

# Efficient *in vitro* inhibition of HIV-1 *gag* reverse transcription by peptide nucleic acid (PNA) at minimal ratios of PNA/RNA

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## ABSTRACT

We have tested the inhibitory potential of peptide nucleic acid (PNA) on *in vitro* reverse transcription of the HIV-1 *gag* gene. PNA was designed to target different regions of the HIV-1 *gag* gene and the effect on reverse transcription by HIV-1, MMLV and AMV reverse transcriptases (RTs) was investigated. We found that a bis-PNA (parallel antisense 10mer linked to antiparallel antisense 10mer) was superior to both the parallel antisense 10mer and antiparallel antisense 10mer in inhibiting reverse transcription of the gene, thus indicating triplex formation at the target sequence. A complete arrest of reverse transcription was obtained at ~6-fold molar excess of the bis-PNA with respect to the *gag* RNA. At this molar ratio we found no effect on *in vitro* translation of *gag* RNA. A 15mer duplex-forming PNA was also found to inhibit reverse transcription at very low molar ratios of PNA/*gag* RNA. Specificity of the inhibition of reverse transcription by PNA was confirmed by RNA sequencing, which revealed that all tested RTs were stopped by the PNA/RNA complex at the predicted site. We propose that the effect of PNA is exclusively due to steric hindrance, as we found no signs of RNA degradation that would indicate PNA-mediated RNase H activation of the tested RTs. In conclusion, PNA appears to have a potential to become a specific and efficient inhibitor of reverse transcription *in vivo*, provided sufficient intracellular levels are achievable.

## INTRODUCTION

Replication of human immunodeficiency virus (HIV) invariably occurs through reverse transcription of the viral genomic RNA upon entry of the viral core into the host cell, as is typical for all

retroviruses. Synthesis of the first cDNA strand, the minus strand, is initiated at a tRNA primer, which binds to the primer binding site located close to the 5'-end of the genomic RNA. The first cDNA transcript ends at the 5'-end of the RNA, which is characterized by a sequence R, also present at the 3'-end of the RNA. Removal of the RNA, by the RNase activity of the HIV-1 reverse transcriptase (RT), allows binding of the cDNA from the 5'-end to the R region at the 3'-end of the RNA and perpetuation of minus strand cDNA synthesis. Initiation of second (plus) strand DNA synthesis takes place at the polypurine tract (PPT) located near the 3'-end of the genomic RNA. The PPT shows some resistance to RNase H during reverse transcription, thereby allowing plus strand cDNA synthesis to initiate from the PPT-cDNA hybrid. Completion of plus strand cDNA synthesis is accomplished after one more step of strand transfer (1). Targeting the process of cDNA synthesis appears to be a logical approach for anti-retroviral therapy. Although a broad spectrum of anti-RT compounds extending from nucleoside analogues (azidothymidin and dideoxyinosin) to non-nucleoside inhibitors, such as tetrahydroimidazo[4,5,1-*jk*][1,4]benzodiazepine-2(1*H*)-one and tetrahydroimidazo[4,5,1-*jk*][1,4]benzodiazepine-2(1*H*)-thione derivatives, nevirapine, pyridinone and bisarylpiperazine, is currently available, emergence of viral strains resistant to these inhibitors has limited their use in the treatment of AIDS (2,3). Another area of possible anti-RT approaches is represented by antisense DNA oligonucleotides and oligonucleotide analogues. Strategies aimed at blocking HIV-1 RT have so far included competitive inhibition of the formation of RT/primer/template complex by template/primer DNA oligonucleotides (2), degradation of the template RNA through antisense DNA oligo-RNA hybrids, triggering the RNase H activity of HIV-1 (4-6), inhibition of plus strand cDNA synthesis by triplex binding of the PPT-cDNA hybrid (1), inhibition of HIV-1 U5 long terminal repeat (LTR) integration by triplex binding of a polypurine stretch in the integrase binding site (7) and direct inhibition of cDNA extension by antisense template hybrids (8). Although some promising

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results have been obtained *in vitro*, such obstacles as inefficient *in vivo* deliverance, short half-lives of unmodified oligonucleotides in serum and cytoplasm and reduced specificity/affinity of modified oligonucleotides preclude a successful clinical application of antisense-based inhibition of HIV RT (9). Thus, the introduction of major improvements in molecular design is needed to render antisense oligonucleotides a viable therapeutic alternative.

In this study we set out to investigate the potential of peptide nucleic acid (PNA) as an inhibitor of HIV-1 RT. PNA is a DNA analogue in which the phosphate backbone has been substituted for an achiral and uncharged pseudopeptide backbone composed of *N*-(2-aminoethyl)glycine (10). This structure is virtually resistant to degradation by nucleases and proteases and ensures that PNA retains its full stability in plasma and cell extracts (11). Furthermore, PNA forms exceedingly stable triplexes with homopurine DNA or RNA targets in which one strand is engaged in conventional Watson-Crick pairing while the other PNA strand is bound in the major groove of the PNA-DNA duplex helix by Hoogsteen pairing (10). Triplex binding of PNA to a DNA or RNA target has been shown to inhibit the elongation process of the ribosome (12,13) and DNA polymerase (12) and preliminary experiments have also shown inhibition of MMLV reverse transcriptase (12) *in vitro*. In the present paper we have investigated the effect on the function of several RTs, including HIV-1 RT, of PNAs bound by triplex formation or duplex hybridization to selected regions of HIV-1 *gag* RNA.

## MATERIALS AND METHODS

### Construction of plasmid pSP64-*gag*-polyA and *in vitro* synthesis of *gag*-poly(A)<sup>+</sup> RNA

To test the inhibitory potential of PNA on reverse transcription, first plasmid pSP64-*gag*-polyA, enabling *in vitro* synthesis of HIV-1-specific poly(A)<sup>+</sup> RNA, was constructed. This was accomplished by inserting the 1.7 kb *Bgl*I fragment, containing ~0.4 kb of non-coding proviral/chromosomal DNA in addition to 1.3 kb of the *gag* gene, from the HIV-1 full-length clone pNLBT (14) into the *Bam*HI polylinker site of plasmid pSP64polyA (Promega). In the resulting construct the insert came under control of the plasmid SP6 promoter and the end was bounded by a 30 base long poly(A)<sup>+</sup> tail and a single *Eco*RI site.

For *in vitro* transcription the plasmid was linearized with *Eco*RI and 2 µg were used in a reaction which consisted of 80 µl transcription buffer (Boehringer Mannheim), 0.5 mM of each of the four nucleotides ATP, UTP, CTP and GTP (Pharmacia), 0.25 mM DTT (Boehringer Mannheim), 0.1 µg/ml tRNA (*Escherichia coli*; Boehringer Mannheim), 0.05 µg/ml heparin (Sigma), 100 U RNA polymerase (Pharmacia) and 25 U human placental RNase inhibitor (RNAguard; Pharmacia). The transcription reaction was kept for 40 min at 37°C, after which 5 U RNase-free DNase I (Boehringer Mannheim) were added and the incubation continued for 15 min at 37°C. For some *in vitro* translation experiments the RNA was artificially capped by adding 0.2 A<sub>250</sub> units m<sup>7</sup>G(5')ppp(5')G (Boehringer Mannheim) and by lowering the GTP concentration to 0.1 mM. Purification of the *gag*-poly(A)<sup>+</sup> transcript was done using a mRNA purification kit (Dyna) according to the manufacturer's instructions. The recovered RNA was quantified spectrophotometrically and

analysed by agarose gel electrophoresis. Single reactions typically yielded 3–4.5 µg full-length 1.7 kb transcript.

### Assay for inhibition of the reverse transcriptase activity

Synthesis of the first strand cDNA was primed with a DNA oligonucleotide primer (A318, 5'-ATCACTTCTGG GCTGAA-AGC-3'), which is complementary to positions 486–505 in the *gag* open reading frame (ORF) of the *gag*-poly(A)<sup>+</sup> transcript (Fig. 1). After incubation of the template RNA (200–250 ng, giving a final molarity of ~0.05 µM in the assay) for 10 min at 80°C, the primer (10 pmol) as well as PNA or antisense oligonucleotides were allowed to anneal for 5 min at 37°C before the extension reaction was carried out at 37°C for 60 min. The reaction contained 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl<sub>2</sub>, 10 mM DTT, 5 U human placental RNase inhibitor, 2 mM dATP, dTTP and dGTP, 0.2 mM dCTP, 0.5 µCi [α-<sup>32</sup>P]dCTP (800 Ci/mmol; Amersham) and 5–8 U HIV RT (Amersham) or AMV RT (Pharmacia) or 100 U MMLV RT (Gibco) in a final volume of 10 µl. After incubation the cDNA was ethanol precipitated and an aliquot was run on a 6% denaturing polyacrylamide gel. Quantitation of the cDNA yield was in an InstantImager (Packard).

### RNA sequencing

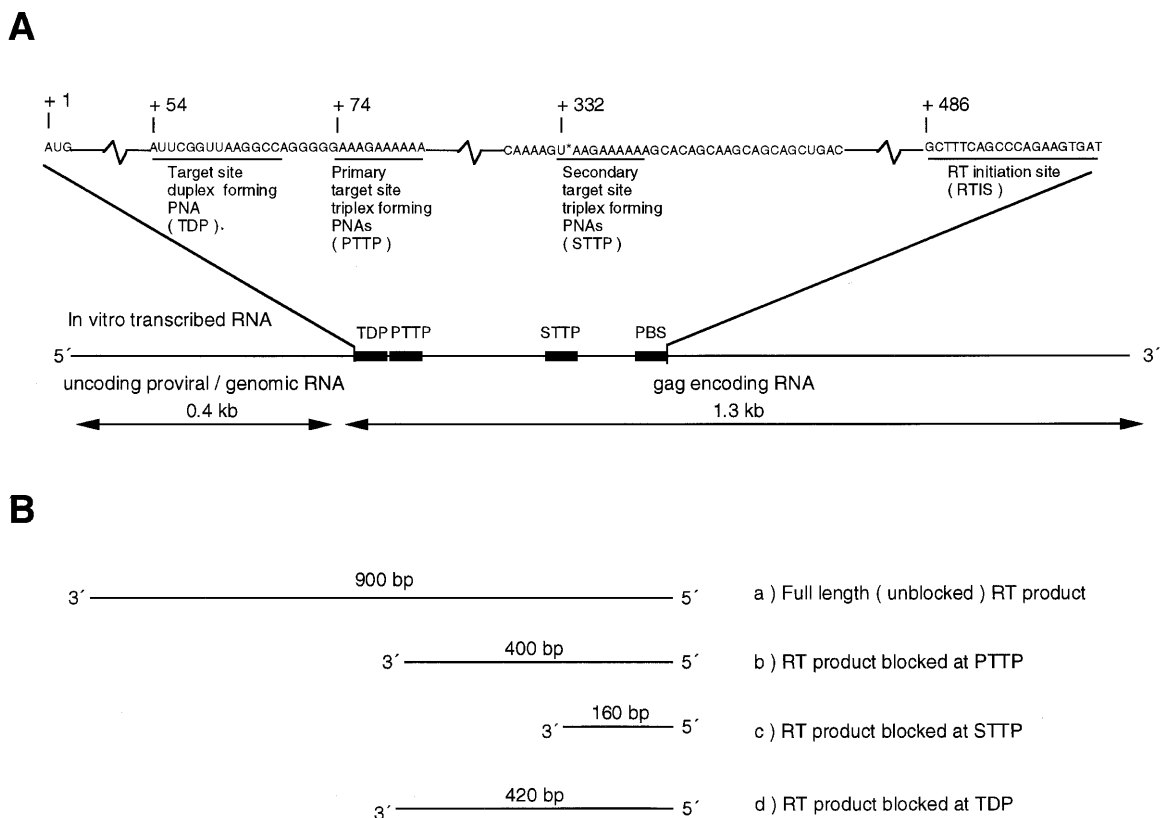
To confirm the exact location of the blocking site, RNA sequencing of the *gag*-poly(A)<sup>+</sup> RNA was performed in parallel reactions containing no PNA or PNA 897 at a concentration of 0.3 µM. The procedure consisted of the same steps as the reverse transcriptase assay with the modifications that the A318 primer was 5'-end-labeled and the concentration of all four nucleotides was 1.3 mM. Further, 0.5 mM of either ddGTP, ddATP, ddTTP or ddCTP were present in four separate termination reactions. Radioactive labelling of the primer and subsequent purification were done using polynucleotide kinase (Pharmacia), [γ-<sup>32</sup>P]ATP (Amersham) and Microspin G-25 columns (Pharmacia) according to the manufacturer's instructions. The sequencing products were resolved by denaturing PAGE (6%) and visualized on X-ray film by autoradiography.

### Assay for RNase H activity

To determine the potential of PNA-RNA complexes to trigger RNase H activity of HIV-1 RT, a radioactively labelled transcript from the pSP64-*gag*-polyA construct was prepared by performing *in vitro* transcription as presented above, with the exception that 1 µCi [α-<sup>32</sup>P]CTP (2000 Ci/mmol; Amersham) was added and at the same time the CTP concentration was lowered to 0.05 mM. The RT reaction was done exactly as described for the RT inhibition assay, except that [α-<sup>32</sup>P]dCTP was omitted. Any changes in the size distribution of RNA were analysed by fractionation on a 5% denaturing polyacrylamide gel.

### *In vitro* translation

For *in vitro* translation, a transcription/translation vector, pBAB72, enabling *in vitro* synthesis of full-length *gag* polyprotein p55 was obtained through the NIH AIDS Research and Reference Reagent Program (15). pDAB72 was linearized with *Bam*HI and 2 µg of the linearized template DNA was transcribed as described above for transcription of the pSP64-*gag*-polyA vector, with the exception that T7 RNA polymerase (Pharmacia)



**Figure 1.** Schematic diagram of the 1.7 kb *in vitro* transcript used for the study of RT inhibition. Positions of the primary and secondary targets for the PNAs used in this study are shown along with the position of the RT initiation site (RTIS). The terminal mismatch in STTP for the triplex-forming PNAs is indicated by an asterisk (A). The expected lengths of reverse transcription products truncated at the PNA target sites are also indicated (B).

was used instead of SP6 RNA polymerase. Transcription of the pDAB72 vector gave rise to a RNA product of ~1.5 kb, consisting, from the 5'-end, of 11 ribonucleotides of non-coding RNA flanked by the full-length *gag* and a portion of the *pol* ORF. The transcript was analysed in and subsequently purified from a 1% low melting temperature agarose gel (NuSieve; FMC BioProducts). For purification, the RNA band was excised and melted at 67°C in 3 vol. sterile water followed by phenol/chloroform extraction (1:1) and standard ethanol/sodium acetate precipitation. Finally, the RNA was resuspended in DEPC-treated water and quantified spectrophotometrically. For *in vitro* translation, 200–250 ng of this RNA (amounting to a final concentration of ~0.02 μM in the assay) was employed for peptide synthesis by a rabbit reticulocyte translation system (Boehringer Mannheim), according to the manufacturer's instructions. Translation was carried out in the presence of [<sup>35</sup>S]methionine (Amersham) and translation products were analysed by 10% SDS-PAGE and visualized by electronic autoradiography in an InstantImager.

#### Synthesis of PNA, RNA, primers and modified DNA oligonucleotides

PNAs were synthesized as described (16–18). The PNAs were purified by HPLC and their identity was confirmed by MALDI-TOF mass spectrometry. The bis-PNA, PNA 897, was linked by eg1:8-amino-3,6-dioxaoctanoic acid.

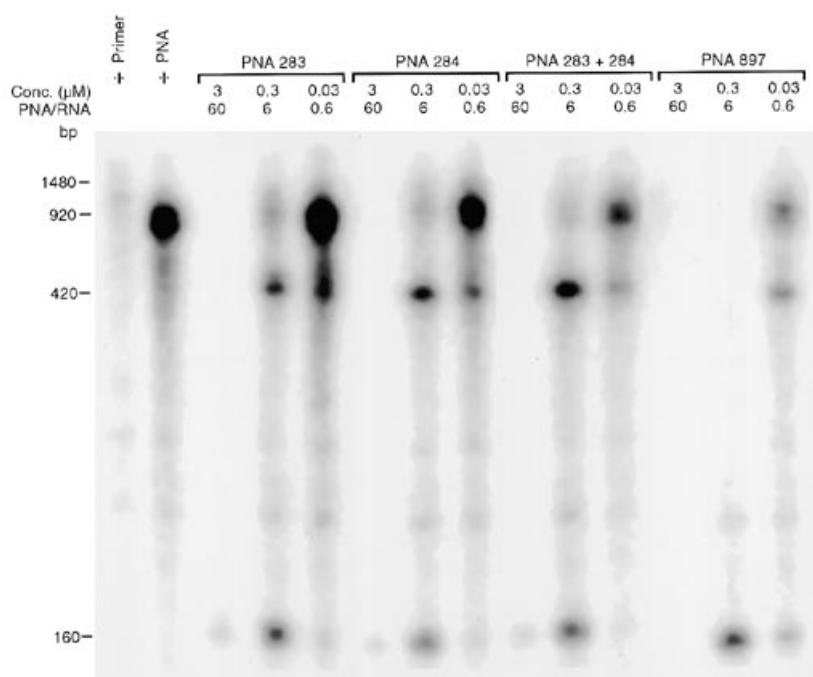
Primer and oligophosphorothioates were supplied by DNA Technology (Denmark). The primer was synthesized by phosphoramidite chemistry and phosphorothioates were synthesized as has been described (19) and purified by HPLC.

## RESULTS

### PNAs designed to form triplex hybrids with *gag* RNA inhibit cDNA synthesis at very low molar ratios of PNA/RNA

First, we tested the inhibitory potential on RT activity of 10mer PNAs designed to form triplex hybrids with a highly conserved polypurine region of the HIV-1 *gag* sequence. [Please note that the purine-rich region of the *gag* region of the HIV-1 genome is not to be mistaken for the purine-rich region known as the polypurine tract (PPT) which occurs in the coding region of the integrase and also adjacent to the 3'-LTR.]

The three PNAs employed for this purpose were: PNA 283, H-T<sub>6</sub>CT<sub>3</sub>-NH<sub>2</sub>, which is complementary to position 74–83 of the HIV *gag* ORF (primary target for the tested triplex-forming PNAs; PTTP) and is also predicted to hybridize to position 332–341 of the HIV *gag* ORF (secondary target for the tested triplex-forming PNAs; STTP) with only one terminal mismatch (a U instead of an A at the 5'-end) when compared with positions 74–83; PNA 284, H-T<sub>3</sub>CT<sub>6</sub>-NH<sub>2</sub>, which is antiparallel to PNA 283; PNA 897, H-T<sub>6</sub>CT<sub>3</sub>(eg1)<sub>3</sub>T<sub>3</sub>JT<sub>6</sub>-LysNH<sub>2</sub> (J is pseudoiso-



**Figure 2.** Effect of triplex-forming PNAs on reverse transcription of HIV-1 *gag* RNA. Reverse transcription was carried out with HIV-1 RT as described in Materials and Methods and the result was visualized by electronic autoradiography (InstantImager; Packard). The effects of PNAs 283, 284 and 897 and PNAs 283 and 284 in combination are shown. All PNAs or combinations thereof were tested in the concentration range 0.03–3 µM, i.e. at molar ratios of PNA/RNA ranging from 0.6 to 60.

cytosine), which is a bis-PNA consisting of both PNA 283 and PNA 284 motifs covalently linked.

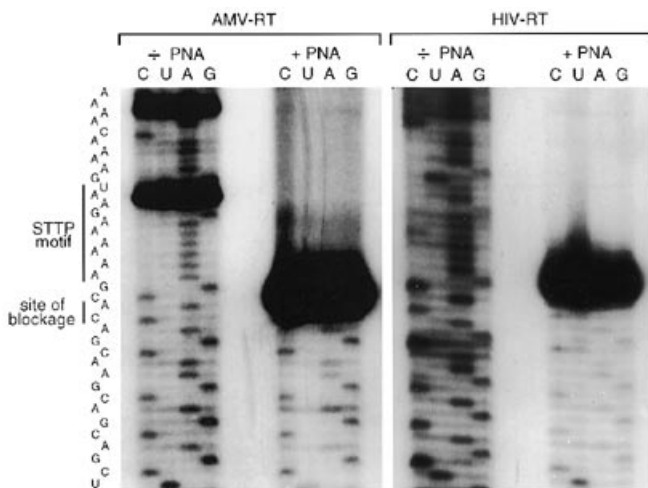
The position of the A318 primer binding site (RT initiation site; RTIS) and the relative positions of PTTP and STTP for the triplex-forming PNAs are summarized in Figure 1A. As is obvious from Figure 1B, the predicted size of the unblocked RT product is 900 bp (corresponding to cDNA synthesis of 500 bases of the *gag* coding region and ~400 bases of the non-coding proviral/plasmid sequences), whereas RT product blocked at PTTP would have a size of 400 bp and blockage at STTP is expected to give a product of 160 bp.

The effect on reverse transcription was examined with PNAs 283, 284 and 897 individually and with PNAs 283 and 284 in combination. All PNAs, or combinations thereof, were tested in overall concentrations ranging from 0.03 to 3 µM, i.e. at molar ratios of PNA/RNA ranging from 0.6 to 60. The inhibitory effect on HIV-1 RT activity is shown in Figure 2. When the PNAs were used at 3.0 µM concentration, almost complete and therefore presumably non-specific inhibition of the reaction was observed in all cases. At 0.3 µM concentration, however, PNAs 283 and 284, and the combination thereof, resulted in three bands. These bands were consistent with the predicted size of the full-length product (900 bases), an RT product blocked at PTTP (of ~400 bases) and an RT product blocked at STTP (of ~160 bases). PNA 897 resulted only in appearance of the latter band, indicating a total block at STTP. At a concentration of 0.03 µM, which represents a molar ratio of 0.6 with respect to the template RNA, PNAs 283, 284 and the combination of 283 and 284 resulted mainly in the full-length RT product, while blocking with PNA 897 resulted in the full-length cDNA as well as truncated cDNAs, predominantly of the size (400 bp) expected for blockage at PTTP. A similar pattern of inhibition was obtained when MMLV

RT was used instead of HIV RT (data not shown). The above results strongly indicate that the tested RTs may become blocked through triplex binding of the PNAs at the two predicted sites in the *gag* RNA. The fact that antiparallel antisense PNA 284 is as efficient as the parallel antisense PNA 283 and that their combination slightly increases the efficiency of inhibition implies formation of triplex structures at the target sequences. Moreover, the notion that triplex clamping at the targeted sites may represent a paramount obstacle to reverse transcription was corroborated by the bis-PNA (PNA 897), which was found to be the most potent inhibitor.

#### RNA sequencing confirms the specificity of PNA interaction with its target region

To confirm the sequence specificity of the studied PNAs and to investigate the exact position at which reverse transcription is stalled by these antisense oligomers we performed sequencing of the RNA template using HIV-1, MMLV and AMV RTs. The representative data obtained with AMV RT and HIV RT are shown in Figure 3. Similar results were also produced with MMLV RTs and show in accordance that DNA synthesis is blocked at 2–4 nt before the target sequence (in this case STTP). Thus, inhibition of reverse transcription appears to be accomplished in a site-specific manner through steric hindrance by the annealed PNAs. Moreover, the sequencing experiments also revealed an interesting difference in the ability of the tested RTs to act as polymerases for RNA sequencing of *gag* RNA. While AMV RT resulted, over a wide range, in the most readable sequence (probably due to equally efficient incorporation of the four ddNTPs), HIV RT was superior to both AMV RT and MMLV RT in resolving secondary structures of the RNA that



**Figure 3.** Effect of PNA 897 on the sequencing of HIV-1 *gag* RNA. The *in vitro* produced RNA was sequenced as described in Materials and Methods. Sequencing was in parallel reactions with or without PNA using either HIV-1 RT or AMV RT as DNA polymerase. The termination reactions were loaded (reading from left to right) in the order G, A, T and C, corresponding to C, U, A and G in the template RNA, as indicated above the lanes of the autoradiogram. The sequence of the RNA is indicated to the left of the autoradiogram. The target site for the PNA (the STTP motif) and the apparent site of PNA blockage are also indicated.

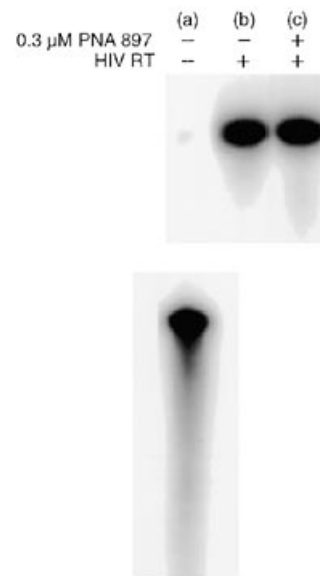
otherwise resulted in compression of the sequencing products. Such compression could be seen at two places above the site of blockage when sequenced with AMV RT, while no or only slight compression was seen when the reaction was driven by HIV-1 RT (Fig. 3).

#### HIV-1 RT inhibition by PNA is not caused by activation of RNase H

In an attempt to provide additional evidence that steric hindrance is the major (or only) mechanism mediating the effect of PNAs, we tested susceptibility of the inhibitory complexes to RNase H activity of the HIV-1 RT. The results shown in Figure 4 demonstrate integrity of the template RNA in the presence of PNA 897 and HIV-1 RT. Similar results were obtained with MMLV RT (data not shown). These findings rule out the possibility that RNase H activity could participate in the inhibitory mechanism of PNA 897 and are consistent with earlier reports showing that RNA/PNA hybrids are not substrates for RNase H (13). Notably, it was found that both the tested RTs bound all the employed RNAs resulting in complete retardation of the RNA when analysed by gel electrophoresis.

#### 15mer duplex-forming PNA inhibits RT as efficiently as the 10mer PNAs forming triplex hybrids with the target RNA

It has recently been shown that only triplex-forming PNAs and not duplex-forming PNAs are capable of producing full inhibition of *in vitro* translation elongation (13). To test if triplex-forming PNAs are also required in order to fully inhibit RT activity, a 15mer PNA of sequence H-TGGCCTTAACCGAAT-LysNH<sub>2</sub> targeted to positions 54–69 (TDP in Fig. 1) in the HIV-1 *gag* ORF was analysed for its potential to inhibit reverse transcription

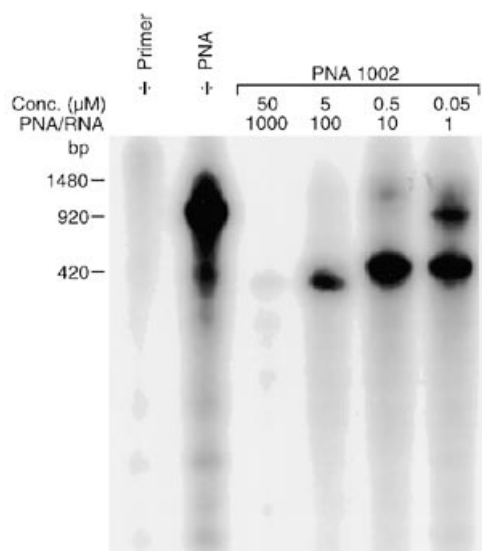


**Figure 4.** Analysis of template RNA integrity in the presence of HIV-1 RT and antisense PNA. A radioactively labelled HIV-1 *gag* RNA *in vitro* transcript was incubated either without PNA and HIV-1 RT (a), in the presence of HIV-1 RT (b) or in the presence of both PNA and HIV-1 RT (c) under the same conditions as for the reverse transcription reactions, except that addition of [ $\alpha$ -<sup>32</sup>P]dCTP was omitted. RNAs were analysed by fractionation on a 5% denaturing polyacrylamide gel. The data show that PNA did not trigger any degradation of the RNA that would be expected as a result of HIV-1 RT RNase H activity. Notably, it is seen from the mobility shift that all the employed RNA was bound by the RT.

of the *gag* template RNA. As demonstrated by the data in Figure 5, we found that the duplex-forming PNA gave complete inhibition at molar ratios of PNA/RNA of 10 or more, while a ratio of 1 resulted in inhibition of ~70%. These results clearly show that triplex clamping of the RNA by the PNA is not necessary to obtain complete inhibition of RT polymerase activity and further that a 15mer duplex-forming PNA oligomer can inhibit cDNA synthesis at molar ratios of PNA/RNA comparable with the best results obtainable with triplex-forming 10mer PNAs.

#### Phosphorothioates have no effect on RT activity at concentrations sufficient for complete inhibition with PNA

With the aim of comparing the anti-RT performance of PNAs with other relevant DNA analogues, we tested the effect on RT activity of two phosphorothioate oligonucleotides which were analogous in their base composition to PNAs 283 and 284. The two phosphorothioates were tested separately and in combination in the RT activity assay under identical conditions as were used for the PNAs (3.0–0.03  $\mu$ M). When tested at concentrations of 0.3  $\mu$ M (an oligonucleotide/RNA ratio of 6) or less the phosphorothioates failed to demonstrate any effect on HIV-1 RT activity. When tested at a concentration of 3.0  $\mu$ M, only the combination of the two phosphorothioates was able to inhibit the RT reaction. There was a slight reduction in the full-length RT product but no truncated product, as would be expected for specific inhibition (results not shown).



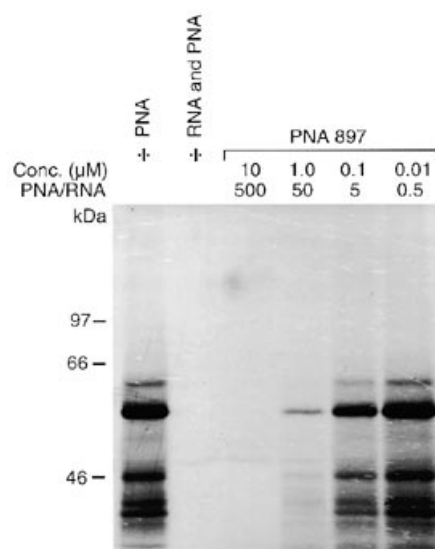
**Figure 5.** Effect of a duplex-forming PNA on reverse transcription of HIV-1 *gag* RNA. Reverse transcription was carried out with HIV-1 RT as described in Materials and Methods and the result was visualized by electronic autoradiography (InstantImager; Packard). PNA 1002 was tested over the range of concentrations 0.05–50  $\mu\text{M}$ , i.e. at molar ratios of PNA/RNA ranging from 1 to 1000.

#### RT inhibition by PNA takes place at molar ratios of PNA/RNA that have no influence on translation

Finally, we set out to investigate the effect of bis-PNA on *in vitro* translation of the *gag*-poly(A)<sup>+</sup> RNA. Our data show that PNA 897 at a concentration of 0.1  $\mu\text{M}$  (molar ratio of PNA/RNA of ~5) or less had little or no effect on *in vitro* translation. Concentrations of PNA 897 in excess of 1  $\mu\text{M}$  resulted in complete inhibition of the translational process (Fig. 6). The complete absence of translational product is to be expected, as one of the targets for the PNA is situated only 50 bases from the start codon (Fig. 1) and a truncated translational product of only 18 amino acids is below the limit of detection in the employed reticulocyte translation system. Collectively, our data suggest that over a specific range of concentrations PNA can be utilized to selectively block reverse transcription without having an adverse, even specific, effect on translation.

#### DISCUSSION

Motivated by an urgent need to find methods for effectively suppressing expression of pathogenic genes such as oncogenes and retroviral genes, intensive research into the gene suppressing capacity of synthetic oligonucleotides and oligonucleotide analogues has been carried out, especially in the last decade. Although promising results have been obtained in a variety of cell-based or cell-free *in vitro* systems, no successful clinical application of the antisense technology has been reported so far (20). Remarkably, however, a recent report from Monia *et al.* showed that almost complete and specific inhibition of *C-raf-1* kinase gene expression can be achieved both *in vitro* and *in vivo* using a carefully selected antisense phosphorothioate applied in nanomolar concentrations (21). The strong effect on *C-raf-1*



**Figure 6.** Effect of PNA 897 on *in vitro* translation of HIV-1 *gag* RNA. *In vitro* translation was performed as described in Materials and Methods and subsequently analysed by SDS-PAGE on a 10% gel. The results shown were obtained with a capped transcript. Similar results were obtained independently of capping the RNA. PNA 879 was tested in concentrations ranging from 0.01 to 10  $\mu\text{M}$ , corresponding to molar ratios of PNA/RNA of 0.5–500. As appears from the autoradiogram, PNA 897 at a concentration of 0.1  $\mu\text{M}$  or less had only a slight effect on *in vitro* translation, while at concentrations in excess of 0.1  $\mu\text{M}$  an inhibition of the translational process was evident.

kinase gene expression is believed to depend on RNase H cleavage of the target mRNA triggered by binding of the antisense phosphorothioate to an optimal hybridization site within the structured mRNA molecule. Whether expression of HIV proteins can be inhibited *in vivo* with similar specificity and potency at similar low concentrations of phosphorothioates upon extensive screening of antisense candidates remains to be shown. So far, efficient suppression of HIV-1 in culture has mostly been reported at concentrations of antisense oligomers above 1  $\mu\text{M}$  and many reports have described non-specific inhibition of gene expression and toxic effects of antisense oligomers at these concentrations (9,20,22). Further, it has recently become evident that efficient inhibition of *de novo* infection with HIV-1 obtained *in vitro* with a variety of antisense phosphorothioates is to a large extent due to a completely non-specific process in which the phosphorothioates interfere with binding of HIV-1 to its target cells (20). However, even if it becomes evident that expression of HIV-1 proteins can be efficiently suppressed *in vivo* by one or more carefully selected antisense phosphorothioates at non-toxic and obtainable concentrations, the risk of HIV-1 developing escape mutants is high. Thus, the search for a more generally applicable antisense strategy seems necessary. Facing this challenge, we sought to examine the inhibitory effect of PNA on *in vitro* reverse transcription of the *gag* gene of HIV-1. Bearing in mind practical implications, we intended to specifically maximize inhibition of HIV-1 RT-driven cDNA synthesis, nonetheless, for comparative purposes we also employed some of the best characterized RTs of animal retroviruses. Our results clearly demonstrated that complete inhibition of RT-driven cDNA synthesis is attainable even at a RNA/PNA molar ratio of only 6, which is at least an order of

magnitude less than that accomplished with phosphorothioates of similar base composition. Similar superiority of PNAs over analogous phosphorothioates has recently been demonstrated in a study comparing their ability to inhibit telomerase activity in cell extracts and permeabilized cells (23). We have also confirmed previous reports that PNA/RNA hybrids are not a substrate for RNase H (13), thus indicating steric blockage of the RT as the direct cause of the observed inhibition. The most efficient PNA employed in our study was a bis-PNA capable of clamping the target in a triplex complex. Importantly, however, a 15mer PNA designed only to engage in duplex formation with the target was also found to be very efficient in blocking RT-driven cDNA synthesis. Furthermore, we found that PNA at molar PNA/RNA ratios sufficient to completely block cDNA synthesis had no effect on *in vitro* translation. This indicates that if used at such concentrations, RT activity can be targeted without risking non-specific and toxic interference with translation. Considering the high mutation rate of HIV-1, allowing the virus to escape most tested nucleotide analogues and virus enzyme inhibitors, it is interesting to mention that in addition to the completely complementary primary target motif, the triplex-forming PNAs also bound with high affinity to a secondary region which exhibited a single mismatch. This observation indicates that triplex-forming PNAs might retain high targeting potential even in the face of the well-recognized rapid HIV-1 mutation rate. From the therapeutic point of view, such a property, along with the previously demonstrated low intracellular toxicity and extraordinary stability (10,12), makes PNA a very promising candidate for inhibition of RT activity *in vivo*. It is important to emphasize that our study has been exclusively concerned with inhibition by PNA of the RT elongation process. Our finding that a duplex-forming PNA is very efficient in inhibiting RT elongation opens the possibility that PNAs could also be used as an inhibitor of the initial priming process of HIV-1 cDNA synthesis. A study of the potential of PNA as an inhibitor of HIV-1 RT initiation would need a system in which the RT is primed by tRNA, as is the case *in vivo*, and further the influence of nucleocapsid protein (NC) would have to be investigated, as this viral protein is known to be important for efficient and specific priming of HIV-1 RT *in vivo* (24,25). The influence of factors such as NC and topoisomerase I on HIV-1 RT elongation is currently being investigated (26–28) and a more comprehensive study on the effect of PNA on RT processivity in different parts of the HIV-1 genome would also have to consider the possible implications of such factors.

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## REFERENCES

- Volkmann,S., Danull,J. and Moelling, K. (1993) *Biochimie*, **75**, 71–78.
- Idriss,H. and Stammers,D.K. (1994) *J. Enzyme Inhibition*, **8**, 97–112.
- Pauwels,R., Andries,K., Desmyter,J., Schols,D., Kukla,M.J., Breslin,H.J., Raeymaeckers,A., Gelder,J.V., Woestenborghs,R., Heykants,J., Schellekens,K., Janssen,M.A.C., Clercq,E.D. and Janssen,P.A.J. (1990) *Nature*, **343**, 470–474.
- Dudding,L.R., Harington,A. and Mizrahi,V. (1990) *Biochem. Biophys. Res. Commun.*, **167**, 244–250.
- Hatta,T., Takai,K., Yokoyama,S., Nakashima,H., Yamamoto,N. and Takaku,H. (1995) *Biochem. Biophys. Res. Commun.*, **211**, 1041–1046.
- Hatta,T., Kim,S.G., Nakashima,H., Yamamoto,N., Sakamoto,K., Yokoyama,S. and Takaku,H. (1993) *FEBS Lett.*, **330**, 161–164.
- Bouziane,M., Cherny,D.I., Mouscadet,J.F. and Auclair,C. (1996) *J. Biol. Chem.*, **271**, 10359–10364.
- Bordier,B., Hélène,C., Barr,P.J., Litvak,S. and Sarih,C. L. (1992) *Nucleic Acids Res.*, **20**, 5999–6006.
- Stein,C.A., Tonkinson,J.L. and Yakubov,L. (1991) *Pharmacol. Ther.*, **52**, 365–384.
- Nielsen,P.E., Egholm,M. and Buchardt,O. (1994) *Bioconjugate Chem.*, **5**, 3–7.
- Demidov,V.V., Potaman,V.N., Frank,K.M., Egholm,M., Buchardt,O., Sonnichsen,S.H. and Nielsen,P.E. (1994) *Biochem. Pharmacol.*, **48**, 1310–1313.
- Hanvey,J.C., Peffer,N.J., Bisi,J.E., Thomson,S.A., Cadilla,R., Josey,J.A., Ricca,D.J., Hassman,C.F., Bonham,M.A., Au,K.G., Carter,S.G., Bruckenstein,D.A., Boyd,A.L., Noble,S.A. and Babiss,L.E. (1992) *Science*, **258**, 1481–1485.
- Knudsen,H. and Nielsen,P.E. (1996) *Nucleic Acids Res.*, **24**, 494–500.
- Zachar,V., Spire,B., Hirsch,I., Chermann,J.C. and Ebbesen, P. (1991) *J. Virol.*, **65**, 2102–2107.
- Erickson,V.S., Manfredi,J., Viitanen,P., Tribe,D.E., Tritch,R., Hutchison,C., Loeb,D.D. and Swanstrom,R. (1989) *Aids Res. Hum. Retroviruses*, **5**, 577–591.
- Christensen,L., Fitzpatrick,R., Gildea,B., Petersen,K.H., Hansen,H.F., Koch,T., Egholm,M., Buchardt,O., Nielsen,P.E., Coull,J. and Berg,R.H. (1995) *J. Peptide Sci.*, **3**, 175–183.
- Dueholm,K.L., Egholm,M., Behrens,C., Christensen,L., Hansen,H. F., Vulpius,T., Petersen,K., Berg,R.H., Nielsen,P.E. and Buchardt,O. (1994) *J. Org. Chem.*, **59**, 5767–5773.
- Egholm,M., Christensen,L., Dueholm,K.L., Buchardt,O., Coull,J. and Nielsen,P.E. (1995) *Nucleic Acid Res.*, **23**, 217–222.
- Iyer,R.P., Egan,W., Regan,J.B. and Beaucage,J. (1990) *J. Am. Chem. Soc.*, **112**, 1253–1254.
- Gura,T. (1995) *Science*, **270**, 575–577.
- Monia,B.P., Johnston,J.F., Geiger,T., Muller,M. and Fabbro,D. (1996) *Nature Med.*, **2**, 668–675.
- Ghosh,M.K. and Cohen,J.S. (1992) *Prog. Nucleic Acid Res. Mol. Biol.*, **42**, 79–126.
- Norton,J.C., Mieczyslaw,A.P., Wright,W.E., Shay,J.W. and Corey,D.R. (1996) *Nature Biotechnol.*, **14**, 615–619.
- Li,X., Quan,Y., Arts,E.J., Li,Z., Preston,B.D., de-Rocquigny,H., Roques,B.P., Darlix,J.L., Kleiman,L., Parniak,M.A. and Wainberg,M.A. (1996) *J. Virol.*, **70**, 4996–5004.
- DeStefano,J.J. (1996) *J. Biol. Chem.*, **271**, 16350–16356.
- Ji,X., Klarmann,G.J. and Preston,B.D. (1996) *Biochemistry*, **35**, 132–143.
- Rodriguez-Rodriguez,L., Tsuchihashi,Z., Fuentes,G.M., Bambara,R.A. and Fay,P.J. (1995) *J. Biol. Chem.*, **270**, 15005–15011.
- Takahashi,H., Matsuda,M., Kojima A., Sata,T., Andoh,T., Kurata,T., Nagashima,K. and Hall,W.W. (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 5694–5698.