Destabilization of tRNA$\textsubscript{3}^{\text{Lys}}$ from the primer-binding site of HIV-1 genome by anti-A loop polyamide nucleotide analog

Neerja Kaushik, Tanaji T. Talele, Raymond Monel$^1$, Paul Palumbo$^1$ and Virendra N. Pandey*

Department of Biochemistry and Molecular Biology, Center for the Study of Emerging and Re-Emerging Pathogens and $^1$Department of Pediatrics, Division of Allergy, Immunology and Infectious Diseases, UMDNJ-New Jersey Medical School, 185 South Orange Avenue, Newark, NJ 07103, USA

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ABSTRACT

Initiation of human immunodeficiency virus type 1 (HIV-1) reverse transcription occurs by extension of the cellular tRNA$\textsubscript{3}^{\text{Lys}}$ which anneals to the primer-binding site (PBS) on the 5′ non-translated region of the viral RNA genome. The A-rich sequence (A-loop) upstream of the PBS interacts with the anticodon loop of tRNA$\textsubscript{3}^{\text{Lys}}$ and has been proposed to be essential for conferring specificity to tRNA$\textsubscript{3}^{\text{Lys}}$ for priming the initiation of HIV-1 reverse transcription. We observed that polyamide nucleic acid targeted to the A-loop sequence (PNAAL) exhibits high binding specificity for its target sequence. The PNAAL pre-bound to the A-loop sequence prevents tRNA$\textsubscript{3}^{\text{Lys}}$ priming on the viral RNA consequently blocking in vitro initiation of reverse transcription. Further, PNAAL can efficiently disrupt the preformed [tRNA$\textsubscript{3}^{\text{Lys}}$–viral RNA] complex thereby rendering it non-functional for reverse transcription. The endogenous reverse transcription in disrupted HIV-1 virions containing packaged tRNA$\textsubscript{3}^{\text{Lys}}$ and its replicating enzyme RT was significantly inhibited by PNAAL, thus providing direct evidence of the involvement of the A-loop region of viral RNA genome in tRNA$\textsubscript{3}^{\text{Lys}}$ priming process. These findings suggest the potential of the A-loop region as a critical target for blocking HIV-1 replication.

INTRODUCTION

Currently utilized therapies for AIDS involve inhibitors which block viral maturation and reverse transcription of viral RNA into double-stranded DNA (1–3). However, the rapid emergence of drug-resistant strains has considerably overshadowed the benefits of the clinically available anti-human immunodeficiency virus type 1 (HIV-1) drugs. Selection of the dominant, pre-existing drug-resistant variants and the abundance of latently infected cells (which possess integrated proviral DNA) are the potential barriers encountered to effective drug therapy (2–4). A potentially important approach to overcome this problem is to target those regions of the viral genome that are essential for viral replication but averse to mutational changes.

The unique 5′ (R-U5-PBS) non-translated region (1–333 nt) of the HIV-1 genome containing several critical domains essential for viral replication may be an ideal target for drug intervention. These critical domains comprise of: (i) primer-binding site (PBS; nt 183–201), essential for tRNA$\textsubscript{3}^{\text{Lys}}$ primed initiation of reverse transcription (5–9); (ii) the A-loop region, located upstream of the PBS (nt 168–173) is essential for the selection and interaction of tRNA$\textsubscript{3}^{\text{Lys}}$ primer (10–12); (iii) the long terminal repeat (LTR) sequences at the 5′ and 3′ ends, essential for viral transcription and integration (13); and (iv) the trans-activation response element (TAR), essential for viral gene expression via transcriptional activation (14–16) and probably having some additional role in the initiation of reverse transcription (17,18). These regulatory sequences in the 5′ non-translated region are averse to mutational changes and, therefore, can be potential targets for arresting viral replication.

We have earlier shown that a polyamide nucleic acid (PNA) targeted to the PBS region of the viral genome, blocks the initiation of reverse transcription (19). We have further demonstrated that PNA targeted to the TAR sequence of the viral RNA genome is able to prevent Tat–TAR interaction by efficient sequestration of the TAR and block Tat-mediated trans-activation of HIV-1 LTR transcription (20). PNAs are DNA homologs containing a peptide backbone of 2-aminoethylglycine units to which purine and pyrimidine bases are linked. PNAs are resistant to degradation by nucleases and proteases (21) and are able to bind the target region in the duplex DNA by invasion and displacement of one of the DNA strands (19,22,23). The high stability of PNA–nucleic acid complexes is reduced dramatically by single base pair mismatches, suggesting that PNAs recognize their targets in a sequence-specific manner (19,21,23). PNA, with a terminal DNA nucleotide, is also recognized as a bona fide primer by HIV-1 reverse transcriptase (RT), resulting in abortive reverse transcription products (19,24,25).

In this communication, we present evidence to demonstrate that a 15mer PNA, targeted to the A-loop sequence (named as PNAAL) upstream of the PBS region, can specifically

*To whom correspondence should be addressed. Tel: +1 973 972 0660; Fax: +1 973 972 8657; Email: pandey@umdnj.edu
sequester the target sequence and effectively inhibit the initiation of reverse transcription. We chose the A-loop region as a target as it has been shown to be critical for stabilization of the tRNA<sub>3Lys</sub> primer on the viral genome (10–12, 26–29). A scrambled PNA sequence was used in all experiments as a negative control to determine the specificity of PNA-Al. Our results indicate that PNA-Al either prevents the tRNA<sub>3Lys</sub> primer forming a complex with the PBS region of the viral genome or destabilizes the tRNA<sub>3Lys–PBS</sub> complex and inhibits both initiation of reverse transcription and elongation of initiated primer in vitro. The PNA-Al was equally effective in blocking the process of reverse transcription in isolated HIV-1 virions. The significance of these findings may lead to advances in HIV-1 therapeutic research.

**MATERIALS AND METHODS**

DNA modifying enzymes were purchased from Promega or Roche Biochemicals. Tritiated dNTPs, [<sup>32</sup>P]-ATP and [α-<sup>32</sup>P]dNTPs were the products of Dupont-New England Nuclear Inc. The DNA oligomers were synthesized at the Molecular Resource Facility at UMDNJ. PNA oligomers were synthesized at the Applied Biosystems Inc. All other reagents were of the highest available purity grade and purchased from Fisher, Millipore Corp., and Bio-Rad.

**Plasmid and clones**

The expression vector pKK-RT66 was constructed in this laboratory and used to purify the wild type HIV-1 RT (19). An HIV-RNA expression clone pHIV-PBS was a generous gift from Dr M. A. Wainberg (30). This clone contains a 947 bp HIV-RNA expression clone pHIV-PBS was a generous gift laboratory and used to purify the wild type HIV-1 RT (19). An

**Isolation of p66/51 HIV-1 RT**

The recombinant clone pKK-RT66 encoding the wild type p66 HIV-1 RT was expressed in JM109 and purified as described before (19). The heterodimeric HIV-1 RT (p66/p51) was generated by the proteolytic cleavage of the p66/p66 homodimer as described below. The proteolytic buffer in a total volume of 2 ml contained 0.1 M potassium phosphate, pH 7.5, 1.0 M NaCl, 1 mM dithiothreitol (DTT), 1 mM EDTA, 25 μM of p66/p66 (6.1 mg) and 25 μM of HIV-1 protease (1.15 mg). The mixture was incubated at 4°C and the extent of proteolytic cleavage was monitored by SDS–PAGE followed by Coomassie blue staining. Following complete cleavage of the second p66 subunit (~16 h incubation), the reaction mixture was diluted 20-fold with 20 mM potassium phosphate buffer, pH 8.0, and applied to a 5 ml phosphocellulose column pre-equilibrated with a buffer containing 20 mM potassium phosphate, pH 8.0, 50 mM NaCl and 1 mM DTT. After washing the column with 10 column vol of the same buffer, the heterodimeric RT was eluted with 20 ml of this buffer containing 250 mM NaCl and concentrated by precipitating with ammonium sulfate at 70% saturation. The precipitate was dissolved in a small volume of buffer consisting of 50 mM Tris–HCl pH 7.5, 100 mM NaCl and 1 mM DTT, followed by dialysis against the same buffer containing 50% glycerol. The enzyme preparation was stored at −70°C. Protein concentrations were determined by using the Bio-Rad colorimetric kit as well as by spectrophotometric measurements using ε<sub>280</sub> = 2.62 × 10<sup>5</sup> M<sup>−1</sup> cm<sup>−1</sup> for p66/51 heterodimers (31).

**Isolation of HIV-1 virions**

The lymphocyte cells, CEM CD4+ (12D7), were grown in complete RPMI-1640 medium. The cells in the log phase were harvested, and washed with equal volumes of phosphate buffered saline (PBS) without Ca<sup>2+</sup> or Mg<sup>2+</sup>. Cells (10<sup>6</sup> cells/ml) were re-suspended in RPMI-1640 medium (without fetal calf serum, t-glutamine or penicillin/streptomycin). A portion of the cell suspension (250 μl, 2.5 × 10<sup>6</sup> cells) mixed with plasmid plasmid DNA (3 μg) was electroporated at 230 V and then plated in 10 ml of complex RPMI-1640 media (32). Cells were subsequently incubated at 37°C in 5% CO<sub>2</sub> containing humidified air for 3–4 days. The cells were centrifuged at 1200 r.p.m. for 7 min and the supernatant was used to monitor the levels of p24 antigen using the ELISA p24 antigen kit (ABBOTT Laboratories). The culture supernatant was further centrifuged at 30 000 g for 90 min to pellet the HIV-1 virions. The HIV-1 virion pellet was disrupted in a buffer containing 50 mM Tris–HCl pH 7.5, 100 mM NaCl and 0.1% NP-40 and used as a source of endogenous RT as well as tRNA<sub>3Lys</sub>-primed HIV-1 viral RNA genome.

**Construction of the HIV-1 RNA expression clone pU5-PBS for gel retardation analysis**

We constructed an HIV-1 RNA expression clone, pU5-PBS, in order to generate a shorter HIV-1 RNA transcript for analyzing its binding affinity to the A-loop PNA. This clone contains a 183 bp fragment corresponding to nt 473–656 of pHXB2 HIV-1 proviral clone and was used to transcribe the 495 base-long HIV-1 RNA template.

**Preparation of R-U5-PBS HIV-1 RNA template**

Two plasmids, pHIV-PBS and pU5-PBS, were used to transcribe the 495 and 200 base-long U5-PBS RNA templates, respectively. The 495 base-long RNA transcript contains 11 nt from the plasmid vector flanking the S' terminus and the remaining 484 nt correspond to part of the non-coding and gag-coding regions, the primer binding sequence, U5 and part of the R region (57 nt). The 200 base-long RNA transcript contains 183 nt corresponding to the HIV-1 region comprising of the primer-binding sequence, U5 and part of the R region. The additional 17 nt flanking the 5' terminus are derived from the plasmid vector. The plasmid pHIV-PBS was linearized by AccI and pU5-PBS by XhoI restriction enzymes and transcribed using T7 RNA polymerase and other reaction components from Roche Biochemicals. After in vitro transcription reaction, the DNA template was removed by DNase I digestion and the RNA transcripts were purified by phenol–chloroform extraction and alcohol precipitation. The RNA products were dissolved in DEPC-treated water containing 10 mM DTT and further purified by G-50 spin column and stored at −70°C.
Preparation of ³²P-labeled U5-PBS RNA template

The plasmid pU5-PBS linearized with XhoI was transcribed using T7 RNA polymerase (Roche Molecular Biochemicals) to prepare ³²P-labeled 200 base-long U5-PBS RNA. The RNA transcript was labeled internally by including [α-³²P]UTP (3000 C/mmol; Amersham Life Sciences) and purified by phenol–chloroform extraction and alcohol precipitation. The RNA product was dissolved in DEPC-treated water containing 10 mM DTT, further purified by G-50 spin column and stored at -70°C.

Gel retardation assay

The internally ³²P-labeled 200 base-long U5-PBS RNA (5000 Cerenkov c.p.m.) was incubated at varying molar ratios of PNAAL or scrambled PNA for 2 h at 37°C in a binding buffer containing 30 mM Tris–HCl pH 8.0, 60 mM KCl, 5.5 mM MgCl₂, 10 mM DTT, 10% glycerol, 0.01% NP-40 and 500 ng of r (I-C), in a final volume of 15 µl (20). Two microliters of RNA gel loading dye (0.27% bromophenol blue and 20% glycerol) was added to the samples and subjected to native gel retardation analysis on an 8% polyacrylamide gel in Tris–borate–EDTA (TBE) buffer containing 5% glycerol. The gels were routinely pre-run at 100 V for 30 min at 4°C in TBE buffer, pH 8.2. The RNA–PNA complexes were resolved from the free RNA at a constant voltage of 150 V at 4°C and subjected to phosphorimager analysis.

Labeling of tRNA₃Lys

The high-pressure liquid chromatography-purified human placental tRNA₃Lys obtained from BIOS&T was 3’ end labeled with ³²PpCp by T4 RNA ligase according to the standard protocol (33). The labeled product was extracted three times with phenol–chloroform, precipitated with alcohol, lyophilized and suspended in TE buffer. This was further purified on a NAP-10 column to remove the unincorporated radiolabeled nucleotides.

Destabilization of tRNA₃Lys–U5-PBS RNA complex by PNAAL

The 495 base-long U5-PBS RNA transcript was incubated with the labeled tRNA₃Lys (2 × 10⁶ c.p.m.) at 37°C for 1 h in a buffer containing 50 mM Tris–HCl pH 7.8, 1 mM DTT and 60 mM KCl. This was followed by further incubation for another 1 h in the presence and absence of PNAAL or scrambled PNA. Two microliters of RNA gel loading dye were added to 10 µl of the reaction mixture. The tRNA₃Lys–U5-PBS RNA complex formed was resolved by electrophoresis on an 8% polyacrylamide gel at 4°C on a 6% non-denaturing polyacrylamide gel at 150 V for 3 h in 89 mM Tris–borate buffer, pH 8.2. The gel was dried and analyzed by phosphorimager.

Reverse transcription of U5-PBS RNA template primed with 17mer DNA or tRNA₃Lys

An aliquot of the 495 base-long U5-PBS RNA template was annealed with either the labeled 17mer DNA primer complementary to the PBS or with the natural tRNA₃Lys. The molar ratio of RNA template to 17mer DNA primer or to tRNA₃Lys was 2:1. Reverse transcription reactions were carried out by incubating 2.5 nM of U5-PBS RNA–tRNA₃Lys template primer with 50 nM of HIV-1 RT in a reaction mixture containing 25 mM Tris–HCl pH 7.8, 10 mM DTT, 100 µg/ml BSA, 5 mM MgCl₂ and 50 µM of dATP, dTTP, dGTP and 2 µM of [α-³²P]dCTP (0.25 µCi/pmol). In experiments with U5-PBS RNA template primed with 5’-³²P-labeled 17mer DNA primer, the unlabeled dNTPs were supplemented in the reaction at a final concentration of 50 µM (each). The reaction was initiated by the addition of enzyme and terminated by the addition of equal volume of Sanger’s gel loading solution (34). The products were resolved on an 8% polyacrylamide–urea gel.

The U5-PBS RNA pre-bound with tRNA₃Lys or 17mer DNA primer was incubated with PNAAL complementary to the A-loop region upstream of the PBS, at varying molar ratios in a buffer containing 50 mM Tris–HCl pH 7.8, 10 mM DTT, 60 mM KCl and 5 mM MgCl₂ at 37°C for 2 h. In another set of experiments, both PNAAL and tRNA were allowed to compete for binding to U5-PBS RNA. For this, U5-PBS RNA was incubated with PNAAL at varying molar ratios along with tRNA₃Lys at 37°C for 2 h. The extent of DNA synthesis catalyzed by HIV-1 RT in the presence or absence of the PNAAL or scrambled PNA was assessed as described above.

Effect of PNAAL on tRNA₃Lys-derived endogenous reverse transcription in disrupted HIV-1 virions

The tRNA₃Lys-derived endogenous reverse transcription in disrupted HIV-1 virions was carried out as described below. An aliquot (7 µl) of disrupted virions was incubated in a reaction mixture containing 50 mM Tris–HCl pH 7.8, 10 mM DTT, 60 mM KCl, 5 mM MgCl₂, 0.01% NP-40, 100 µg/ml BSA, 20 µM each of dATP, dGTP, dTTP and 1 µM of [α-³²P]dCTP (0.5 µCi/pmol) in a total volume of 15 µl. The reaction was carried out at 37°C for 30 min and quenched with 20 mM EDTA. The products were alcohol precipitated and analyzed on an 8% denaturing polyacrylamide–urea gel followed by phosphorimaging.

The effect of PNAAL or scrambled PNA on the endogenous reverse transcription in the disrupted virions was assessed by pre-incubating the disrupted virions at varying concentrations of PNA at 4°C for 16 h. The control sample was incubated under identical conditions in the absence of PNA. The extent of endogenous reverse transcription in the disrupted virions was monitored as described above.

RESULTS AND DISCUSSION

The initiation of HIV-1 reverse transcription occurs by extension of the cellular tRNA₃Lys primer annealed near the 5’ non-translated region of the viral RNA genome at a site called the PBS. The 18-nt PBS sequence, located downstream of the U5 region of the 5’ LTR, spans from nt 183–201 of the viral RNA genome and is complementary to the 3’ terminal nucleotides of the primer tRNA₃Lys (7). The A-loop region upstream of the PBS has been implicated in the selection and stabilization of tRNA₃Lys on the viral genome (10–12). In this study, we have employed a polyamide nucleotide analog (PNAAL) to target the A-loop region in order to examine its influence on tRNA₃Lys priming and on the process of reverse transcription. The sequences of PNAAL and non-specific scrambled PNA used in the experiments are shown in Figure 1A. Both PNAAL and scrambled PNA contain purine and pyrimidine bases linked with polyamide backbone in lieu of sugar–phosphate.
HIV-1 utilizes only rRNA, for the initiation of reverse transcription (9,35,36). The sequences upstream of the PBS are complementary to the anticodon loop and the TΨC loop and arm of tRNA, (Fig. 3), that have been proposed to be essential for conferring specificity for utilizing tRNA, for the initiation of reverse transcription (12). Among these, the A-loop region upstream of the PBS seems to interact with the U-rich anticodon (USUU) of the tRNA, primer (12,29,37). Mutational studies in this region have shown that the A-loop is essential for maintaining the selective use of the tRNA, primer (10,12,38). We therefore reasoned that sequestering of this important region may have a strong destabilizing effect on the formation of the [enzyme–tRNA,–viral RNA] complex and, in turn, may block the initiation of HIV-1 reverse transcription.

We therefore examined the formation of the [tRNA,–viral RNA] complex in the presence or absence of PNAAL or scrambled PNA. Results obtained from gel retardation studies are shown in Figure 4. As seen in Figure 4, the preformed [tRNA,–viral RNA] complex is disrupted by the interaction of PNAAL with its target sequence on the viral genome (Fig. 4, lanes 3 and 4). The non-specific scrambled PNA had no influence on this interaction (Fig. 4, lanes 5 and 6). It is possible that interaction between the tRNA, and PBS in the virion may be stabilized to a much higher ordered structure by interacting with the A-loop region of the viral RNA genome (39), and perturbation in this loop structure due to its interaction with the complementary PNAAL may have a strong destabilizing effect on the formation of the [tRNA,–viral RNA] complex. This interaction may, in turn, prevent initiation of HIV-1 reverse transcription and the transition of initiated primer to elongation.

In order to ascertain this possibility, reverse transcription of U5-PBS HIV-1 RNA primed with either 5′-32P-labeled 17mer DNA primer or with tRNA, by HIV-1 RT was examined in the absence or presence of PNAAL. Incubation of PNAAL with U5-PBS HIV-1 RNA pre-bound with the 17mer DNA primer had no effect on the initiation of reverse transcription but caused complete blockage of elongation near the A-loop region (Fig. 5). At molar ratios of 1:2, or higher, of template to PNAAL, extension of 32P-labeled DNA primer was aborted after processive addition of 9 nt upstream of the PBS region, the starting sequence for interaction of PNAAL (Fig. 5, lanes 4–6). During reverse transcription, HIV-1 RT has the ability to displace the RNA or DNA oligomers bound upstream on the template due to its strand displacement activity (19). However, the accumulation of products at the ninth template nucleotide position shows that it is unable to displace PNAAL due to strong binding of PNAAL to its target sequence. Similar inhibition of strand displacement activity of herpes simplex DNA helicase has been noted with the PNA–DNA complex (40).

As tRNA, is the natural primer for HIV-1 reverse transcription, we examined the effect of PNAAL on tRNA, primed initiation of reverse transcription catalyzed by HIV-1 RT. As expected, reaction incubation of PNAAL with the U5-PBS HIV-1 RNA pre-bound to tRNA, resulted in a significant inhibition of reverse transcription (Fig. 6, left panel, lanes 2–4). At a 1:4 ratio of template to PNAAL, complete abolition of initiation of reverse transcription was observed (Fig. 6, left panel, lane 4), thus indicating the ability of PNAAL molecules to destabilize the bound tRNA, primer by

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FIGURE 1. Sequence and general structure of scrambled and PNAAL targeted to the A-loop region of U5-PBS HIV-1 RNA. (A) General structure of PNA. (B) Secondary structure of the HIV-1 RNA genome corresponding to the primer binding site and A-loop region.

FIGURE 2. Specificity of PNAAL to its target sequence on the HIV-1 RNA. The binding specificity of PNAAL and scrambled PNA to the A-loop sequence was assessed by gel mobility shift of the 32P-labeled 200 base-long U5-PBS HIV-1 RNA as described under Materials and Methods. Lanes 1–7 represent molar ratios of U5-PBS RNA to PNAAL of 1:0, 1:0.2, 1:0.4, 1:1, 1:2.5, 1:5 and 1:10, respectively. Lanes 8–10 represent experiment with scrambled PNA at molar ratio of U5-PBS RNA to scrambled PNA of 1:2.5, 1:5 and 1:10, respectively.

backbone. The target sequence corresponding to the stem–loop upstream of the PBS region where PNAAL binds is shown in Figure 1B. To ascertain the ability of PNAAL to interact with the A-loop region, gel retardation assays were performed with 32P-labeled 200 base-long U5-PBS RNA and PNAAL (Fig. 2). Incubation of PNAAL with U5-PBS RNA, resulted in the formation of a specific RNA–PNAAL complex that could be detected by native PAGE. A shift in U5-PBS RNA mobility was noted with the appearance of a band that ran slower on the gel (Fig. 2, lanes 2–7). This slower moving complex was not present when scrambled PNA was used in place of PNAAL (Fig. 2, lanes 8–10).

During assembly of the HIV-1 virion, several cellular tRNAs including tRNA, are packaged into the virion particle but
invading the stem–loop target sequence on the viral RNA. Under similar conditions, scrambled PNA had no influence on the initiation and elongation process (Fig. 6, left panel, lanes 5–7). The inhibitory effect of PNA AL was more prominent when it was incubated with U5-PBS RNA along with tRNA3Lys (Fig. 6, right panel, lanes 2–4), suggesting that PNA AL can effectively inhibit reverse transcription by interfering with the priming of tRNA3Lys on the viral RNA or by displacing the primed tRNA3Lys from the template. The observed inhibition was concentration dependent with respect to PNA AL. A 1:4 molar ratio of template to PNA AL, complete blockage of initiation of reverse transcription was noted (Fig. 6, right panel, lane 4).

As the inhibitory function of PNA AL on the initiation of tRNA3Lys primed reverse transcription and its subsequent extension was observed in vitro in the absence of other viral components/proteins, it was of interest to see whether such inhibition could also be seen with the isolated HIV-1 virion particles. The disrupted HIV-1 virions containing endogenous tRNA3Lys-primed viral RNA genome and reverse transcriptase were incubated at various concentrations of PNA AL on ice for indicated time points, and then supplemented with four dNTPs plus [α-32P]dCTP to initiate the reaction. It was observed that at all the concentration ranges of PNA AL (50 nM to 2.5 µM) the initiation of reverse transcription was significantly reduced and the elongation of initiated product was efficiently blocked (Fig. 7, lanes 2–9). As expected, scrambled PNA had no influence on the endogenous reverse transcription. These results suggest that the interaction of PNA AL with the A-loop region on the viral genome may block the base pairing interaction between the anticodon of tRNA3Lys and A-loop, thereby destabilizing

Figure 3. Schematic representation showing the interaction of tRNA3Lys with the A-loop region of HIV-1 RNA. (A) The secondary structure of tRNA3Lys. The anticodon loop spans from nt 33 to 36. (B) Priming of the PBS region of U5-PBS RNA with the 3′ terminal 18 nt of tRNA3Lys. For clarity, the interaction of the anticodon loop of tRNA3Lys with the A-loop sequence of U5-PBS RNA is depicted by half bracket.

Figure 4. Destabilization of the interaction between tRNA3Lys and U5-PBS RNA by PNA AL. The labeled tRNA3Lys was incubated with the 495 base-long U5-PBS HIV-1 RNA in the presence or absence of PNA AL or scrambled PNA as described in Materials and Methods. The [U5-PBS RNA–tRNA3Lys] complex formed in the absence or presence of PNA AL was resolved by non-denaturing gel retardation analysis. Lane 1, 32P-labeled tRNA3Lys alone; lane 2–4, pre-formed [tRNA3Lys–U5-PBS RNA] complex incubated in the presence of PNA AL at molar ratios of U5-PBS RNA to PNA AL of 0.0, 1:5 and 1:10, respectively. Lanes 5 and 6 represent incubation of [tRNA3Lys–U5-PBS RNA] complex in the presence of scrambled PNA at molar ratios of U5-PBS RNA to scrambled PNA of 1:5 and 1:10, respectively. The positions of free tRNA3Lys as well as tRNA3Lys complexed with U5-PBS RNA are shown on the left.
the tRNA_{3Lys} priming on the viral genome. The observation of two major products longer than 400 nt in reactions with disrupted virions suggests that these products may have been generated as a result of strand transfer during reverse transcription. Surprisingly, the labeled endogenous tRNA ran below the expected 77 nt position. It is possible that some population of the endogenous tRNA_{3Lys} may have undergone cleavage of 5–6 nt either from the 5′ or the 3′ terminus during incubation with PNAAL or scrambled PNA. Prior to the initiation of reverse transcription, thus resulting in the shorter species.

It has been shown that dethiolation of mcm 5S2U at position 34 (26) or its modification from SUU to the suppressor anticodon CUA destabilizes the interaction between tRNA_{3Lys} and the viral RNA genome (41). The mutant tRNA_{3Lys} with CUU anticodon was efficiently packaged in vivo but was unable to initiate reverse transcription. The inability of in vivo packaged mutant tRNA_{3Lys} (CUU) to prime reverse transcription was suggested to be a consequence of its inability to interact with this A-rich loop of the viral genome. Wakefield et al. (9) have shown that the A-loop region is involved in primer tRNA_{3Lys} placement. Infection of cells with HIV-1 containing a PBS complementary to tRNA_{His} rather than tRNA_{3Lys} was found to quickly revert back to the original PBS complementary to tRNA_{3Lys}. However, when the A-loop was changed such that it was complementary to tRNA_{His} anticodon, the tRNA_{His} became stabilized in the viral population (12). It is obvious that the integrity of the A-loop structure is essential for initiation of reverse transcription and could be a prime target for halting viral replication. Our results show that polyamide nucleic acid, designed to complement the A-loop region, is able to block initiation of tRNA_{3Lys} primed reverse transcription but not with oligonucleotide primed reaction. However, the extension of oligonucleotide primed synthesis is completely terminated at the A-loop region suggesting that HIV-1 RT is unable to displace the PNA bound to the A-loop region.

The stability of PNAs in biological fluids together with its unique sequence specificity suggests that these synthetic poly-nucleobase molecules may have great potential as antisense agents in vivo. An antisense PNA targeted to the coding region of Ha-ras mRNA was found to interfere with translation initiation complex and arrest polypeptide chain elongation (42). Recently, this approach has been used to block telomerase activity by PNA targeted to telomerase RNA leading to progressive telomere shortening and causing immortal human breast epithelial cells to undergo apoptosis and cell death (43,44). Telomeres regained their initial length following removal of anti-telomerase PNA from the cell culture (45). The antisense and antigenic activity of PNAs targeted to AUG start codon and 5′ UTR of human B-cell lymphoma (Bcl)-2 gene,
viral proteins were incubated at varying concentrations of PNA and then subjected to transcription in disrupted HIV-1 virions. The disrupted HIV-1 virions containing endogenous reverse transcriptase products in the absence of PNA. Lanes 2–9 represent the reaction products were extracted with phenol–chloroform, precipitated with alcohol and analyzed on an 8% denaturing gel followed by visualization on a phosphorimager. Lane 1 is the control lane showing the endogenous reverse transcription process as described in Materials and Methods. The reverse transcription products in the absence of PNA. Lanes 2–9 represent the reactions carried out at PNA concentrations of 50, 100, 250, 500 and 750 nM and 1.2 and 2.5 μM, respectively. Lane 10 shows the reaction in the presence of 2.5 μM scrambled PNA. Molecular markers (M) are shown in the leftmost lane. The two major bands migrating above the 400 nt position represent products of strand transfer reaction.

were able to block (Bcl)-2 protein synthesis in a cell-free system. Repeated i.c.v. administration of PNA targeted to a region of the rat delta opioid receptor, significantly inhibited the antinociceptive response and locomotor response to delta opioid receptor agonists (47). Despite the extremely favorable and encouraging antisense activity of PNAs, their biodelivery into the cells remains a major obstacle. A PNA–DNA hybrid mimicking the NF-kappaB binding site of HIV-1 promoter has been reported (48). Recently, several studies have reported methods for effective biodelivery of PNAs and their antisense effects in cells. Certain peptide–PNA conjugates have been shown to be efficiently taken up by the cells and exhibit antisense activity on the targeted gene (49–56). A similar approach to improve cellular uptake of PNAs is currently in progress in our laboratory. A cocktail of such modified PNA targeted to various critical regions of the HIV-1 genome may be a potential inhibitor of HIV-1 replication.

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