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Pyrosequencing of small non-coding RNAs in HIV-1 infected cells: evidence for the processing of a viral-cellular double-stranded RNA hybrid

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ABSTRACT

Small non-coding RNAs of 18–25 nt in length can regulate gene expression through the RNA interference (RNAi) pathway. To characterize small RNAs in HIV-1-infected cells, we performed linker-ligated cloning followed by high-throughput pyrosequencing. Here, we report the composition of small RNAs in HIV-1 productively infected MT4 T-cells. We identified several HIV-1 small RNA clones and a highly abundant small 18-nt RNA that is antisense to the HIV-1 primer-binding site (PBS). This 18-nt RNA apparently originated from the dsRNA hybrid formed by the HIV-1 PBS and the 3′ end of the human cellular tRNAlys3. It was found to associate with the Ago2 protein, suggesting its possible function in the cellular RNAi machinery for targeting HIV-1.

INTRODUCTION

Small non-coding RNAs of 18–25 nt in length are important in the RNA interference (RNAi) mechanism for controlling gene expression (1,2). An intermediate step in the RNAi pathway is the processing of precursor double-stranded (ds) RNAs into siRNA or miRNA by the RNase III Dicer (3). Dicer recognizes and cleaves dsRNA substrates into products of ~18–25 nt in length. Dicer substrates can be long linear dsRNAs or hairpin RNAs that have either perfectly complementary or imperfectly complementary stems. The Dicer-cleaved siRNAs can enter an Ago2-containing RNA-induced silencing complex (RISC). This si–RISC complex can target and cleave a mRNA that is recognized by base complementarity to the guide siRNA. Alternatively, Dicer-produced miRNAs can associate with RISC to form a mi–RISC complex which can act to silence the translation of mRNA targets [review (4) for further detail].

Dicer processes cellular miRNAs (5) and siRNAs (6). However, several mammalian viruses, including HIV-1 (7–10), encode viral miRNAs which are also processed by Dicer (11). To characterize small RNAs in HIV-1 infected cells (12,13), we performed small RNA cloning followed by high throughput nucleotide pyrosequencing. This approach identified many clones with discrete HIV-1 sequences and also a highly abundant clone containing a cellular 18-nt non-coding RNA (PBSncRNA) sequence which is antisense to the HIV-1 primer-binding sequence (PBS). The latter finding of a PBSncRNA in HIV-1 infected cells is in agreement with two recent reports on the identification of similar PBS-complementary short ncRNAs to endogenous retroviruses (14,15). HIV-1 PBSncRNA was found to be associated with an Ago2 protein intracellularly, suggesting that it is potentially active in the cell's RNAi pathway against HIV-1.

MATERIALS AND METHODS

Cell culture

HIV-1 latently infected human monocyte cell line, U1 and human T-cell line, MT4, were cultured in RPMI 1640 medium with 10% fetal calf serum (FCS) and 2 mM L-glutamine. In U1 cells, HIV-1 virus production was induced by treatment with 1 μM phorbol myristate acetate (PMA). HeLa and 293T cells were propagated at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and 2 mM L-glutamine. To generate an AGO2-over expressing cell line, 293T cells were transfected with either pFLAG-AGO2 or an empty vector together with pRS (Origene). Stable transformants ('293T-AGO2' for a line stably expressing pFLAG-AGO2 and '293T-control' for a control cell line) were selected with 1 μg/ml puromycin.

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Plasmids and siRNAs

DNA oligonucleotides corresponding to the PBS sequence (5′-CTAGTTGGCGCCCGAACAGGGACA-3′; 5′-AGC TTGTCCCTGTTCGGGCGCCAA-3′) were hybridized and cloned into the *Spe* I and *Hind* III sites of pMIR-REPORT-Luc (Ambion). Control plasmid pMIR-REPORT-β-gal (Ambion) encoding β-galactosidase was used for luciferase assay normalization. PBS siRNA (5′-GUCCCUGUUCGGGCGCCAdTdT-3′; 5′-UGGCG CCCGAACAGGGACdTdT-3′), PBS mut siRNA (5′-A CCCCUGGUCGGGCGCAAdTdT-3′; 5′-UUGCGCCC GACCAGGGGUdTdT-3′), to knock down Dicer protein si-Dicer (5′-UGCUUGAAGCAGCUCUGG AdTdT-3′ and 5′-UCCAGAGCUGCUUCAAGC AdTdT-3′) (16) and si-control (5′-CUUUAAGCUCCCU GAGCGUUU-3′ with 5′-ACGCUCAGGGAGCUUAA AGUG-3′) RNAs were synthesized by Invitrogen. The expression plasmid for FLAG-AGO2 was prepared by PCR using pIRESneo-FLAG/HA-AGO2 (17) as a template.

Reporter assays

HeLa and 293T cells were co-transfected using Lipofectamine 2000 (Invitrogen) with PBSncRNA or PBSncRNA mutant and a pMIR-REPORT-Luc reporter plasmid with (LucPBS) the addition of a single PBSncRNA-complementary target site. The pMIR-REPORT-β-gal plasmid was also added to the transfection as a normalization control. Forty-eight hours after transfection, cells were washed twice with 1× phosphate-buffered saline and then lysed in 1× luciferase lysis buffer (Promega). Luciferase assay substrate (Promega) was used according to the manufacturer's protocol, and activity was measured in an Opticom II luminometer (MGM Instruments). Normalization of luciferase activity was based on β-galactosidase activity measured with Galacto-Star as described by the manufacturer (Tropix, Bedford, MA, USA). All luciferase values represent averages ± SD from at least three independent transfections.

Reverse transcriptase assay and viral infection

Media collected from pNL4-3 transfected HeLa cells, or 293T cells, or from PMA-treated U1 cells were filtered using 0.45-μm membrane. Virus was quantified by Reverse transcriptase (RT) assay (18). In total, 10^6–10^7 cpm RT units of virus prepared from transfected HeLa cells were used to infect 5×10^6 MT4 cells. After 2 h of exposure to virus, cells were washed twice with phosphate buffered saline and resuspended into RPMI and cultured for 48 h. Supernatant RT was quantified, and the cells were harvested for small RNA isolation using the mirVana miRNA isolation kit (Ambion).

In vitro transcription

A T7 promoter driven 5′-UTR region (from 584 to 710 nts of pNL4-3) was PCR amplified by Taq polymerase (Clontech) using DNA primers (T7 sense primer 5′-TAA TACGACTCACTATAGGGAGAGTAACTAGAGAT CCCTCAGACCCTTTTAGTCAGTGTG-3′ and reverse primer 5′-TTCAGCAAGCCGAGTCCTGCGTCGA-3′). The T7 promotor sequence is underlined. *In vitro* transcription of the PCR product was carried out using MAXIscript® T7 Kit (Ambion) according to the manufacturer's protocol.

In vitro reverse transcription and *in vitro* Dicer assays

Ten nanograms of the *in vitro* synthesized RNA template (T7PBS) was hybridized with total tRNA (BioS&T) using conditions described in Beerens and Berkhout (19). Reverse transcription of the mixture was carried out in the presence of 20 ng reverse primer (described earlier) and SuperScript III (Invitrogen). Under the same hybridization conditions, a mixture containing the total tRNA and T7PBS was subjected to digestion with 1 U of recombinant Dicer (Genlantis) for 12 h at 37°C. The diced RNA was resolved in 15% acrylamide—8 M urea gel.

Preparation of AGO2-associated small RNA

293T-AGO2 or 293T-control cells were transfected with pNL4-3 or pUC18 as a negative control. At 2 days post-transfection, cells were harvested with IP buffer [50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% NP-40, 1.5 mM $MgCl_2$, 0.2 mM PMSF, 1 mM DTT, protease inhibitor (complete, Roche) and 100 U/ml SUPERase-in (Ambion)] followed by immunoprecipitation with anti-FLAG agarose (Sigma), anti-AGO2 (Cell Signaling) or mouse normal IgG (as control, Zymed laboratories) with protein G/A agarose (Calbiochem). After six washes, small RNAs in the immunoprecipitates were recovered with mirVana miRNA isolation kit (Ambion). To investigate the effect of RT on the production of PBSncRNA, 5 μM of the non-nucleoside reverse transcription inhibitor (nevirapine) was added 2 h before the cells were transfected, and the drug was maintained throughout the entire transfection process.

Northern blot analysis

RNAs were separated using 15% polyacrylamide–8 M urea gel and electrotransferred for 1 h at 200 mA to nylon membranes. After UV crosslinking, membranes were prehybridized for 1 h at 68°C in Ultrahyb (Ambion) and incubated overnight at 37°C with cognate probes. Membranes were washed extensively at room temperature and autoradiographed.

Small RNA cloning

The cloning of small RNAs from HIV-1 infected MT4 cells was conducted as described by Lau *et al.* (20). HIV-1 virus (>1 MOI) derived from transfection of molecular clone pNL4-3 into 293T cells was used to infect MT4 cells for 2 days. One-hundred micrograms of size-enriched small RNAs (<200 nt) were prepared from the infected and uninfected cells and used for cloning followed by sequencing. The classification of small RNA sequences was based on sequence analysis using the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/), the

miRNA registry database (http://microrna.sanger.ac.uk/sequences/), the rRNA database (http://bioinformatics.psb.ugent.be/webtools/rRNA/blastrrna.html) and the human tRNA database (http://lowelab.ucsc.edu/GtRNAdb/), and the piRNA database (http://research.imb.uq.edu.au/rnadb/FastaDownloads/382.aspx).

Quantitative real-time PCR

Small RNAs (<200 nt) were isolated using mirVana miRNA isolation kit (Ambion). miRNA quantification was as described earlier (21,22). RNA was polyadenylated with ATP by poly(A) polymerase at 37°C for 1 h using RNA tailing kit (Ambion) and reverse transcribed using 0.5 μg of poly(T) adapter primer (Invitrogen). For each PCR, equal amounts of cDNA (first normalized using the snU6 RNA) were mixed with SYBR Green PCR mix (ABI) and 5 pmol of forward primer (designed on the entire tested miRNA sequence) and reverse primer (based on the adaptor sequence). Amplification was done under the condition of 15 s at 95°C and 1 min at 60°C for 55 cycles in an Opticon real-time PCR detection system (Bio-Rad) or 7300 Real Time PCR System (ABI). (Supplementary Figure S1). Random pNL4-3 primers (HIVs3263 and HIVs5543) starting from 3263 nt (5′-ATGGACAGTACAGCCTATAGTGCT-3′) and 5543 (5′-AACTGACAGAGGACAGATGGAA-3′) were used as specificity controls.

To quantify luciferase mRNA and HIV-1 genomic RNA, total RNA from transfected cells was harvested by using the mirVana miRNA isolation kit (Ambion) following the manufacturer's protocol. Fifteen micrograms of total RNA were first treated with RQ1 RNase-free DNase (Promega) at 37°C for 30 min followed by phenol/chloroform extraction and ethanol precipitation. One microgeam of the resuspended RNA was used for RT reaction. The final product was then phenol/chloroform extracted and ethanol precipitated, and 1 ng of the product was used for quantification. The qPCR reaction was the same as described earlier. To detect luciferase mRNA, forward primer 5′-CTCGGGTGTAATCAGAAT-3′ and backward primer 5′-TTGCTAGTACCAACCCTA-3′ were used. To detect HIV-1 genomic RNA, forward primer 5′-CTCTCTGGCTAACTAGGGAAC-3′ and backward primer 5′-CAAGCCGAGTCCTGCGTCGAGAGATC-3′ were used. The results were normalized to the amount of GAPDH mRNA measured using forward primer 5′-GCTCACTGGCATGGCCTTCCGTGT-3′ and backward primer 5′-TGGAGGAGTGGGTGTCGCTGTTGA-3′.

RESULTS

Pyrosequencing of small RNAs in HIV-1 infected T-cells

Viral miRNAs have been reported for several viruses (23,24). To understand better the small RNA profile of HIV-1 NL4-3 infected cells, we sequenced size-fractionated small RNAs from virus infected MT4 T-cells. The MT4 cells were infected with HIV-1; and 2 days later, small RNAs were harvested and cloned (20). In total 47 773 discrete clones were sequenced (1 004 656 nt) and analyzed. Consistent with other studies (15,25) which identified miRNA as the largest (~60%) constituent of small RNAs, 52% of our small RNAs were miRNAs (Figure 1A). In total, 24 892 miRNA clones corresponding to 398 discrete miRNA species (Supplementary Table S1) were identified. The relative distribution of our sequenced miRNA species is consistent with the published literature on miRNAs expressed T-cells (26). For instance, miRNAs including the miR-17-92 cluster (different members in our library varied between 57 and 1276 clones), miR-21 (9521 clones) and miR-155 (409 clones) were highly represented in our sampling. Nonetheless, 27% (107/398) of all the discrete miRNAs identified in our study were cloned only once (Figure 1B). In other studies, many *bona fide* cellular miRNAs have also been reported to be cloned as single copies (27–30). For example, Berezikov *et al.* (30) reported that in their library of 19 291 mouse and 23 351 human small RNA clones, 28% of the known miRNAs were detected either only once or not at all. In a separate respect, our miRNA-cloning sensitivity may be slightly better than that reported in some other studies (26) which identified and cloned from Jurkat T-cell RNAs many fewer discrete miRNA species [i.e. only 592 Jurkat miRNA clones were sequenced by Landgraf *et al.* to reveal 60 individual miRNAs (26)]. As noted elsewhere, the results from various studies should be interpreted with caution since differences could be due to dissimilar cell types, methodologies, tissue culturing conditions and sample size (31). Separately, of interest, 7.58% (3618 clones representing 151 unique sequences) of our small RNAs were piRNAs (Figure 1A). While piRNAs are generally considered to be restricted in expression to germ line cells (32,33), our identification of piRNAs agrees with a report of Ago2- and Ago3-associated piRNAs in Jurkat cells (34). The role of piRNAs in somatic cells requires further study.

Characterization of HIV-1 small ncRNAs

Amongst our 47 773 clones, 125 HIV-1 entities with sequence lengths of 15–26 were found (Supplementary Table S2). Like cellular miRNAs, the largest class of these viral clones encompassed those sequences (113) that were cloned only once (six others were cloned twice and one was cloned thrice). Because cloning proficiencies may be operator-dependent, the functional implications and quantitative interpretation of sequences that were cloned singly versus doubly or triply are not clear, especially since a plurality of authentic cellular miRNAs was cloned only once by us (Supplementary Table S1). In the singly cloned HIV-1 sequences, three observations are noteworthy. First, we cloned a TARncRNA (Figure 2A, left) that agrees with the reported isolation of up to 17 TAR miRNA clones by others (10). Second, we cloned a NEFncRNA sequence (Figure 2A, middle) which is complementary to a previously described NEF miRNA (hiv1-miR-N367) [(9,35) discussed in more detail below]. Third, we cloned a ncRNA sequence (vsiRNA*) (Figure 2B, left) which matches as the miRNA* strand for a previously reported HIV-1 vsiRNA sequence (36).

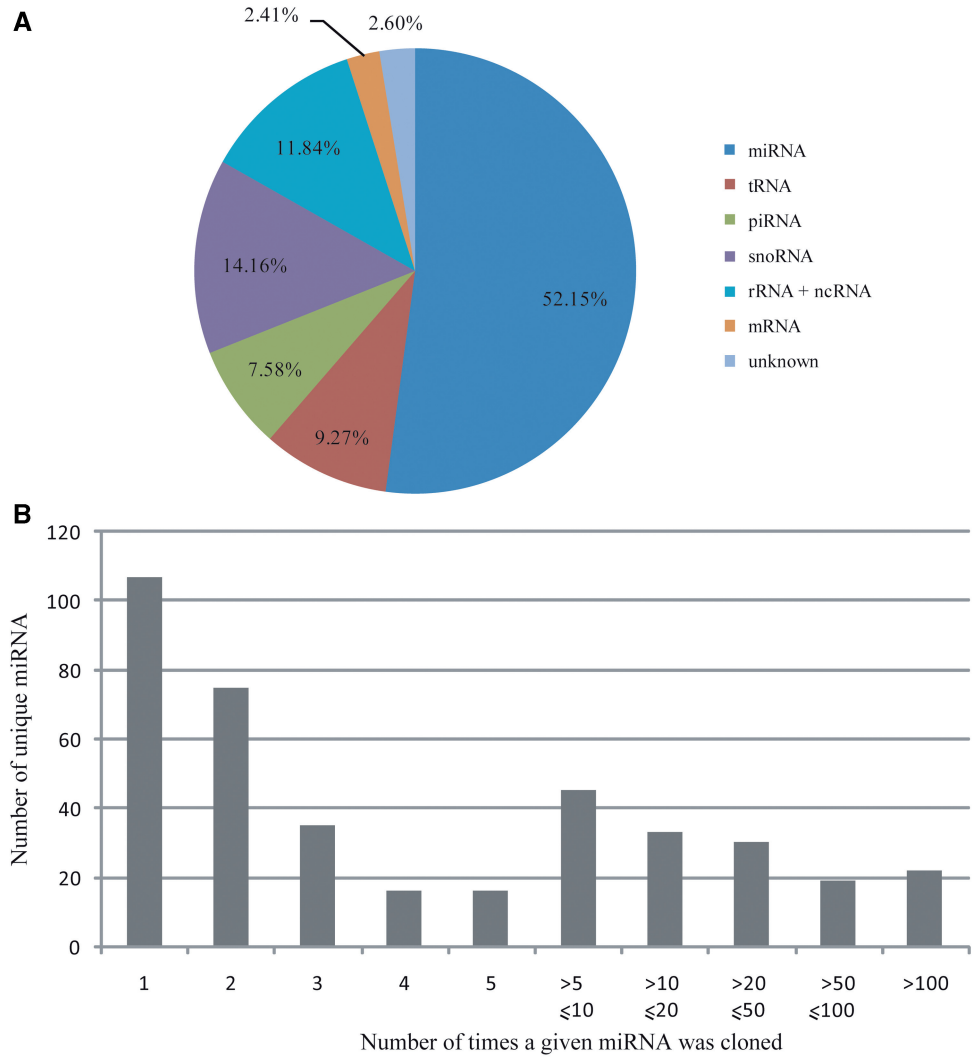

Figure 1. Pyrosequencing of small RNAs from HIV-1 infected T cells. (**A**) A pie chart indicates the distribution of different cellular small RNAs. (**B**) Distribution frequency showing the number of times that different miRNAs were cloned.

These three cloned examples, although difficult to interpret by themselves, appear to independently confirm earlier published observations on small HIV-1 non-coding RNAs. We also identified six HIV-1 RNA clones which share an identical core sequence but diverged slightly in their end nucleotides (Figure 2B, right, RREncRNA). The RREncRNA maps proximal to the vsiRNA-vsiRNA* sequence within the highly secondary structured HIV-1 RRE stem I (Figure 2B). The variations in the termini of RREncRNA clones may have arisen from inexact Dicer end processing which has been noted by others (14). The functional importance of TARncRNA, NEFncRNA, vsiRNA/vsiRNA* and RREncRNA in HIV-1 infected cells remains to be investigated.

It is technically challenging to clone low copy small RNAs. Cloning, *per se*, may not be a quantitatively reliable metric. In contrast, sequence specific real time RT–PCR is a well-established measure of RNA copy number. To assess better the abundance of HIV-1 small RNAs, we next employed real-time quantitative RT–PCR (RT–qPCR) to characterize TARncRNA, RREncRNA and NEFncRNA (Figure 3). As expected, TARncRNA, RREncRNA and NEFncRNA were detected in HIV-1 NL4-3 infected MT4, but not in uninfected control cells (data not shown). As negative detection controls, we randomly chose HIV-1 primer pairs that would measure ncRNAs, if any, which corresponded to NL4-3 sequences located at 3263 and 5543. These control primers produced no detectable signals in virus infected MT4 cells (data not shown). In contrast, sequence-specific RT–qPCR did quantify 4×10^4 copies of TARncRNA, 187 copies of RREncRNA and 607 copies of NEFncRNA, per 10^6 copies of cellular small U6 RNA. Based on published data (37), there are $\sim 10^5$ copies of U6 RNA per cell. Hence, TARncRNA, RREncRNA and NEFncRNA, normalized to U6, are calculated to be roughly 3×10^3, 19 and 61 copies per cell, respectively. We note that several factors may influence the copy number detection of small RNAs. For instance, it is possible that fewer than 100% of the MT4 cells were infected by HIV-1; this would

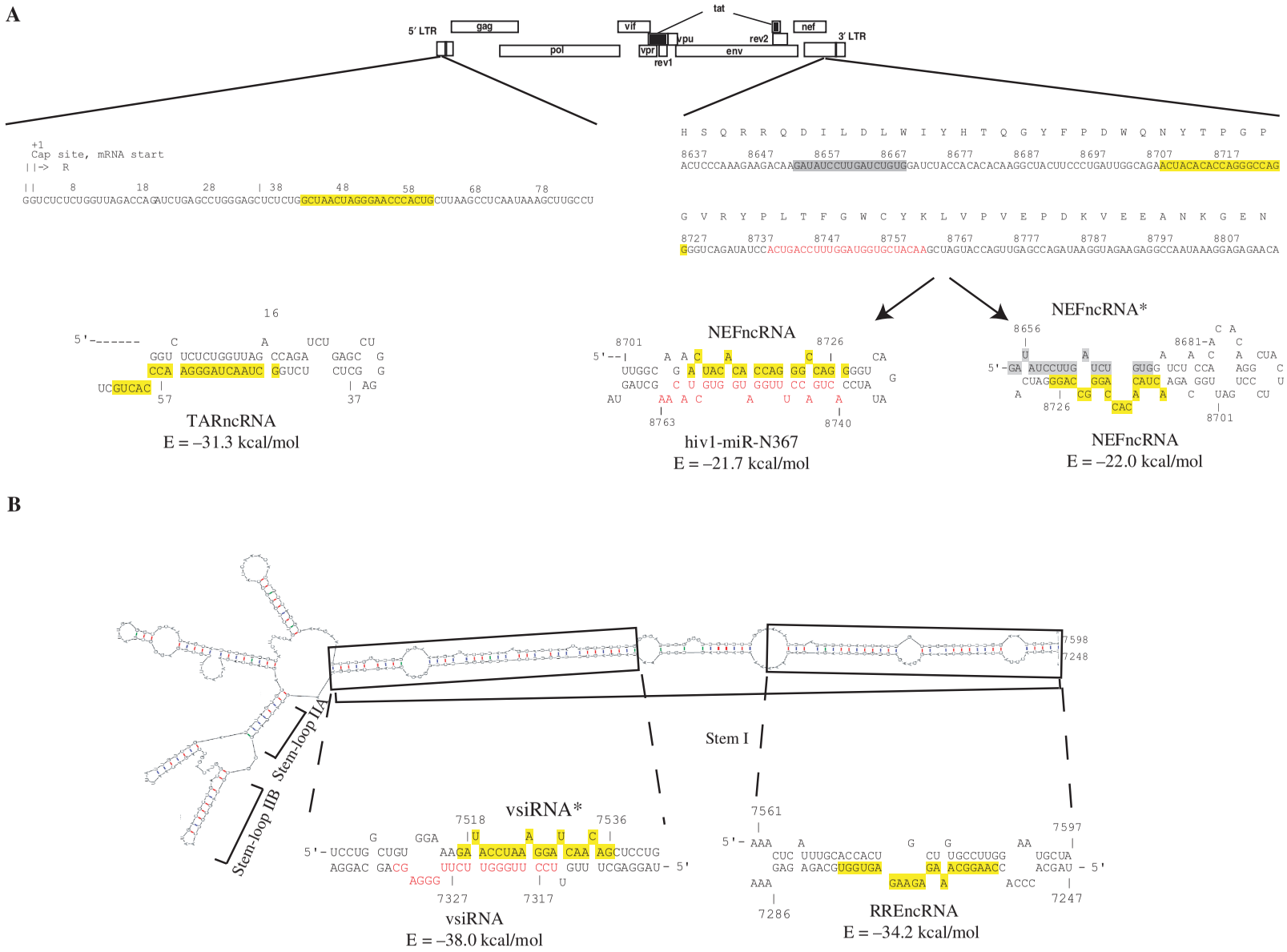

Figure 2. Characterization of HIV-1 small non-coding RNAs. (**A**) TARncRNA (highlighted in yellow) maps to the LTR (Left). The predicted TAR hairpin structure and the location of the TARncRNA are shown. NEFncRNA, NEFncRNA* (highlighted in yellow and grey) and hiv1-miR-N367 (red) (9,35) are indicated in the Nef coding sequence (top). Two alternate hairpin structures can be predicted for these sequences. In one structure, NEFncRNA is complementary to the previously cloned NEFmiRNA, hiv-1-miR367. In the second structure, NEFncRNA is in a hairpin with NEFncRNA* sequence which was also cloned in our small RNA library. Both structures share similar calculated free energies. (**B**) Mfold (68) prediction of the HIV-1 pNL4-3 RRE structure (top). RRE stem I is the apparent precursor for a vsiRNA*-vsiRNA (red) (36) duplex and for the RREncRNA (highlighted in yellow). Both vsiRNA* and RREncRNA were cloned in the current study. Free energies (E) of the vsiRNA*-vsiRNA and RREncRNA structures are −38.0 and −34.2 kcal/mol, respectively.

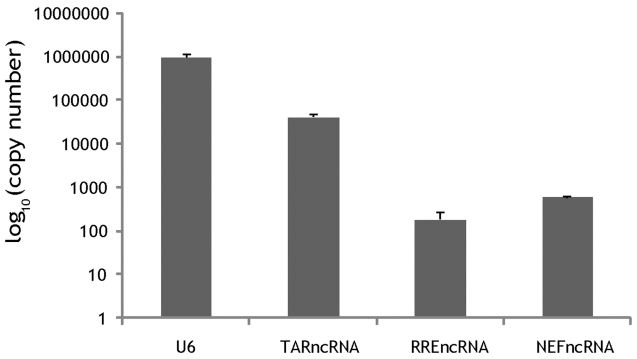

Figure 3. Quantification of HIV-1 small RNAs by real-time RT–PCR. The HIV-1 small RNAs (TARncRNA, RREncRNA and NEFncRNA) were verified by quantitative RT–PCR using U6 small RNA for normalization. Methodology in Supplementary Figure S1.

affect the copy number per cell calculation. Additionally, the stability of viral RNA structures and the likelihood that RNAs with secondary structures are bound by RNA-binding proteins (e.g. TAR- or RRE-binding proteins) may affect the accessibility of these sequences to processing factors.

Small RNA processed from a PBS–tRNAlys3 duplex

Curiously, the most individually abundant HIV-1-related clone (19 clones were isolated) from the infected MT4 cells was an 18-nt ncRNA (PBSncRNA, Figure 4A) with a perfectly matched antisense sequence to the viral PBS. During HIV-1 replication, an RNA duplex is formed between the PBS and cellular tRNAlys3 (Figure 4B) with the 3′-end of PBS-bound tRNAlys3 used as the primer for reverse transcription. It is known that tRNA maturation requires the addition of the CCA nucleotides to the 3′ terminus via the action of CCA-adding enzyme (tRNA nucleotidyltransferase) (38). The detection of CCA sequence in our PBSncRNA (Figure 4B) is consistent with its origin from mature tRNAlys. Indeed, the cloning of the 18-nt PBSncRNA as a discrete moiety suggests that the PBS–tRNAlys3 hybrid might be processed by an RNase III enzyme such as Dicer.

It is possible that the 18-nt PBSncRNA could be an artifactual product of random tRNAlys3 degradation. Two observations argue against this explanation. First, the abundance of processed PBSncRNA was correlated with HIV-1 expression. Thus increasing amounts of PBSncRNA were detected when we transfected escalating amounts of HIV-1 molecular clone pNL4-3 into 293T cells (Figure 4C). This correlation with HIV-1 expression was also illustrated by the finding that 19 copies of PBSncRNA were cloned from HIV-1 infected MT4 cells (Figure 4A) while in the corresponding uninfected MT4 cells only a single PBSncRNA clone was recovered (data not shown). In northern blot analysis, detection of PBSncRNA using a sequence-specific probe was observed in HIV-1 pNL4-3 transfected (but not mock transfected) HeLa cells and in PMA-induced (but not mock induced) HIV-1 latently infected U1 cells (Figure 4D, lanes 2 and 4).

Second, in the sequencing data from our infected MT4 cells, we quantified the copy number of PBSncRNA versus other similar 18-nt ncRNAs that could have arisen from nine other comparably abundant tRNA species (Figure 4E). If the PBSncRNA were a product of random degradation of tRNAly3, then one might expect the other 3′ tRNA 18-nt fragments from comparably abundant tRNAs to be produced in closely equivalent copy numbers. However, the tRNAlys3 PBSncRNA was by far the most abundant moiety in HIV-1 infected MT4 cells, consistent with it arising from a viral PBS–tRNAlys3 dependent processing event (Figure 4E). Indeed, consistent with this notion, two other groups have recently described the unexpected cloning and pyrosequencing of an abundant small ncRNA originating apparently from the processing of an RNA hybrid formed from a tRNA primer with the PBS from cell endogenous retroviruses (14,15). For example, Kawaji *et al.* cloned and identified in high abundance the discrete 3′-end of tRNAGluCUC RNA sequence 5′-UCGUUUCCCGGUCAGGGAAC CA-3′ which is wholly complementary to the PBS (5′-UG GUUCCCUGACCGGGAAACGA-3′) of Harlequin (ERV1) (15). Similarly, Calabrese *et al.* (14) identified an abundantly expressed short ncRNA sequence which is anti-sense to the PBS of the transposon repeat, commonly found in the early mouse embryo and ES cells (39–41). Thus, these collective findings suggest that tRNA-viral PBS hybrids could be ubiquitously processed into discrete intracellular ncRNA species perhaps using either RNAse III dependent or independent paths.

Next, we queried if a PBS–tRNAlys3 duplex could be an *in vitro* substrate for Dicer. To address this question, we performed Dicer assays using a T7 synthesized ^{32}P-radiolabeled HIV-1 RNA that contains the PBS sequence (T7PBS; Supplementary Figure S2). The processing of the T7PBS RNA after annealing with a primer pool of total cellular tRNAs with or without added Dicer was monitored. A mostly unprocessed T7PBS RNA (127 bp) was observed when neither tRNA nor Dicer was added (Figure 5A, lane 3). The addition of Dicer-alone to the T7PBS RNA was insufficient to produce a processed 18-nt band (Figure 5A, lane 2). However, the specific processing of the T7PBS RNA to an 18-nt RNA occurred when both tRNA and Dicer were added to the reaction (Figure 5A, lane 1, see * band). These results suggest that Dicer can recognize and process a T7PBS–tRNAlys3 hybrid into a ncRNA product, although further investigation is needed to elucidate how this processing might occur inside cells.

We also considered the possibility that the PBSncRNA could be generated by the RNaseH cleavage activity of the HIV-1 RT protein (42,43). We do not favor this explanation for the following reasons. First, PBSncRNA was produced in a single-cycle transfection of the HIV-1 NL4-3 molecular clone into cells (Figure 4C). This type of assay does not involve a reverse transcription step. Second, to check more directly that PBSncRNA is not a result of RNaseH activity during a reverse transcription step, we added nevirapine, a non-nucleoside reverse transcriptase inhibitor, before and during the transfection of pNL4-3 into 293T-AGO2 cells (Figure 5B).

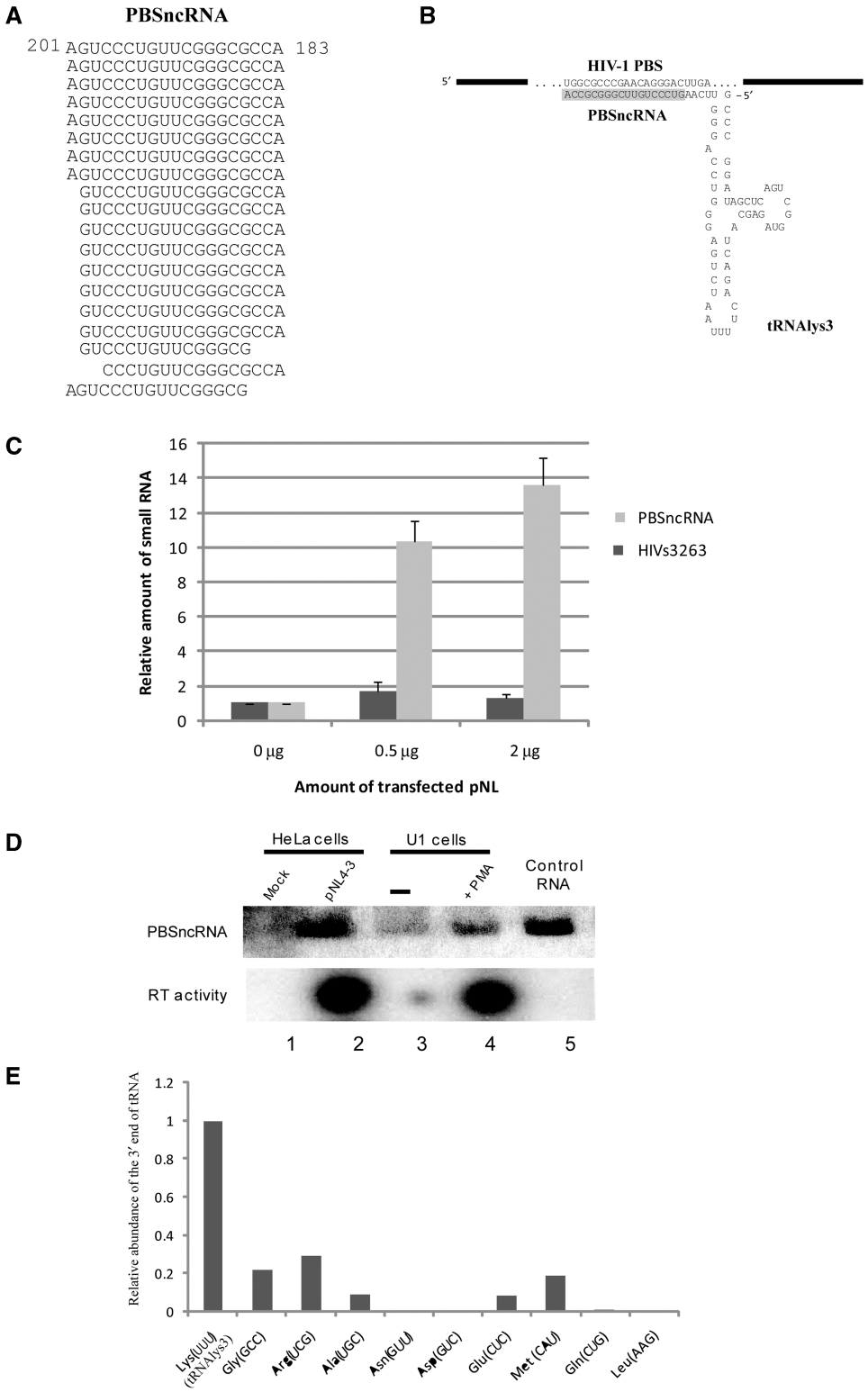

Figure 4. Detection of PBSncRNA that is antisense to the HIV-1 PBS. (**A**) Multiple copies of PBSncRNAs were cloned from HIV-1 infected MT4 cells. The numbering corresponds to pNL4-3 sequence. (**B**) A schematic diagram showing the duplex formed between the HIV-1 PBS with the 3′-end of tRNAlys3. PBSncRNA sequence is highlighted in grey. (**C**) Immunoprecipitation of the AGO2 protein followed by primer-specific RT–qPCR of the AGO2-associated RNA shows that the amount of PBSncRNA quantified in 293T-AGO2 cells is dose-dependent on the amount of transfected pNL4-3 (pNL). (**D**) Northern blot analysis demonstrating increased detection of PBSncRNA in PMA-induced HIV-1 latently infected U1 cells, and in HIV-1 molecular clone (pNL4-3)-transfected HeLa cells (upper panel). Production of HIV-1 from the indicated cells was verified by measuring RT activity (bottom panel). (**E**) Quantification of the predicted processing of the 3′-end of the indicated tRNAs in HIV-1 infected MT4 cells. The relative detection of different 3′-end—tRNA fragments from the sequencing data is compared. The value of the 18-nt PBSncRNA from Lys(UUU) (tRNAlys3) was set as 1. tRNA(UUU) = tRNAlys3.

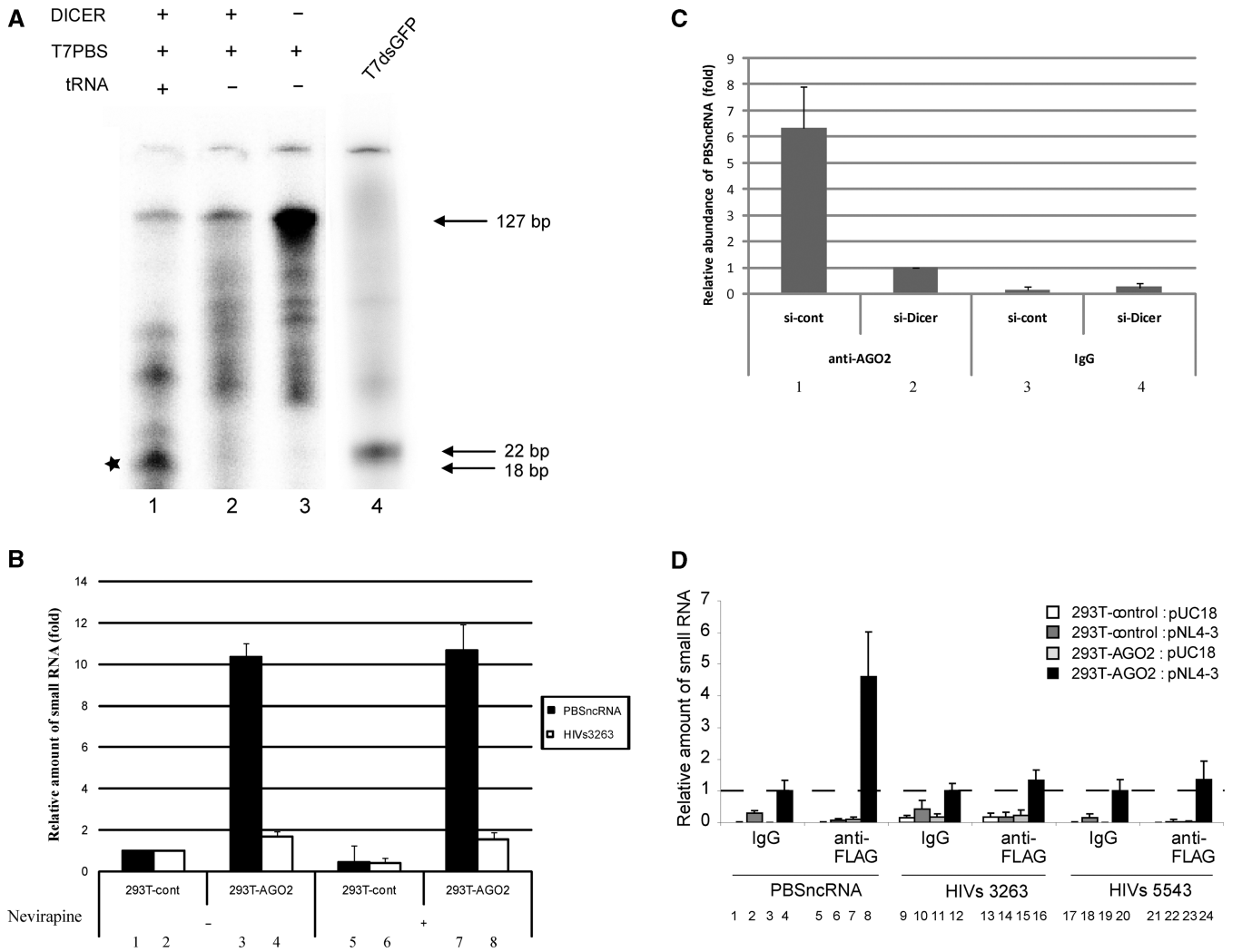

Figure 5. Evidence for the *in vitro* processing and intracellular RISC-incorporation of PBSncRNA. (**A**) *In vitro* Dicer assay of the HIV-1 PBS–tRNA hybrid (the PBS containing radiolabeled transcript was made as described in Supplementary Figure S2) produced a small RNA of 18 nt (asterisk; lane 1). This small RNA was not observed in the absence of either tRNA (lane 2) or tRNA + Dicer (lane 3). As a positive control, *in vitro* T7 RNA polymerase synthesized and then annealed ds GFP RNA (T7dsGFP) was processed *in vitro* by Dicer, which yielded the expected small RNAs of ~22 bp (lane 4). (**B**) Non-nucleoside RT inhibitor (Nevirapine) does not affect the expression of PBSncRNA in a single-cycle transfection of an HIV-1 molecular clone. We transfected pNL4-3 into nevirapine treated (+; lanes 5–8) or untreated cells (−; lanes 1–4) and subsequently quantified PBSncRNA. Similar expression levels of PBSncRNA was detected in nevirapine treated (lane 7) and untreated (lane 3) 293T-AGO2 cells. Controls include the immunoprecipitation using anti-FLAG in control cell line (293T-cont) (lanes 1 and 5) and the attempted detection of a randomly selected HIV-1 sequence (HIVs3263) (lanes 2, 4, 6 and 8). (**C**) siRNA knock down of Dicer reduced the expression of PBSncRNA. PBSncRNA was quantified in 293T-AGO2 cells transfected with control-siRNA (si-cont) or Dicer-siRNA (si-Dicer) and co-transfected with pNL4-3. The amount of AGO2-associated PBSncRNA was then quantified by RT–qPCR after immunoprecipitation with anti-AGO2 (lanes 1 and 2). Control immunoprecipitation was also performed with an irrelevant IgG (lanes 3 and 4). (**D**) Immunoprecipitation (IP) of FLAG-Ago2 using anti-FLAG or a control IgG antibody was performed in a 293T-AGO2 cell line or in a control 293T parental cell line as described in the text. Quantitative real-time RT–qPCR detection of the HIV-1 PBSncRNA or two randomly chosen HIV-1 sequences (HIVs3263 and HIVs5543) from the IP products was performed. The value of the RT–qPCR result from the IP of 293T-AGO2 transfected with pNL4-3 was set as 1.

The amount of PBSncRNA produced was essentially the same in the presence or absence of nevirapine suggesting that its production is RT-independent. Finally, we also performed siRNA knock down of Dicer. By knocking down Dicer, we indeed observed a reduction in the amount of PBSncRNA compared to control cells (Figure 5C).

Association of PBSncRNA with Ago2-RISC

What could be a possible fate for the PBSncRNA? Would it functionally engage intracellular RISC? The Ago2 protein is a central component of RISC (44), and above results in Figure 5C suggest that PBSncRNA can engage RISC. To check in greater detail how PBSncRNA can function with Ago2, a 293T cell line stably over-expressing FLAG-tagged Ago2 (293T-AGO2) was constructed. 293T-AGO2 cells were transfected with pNL4-3, and Ago2 was immunoprecipitated using anti-FLAG. The presence of PBSncRNA in the immunoprecipitate was assessed by real-time RT–qPCR. As a control, transfection of pNL4-3 into the parental 293T cell line was also immunoprecipitated in parallel

with either anti-FLAG or with an irrelevant IgG control antibody (Figure 5D; lanes 1–8). A significant recovery of PBSncRNA from pNL4-3/293-AGO2 cells was detected by RT–qPCR in the IP with anti-FLAG, but not in the IP with IgG control antibody (Figure 5D; compare lane 4–8); an insignificant amount of PBSncRNA was seen in the immunoprecipitates from control and mock transfected cells (Figure 5D; lanes 1–3 and lanes 5–7). To check further for specificity of detection, we selected two random HIV-1 sequences (HIVs3263 and HIVs5543) for use in control RT–qPCR assays (Figure 5D; lanes 9–24). In both instances, no specific recovery was detected by these sequences in the immunoprecipitations (Figure 5D; lanes 1, 3, 6 and 7). Taken together, the results support the interpretation of an intracellular association of PBSncRNA with Ago2-RISC.

Over-expression of PBSncRNA modulated HIV-1 replication

If PBSncRNA-associates with Ago2, could this interaction result in an active RISC? We, next, constructed a reporter plasmid containing a single PBS-target sequence positioned downstream of a luciferase cDNA (LucPBS; Figure 6A). Transfection of LucPBS with a synthetic PBSncRNA at 5 nM into HeLa cells produced a 40% inhibition of luciferase activity (Figure 6B). Increasing the amount of transfected PBSncRNA to 50 nM increased luciferase inhibition to 80% (Figure 6B). As control, a parallel transfection of a mutant PBSncRNA (PBSncRNA mutant) did not inhibit luciferase expression, supporting that the observed PBSncRNA-silencing was sequence specific.

To query the role of PBSncRNA-silencing in viral biology, we examined how it might affect single-cycle of HIV-1 replication. HIV-1 molecular clone pNL4-3 was co-transfected into 293T cells with PBSncRNA or PBSncRNA mutant, and virus production was measured by checking for the release of RT into the culture supernatant. Consistent with the results from the luciferase reporter assays (Figure 6B), co-transfection of the PBSncRNA, but not the PBSncRNA mutant, produced a dose-dependent inhibition of virus RT production (Figure 6C). Next, to ask whether the silencing effect of PBSncRNA is through destabilization of the targeted RNA or through a mechanism of translational inhibition, RT–qPCR was performed to quantify HIV-1 RNA with or without PBSncRNA transfection (Supplementary Figure S3). The HIV-1 RNA copies were significantly decreased by transfected PBSncRNA in a dosage-dependent manner (Supplementary Figure S3). Finally, to ask if cell endogenous PBSncRNA exhibits a similar anti-HIV-1 effect as observed with over expressed PBSncRNA, we reasoned that knocking down the ambient level of PBSncRNA in cells could make these cells more susceptible to HIV-1 replication. We employed synthetic RNAs (antagomirs) designed to knock down PBSncRNA. When these antagomirs were introduced into cells, replication of HIV-1 in the transfected cells was notably augmented (Figure 6D).

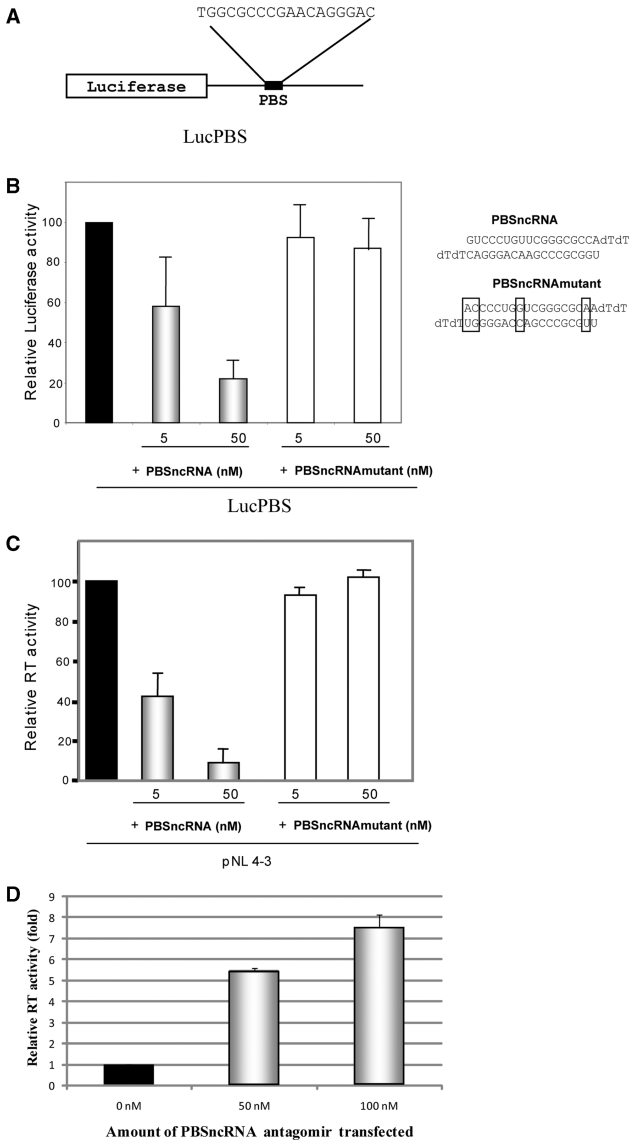

Figure 6. Over expressed PBSncRNA or knock down of physiologically expressed PBSncRNA can modulate viral replication. (**A**) A schematic diagram of the LucPBS construct. A single copy of HIV-1 PBS sequence (TGGCGCCCGAACAGGGAC) was positioned downstream of the luciferase coding sequence. (**B**) Functional studies employing synthetic dsRNA oligonucleotide corresponding to the PBSncRNA or its mutated form, PBSncRNAmutant. Mutated nucleotides are boxed. Different concentrations (5 or 50 nM) of the PBSncRNA and PBSncRNAmutant were separately co-transfected with LucPBS construct (described in A) into HeLa cells. The PBSncRNA inhibited up to ~80% the luciferase expression. (**C**) Similar experiments were performed in 293T cells using HIV-1 molecular clone (pNL4-3) in a single round replication assay (see text). The PBSncRNA effectively inhibited up to ~80% of HIV-1 replication while no inhibition was observed with PBSncRNAmutant. (**D**) De-repression of HIV-1 replication by knock down of cell endogenous PBSncRNA using RNA 'antagomirs'. Co-transfection of pNL4-3 and increasing amount of antagomirs, complementary to the PBSncRNA, in 293T cells increased HIV-1 replication as measured by RT activities.

Collectively, the findings indicate that both over expressed and physiologically expressed PBSncRNA can serve modulating influences on HIV-1 replication in cells.

DISCUSSION

Here, we have performed high throughput pyrosequencing of cloned RNAs from HIV-1 infected MT4 cells. We sequenced 47 773 clones which included 128 viral small RNA entities. In examining the results, several reasons suggest (although they do not fully exclude) that most of our viral RNA sequences are unlikely to be products of random degradation. First, we employed a small RNA cloning technique which requires the presence of intact 5′ phosphate and 3′ OH ends (26). Degraded RNAs have non-phosphorylated 5′-ends and are unlikely to be cloned by this technique. Second, degradation products tend to have a wide-distribution of lengths due to random cleavage. The lengths of our cloned small RNA sequences are narrowly restricted. Third, the presence of mRNA sequences in a small RNA clone library can represent an internal measure of degradation products. In our library, only 2.41% of the small RNAs were mRNA-derived (Figure 1A), a number consistent with other studies, suggesting a limited contribution of degradation to our cloning. Finally, in the case of PBSncRNA [which has related examples described by others (14,15)], we showed an association of this moiety with Ago2. This type of association is compatible with a functionally relevant interaction which is unlikely to occur for RNAs generated by random degradation.

Several studies have reported on the cloning and sequencing of small RNAs encoded by viruses (23,24). Small viral RNAs have most prevalently been described in Herpesvirus-infected cells (45). In HIV-1 research, a recent study has performed small RNA-cloning and pyrosequencing using the Ach2 'latent' HIV-1-infected cell line and found minimal representation of viral sequences (26). However, the Ach2 'latent' cell results could be problematic because the HIV-1 provirus in Ach2 cells contains a mutated TAR which renders the LTR non-responsive to the viral transcriptional activator, Tat. Indeed, it was shown previously that when the same TAR-mutation in Ach2 cells was transferred into an otherwise infectious molecular clone of HIV-1, the virus became crippled for replication, and no viral production was measured (46). Our study differs from the Ach2-work in employing the productive infection of MT4 cells with a replication competent HIV-1 virus. As noted earlier, our cloning of a wider-range of T-cell specific miRNAs possibly indicate a greater sensitivity than that reported by Landgraf *et al.* who sequenced 1098 Ach2-miRNA clones (26) while we sequenced 24 892 MT4-miRNA clones. Nonetheless, one should be cautious about interpreting diverse results since each infection system and cloning method could have inherent constraints which influence the precision of measurements (47). Likely, with further improved sensitivity of cloning and sequencing, small viral ncRNAs could be discovered for an increasing number of mammalian viruses (48). Indeed, in a recent small RNA cloning and pyrosequencing study of four HTLV-1 infected/transformed cell lines, we have also identified many discrete small viral ncRNAs (Yeung, unpublished observations). It remains a future challenge to determine the biological implications of small viral ncRNAs in infected cells.

Although we do not yet understand the functions of small HIV-1 ncRNAs, the identities of some clones are consistent with the expectations from previous findings (7–9). Bennasser *et al.* (49) and Omoto *et al.* (9) had earlier predicted two potential miRNA-like hairpin structures for TAR and NEF, respectively. Other investigators have subsequently confirmed the existence and cloning of a TAR miRNA (7,8,10), and our cloning (Figure 2A; Supplementary Table S2) and RT–qPCR results (Figure 3) are concordant with these findings. Omoto *et al.* (9,35) had also cloned one arm of a Nef-miRNA (hiv1-mir-N367) hairpin (Figure 2A, right). Interestingly, our NEFncRNA now appears to be the hiv1-mir-N367* strand complementary to Omoto's Nef-miRNA (hiv1-mir-N367) sequence. Provocatively, NEFncRNA can also assume an alternative hairpin structure (Figure 2A, right, NEFncRNA-NEFncRNA*); and the NEFncRNA* strand of this alternate hairpin was also recovered in our cloning. In our viral RNA sequences, we further identified a clone for a putative complementary arm (vsiRNA1*) of a RNA-duplex that contains the previously reported vsiRNA1 (36). The vsiRNA1*-vsiRNA1 duplex maps to a highly secondary structured RRE segment, which apparently also produces a cloned RREncRNA (Figure 2B, right). Pending a better comprehension of the roles played by these RNA sequences, one interpretation is that these HIV-1 small ncRNAs are products of processing by the cellular RNAi machinery, and that their presence agrees with evidence elsewhere that RNAi serves an innate antiviral defense in mammalian cells (31,50).

Our pyrosequencing study also identified a small 18-nt antisense ncRNA (PBSncRNA) that is fully complementary to the HIV-1 PBS (Figure 4). The abundance of PBSncRNA is HIV-1 expression dependant, consistent with a mechanism whereby much of this moiety is processed from a PBS–tRNAlys3 duplex by Dicer (Supplementary Figure S4). Although we were initially surprised by this cloning, a closer inspection of the literature revealed two recent studies which also described the abundant cloning of discrete analogous PBSncRNAs complementary to the PBS of endogenous retroelements (14,15). Together, these three PBSncRNA findings fit a general notion that retroviral PBS–tRNA hybrid duplex may be ubiquitously processed by the cell to yield ncRNAs. The deliberate over expression of a HIV-1-PBS-targeting ncRNA [Figure 6; and Han *et al.* (51)], like other PBS-targeting molecules (52), can modulate viral replication. We also observed that the knock down of physiologically expressed PBSncRNA in cells increased the replication of HIV-1 in these cells (Figure 6D) suggesting that ambiently expressed PBSncRNA can target intracellularly viral genomic sequence (53).

The suggestion that small HIV-1 ncRNAs and PBSncRNA represent evidence that mammalian cells employ RNAi to defend exogenous retroviral infection agrees with the current view that RNAi is utilized by cells to suppress mammalian endogenous retroviruses (40,41,54–56). The findings also reconcile accumulating

evidence that mammalian viruses including HIV-1 encode functional RNAi suppressors (which viruses should encode if cells use RNAi-against viruses). Examples of these suppressors include hepatitis C virus (HCV) core and envelope protein 2 (57,58), vaccinia virus E3L (59), Ebola virus VP35 (60), primate foamy virus Tas (61), influenza A virus NS1 (59,62,63) and HIV-1 Tat (36,50,64). Some of these suppressors may serve to moderate Dicer activity (36,65), and/or sequester dsRNA (59,62,63,66). Either mechanism could limit the capacity by the cell to process viral sequences into small ncRNAs, perhaps in part explaining some of the challenges in detecting small viral ncRNAs in mammalian cells. In the big picture, studying the strike-counter strike interplay in nucleic acid-based RNAi restriction between viruses and their hosts (67) may shed further understanding on viral sequence evolution and viral diversity.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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