Polyamide Nucleic Acid Targeted to the Primer Binding Site of the HIV-1 RNA Genome Blocks in Vitro HIV-1 Reverse Transcription †

ARTICLE *in* BIOCHEMISTRY · FEBRUARY 1998

Impact Factor: 3.02 · DOI: 10.1021/bi972197m · Source: PubMed

Polyamide Nucleic Acid Targeted to the Primer Binding Site of the HIV-1 RNA Genome Blocks *in Vitro* HIV-1 Reverse Transcription†

Reaching Lee, Neerja Kaushik, Mukund J. Modak, Ravi Vinayak,‡ and Virendra N. Pandey*

Department of Biochemistry and Molecular Biology, UMD−New Jersey Medical School, Newark, New Jersey 07103

Received September 5, 1997; Revised Manuscript Received November 5, 1997

ABSTRACT: We report here that polyamide nucleic acid (PNA) as well as a polyamide nucleic acid−DNA chimera complementary to the primer binding site of the HIV-1 genome can completely block priming by $tRNA_3^{Lys}$ and consequently the *in vitro* initiation of reverse transcription by HIV-1 RT. Conventional heating and cooling is not required for annealing PNA analogs to the complementary nucleotide sequence as effective blockage of reverse transcription results from their invasion in the duplex region of preprimed U5-PBS HIV-1 RNA template-primer and was seen even at ambient temperature. Further, the extension of the initiated nascent (−) strand DNA can also be blocked by inclusion of another PNA, targeted to upstream sequences in the U5 region of the viral RNA. Interestingly, a PNA chimera having only two DNA nucleotides annealed with the U5-PBS RNA is recognized as a bonafide primer by HIV-1 RT, as the 3′OH end of the chimeric molecule is extended by the enzyme in the presence of dNTPs. A significant observation was that RNA/PNA or RNA/(PNA−DNA) hybrids were entirely resistant to the RNase H activity of HIV-1 RT. Furthermore, PNA invasion into the RNA/DNA hybrid completely prevented the cleavage of the RNA strand, suggesting that the RNase H activity of HIV-1 RT which was required in reverse transcription may also be inhibited by the PNA oligomer. These observations suggest that oligomeric PNAs targeted to various critical regions of the viral genome are likely to have strong therapeutic potential for interrupting multiple steps involved in the replication of HIV-1 and warrant serious investigation especially in the area of an effective delivery system.

One of the major currently utilized therapies for AIDS is the use of inhibitors which block reverse transcription of viral RNA into double-stranded DNA. Several nucleoside analogs and non-nucleoside inhibitors exhibiting inhibitory activity on the viral reverse transcriptase have been deployed as chemotherapeutic agents (*1*). Recently, protease inhibitors have been developed which appear to block the process of viral maturation (*2*, *3*). However, the rapid emergence of mutant viral strains resistant to these inhibitors continues to pose a constant threat to containing this disease. Another potentially important approach is the use of antisense nucleic acid complementary to the target sequence of HIV-1 genomic RNA to block both mRNA translation and viral replication (*4*). Although this approach is very promising, the uptake and the instability of the antisense oligonucleotides in the cells are problems yet to be overcome (*5*). We have employed a new class of antisense nucleic acid analog, polyamide nucleic acid (PNA),[1] targeted to the primer binding site of U5-PBS HIV-1 genomic RNA that blocks the initiation of reverse transcription and its subsequent elongation. The PNAs are DNA analogs (Figure 1) in which the entire sugar phosphate backbone is replaced by *N*-(2-aminoethyl)glycine units (*6*). These analogs have been shown to be highly stable in human serum and cell extracts (*7*). The PNAs have been shown to recognize their complementary target in double-stranded DNA and are capable of forming exceptionably stable complexes by strand displacement (*6*, *8*). These analogs are able to form stable triplexes with dsDNA which are resistant to denaturation at temperatures where DNA duplex of identical sequence exhibits melting (*9*). Despite the high stability of the PNA−DNA complexes, single base pair mismatches greatly reduce the melting temperature, suggesting that PNAs are able to recognize dsDNA in a sequence-specific manner (*10*, *11*). PNA has been used as a sequence-specific blocker of DNA-recognizing enzymes, mostly restriction endonucleases (*12*). Because of the unique properties of PNA molecules to bind tightly to both RNA and DNA, we have utilized them in the study of the reverse transcriptase system. Very recently, Koppelhus et al. (*13*) reported *in vitro* inhibition of the

† This research was supported in part by a grant from the National Cancer Institute (CA72821 to V.N.P.).

* Address correspondence to this author. Telephone: 973-972-0660. FAX: 973-972-5594.

‡ Present address: PE Applied Biosystems, 850 Lincoln Center Dr., Foster City, CA 94404.

[1] Abbreviations: PNA, polyamide (peptide) nucleic acid; DiPEA, diisopropylethylamine; DMF, dimethylformamide; HBTU, benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HIV-1 RT, human immunodeficiency virus type 1 reverse transcriptase; IMAC, immobilized metal affinity chromatography; IDA−Sepharose, iminodiacetic acid−Sepharose; MuLV, murine leukemia virus; PBS-RNA template, HIV-1 genomic RNA template corresponding to the primer binding sequence region; PBS-DNA template, HIV-1 genomic DNA template corresponding to the RNA-PBS sequence; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DTT, dithiothreitol; IPTG, isopropyl β-thiogalactopyranoside; dNTP, deoxyribonucleoside triphosphate; dATP, dGTP, dCTP, and dTTP represent nucleoside triphosphates of deoxyadenosine, deoxyguanosine, deoxycytidine, and thymidine, respectively.

S0006-2960(97)02197-1 CCC: $15.00
Published on Web 01/01/1998

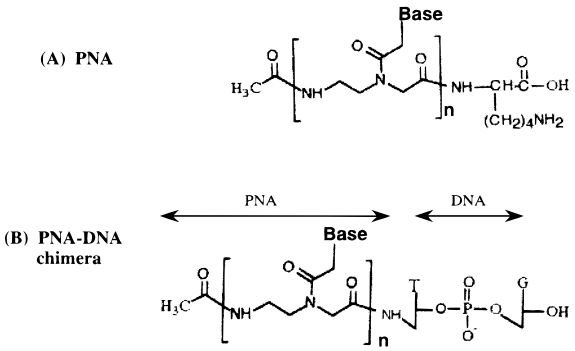

FIGURE 1: General structure for PNA (A) and PNA−DNA chimera (B). In the structure of PNA (A) and $(PNA)_{19}-(DNA)_2$ chimera (B), b represents purine (adenine, guanine) and pyrimidine (cytosine, thymine) bases linked with polyamide backbone, and n represents the number of these bases in the molecule. In the PNA molecule, a single Lys amino acid was attached at the end of the sequence while the $(PNA)_{19}-(DNA)_2$ chimeric molecule contained 19 bases in the form of PNA and a dinucleotide (T and G) with a 3′OH terminus.

polymerase activity of HIV-1 RT by PNA. While our results are in general agreement with this report, we have further analyzed the inhibitory effects of sequence-specific PNAs to show that they can effectively block reverse transcription by invading the duplex region of U5-PBS HIV-1 RNA primed with DNA primer or $tRNA_3^{Lys}$, a natural primer for viral replication. Interestingly, the effective binding/annealing of these analogs to the target sequences occurs at ambient temperature without the conventional heating−cooling process.

We describe here the results of our investigation with PNA and PNA−DNA oligomeric chimera that have sequence complementarity with the primer binding sequence (PBS) of the HIV-1 genomic RNA. PBS is the region where the 3′-end of the $tRNA_3^{Lys}$ anneals and allows reverse transcriptase to initiate the first strand DNA synthesis. We find that both PNA and PNA−DNA chimera bind to the primer binding site of the viral RNA genome and cause complete blockage of DNA synthesis. We also show that PNA is an effective blocker of the strand transfer reaction as PNA bound to the RNA template cannot be displaced by the strand displacement activity of HIV-1 RT. Furthermore, RNA−PNA hybrids are completely insensitive to the RNase H activity of HIV-1 RT. Our results strongly suggest the utility of PNAs as potential blockers of the multistep process of reverse transcription and that serious efforts be devoted to the development of an effective delivery system to introduce these analogs in cells to assess their *in vivo* efficacy.

MATERIALS AND METHODS

DNA-modifying enzymes were from Promega or Boehringer Mannheim. Tritiated dNTPs, [γ-^{32}P]ATP, and [α-^{32}P]dNTPs were the products of Dupont−New England Nuclear Inc. Synthetic oligomeric primers were obtained from Bio-Synthesis Inc. and were further purified by polyacrylamide gel electrophoresis. The DNA phosphoramidites, RNA phosphoramidites, ancillary reagents for DNA and RNA syntheses, diisopropylethylamine (DiPEA), dimethylformamide (DMF), and benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) were obtained from Applied Biosystems. All other reagents were of the highest available purity grade and purchased from Fisher, Millipore Corp., Boehringer Mannheim, and Bio-Rad.

Construction of Expression Plasmids. DNA manipulations were carried out according to standard protocol (*14*). We used the prokaryotic expression plasmid pKK223-3 (Pharmacia) for expression of the recombinant HIV-1 RT. This expression vector carries the strong *tac* promoter upstream of the multiple cloning site. The *Eco*R1 and *Hin*dIII fragments (1.68 kb) of pRC-RT plasmid (*15*) containing the full-length coding sequence of p66 HIV-1 RT were cloned into pKK223-3. The recombinant plasmid (pKK-p66) was introduced into JM109 for expression of the P66 HIV-1 RT. The 1.32 kb p51 coding region (codons 1−440) was amplified from pRC-RT by PCR, and the *Eco*R1 and *Hin*dIII sites were introduced at the 5′ and 3′ ends, respectively, along with a TAA stop codon at the 3′ end. The upstream and downstream p51 primer sequences were TATGGGGCGAA-TTCCCCATTAGCCCTATTG and TATAGGGCAAGCTTT-TAGAAGGTTTCTGCTCC, respectively. The amplified fragment was restriction-digested with *Eco*R1 and *Hin*dIII and ligated with PKK223-3 restriction-digested with the same two enzymes to construct PKK-p51. The p51 coding sequence of this plasmid ends with amino acid Phe440. The recombinant clones were introduced into *E. coli* JM109 for expression.

Overexpression and Isolation of p66/51 HIV-1 RT. E. coli JM109 carrying the recombinant plasmid was grown at 37 °C in Luria broth containing ampicillin (100 μg/mL). Induction by IPTG was performed at 0.5 OD_{595} as described before (*16*, *17*). The p66/51 heterodimeric enzyme was prepared by combining the cell pellets of the p66 and p51 clones at an appropriate ratio (*17*). The heterodimeric enzyme from the cell free lysate was prepared as described by Hsieh et al. (*18*). The purified protein was predominantly in the p66/p51 heterodimer form as judged by SDS−PAGE. The heterodimers were also prepared by reconstitution of the separately purified subunits. Protein concentrations were determined by using the BioRad colorimetric kit as well as by spectrophotometric measurements using $\epsilon_{280} = 2.62 \times 10^5$ M^{-1} cm^{-1} for p66/51 heterodimers (*19*).

U5-PBS Viral RNA Template. The HIV-RNA expression clone pHIV-PBS was a generous gift from Dr. M. A. Wainberg (*20*). It was used for the preparation of HIV-1 genomic RNA. This clone contains a 947 bp fragment of HIV-1 proviral genome (from +473 to + 1420) which includes the PBS region. The plasmid pHIV-PBS was linearized with *Acc*I and was transcribed using T7 RNA polymerase. The enzyme, buffer, and rNTP solutions were from Boehringer Mannheim, and the transcription reaction was carried out according to the manufacturer's protocol. Following the transcription reaction, 20 units of DNase I (RNase free) was added and incubated for 15 min to remove the DNA. The reaction mixture was extracted with phenol/chloroform, and the U5-PBS RNA transcripts were precipitated with ethanol, lyophilized, and redissolved in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1 unit/μL RNasin.

Preparation of Synthetic ^{32}P-Labeled $tRNA_3^{Lys}$. A $tRNA_3^{Lys}$ gene clone (pTL9) in pUC8 was obtained from Dr. S. F. J. Le Grice (*21*). A 256 bp region of the clone containing the T7 promoter upstream of the full-length tRNA gene was

Chart 1

1. HIV-1 U5-PBS RNA containing primer binding site.

<----------PBS----------->
3'- CAG GGA CAA GCC CGC GGU GAC GAU CUC UAA AAG GUG UGA CUG AUU UUC CCA GAC UCC CUA GAG AUC AAU GGU CUC AGU GUG UUG UCU GCC CGU GUG UGA UGA ACU UCC UGA GUU CCG UUC GAA AUA ACU CCG AAU UCG UCA CCC AAG GGA UCA UCG GUC UCU CGA GGG UCC GAG UCU AGA-5'

2. 18 mer DNA PBS primer.

5'-GTCCCTGTTCGGGCGCCA-3'

3. 22 mer DNA PBS primer.

5'-GTCCCTGTTCGGGCGCCACTGC-3

4. 36 mer DNA blocker(complementary to region +7 through +42 upstream of PBS).

5'-AGAGATTTTCCACACTGACTAAAAGGGTCTGAGGGA-3'

5. 21 mer **(PNA)$_{19}$-(DNA)$_2$** chimera complementary to PBS.

<------(PNA)$_{19}$-------><-(DNA)$_2$->

gtccctgttcgggcgccac-TG-3'OH

6. 21 mer PNA complementary to PBS.

<------(PNA)$_{21}$---------->

gtccctgttcgggcgccactg-Lys

7. (PNA)$_{20}$ Blocker.

tgagggatctctagttacca -Lys

(complementary to 34 nucleotide upstream of PBS).

8. 30 mer RNA corresponding to the U5-PBS region of the HIV-1 RNA genome.

3'-CAGGGACAAGCCCGCGGUGACGAUCUCUAA-5'

amplified by polymerase chain reaction as described by Richter et al. (*22*). The PCR product was used as the template for *in vitro* transcription of $tRNA_3^{Lys}$ using T7 RNA polymerase (*23*). For internal labeling of $tRNA_3^{Lys}$, 200 μM [α-^{32}P]UTP (specific activity 1 μCi/60 pmol; reaction volume 30 μL) was included in the transcription reaction. Following the transcription, the labeled tRNA was purified by denaturing 8% polyacrylamide−urea gel electrophoresis. The gel-purified ^{32}P-labeled $tRNA_3^{Lys}$ was stored at −70 °C in a solution containing 10 mM Tris-HCl, pH 7.0, and 1 unit/μL RNasin.

U5-PBS RNA Template Primed with 18-mer DNA or $tRNA_3^{Lys}$. An aliquot of the transcribed U5-PBS RNA (496 base long) was annealed either with 18-mer DNA primer complementary to the PBS (see Chart 1) or with synthetic $tRNA_3^{Lys}$. The molar ratio of RNA template to 18-mer DNA primer and to tRNA was approximately 1:1 and 2:1, respectively. Reverse transcription reactions were carried out by incubating 2.5 nM U5-PBS RNA/18-mer template-primer with 50 nM heterodimeric HIV-1 RT in a reaction mixture containing 25 mM Tris-HCl, pH 7.5, 10 mM DTT, 100 mg/mL BSA, 5 mM $MgCl_2$, 50 μM each of dATP, dTTP, and dGTP, and 5 μM [α-^{32}P]dCTP in a final volume of 5 μL. In some of the experiments, U5-PBS RNA template was primed with 5′-^{32}P-labeled 18-mer DNA primer, and the unlabeled dNTPs were supplemented in the reaction at 50 μM (each) concentration. The reaction was initiated by addition of the enzyme and terminated at different time intervals by the addition of an equal volume of Sanger's gel loading solution (*24*). The products were resolved on a 16% polyacrylamide−urea gel.

Synthesis of PBS-Targeted PNA and $(PNA)_{19}-(DNA)_2$ Chimera. The synthesis of the 21-mer PNA [5′-(N)>gtccctgttcgggcgccatg-Lys] and $(PNA)_{19}-(DNA)_2$ chimera [5′(N)>gtccctgttcgggcgccaT*G-3′OH] corresponding to the primer binding sequence (U5-PBS) of the HIV genome was carried out on an ABI 394 DNA/RNA synthesizer using reported protocols (*25*, *26*). The PNA bases are designated by lower case letters while DNA sequences are shown as upper case letters. In the first step of the synthesis of the chimera, 3′-*O*-phosphoramidite of 5′-deoxy-5′-*N*-(monomethoxytrityl)aminothymidine (T*) (*27*) was dissolved in acetonitrile to a concentration of 0.12 M and coupled onto the 3′-DNA support (G) using standard DNA synthesis cycles. Next, the PNA monomers were dissolved in 1:1 DMF/acetonitrile to a concentration of 0.2 M. To each of these monomer solutions was added an equal volume of a 0.2 M solution of HBTU (*O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate) in 1:1 DMF/acetonitrile. The coupling was effected in the presence of a 0.2 M solution of diisopropylethylamine (DiPEA) in 1:1 dimethylformamide (DMF)/acetonitrile. Finally, the 5′ (N) end of the PNA part was capped by acetylation using standard capping reagents, and the chimera was cleaved from the support (1 h, ambient temperature, ammonium hydroxide). The exocyclic amino groups were deprotected by heating the ammonium hydroxide solution at 55 °C for 12−16 h. The solution was then concentrated to half the original volume and then desalted by gel-filtration.

Annealing of PNA and $(PNA)_{19}-(DNA)_2$ Chimeric Primer to U5-PBS RNA or U5-PBS DNA Template. The 21-mer PNA−DNA chimera complementary to the PBS region contained 19 bases in the form of PNA and 2 bases with a 3′OH end in the form of DNA. The chimera was incubated with U5-PBS RNA or U5-PBS DNA template (see Chart 1) in the molar ratio of 1:1 in a solution containing 50 mM Tris−HCl, pH 7.5, 10 mM DTT, and 5 mM $MgCl_2$ either at ambient temperature (25 °C) or at 37 °C for the indicated period and used in the extension reaction.

In some of the experiments, PNA was incubated with PBS-RNA template already primed either with 5′-^{32}P-labeled 18-mer DNA or with $tRNA_3^{Lys}$. The extent of DNA synthesis catalyzed by HIV-1 RT in the presence and absence of the PNA or its chimeric analog was assessed as described above. A typical extension reaction was performed by incubating a 5 nM sample of the primed PBS template with the desired amount of PNA or $(PNA)_{19}-(DNA)_2$ chimera in 7 μL of 20 mM Tris-HCl, pH 7.5, and 50 mM NaCl for 60 min at 37 °C. Subsequently, 1 mL of 10× reaction buffer (500 mM Tris-HCl, pH 7.5, 10 mM DTT, 0.1% BSA, 50 mM $MgCl_2$) was added together with 50 μM each of dATP, dTTP, and dGTP and 5 μM [α-^{32}P]dCTP. In experiments with 5′-^{32}P-labeled 18-mer PBS primer, the unlabeled dNTPs were used at 50 μM concentration.

Utilization of the $(PNA)_{19}-(DNA)_2$ Chimera as the Primer for the Polymerase Reaction Catalyzed by HIV-1 RT. The $(PNA)_{19}-(DNA)_2$ chimera was annealed with U5-PBS RNA or U5-PBS DNA template by incubating equimolar concentrations of the chimera and the template at 25 °C for 2 h. The resulting hybrid was then used as the template-primer for the polymerase reaction catalyzed by HIV-1 RT under standard reaction conditions (*16*, *17*). The extension reactions contained 50 mM Tris-HCl, pH 7.5, 10 mM DTT, 100 nM RNA/$(PNA)_{19}-(DNA)_2$ or DNA/$(PNA)_{19}-(DNA)_2$ hybrid as template-primer, 5 mM $MgCl_2$, 50 μM each of dATP,

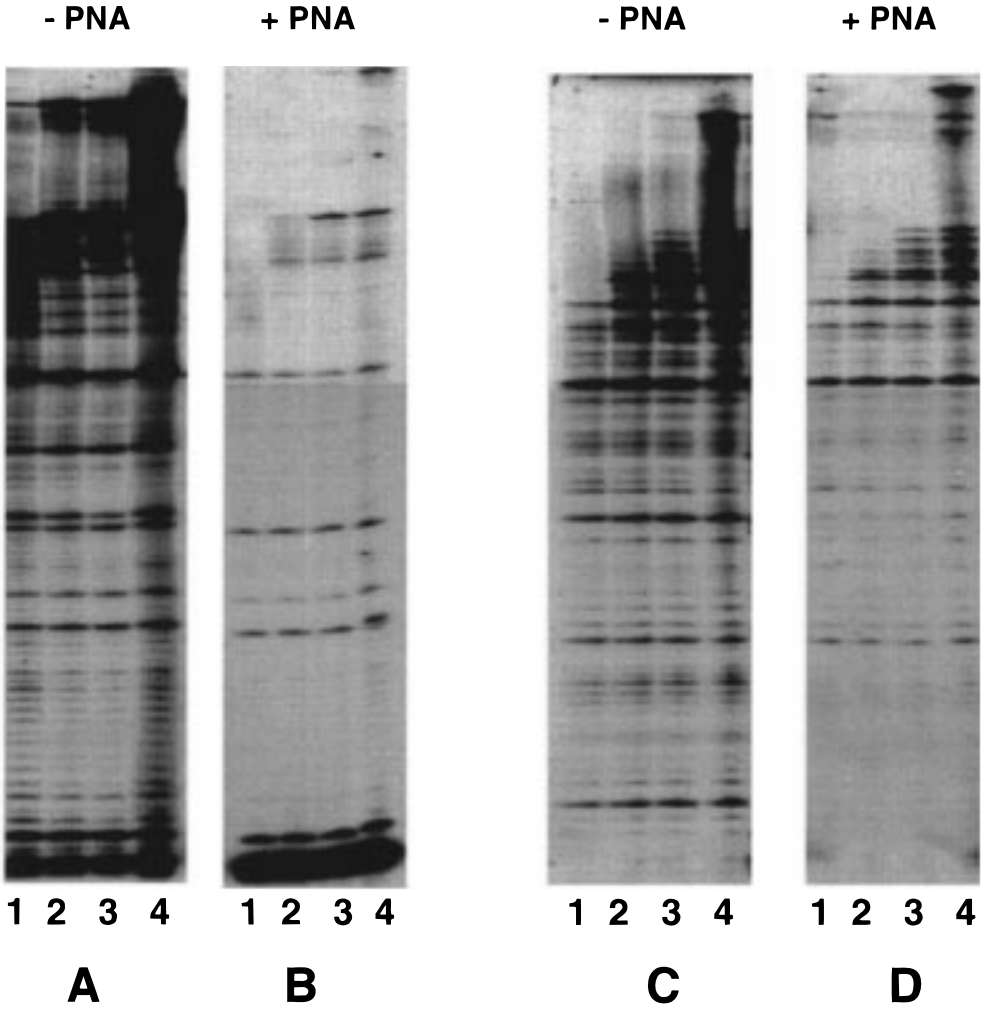

FIGURE 2: Initiation and extension of reverse transcription on primed HIV-1 RNA template are blocked by polyamide nucleic acid complementary to the PBS region. The HIV-1 RNA template annealed with either 5′-^{32}P-labeled or unlabeled oligomeric 18-mer DNA primer in a 2:1 molar ratio was used in the reaction. The template-primer was preincubated at 37 °C for 1 h in the absence or presence of PNA or the PNA chimera. With labeled template-primer (panels A and B), the reverse transcription reaction was initiated by the addition of 80 μM dNTPs (20 μM each) while with unlabeled template-primer (panels C and D), 5 μM α-^{32}P-labeled dCTP along with 20 μM each of dATP, dGTP, and dTTP was added in a total reaction volume of 10 μL under standard reaction conditions as described under Materials and Methods. The reactions were carried out at 37 °C for 5, 10, 15, and 30 min, and the reaction products were analyzed on a denaturing 8% polyacrylamide−urea gel. Panels A and B represent experiments where the primer was prelabeled with ^{32}P at the 5′ position. Panels C and D represent experiments in which the unlabeled primer was labeled during the extension reaction by incorporation of ^{32}P-labeled dCTPs. Lanes 1 through 4 in each panel represent the extension reaction at 37 °C for 5, 10, 15, and 30 min, respectively.

dTTP, and dGTP, 5 μM [α-^{32}P]dCTP (0.2 μCi/pmol), and 42 nM HIV-1 RT in a total volume of 10 μL. The reaction mixture was incubated for 30 min at room temperature and terminated by the addition of an equal volume of Sanger's gel loading solution. The samples were heated at 100 °C for 5 min and resolved by denaturing 8% polyacrylamide−urea gel electrophoresis followed by autoradiography.

Reverse Transcriptase/DNA Polymerase Reactions. The reactions were carried out in a final volume of 5−10 μL containing 50 mM Tris-HCl, pH 7.5, 10 mM dithiothreitol, 0.1 μg/mL bovine serum albumin, 5 mM $MgCl_2$, 2.5 nM 5′-^{32}P-primer (10^4 Cerenkov CPM) annealed with RNA or DNA template, and 20 μM of each of the dNTP substrates. With unlabeled template-primers, 20 μM each of dATP, dGTP, and TTP and 2 μM [α-^{32}P]dCTP (0.1−0.2 μCi/pmol) were used. The molar ratio of primer to template was 1:1 unless otherwise indicated. Any variation used in a specific experiment is as described in the corresponding figure legend. Unless otherwise indicated, all reactions were carried out at 37 °C. Reactions were quenched by the addition of Sanger's gel loading dye, and the reaction products were analyzed by denaturing polyacrylamide−urea gel electrophoresis (*17*).

Ribonuclease H Activity on the (PNA−DNA)/RNA Hybrid. We used a 30-meric synthetic U5-PBS RNA template corresponding to the PBS region of the HIV genome for assessing the RNase H activity. The RNA template was 5′ labeled and purified by 8% denaturing polyacrylamide−urea gel electrophoresis. The labeled template was annealed with 22-mer complementary DNA primer or $(PNA)_{19}-(DNA)_2$ chimera or with 21-mer PNA. The individual hybrids were separately incubated with the enzyme under standard reaction conditions. The reaction mixture (10 μL) contained 5 nM labeled hybrid (10^4 Cerenkov CPM), 80 mM NaCl, 5 mM $MgCl_2$, 50 mM Tris-HCl, pH 8.0, 0.1 mg/mL BSA, and 100 ng of enzyme. Reactions were carried out at 37 °C, and the reaction products were analyzed on a 16% denaturing polyacrylamide−urea gel.

RESULTS

Effect of the PNA and PNA−DNA Chimera on the Primer Extension Reaction Catalyzed by HIV-1 RT. A time course of extension of 5′-^{32}P-labeled and unlabeled 18-mer PBS DNA primer annealed with U5-PBS HIV-1 RNA template containing the primer binding site (PBS) by HIV-1 RT was examined in the absence and presence of PNA or its chimeric derivative. The PNA and its derivative $(PNA)_{19}-(DNA)_2$ chimera contained sequences complementary to the PBS region of the viral RNA genome. The first 19 bases of $(PNA)_{19}-(DNA)_2$ chimera are linked with a polyamide backbone, while the last 2 bases of the chimera are in the form of a DNA dinucleotide with a free 3′OH terminus. It was observed that when both PNA and $(PNA)_{19}-(DNA)_2$ chimera were preincubated for 1 h at 37 °C with U5-PBS HIV-1 RNA template already primed with 5′-^{32}P-18-mer PBS primer, they significantly inhibited the extension of the primer by HIV-1 RT. Results obtained in the absence and presence of PNA are shown in Figure 2A and Figure 2B, respectively. The inhibitory effect of PNA (or PNA chimera) on the extension reaction was seen more prominent with unlabeled 18-mer PBS primer and [α-^{32}P]dNTP substrate (Figure 2C,D). As shown in the figure, a marked reduction in the extension of the primer indicates the ability of the PNA molecules to invade the duplex region of the targeted sequence and thereby block the extension reaction (Figure 2D, lanes 1−4). Interestingly, the primer extension was found to be significantly reduced even when the preincubation of these analogs with the targeted template-primer was carried out at 25 °C. As shown in Figure 3, preincubation periods of as low as 4 h at 25 °C blocked the primer extension significantly (Figure 3A,B). However, preincubation periods of 12 h (lane 4) and 16 h (lane 5) with the PNA or PNA−DNA chimera appear to completely block the extension reaction by HIV-1 RT. For control experiments, viral RNA template primed with PBS primer was incubated for 16 h at room temperature and then used in the extension reaction (lanes 1 of panels A and B).

Similar experiments were carried out with U5-PBS DNA template primed with 5′-^{32}P-18-mer PBS primer (Figure 4A,B). The sequence of the 60-mer DNA template used here was the same as the viral U5-PBS RNA template. It was observed that both PNA and the $(PNA)_{19}-(DNA)_2$ chimera could block the extension reaction with DNA template. However, the extent of blockage was more pronounced with the RNA template as compared to the DNA template.

Sequence Specificity of PNAs Targeted to PBS. Since the PNA and its chimeric analog effectively block the extension reaction of HIV-1 RT on primed U5-PBS viral RNA and DNA templates, it was essential to ascertain if the observed inhibitory effect was due to its complementary sequence with respect to the PBS region of the template molecule. Therefore, another oligomeric DNA primer, complementary to sequences upstream of the PBS region, was annealed to U5-PBS RNA template, preincubated with PNA and $(PNA)_{19}-(DNA)_2$ for 5 h and 16 h, and was then used in the extension reaction. The results shown in Figure 5 clearly indicate that the PNA and its chimeric analog, with complementarity to the PBS region, have no influence on the extension of non-PBS DNA primer. As shown in the figure, preincubation of these analogs with the primed U5-PBS RNA template for 5 h (lanes 2, 4) and 16 h (lanes 3, 5) does not block the extension reaction initiated upstream of the PBS region.

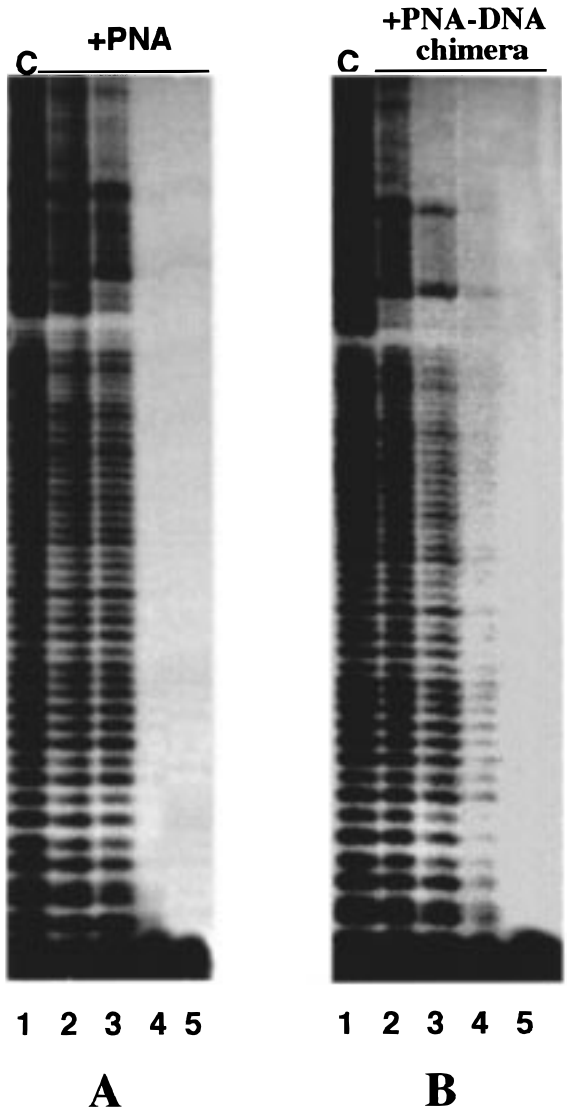

FIGURE 3: PNA and PNA−DNA chimera are able to block the extension reaction when preincubated at ambient temperature with the viral RNA template primed with PBS DNA primer. The HIV-1 RNA template primed with the ^{32}P-labeled 18-mer DNA primer was preincubated with PNA or the $(PNA)_{19}-(DNA)_2$ chimera as described in legend to Figure 1 except that preincubation was carried out at ambient temperature (25 °C) for 4−16 h. The control sample was also preincubated for 16 h without these analogs. Reverse transcription was initiated by the addition of dNTP under standard reaction conditions, and the products were analyzed on a denaturing urea−polyacrylamide gel. Lane 1, control without PNA or the $(PNA)_{19}-(DNA)_2$ chimera; lanes 2 through 5 represent preincubation with the indicated analog for 4, 8, 12, and 16 h at 25 °C prior to initiation of reverse transcription.

Inhibition of Extension of the $tRNA_3^{Lys}$ Primer Annealed with PBS RNA Template. Since $tRNA_3^{Lys}$ is a natural primer for initiation of reverse transcription of the viral RNA genome, we examined the effect of the PBS-targeted PNA molecules on the first strand DNA synthesis with $tRNA_3^{Lys}$-primed viral U5-PBS RNA template. The results of this experiment are depicted in Figure 6. As shown in the figure, both PNA and the PNA chimera can effectively block the synthesis of DNA by interacting with the tRNA-primed PBS region of the viral genome. Addition of a severalfold excess

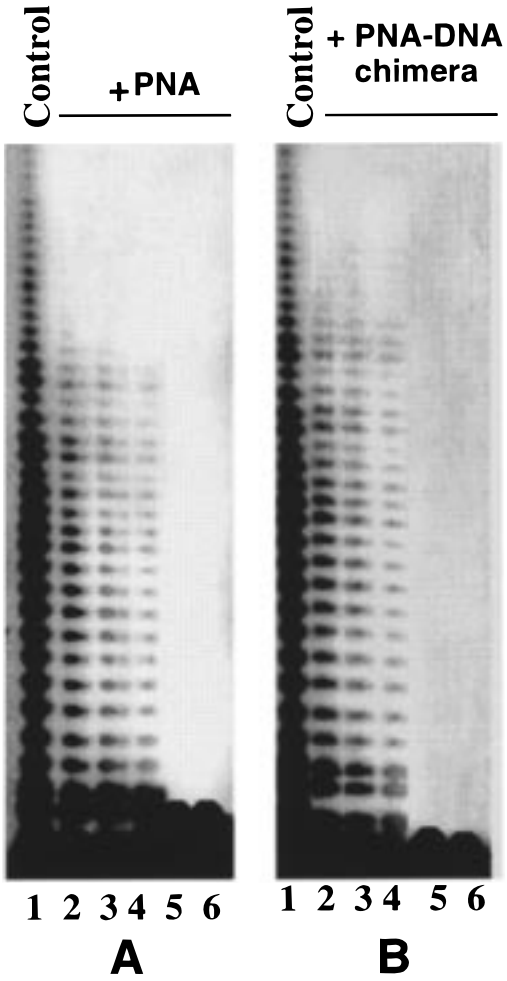

FIGURE 4: Effect of PNA or the PNA−DNA chimera on the polymerase reaction of HIV-1 RT with DNA template primed with PBS DNA primer. A 60-mer DNA template corresponding to viral RNA template was primed with the labeled 18-mer DNA PBS primer and used for this set of experiments. Preincubation of the template-primer with PNA or the $(PNA)_{19}-(DNA)_2$ chimera was carried out at 25 °C for 4−16 h as described in the legend to Figure 2. The polymerase reaction was initiated by supplementing dNTP substrate under standard reaction conditions, and the products were analyzed on a denaturing urea−polyacrylamide gel. Lane 1, 16 h control without the analog; lanes 2 through 6 represent preincubation of template-primer with the indicated analog for 2, 4, 8, 12, and 16 h prior to initiation of the polymerase reaction.

of $tRNA_3^{Lys}$ in the reaction did not revert the inhibition of the extension reaction, suggesting that PNA molecules targeted to PBS are able to either invade or displace the $tRNA_3^{Lys}$-primed duplex region of U5-PBS RNA at room temperature, resulting in blockage of the initiation of reverse transcription.

Is $(PNA)_{19}-(DNA)_2$ Chimera Annealed with Viral RNA Template a Substrate for HIV-1RT? The strong inhibition of primer extension reactions, primed with $tRNA_3^{Lys}$ or DNA primer by both PNA and $(PNA)_{19}-(DNA)_2$ chimera, suggests the ability of the latter to invade the double stranded PBS region of the template, resulting in complete blockage of the extension reaction. However, a possibility that the $(PNA)_{19}-(DNA)_2$ chimera anneals with the RNA template by displacing the original primer and thereby serve as a primer needed to be ruled out. To eliminate this possibility, the U5-PBS RNA annealed with $(PNA)_{19}-(DNA)_2$ chimera at 25 °C for 2 h was used as the template-primer in the

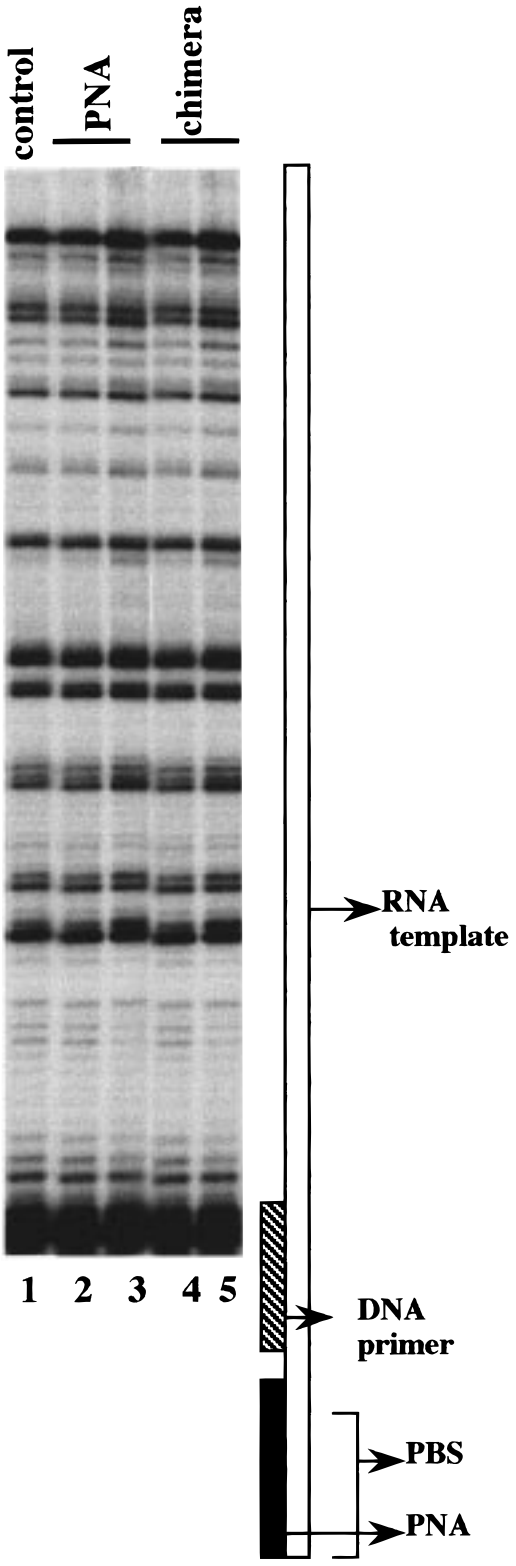

FIGURE 5: PNA targeted to PBS does not influence the extension initiated at a different site upstream of the PBS region. HIV-1 RNA template was annealed with ^{32}P-labeled 17-mer DNA primer upstream of PBS and then preincubated at 37 °C for 4−16 h with PNA-21 targeted to the PBS region downstream to the primed region. The extension reaction was initiated by the addition of dNTP, and the products were analyzed on a polyacrylamide gel as described before. Lane 1, control without PNA; lanes 2 through 5 represent preincubation with PNA-21 for 4, 8, 12, and 16 h prior to the initiation of reverse transcription.

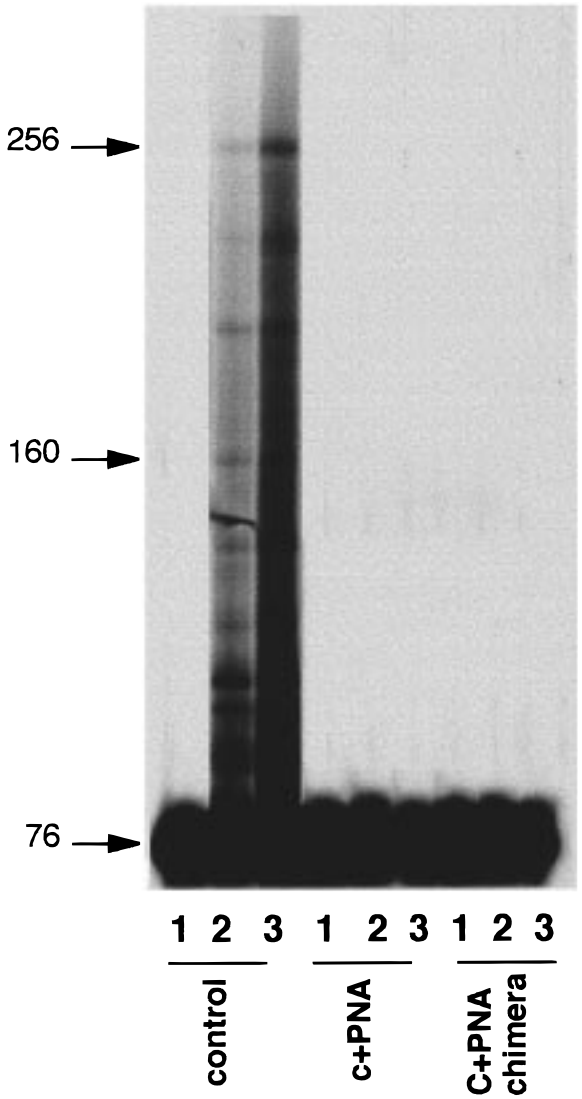

FIGURE 6: Initiation of reverse transcription on $tRNA_3^{Lys}$-primed U5-PBS viral RNA template can be blocked by PBS-targeted PNA. The U5-PBS RNA transcript (496 base long) annealed with internally ^{32}P-labeled $tRNA_3^{Lys}$ (10^5 Cerenkov cpm/lane) was incubated without PNA (control) and with PNA (C+PNA) and PNA chimera (C+PNA chimera) for 16 h at 25 °C. Initiation of reverse transcription and subsequent extension by heterodimeric HIV-1 RT were carried out as described under Materials and Methods. Lane 1 of each panel is the reaction control without HIV-1 RT. Lanes 2 and 3 in each panel indicate the reaction products formed in 30 min and 2 h incubation at 37 °C. The product length is indicated by an arrow on the left panel.

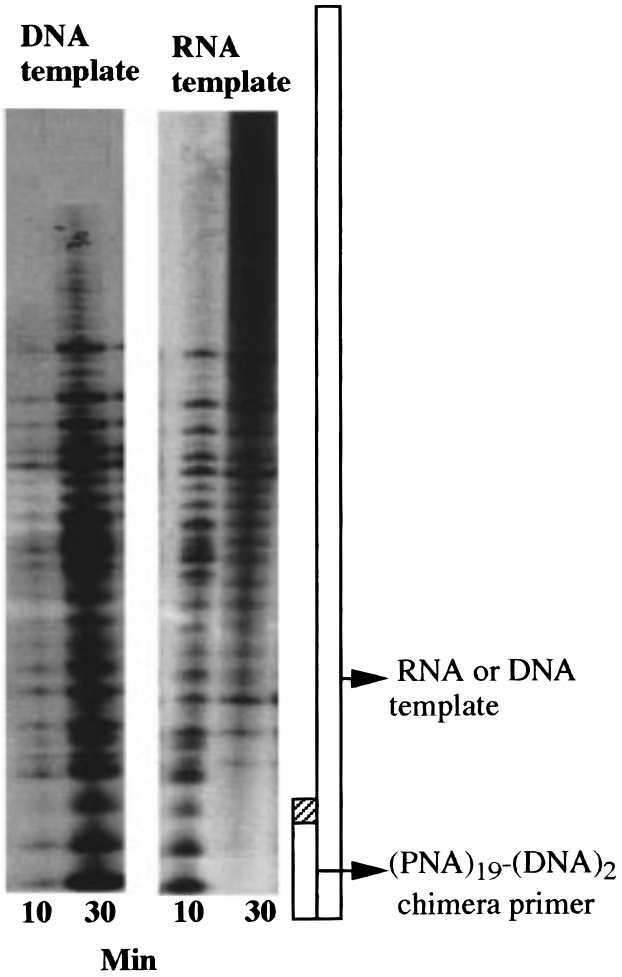

FIGURE 7: PNA−DNA chimera targeted to PBS of U5-PBS viral RNA is a primer substrate for the extension reaction catalyzed by HIV-1 RT. The $(PNA)_{19}-(DNA)_2$ chimera was annealed with U5-PBS RNA as well as with U5-PBS DNA templates by preincubating at 25 °C for 2 h. The annealed hybrids were used as the template-primer for the polymerase reaction catalyzed by HIV-1 RT. The extension reaction was initiated under standard conditions by supplementing all four dNTPs along with α-^{32}P-labeled dCTP as described under Materials and Methods. The reaction products were analyzed by denaturing 8% polyacrylamide−urea gel electrophoresis. Panel A, $(PNA)_{19}-(DNA)_2$ annealed with DNA template; panel B, $(PNA)_{19}-(DNA)_2$ annealed with RNA template.

polymerase reaction catalyzed by HIV-1 RT. The results shown in Figure 7 suggest that $(PNA)_{19}-(DNA)_2$ chimera containing only a dinucleotide with a 3′OH terminus is efficiently extended in a fashion similar to that observed with the normal primer. Thus, the inhibitory effect of the PNA chimera but not that of PNA could conceivably occur via displacement of the normal primer and resumption of synthesis on the PNA chimeric primer. Although the chimeric primer could be extended, the effect of the PNA portion of the chimera appears to interfere with the subsequent step of reverse transcription (see below), i.e., strand displacement and RNase H cleavage.

A PNA Molecule Annealed Upstream to the PBS Region Can Completely Block the Strand Displacement Activity of HIV-1 RT. Since PBS-targeted PNA and its analog were found to be highly effective in blocking the initiation of the first strand DNA synthesis, we examined the effects of a PNA molecule complementary to an upstream sequence of U5-PBS on the extension of PBS DNA primer. The non-PBS-PNA (PNA-20) used in this experiment was complementary to the RNA template between positions +34 and +53 from the primer terminus (see Chart 1). The U5-PBS viral RNA annealed with an 18-mer DNA primer was preincubated with a 2-fold molar excess of PNA-20 for 1−4 h at 25 °C and then used in the extension reactions. It was observed that HIV-1 RT can extend the DNA primer until it encounters the PNA−RNA duplex on the template at a position 34 nucleotides away from the PBS region (Figure 8A, lanes 2−5). The complete termination of the extension reaction at the site of PNA-20 binding is even seen with preincubation periods as low as 1 h and at 25 °C prior to the extension reaction (Figure 8A, lane 2). These observations clearly suggest that the oligo PNA, base-paired to the RNA template, is not susceptible to the DNA strand

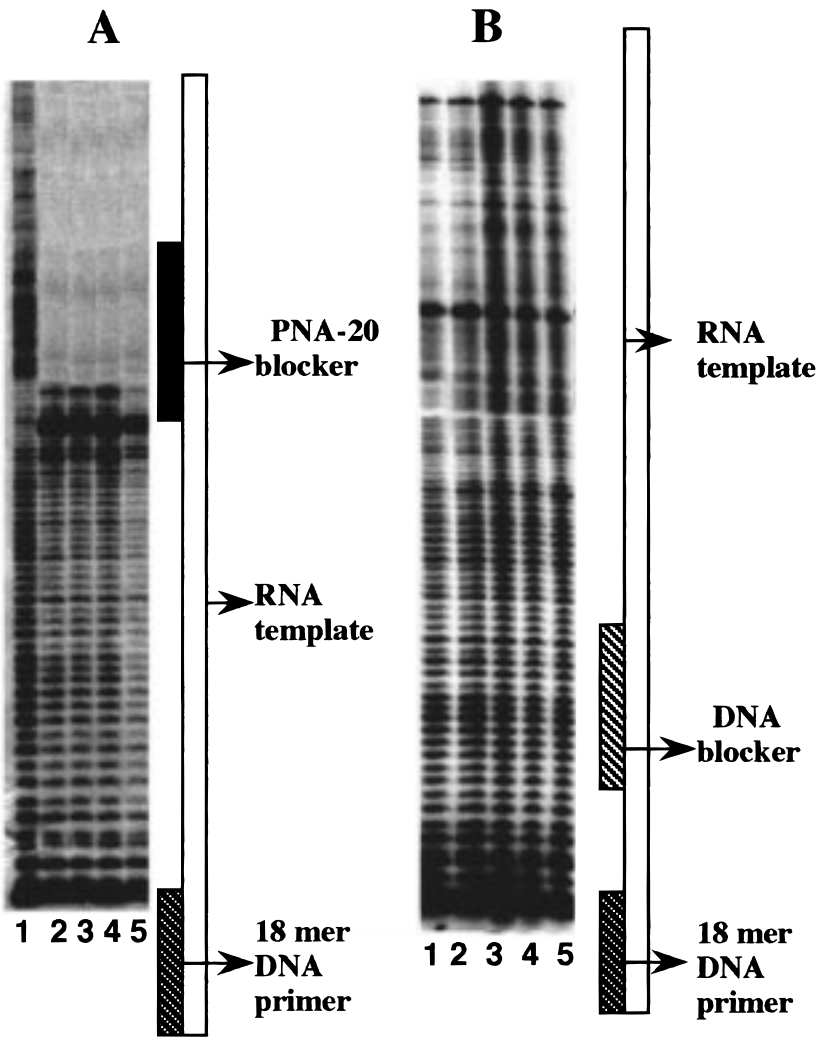

FIGURE 8: Termination of the extension reaction by PNA targeted to sequences upstream of PBS. HIV-1 RNA template primed with ^{32}P-labeled PBS DNA primer was preincubated with PNA-20 for 4−16 h at 25 °C. PNA-20 is complementary to the region 34 nucleotides upstream of the start site (PBS) of the initiation of reverse transcription. The extension reaction was initiated by the addition of dNTP substrates, and the products were analyzed as described before. For comparison, a 36-mer DNA blocker complementary to +7 through +42 nucleotides upstream of PBS was annealed with the primed U5-PBS RNA, and the extension products were analyzed. Panels A and B represent the extension reaction in the presence of PNA-20 blocker and DNA-36 blocker, respectively. Lane 1, control without the blocker; lanes 2 through 5 represent preincubation of template-primer with the blocker for 1, 2, 3, and 4 h, respectively.

displacement activity of the processive enzyme. However, a 36-mer DNA blocker (see Chart 1) annealed upstream to the PBS region between +7 and +42 template nucleotides from the primer terminus can be effectively displaced by the enzyme during the extension reaction (Figure 8B).

Melting Temperature for PNA−RNA and PNA−DNA Duplexes. Since the strand displacement activity of HIV-1 RT is ineffective in displacing the PNA blocker during DNA synthesis, it was reasoned that the binding of these molecules to the template strand may be very tight and may require exceptionally high energy for strand displacement/melting. We therefore measured the melting temperature (T_m) for these duplexes and compared them with the T_m obtained for normal DNA−DNA and RNA−DNA duplexes of identical sequences. Both 21-mer PNA and 22-mer PBS DNA were separately annealed with 30-mer RNA and 60-mer DNA templates, and their T_m's were determined using a standard protocol. It was observed that the T_m for both $((PNA)_{19}-(DNA)_2)$/RNA and PNA−RNA duplexes was higher by 10−15 °C than that for DNA−DNA and RNA−DNA duplexes

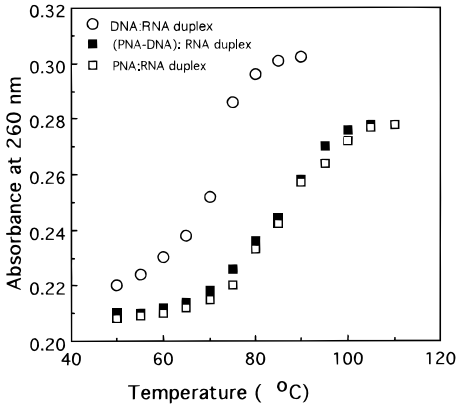

FIGURE 9: T_m curve for DNA−DNA, PNA−DNA, and (PNA−DNA)/DNA hybrid. The respective duplexes were prepared by annealing equimolar amounts of 30-mer RNA template and the respective primer. The temperature was increased in 5 °C increments, and the OD_{260} was recorded.

(Figure 9). We could not completely separate PNA and its analog from the corresponding template strand by heating at 100 °C in the formamide solution as seen from the

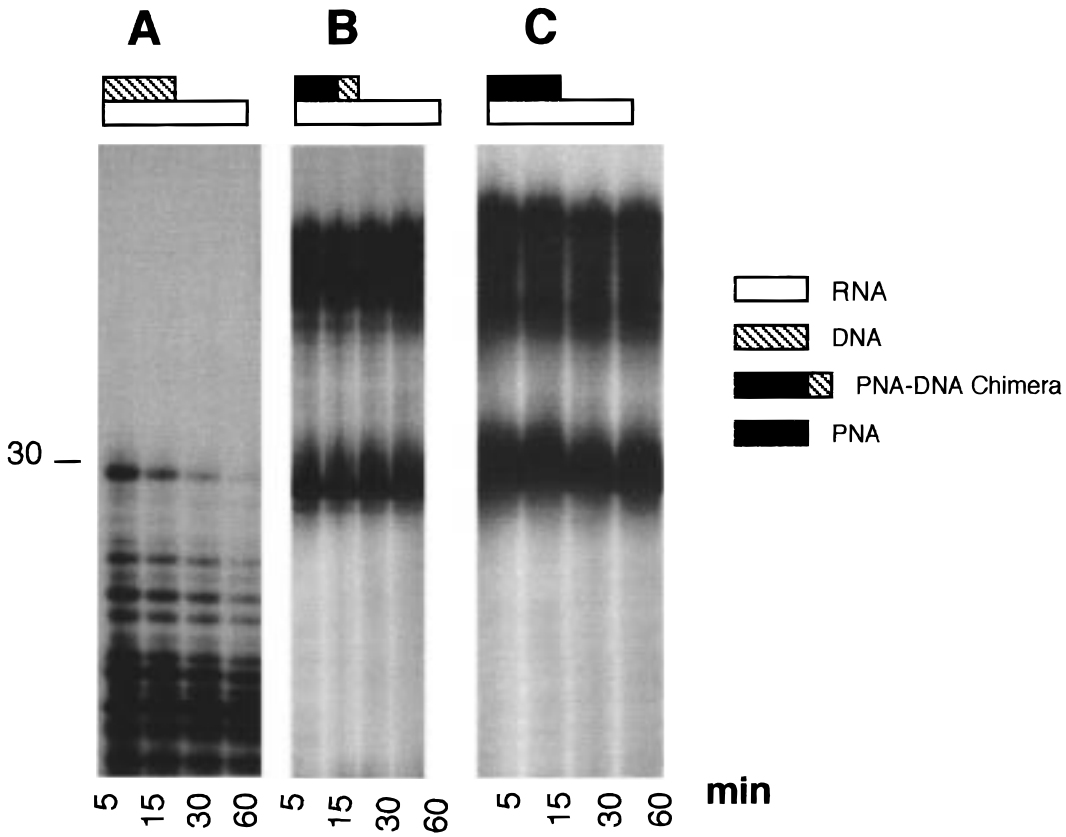

FIGURE 10: PNA−RNA and $(PNA)_{19}-(DNA)_2$/RNA hybrids are insensitive to the RNase H activity of HIV-1 RT. A synthetic 5′-^{32}P-labeled 30-mer RNA corresponding to HIV-1 U5-PBS RNA template was individually annealed with a 2 mol excess of complementary 22-mer DNA, 21-mer PNA, or 21-mer $(PNA)_{19}-(DNA)_2$ chimera. Annealing was carried out either by heating the mixture at 70 °C followed by slow-cooling to room temperature or by incubating the mixture at 37 °C for 60 min. The resulting hybrids were incubated with heterodimeric HIV-1 RT for various time points under standard reaction conditions. The cleavage products of hybrid RNA from RNA−DNA (panel A), $(PNA)_{19}-(DNA)_2$/RNA (panel B), and PNA−RNA (panel C) were monitored by denaturing polyacrylamide−urea gel electrophoresis as described under Materials and Methods.

significant retardation of the template strand on the autoradiogram of the polyacrylamide−urea gel (see Figures 10).

The $(PNA)_{19}-(DNA)_2$/RNA Hybrid Is Insensitive to the RNase H Activity of HIV-1 RT. Since $(PNA)_{19}-(DNA)_2$ chimera which was annealed with the complementary U5-PBS RNA template could be extended by HIV-1 RT, the next step was to examine whether the hybrid containing the chimera and RNA was susceptible to the RNase H activity of the enzyme. A synthetic 5′-^{32}P-labeled 30-mer U5-PBS RNA was annealed with (a) $(PNA)_{19}-(DNA)_2$ chimera, (b) 21-mer PNA, or (c) 22-mer DNA. The individual hybrid substrate was then incubated with the heterodimeric HIV-1 RT under standard reaction conditions. The cleavage products of the hybrid RNA molecule were monitored by denaturing polyacrylamide urea gel electrophoresis. The results depicted in Figure 10 indicate that the RNA template annealed with either PNA (panel B) or PNA−DNA chimera (panel C) was completely resistant to the RNase H cleavage activity of HIV-1 RT, while the standard RNA−DNA hybrid comprised of 30-mer RNA and 22-mer PBS DNA was rapidly cleaved into smaller fragments under similar conditions (panel A). Similar results were also obtained with preformed RNA−DNA hybrids preincubated with PNA or PNA chimera for 10 h at 25 °C, suggesting that PNA is able to invade the duplex hybrid region to block the RNase H cleavage activity (data not shown).

DISCUSSION

The results presented here suggest that PNA and PNA−DNA chimera targeted to the PBS region of the HIV genome can effectively inhibit the initial priming process of HIV-1 cDNA synthesis. These nucleic acid analogs also inhibit the strand displacement activity of the enzyme and the elongation process of DNA synthesis. An increase of 10−15 °C in the T_m for both PNA−RNA and (PNA−DNA)/RNA duplexes compared with their respective oligomeric DNA revealed a selectively higher affinity of these analogs to the PBS region. This increase in the T_m of the duplex may be exploited in sequestering the PBS region on the HIV-1 genomic RNA, thus making it unavailable for initiation of the first strand DNA synthesis. This possibility was examined by using the 5′ U5-PBS genomic RNA of HIV-1 primed with 18-mer DNA oligomeric primer. As expected, both PNA and its $(PNA)_{19}-(DNA)_2$ chimeric derivative targeted to the PBS region were able to invade the duplex region to form a triplex structure, resulting in a near-complete inhibition of primer extension (Figures 2, 3, and 4). The results were more prominent when the synthetic $tRNA_3^{Lys}$ primer was annealed to the PBS region. Preincubation of the RNA−tRNA template-primer with PNA rendered the tRNA ineffective in initiating DNA synthesis (Figure 6). Interestingly, PNA and its derivatives exhibit this effect at ambient temperature (25 °C), a prerequisite for a genome-targeted drug, and do not require the conventional heating−cooling process for

binding/annealing to the target sequences. It was further ascertained that the binding of these analogs to the HIV-1 RNA genome is sequence-specific and occurred by annealing to the targeted PBS region of the template; a PNA complementary to a region other than the PBS region was ineffective in blocking the initiation of reverse transcription (Figure 5). Furthermore, a PNA molecule complementary to the upstream region of PBS promptly caused termination of DNA synthesis initiated from the PBS, upon encountering the PNA molecule (Figure 8A). It was also observed that the enzyme carrying out reverse transcription along the RNA template was unable to displace a PNA molecule bound to a target sequence upstream of the U5- PBS region although it readily displaced a DNA blocker of a larger size annealed between +7 and +42 nucleotides from the primer terminus (Figure 8B). These results suggest that PNA bound to its target site is resistant to the strand displacement activity of HIV-1 RT. Significantly, the RNase H activity of HIV-1 RT is also inhibited by PNA and $(PNA)_{19}-(DNA)_2$ chimera as judged by the total resistance of the hybrid of 21-mer PNA/30-mer RNA as well as $(PNA)_{19}-(DNA)_2$/RNA to RNase H cleavage (Figure 10). The oligomeric RNA, annealed to a complementary DNA in lieu of PNA, serves as substrate for the RNase H activity of the enzyme.

Although PNA molecules have been shown to recognize complementary sequences and follow the Watson−Crick base-pairing rule, they can form both parallel and antiparallel duplexes (*28*). In the present study, we have shown that the $(PNA)_{19}-(DNA)_2$ chimera anneals with the RNA template in an antiparallel fashion, as judged by the extension of the chimera annealed with the U5-PBS RNA template in the polymerase reaction catalyzed by HIV-1 RT. These observations suggest that the duplex comprised of PNA and viral RNA could form a dead end complex from which the genomic RNA cannot be used either for initiation of reverse transcription or for RNase H cleavage. Further, the extension of the (−) strand DNA synthesis can be blocked by placing appropriate PNAs along the RNA template.

Thus, the use of PNAs targeted to multiple sites on the viral genome could be effectively used to ensure complete blockage of the process of reverse transcription. Some possible target sites on the 5′ nontranslated region, in addition to PBS, could include (i) the LTR region to block the strand transfer reaction during reverse transcription, (ii) the dimerization initiation site (DIS) to prevent genomic RNA packaging and possibly in the second strand transfer reaction during reverse transcription (*29*), and (iii) *att* sites in the U3 and U5 regions of linear proviral DNA to block integration with the host genome. The results discussed above strongly suggest that PNAs either by themselves or in the chimeric form (presumably permitting additional modification in the nucleic acid part) are effective blockers of reverse transcription (both RNA-dependent and DNA-dependent DNA polymerase activities). Their inhibitory effect on the RNase H and strand displacement activities of the enzyme further enhances the therapeutic potential of these molecules for halting and aborting the process of HIV-1 replication. In fact, oligomeric PNAs targeted to multiple sites may be expected to provide an additive effect, and such an approach in principle seems quite appealing in adapting these molecules as specific inhibitors of HIV-1 replication as well as for general antiretroviral agents. It becomes pertinent that appropriate vehicles to deliver PNA or PNA−DNA chimera into the host cell be developed so that their *in vivo* utility can be assessed.

ACKNOWLEDGMENT

The authors/R.V. thank Rick Brill (Applied Biosystems) and A. C. van der laan (Leiden University, The Netherlands) for providing some of the PNA monomers.

REFERENCES

1. Larder, B. A. (1993) in *Reverse Transcriptase* (Skalka, A. M., and Goff, S. P., Eds.) p 205, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
2. Wei, X., Ghos, S. K., Taylor, M. E., Johnson, V. A., Emini, E. A., Deutsch, P., Lifson, J. D., Bonhoeffer, S., Nowak, M. A., Hahn, B. H., Sag, M. S., and Shaw, G. M. (1995) *Nature 373*, 117−122.
3. Ho, D. D., Neumann, A. U., Perelson, A. S., Chen, W., Leonard, J. M., and Markowitz, M. (1995) *Nature 373*, 123−126.
4. Kilkuskie, R. E., and Field, A. K. (1997) *Adv. Pharmacol. 40*, 437−483.
5. Crooke, S. (1997) *Adv. Pharmacol. 40*, 1−49.
6. Egholm, M., Buchardt, O., Nielsen, P. E., and Berg, R. H. (1992a) *J. Am. Chem. Soc. 114*, 1895−1897.
7. Demidov, V. V., Potaman, V. N., Frank-Kamenetskii, M. D., Egholm, M., Buchardt, O., Sonnichsen, S. H., and Nielsen, P. E. (1994) *Biochem. Pharmacol. 48*, 1310−1313.
8. Egholm, M., Buchardt, O., Nielsen, P. E., and Berg, R. H. (1992b) *J. Am. Chem. Soc. 114*, 9677−9678.
9. Kim, S. K., Nielsen, P. E., Egholm, M., Buchardt, O., Berg, R. H., and Norden, B. (1993) *J. Am. Chem. Soc. 115*, 6477−6481.
10. Nielsen, P. E., Egholm, M., Berg, R. H., and Buchardt, O. (1991) *Science 254*, 1497−1500.
11. Demidov, V. V., Yavnilovich, M. V., Belotserkovskii, B. P., Frank-Kamenetskii, M. D., and Nielsen, P. E. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 2637−2641.
12. Nielsen, P. E., Egholm, M., Berg, R. H., and Buchardt, O. (1993) *Nucleic Acids Res. 21*, 197−200.
13. Koppelhus, U., Zachar, V., Nielsen, P. E., Liu, X., Eugen-Olsen, J., and Ebbesen, P. (1997) *Nucleic Acids Res. 25*, 2167−2173.
14. Ausubel F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. S., Smith, J. A., and Struhl, K. (1987) *Current protocols in Molecular Biology*, Greene Publishing Associates and Wiley-Intersciences, John Wiley & Sons, New York.
15. Bacerra, S. P., Kumar, A., Lewis, M. S., Widen, S. G., Hughes, S., and Wilson, S. H. (1991) *Biochemistry 30*, 11707−11719.
16. Pandey, V. N., Kaushik, N., Rege, N., Sarafianos, S. G., Yadav, P. N. S., and Modak, M. J. (1996) *Biochemistry 35*, 2168−2179.
17. Kaushik, N., Rege, N., Yadav, P. N. S., Sarafianos, S. G., Modak, M. J., and Pandey, V. N. (1996) *Biochemistry 35*, 11536 −11546.
18. Hsieh, J. C., Zinnen, S., and Modrich, P. (1993) *J. Biol. Chem. 268*, 24607−24613.
19. Kati, W. M., Johnson, K. A., Jerva, L. F., and Anderson, K. S. (1992) *J. Biol. Chem. 267*, 25988−25997.
20. Arts, E. J., Li, X., Gu, Z., Kleiman, L., Parniak, M., and Wainberg, M. A. (1994) *J. Biol. Chem. 269*, 14672−14680.
21. Barat, C., Le Grice, S. F. J., and Darlix, J. (1991) *Nucleic Acids Res. 19*, 751−757.
22. Richter, N. J., Howard, K. J., Cirino, N. M., Wohrl, B. M., and Le Grice, S. F. J. (1992) *J. Biol. Chem. 267*, 15952−15957.
23. Arts, E. J., Ghosh, M., Jacques, P. S., Ehresmann, B., and LeGrice, S. F. J. (1996) *J. Biol. Chem. 271*, 9054−9061.

24. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.
25. Van der Laan, A. C., Brill, R., Kuimelis, R., Kuyl-Yeheskiely, E., van Boom, J. H., Andrus, A., and Vinayak, R. (1997) *Tetrahedron Lett. 38*, 2249−2252.
26. Vinayak, R., van der Laan, A. C., Brill, R., Otteson, K., Kuyl-Yeheskiely, E., Andrus, A., and van Boom, J. H. (1996) 12th International Roundtable on Nucleoside, Nucleotides and their biological Applications, September 15−19, 1996, La Jolla, CA (Proceedings to be published in *Nucleosides Nucleotides*).
27. Smith, L. M., Kaiser, R. J., Sanders, J. Z., and Hood, L. E. (1987) *Methods Enzymol. 155*, 260−301.
28. Wittung, P., Nielsen, P. E., Burchardt, O., Egholm, M., and Norden, B. (1994) *Nature 368*, 561−563.
29. Paillart, J. C., Berthous, L., Ottmann, M., Darlix, J. L., Marquet, R., Ehresmann, B., and Ehresmann, C. (1996) *J. Virol. 70*, 8348−8354.

BI972197M