.

TAR RNA is processed by Dicer

The role of Dicer in miR-TAR-3p production was analysed in HCT-116 cells expressing either wt (Dicer$^{\text{wt}}$) or exon 5-disrupted Dicer (Dicer$^{\text{ex5}}$). This ex5 mutation in the helicase domain reduces Dicer activity (32). Unfortunately, transfection with the TAR$^{\text{wt}}$ encoding HIV-1 plasmid or HIV-1-derived LTR construct did not yield detectable levels of miR-TAR-3p, which may be due to relatively inefficient transcription of these cells (data not shown). We therefore transfected the cells with a TAR$^{\text{mut}}$ expressing LTR construct derived from the HIV-rTA variant that produces significantly more TAR RNA and miR-TAR-3p (Figure 3A). Both the TAR RNA precursor and miR-TAR-3p were detected in Dicer$^{\text{wt}}$ cells (Figure 6A). Importantly, an increased level of TAR RNA and a concomitant reduction of the miR-TAR-3p signal was observed in Dicer$^{\text{ex5}}$ cells. Processing of TAR$^{\text{mut}}$ into miR-TAR-3p was not influenced by Tat, which is in agreement with previous observations (as described above; Supplementary Figure S2B). Analysis of the Dicer-mediated processing of shRNAs in parallel experiments confirmed the high and low Dicer activity in these Dicer$^{\text{wt}}$ and Dicer$^{\text{ex5}}$ cells, respectively (57). These results indicate that Dicer is involved in the processing of the TAR RNAs.

The 3′ end of TAR RNA corresponds with the 3′ end of miR-TAR-3p

To confirm that TAR RNA—like canonical cellular premiRNAs—can bind to Dicer, we transfected 293T cells with the HIV-Tat$^{\text{stop}}$ construct that produces a high level of TAR$^{\text{wt}}$ RNA (Figure 4B) together with a plasmid expressing FLAG-tagged Dicer (43) or the non-related control FLAG-tagged HC V E2 protein. At 48 h after transfection, the Dicer and E2 bound RNAs were isolated by immuno-precipitation. The bound and unbound small RNA fractions were analysed for the presence of the TAR RNA and the cellular Let-7A pre-miRNA by reverse-transcription PCR (Figure 6B), followed by gel electrophoresis (Figure 6C). The TAR RNA and Let-7A pre-miRNA were highly enriched in the Dicer bound fraction, but not in the E2 bound fraction, which indicates that Dicer can bind TAR.

The role of Dicer in miR-TAR-3p production was analysed in HCT-116 cells expressing either wt (Dicer$^{\text{wt}}$) or exon 5-disrupted Dicer (Dicer$^{\text{ex5}}$). This ex5 mutation in the helicase domain reduces Dicer activity (32). Unfortunately, transfection with the TAR$^{\text{wt}}$ encoding HIV-1 plasmid or HIV-1-derived LTR construct did not yield detectable levels of miR-TAR-3p, which may be due to relatively inefficient transcription of these cells (data not shown). We therefore transfected the cells with a TAR$^{\text{mut}}$ expressing LTR construct derived from the HIV-rTA variant that produces significantly more TAR RNA and miR-TAR-3p (Figure 3A). Both the TAR RNA precursor and miR-TAR-3p were detected in Dicer$^{\text{wt}}$ cells (Figure 6A). Importantly, an increased level of TAR RNA and a concomitant reduction of the miR-TAR-3p signal was observed in Dicer$^{\text{ex5}}$ cells. Processing of TAR$^{\text{mut}}$ into miR-TAR-3p was not influenced by Tat, which is in agreement with previous observations (as described above; Supplementary Figure S2B). Analysis of the Dicer-mediated processing of shRNAs in parallel experiments confirmed the high and low Dicer activity in these Dicer$^{\text{wt}}$ and Dicer$^{\text{ex5}}$ cells, respectively (57). These results indicate that Dicer is involved in the processing of the TAR RNAs.

The debate about whether HIV-1 encodes miRNAs is ongoing for 10 years. The fact that miRNA production via the canonical miRNA pathway, involving subsequent microprocessor and Dicer cleavage of the primary transcript, would result in degradation of the viral RNA genome and impede HIV-1 replication was long considered a strong argument against HIV-1 encoded miRNAs. In this study we demonstrate how HIV-1 can produce a miRNA-like small RNA molecule without hampering virus replication. First, we show that HIV-1 produces a small RNA from the 3′ side of the TAR RNA hairpin. Like canonical miRNAs, this miR-TAR-3p is ~21-nt in size, binds to AGO2 and is able to repress the expression of mRNAs bearing the complementary target sequence. Second, we demonstrate that the short TAR RNAs produced upon non-processive transcription from the HIV-1 LTR promoter function as precursor for miR-TAR-3p and are processed by Dicer. miR-TAR-3p is thus not generated via the canonical miRNA pathway in which cleavage of the primary transcript by microprocessor is needed to release the pre-miRNA, but via a non-canonical pathway in which the RNA precursor is produced by pausing and premature termination of RNAP II transcription. By selectively using these short TAR RNAs as precursor, HIV-1 can produce miR-TAR-3p without inducing cleavage of its RNA genome. Third, we demonstrate that the viral Tat protein activates TAR processing into miR-TAR-3p.

The 21-nt miR-TAR-3p that we identified overlapped with earlier suggested 3′ TAR-derived small RNAs (17–21,26). Schopman et al. (20) observed the same miR-TAR-3p read upon deep-sequence analysis of HIV-rTA expressing cells, but other studies identified small RNA molecules that differ in exact size and position (Figure 7A). We did not detect any
Figure 6. Dicer processes TAR RNA into miR-TAR-3p. (A) HCT-116 cells expressing wt or exon5-disrupted Dicer (ex5) were transfected with the plasmid pLTR-2ΔtetO-lucff (36) in which expression of TARmut-containing RNAs is driven by the HIV-rtTA U3 promoter. When indicated, cells were co-transfected with 500 ng pTat. The RNA was analysed after 48 h by northern blotting using the miR-TAR-3p probe. (B) Experimental design for the isolation and characterization of Dicer-bound TAR RNAs from 293T cells transfected with the plasmid expressing FLAG-tagged Dicer and the TARwt-expressing HIV-Tatstop construct. (C) 293T cells were transfected with the HIV-Tatstop construct together with a plasmid expressing FLAG-tagged Dicer (left panels) or FLAG-tagged HCV E2 (right panels). Dicer and E2 bound RNAs were isolated by immunoprecipitation. The TAR RNA (upper panels) and Let-7A pre-miRNA (lower panels) in the bound and unbound small RNA fractions were detected by RT-PCR and gel electrophoresis. The position of the TAR RNA and Let-7A pre-miRNA RT-PCR product is indicated. *, RT-PCR product with only primer and adaptor sequences, lacking any insert sequence. (D) The RT-PCR product corresponding to the TAR RNA in the Dicer bound fraction was TA-cloned and 10 DNA fragments were sequenced to determine the 3′ end of the TAR RNA. The position (indicated with arrowhead) and frequency of the identified 3′ ends are shown.

other previously suggested HIV-1 miRNA (17,48). The differences between our and previous results are likely due to technical differences. The earlier studies used in silico analysis (17) or traditional cloning and sequencing (18), RNase protection (19) or deep sequencing (20,21,26) of total intracellular RNA pools. We combined AGO2-pull down with deep sequencing to specifically analyse small RNA molecules that are loaded into the AGO2/RISC complex. This method resulted in a large number of TAR-derived viral reads for HIV-1 and HIV-rtTA expressing 293T cells (13 945 and 57 721 reads, respectively) that was comparable to the number obtained for cellular miRNAs (33 678 and 18 866, respectively; Table 1). The size of the AGO2-captured viral RNA reads peaked at 21 nt (Figure 2D and data not shown), which is in accordance with the expected size for miRNAs. In the previous sequencing studies that analysed the total small RNA pool, the size of the HIV-1 reads peaked at 18 nt or less (20,26). Possibly, these numerous small RNA reads interfered with the detection of miR-TAR-3p. We observed some heterogeneity in the 5′ and 3′ end of the miR-TAR-3p reads in the sequencing analysis. Such heterogeneity is more frequently observed for cellular and viral miRNAs in deep sequencing analyses (58–60). In the northern blot analysis, a discrete size of 21-nt was observed for miR-TAR-3p.

Wisnant et al. detected TAR-derived RNAs when deep sequencing total intracellular small RNA pools in different HIV-1 infected cells (Figure 7A), but did not identify miR-TAR-3p when analyzing the protein–RNA complexes pulled-down from HIV-1 infected TZM-bl cells by immunoprecipitation using an antibody recognizing AGO1, AGO2 and AGO3 (26). Remarkably, when using a crosslinking-immunoprecipitation technique (PAR-CLIP) to analyse the AGO-bound RNAs in HIV-1 infected C8166 and TZM-bl cells, these authors detected a cluster of small RNAs in the TAR region (∼1000 and ∼2600 reads in C8166 and TZM-bl cells, respectively; Figure 5 in (26)), but these RNAs were not discussed. Importantly, we detected miR-TAR-3p not only in our AGO2-pull down/deep sequencing analysis, but also demonstrate that this small RNA can knock down expression of target-containing mRNAs (Figure 5C), which confirms its association with the AGO2/RISC complex. Moreover, we confirmed the production of this miR-TAR-3p and its TAR precursor in HIV-1 expressing 293T cells (Figures 3A, 4B, 5B) and HIV-1 infected primary T cells (Figure 3F) by northern blot analysis of the intracellular small RNA pool.

We demonstrate that Dicer can bind TAR RNA and that a decrease of the intracellular Dicer activity reduces TAR processing into miR-TAR-3p. These results indicate that Dicer cleaves the TAR precursor. The viral Tat protein was found to stimulate processing of TAR RNA into miR-TAR-3p. Thus, Tat not only activates processive transcription from the LTR promoter but also triggers processing of the TAR RNAs that are formed during non-processive transcription. The observed simultaneous production of full-length HIV-1 RNAs (reflected by the CA-p24 level; Figure 4A) and miR-TAR-3p (Figure 4B) upon Tat induction indicates that activation of processive transcription does not switch off non-processive transcription.
Figure 7. Production of the TAR-encoded miRNA-like small RNA. (A) Alignment of the TAR-derived small RNAs identified in different studies. All studies (except the in silico analysis) characterized the TAR RNAs in HIV-1 expressing cells, but different techniques were used: a–b, SOLiD deep sequencing of AGO2-bound HIV-1 (a) and HIV-rtTA RNAs (b; this study); c, Illumina deep sequencing of HIV-1 RNAs (26); d–f, SOLiD deep sequencing of small HIV-1 (d: ref21; e: 20) and HIV-rtTA RNAs (f: 20); g, RNAse protection analysis (RPA) of HIV-1 RNAs (19); h, cloning and sequencing of small HIV-1 RNAs (18, 22); i, in silico analysis (17). In the upper line the HIV-1 LTR sequence is shown with the TAR region (+1 to +57) boxed in grey and the transcription start site indicated (+1). The dominant 5p and 3p sequence found in each study is shown with the number of reads or clones indicated at the right. (B) Model for HIV-1 TAR RNA and miR-TAR-3p production based on our and previous findings discussed in the text. In the absence of Tat, non-processive RNAP II complexes are formed at the LTR promoter and transcription is initiated but frequently pauses when TAR is formed. Subsequent premature termination of transcription results in the production of short 5′ capped TAR RNAs. These TAR RNAs are not further processed. Possibly, the bended structure of the TAR RNAs prevents Dicer cleavage. In the presence of Tat, binding of Tat to TAR causes straightening of the hairpin structure, which may allow Dicer cleavage. As discussed in the text, alternative scenarios are possible for this Tat-activation of TAR processing. Cleavage of TAR by Dicer results in the production of miR-TAR-5p and miR-TAR-3p. MiR-TAR-3p is loaded onto the AGO2/RISC complex, whereas loading of miR-TAR-5p is blocked by the 5′ cap. Binding of Tat to TAR also recruits the pTEFb complex to the LTR promoter, which results in phosphorylation of the RNAP II complex and activation of processive transcription. Because the TAR stem in the extended transcripts is too short for efficient microprocessor cleavage, full-length RNAs can be produced and virus replication will be allowed.

Interestingly, we observed that whereas wt TAR RNA was processed exclusively in the presence of Tat, the mutated and bulge-deleted TAR RNAs were processed in the absence of Tat. Possibly, TAR processing is controlled by the hairpin structure and Tat-binding results in a cleavage-prone conformation. In this scenario, the bend in the α-helical stem structure caused by the wt 3-nt bulge (52-54) may hinder Dicer cleavage. Binding of Tat to the TAR bulge is known to straighten the hairpin (54–56), which may result in a cleavage-prone RNA conformation. Mutation or deletion of the bulge nucleotides may similarly affect TAR RNA bending and processing. However, alternative scenarios are possible for how Tat affects TAR processing. For example, the 3-nt TAR bulge may bind a protein factor that
prevents Dicer cleavage. Tat binding may displace this inhibitory factor and activate Dicer processing. In this scenario, bulge deletion or mutation may prevent binding of the inhibitory factor and activate processing. Alternatively, Tat may have an indirect effect by influencing the production of a — yet unidentified — cellular factor that stimulates processing of wt TAR. This scenario seems less likely, as it does not explain how bulge deletion or mutation activates TAR processing in the absence of Tat, unless these TAR modifications result in a cleavage-prone RNA conformation that does not require the cellular factor for processing.

It has been shown that microprocessor binds nascent TAR-containing transcripts at the HIV-1 transcription start site and influences RNAP II transcription elongation (21). Microprocessor may similarly bind to cellular nascent RNA transcripts and regulate transcription of cellular genes (21, 61, 62). Gromak et al. demonstrated that this microprocessor function does not depend on the RNA cleavage activity of Drosha (61). It seems likely that microprocessor cleavage is also not involved in TAR RNA and miR-TAR-3p production. First, TAR RNA may not be a good substrate for microprocessor cleavage because the TAR stem consists of only 23 bp, whereas microprocessor requires extended duplexes of ~33 bp for efficient binding and cleavage (4, 26). Furthermore, microprocessor normally cleaves a pri-miRNA stem at ~11 bp from its base and the 3′ end of miR-TAR-3p does not correspond with this position in the TAR hairpin. We demonstrate that TAR RNAs that are produced by non-processive transcription function as miR-TAR-3p precursor. The 3′ end of miR-TAR-3p does correspond with the strong RNAP II transcription pause site that was previously identified in in vitro HIV-1 transcription assays (28) and with the 3′ end of short HIV-1 transcripts produced during non-processive transcription in HIV-Tattop transfected 293T cells (Figure 6D) and latently infected U1 cells, which harbour two HIV-1 copies with defective Tat genes (51). Taken together, these results indicate that the 3′ end of TAR and miR-TAR-3p is created by pausing and subsequent premature termination of transcription.

Cellular promoter-proximal transcripts resulting from RNAP II pausing and subsequent premature transcription termination that fold a hairpin RNA structure can function as miRNA precursor (62, 63). Xie et al. (63) demonstrated that after Dicer cleavage of such capped pre-miRNAs, the capped 5p strand is excluded from loading onto AGO2 and only the 3p strand is efficiently loaded. The 5′ end of HIV-1 transcripts is also capped (64, 65), which may explain why we did not detect small RNAs corresponding to the 5′ side of the TAR stem in the AGO2-bound RNA fraction. Production and processing of the TAR RNA may thus be very similar to that of these cellular pre-miRNAs. The model summarizing how HIV-1 produces miR-TAR-3p is presented in Figure 7B.

The retroviruses BLV (13, 14), BFV (15), SFV (16) and ALV-J (11) were previously shown to produce small RNAs that may function as miRNA. In these studies, deep sequencing analysis of the total intracellular small RNA pool of virus-infected cells demonstrated high abundance of the miRNAs. As discussed above, several studies in which the total small RNA pool of HIV-1 infected cells was similarly analyzed failed to detect miR-TAR-3p. However, we observed miR-TAR-3p at a similar level as cellular miRNAs when sequencing AGO2-captured small RNAs. Assuming that the TAR-derived small RNAs are not preferentially detected, our results thus suggest that miR-TAR-3p is produced at a similar level as cellular miRNAs.

We demonstrate that HIV-1 does not use the canonical miRNA pathway for miR-TAR-3p production, but selectively uses the short TAR RNA transcripts resulting from non-processive RNAP II transcription as the precursor. ALV-J also uses an RNAP II transcript as precursor for the miRNA, but this miRNA is processed via the canonical miRNA pathway, involving successive cleavage of the pri-miRNA transcript by Drosha and Dicer (11). Like HIV-1, BLV, BFV and SFV use a non-canonical pathway for the production of small RNAs (13–16). These viruses produce short sub-genomic RNAP III transcripts that function as miRNA precursor, whereas the extended RNAP II transcripts that also contain the miRNA sequence are not processed (as demonstrated for BLV (13) and SFV (16) and predicted for BFV (15)). The production of designated transcripts that function as precursor thus seems a general retroviral strategy to produce these virus-encoded small RNAs without interfering with genomic and mRNA production.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS
We thank Ted Bradley for his help in the SOLiD deep sequencing and Stephan Heynen for performing CA-p24 ELISA.

FUNDING
Netherlands Organisation for Scientific Research (Chemical Sciences Division; NWO-CW; Top grant). Funding for open access charge: Netherlands Organisation for Scientific Research (NWO; Incentive Fund Open Access).

Conflict of interest statement. None declared.

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