Novel Aptamer Inhibitors of Human Immunodeficiency Virus Reverse Transcriptase

Jeffrey J. DeStefano and Gauri R. Nair

Primer-template-based double-stranded nucleic acids capable of binding human immunodeficiency virus reverse transcriptase (HIV-RT) with high affinity were used as starting material to develop small single-stranded loop-back DNA aptamers. The original primer-templates were selected using a SELEX (Systematic Evolution of Ligands by EXponential enrichment) approach and consisted of 46- and 50-nt primer and template strands, respectively. The major determinant of the ~10-fold tighter binding in selected sequences relative to control primer-templates was a run of 6–8 G residues at the 3' primer end. Sixty, thirty-seven, twenty-seven, and twenty-two nucleotide loop-back single-stranded versions that retained the base pairs near the 3' primer terminus were constructed. Both the 60- and 37-nt versions retained high affinity for RT with K_d values of ~0.44 nM and 0.66 nM, respectively. Random sequence primer-templates of the same length had K_d s of ~20 nM and ~161 nM. The shorter 27- and 22-nt aptamers bound with reduced affinity. Several modifications of the 37-nt aptamer were also tested including changes to the terminal 3' G nucleotide and internal bases in the G run, replacement of specific nucleotides with phosphothioates, and alterations to the 5' overhang. Optimal binding required a 4- to 5-nt overhang, and internal changes within the G run had a pronounced negative effect on binding. Phosphothioate nucleotides or the presence of a 3' dideoxy G residue did not alter affinity. The 37-nt aptamer was a potent inhibitor of HIV-RT *in vitro* and functioned by blocking binding of other primer-templates.

Introduction

RECENT REPORTS IN THE literature have shown that nucleic acids that bind tightly to human immunodeficiency virus reverse transcriptase (HIV-RT) can inhibit HIV replication in cell culture (Joshi and Prasad, 2002; Joshi et al., 2005). This exciting discovery has opened up a future for the possible use of these molecules in anti-HIV therapy (Joshi et al., 2003; Zhang et al., 2004; Held et al., 2006a; James, 2007). Previous studies have focused on single-stranded RNAs that adopted specific structures conducive to strong binding (Joshi and Prasad, 2002; Joshi et al., 2003, 2005). These RNAs were originally identified using an approach developed in the early 1990s called SELEX (Systematic Evolution of Ligands by EXponential enrichment) (Joyce, 1989; Ellington and Szostak, 1990; Tuerk and Gold, 1990). The SELEX method is based on differential binding of nucleic acids to a substrate protein. Initially, a large random pool of RNAs is incubated with a limiting amount of protein. Nucleic acids that bind with higher affinity will preferentially associate with the protein and can be isolated by gel shift, nitrocellulose filter

binding, or more recently, capillary electrophoresis (Mosing et al., 2005). The selected pool is expanded by PCR amplification followed by RNA transcription, and the new pool is subjected to another round of protein binding. After several rounds, nucleic acids with high affinity for the protein, also referred to as aptamers, can be isolated. RNA aptamers first isolated for HIV-RT were usually pseudoknot-type structures (Tuerk et al., 1992). RNA pseudoknot aptamers have been shown to interfere with primer-template binding and are potent inhibitors of reverse transcription (Chen and Gold, 1994; Jaeger et al., 1998; Held et al., 2006b). Single-stranded DNA aptamers with similar properties have also been selected (Schneider et al., 1995; Mosing et al., 2005). For most aptamers, tight binding to RT appears to be governed by the folded structure rather than the sequence of the nucleic acids. This method has since been used to isolate aptamers that can bind several different proteins, including viral therapeutic targets and a few HIV proteins (Gold, 1995; Brody and Gold, 2000; Matsugami et al., 2005; Metifiot et al., 2005; Held et al., 2006a; Kolb et al., 2006). Many of these aptamers

are currently being developed as potential treatments for diseases. One aptamer, Macugen, developed by EyeTech Pharmaceuticals, Inc. (New York, NY U.S.A.) has been approved by the FDA for the treatment of macular degeneration (Macugen) (Nimjee et al., 2005). Other nontherapeutic uses for aptamers are also being explored including their uses in studying the molecular biology of virus replication, as complements to antibodies, and as diagnostic biosensors (Deisingh, 2006; Porschewski et al., 2006; James, 2007).

A variety of factors make these nucleic acid structures an attractive choice for HIV therapy directed against RT. Most notably, they typically bind severalfold more tightly than natural RT substrates (DNA-DNA and RNA-DNA primer-template) and show exceptional specificity for RT (Kensch et al., 2000; Held et al., 2006a). The main disadvantage of DNA aptamer therapy is the lack of a method to deliver the inhibitor to target cells in animal models, especially because host defenses degrade the inhibitor before it is able to sequester the target protein. Modified sugar and phosphate backbones can help protect against degradation, and the potential use of gene vectors for the delivery of RNAs is being explored (Gold, 1995; Brody and Gold, 2000). A new approach where nucleic acids are anchored to small proteins that cargo them across the cell membrane is also promising for both RNA and DNA aptamers and other types of nucleic acid inhibitors (Tripathi et al., 2007). New research suggests that some aptamers can enter cells during viral infection even without the addition of specific proteins or transfection (Matzen et al., 2007; Metifiot et al., 2007). In one case, an aptamer directed against a mouse retrovirus (spleen focus-forming virus) was able to inhibit virus infection not only in cells but also when administered to infected mice (Matzen et al., 2007). Because aptamer binding presumably depends on multiple broad ranging contacts with RT amino acids, it has been suggested that resistant RT mutants would be less likely, although this clearly remains to be proven (Held et al., 2006a). Recent reports describing RT aptamer escape mutants question the veracity of this hypothesis (Fisher et al., 2002; Joshi et al., 2005). However, it should be noted that these escape mutants were replication defective in comparison to wild-type virus.

The natural substrate for RT and other DNA polymerases is a duplex nucleic acid with a recessed 3' terminus (classical primer-template configuration). Recently, we used a novel SELEX-based approach that selected specifically for DNA-DNA primer-template sequences (DeStefano and Cristofaro, 2006). Sequences that bound ~10-fold tighter than random sequences with K_d values in the low-nM range were selected. The 10-fold increase in affinity, though a modest gain in comparison to other aptamers, is notable because the starting material already bound tightly. All the recovered sequences had the same basic motif characterized by a run of 6–8 G residues at the primer 3' terminus. In such, they mimicked the sequence of the polypurine tract (ppt) RNA primer used by HIV to initiate plus strand DNA synthesis. The run of G's at the 3' terminus was pivotal for tight binding as shortening the run reduced binding while substrates with longer runs (up to 26 bases) retained tight binding. In this study, single-stranded loop-back DNAs derived from high-affinity sequences were designed and tested for binding. Substrates as short as 37 nt with a 15-bp hybrid region

and 4 base 5' overhang retained full binding affinity. These small substrates were potent inhibitors of RT *in vitro*.

Materials and Methods

Materials

Wild type HIV-RT (HXB2 strain) was obtained from Worthington Biochemical Corp. (Lakewood, NJ U.S.A.), dNTPs from Roche Applied Sciences (Indianapolis, IN U.S.A.) T4 polynucleotide kinase (PNK) from New England Biolabs (Ipswich, MA U.S.A.), radiolabeled compounds from GE Healthcare Bio-Science Corp. (Piscataway, NJ U.S.A.), Sephadex G-25 spin columns from Harvard Apparatus (Holliston, MA U.S.A.). Oligonucleotides were commercially synthesized by Integrated DNA Technologies, Inc. (Coralville, IA U.S.A.). All other chemicals were from Sigma-Aldrich Co. (St. Louis, MO U.S.A.) or Thermo Fisher Scientific, Inc. (Waltham, MA U.S.A.).

Methods

5' End-labeling of oligonucleotides with T4 PNK. Reactions were carried out using the manufacturer's recommended conditions and supplied buffer. Twenty-five pmol of oligo was labeled using ~33 pmol of [γ - 32 P] ATP (3000 Ci/mmol) for 20 minutes at 37°C. In order to ensure that all 5' ends were phosphorylated, an additional 5000 pmol of cold ATP was added and incubations were continued for 10 minutes. The PNK was then inactivated by incubating at 70°C for 20 minutes and excess nucleotide was removed using a G-25 spin column.

Determination of equilibrium dissociation constants (K_d) with 5' end-labeled substrates. Designed oligonucleotides (see Fig. 2) were 5' end-labeled and mixed with various amounts of HIV-RT (typically 0.13, 0.25, 0.50, 0.75, 1, 2, and 4 nM for tight binding substrates and higher amounts for those binding with lower affinity) in 8 μ L of buffer containing 1 nM oligonucleotide, 50 mM Tris-HCl (pH 8), 1 mM DTT, 80 mM KCl, 6 mM MgCl₂, and 0.1 μ g/ μ L BSA for 5 minutes at room temperature. Reactions were initiated by the addition of 2 μ L dNTPs (100 μ M final in reactions) and heparin "trap" (1 μ g/ μ L final in reactions) in the same buffer as above. The trap was added to sequester RT molecules not bound to the substrate and those that dissociate. This limits extension to a single binding event between the substrate and the enzyme (Peliska and Benkovic, 1992). Samples were incubated for 2 minutes, and were then stopped with an equal volume of 2 $\times$ gel loading buffer (90% formamide, 10 mM EDTA, pH 8, 0.25% each bromophenol blue and xylene cyanol). The reactions were run on a 12% denaturing polyacrylamide gel as described below, and dried gels were imaged using a Bio-Rad FX phosphorimager. The amount of bound enzyme at various concentrations of RT was determined from the proportion of extended products. Measurements of both extended (E) and unextended (U) material were taken and the ratio of E/(U + E) $\times$ 1 (nM concentration of substrate) was used to calculate the level of extended product. This approach helps correct for minor gel loading variations. Controls for the effectiveness of the trap and full extension of the substrate were also performed as described previously (DeStefano and Cristofaro,

2006). Values for the equilibrium dissociation constant (K_d) were determined by plotting the concentration of extended product (nM) vs. the concentration of HIV-RT and fitting the data by nonlinear least square fit to the quadratic equation: $[ED] = 0.5([E]_t + [D]_t + K_d) - 0.5([E]_t + [D]_t + K_d)^2 - 4[E]_t[D]_t)^{1/2}$, where $[E]_t$ is the total enzyme concentration and $[D]_t$ is the total primer-template concentration (Hsieh et al., 1993). Experiments were generally repeated 2–4 times (as indicated in Fig. 2) and averages $\pm$ standard deviations are reported. Note that this approach actually yields an “apparent K_d ” value as it is dependent on a secondary measurement (polymerase extension) to assess binding. This would be a concern if there were secondary binding sites on the substrates that could strongly compete with the 3' recessed terminus for RT, or if a substantial proportion of RT interactions with the 3' terminus were nonproductive with RT dissociating before incorporating nucleotides. Each of these concerns is of very low probability for these substrates.

Determination of equilibrium dissociation constants (K_d) by internal incorporation assay. Assays were performed using the same conditions as described above for the 5' end-labeled determinations. The 60-nt SELEX substrate (see Fig. 2) was either 5' phosphorylated with cold ATP or left unphosphorylated. Reactions were initiated as above except the final concentration of dNTPs was 50 μ M each for dGTP, dCTP, and dTTP, and 1 μ M [α - 32 P] dATP (~110 Ci/mmol) was included. Reactions were stopped with 40 μ L of 25 mM EDTA (pH 8) and excess nucleotide was removed using Sephadex G-50 spin columns. The material was dried and resuspended, then electrophoresed as described above. Only extended products can be quantified by this approach. The level of total substrate was determined using a control without trap.

Preparation of ddG-terminated loop-back substrates. Fifty pmoles of loop-back DNA phosphorylated at the 5' end was incubated with 5 units of Klenow polymerase in 50 μ L of buffer containing 50 mM Tris-HCl (pH 8), 1 mM DTT, 50 mM KCl, 6 mM $MgCl_2$, and 25 μ M ddGTP for 30 minutes at 37°C. The material was extracted and precipitated, then run through two successive Sephadex G-25 spin columns to remove any remaining unused ddGTP. Recovered material comigrated with the original substrate and was not extendable by HIV-RT in the presence of dNTPs (determined by 5' end-labeling a portion of the material at low specific activity with 32 P), indicating that it was 3' terminated with ddG.

Competition binding assay. Reactions contained 10 nM (final concentration) 5' 32 P end-labeled 37 NT SELEX substrate (see Fig. 2D) and 4 nM (final concentration) HIV-RT in 8 μ L of buffer containing 50 mM Tris-HCl (pH 8), 1 mM DTT, 80 mM KCl, 6 mM $MgCl_2$, and 0.1 μ g/ μ L BSA. Various amounts (0, 5, 10, 20, 30, 40, 60, 80, or 160 nM) of cold competitor were also included in the reactions. Samples were incubated at room temperature for 1 hour. Reactions were initiated by the addition of 2 μ L dNTPs (100 μ M final in reactions) and heparin “trap” (1 μ g/ μ L final in reactions) in the same buffer as above. After 2 minutes, reactions were terminated with 10 μ L of 2 $\times$ loading buffer and subjected to electrophoresis on 12% polyacrylamide denaturing gels as described below. The amount of extended product was determined with a Bio-Rad FX phosphorimager. A graph of relative extension (the sample with no competitor added was assigned a value of 1 and all other samples were relative to this) vs. amount of

competitor was plotted. The experiment was repeated with similar results.

Preparation of substrate for RT inhibition and binding inhibition assays. Sixty pmoles of 50-nt template (5'-TTGTAATACGACTCACTATAGGGCGAATTCGAGCTCGGTA CCGGGGATC) and 50 pmoles of 33-nt 5' 32 P end-labeled primer (5'-TTCCCCGGGTACCCGAGCTCGAATTCGCC CTATAG) were mixed in 20 μ L containing 50 mM Tris-HCl (pH 8), 1 mM DTT, and 80 mM KCl. The mixture was heated to 80°C for 2 minutes, then cooled at a rate of 1°C per minute to 30°C. This material was used directly in the assays.

RT inhibition assay. Reactions contained substrate (1:1.2 primer:template, final concentration in reactions was 50 nM in 5' 32 P end-labeled primer) in 30 μ L of buffer containing 50 mM Tris-HCl (pH 8), 1 mM DTT, 80 mM KCl, 6 mM $MgCl_2$, and 0.1 μ g/ μ L BSA. Various amounts (1.25, 2.5, 5, 10, or 50 nM) of inhibitors (either 37 NT SELEX or 37 NT Random each terminated with ddG) were also included in the assays. HIV-RT (0.25 nM final concentration in reactions) was added to the mixture in 5 μ L of the above buffer and incubated at room temperature for 30 minutes. The mixture was placed at 37°C and primer extension was initiated by adding 5 μ L of a supplement containing 800 μ M dNTPs (100 μ M final) in the above buffer. Five microliter aliquots were removed at 2, 5, 10, 15, and 20 minutes and add to 5 μ L of 2 $\times$ gel loading buffer. Samples were run on a 10% denaturing gel as described below and quantified using a phosphorimager as described above. A graph of the concentration of extended primer (nM) vs. time was plotted. The experiment was repeated with similar results.

Binding inhibition assay. Reactions contained substrate (1:1.2 primer:template, final concentration in reactions was 10 nM of 5' 32 P end-labeled primer) and 4 nM HIV-RT in 8 μ L of buffer containing 50 mM Tris-HCl (pH 8), 1 mM DTT, 80 mM KCl, 6 mM $MgCl_2$, and 0.1 μ g/ μ L BSA. Various amounts (0, 2.5, 5, 10, 20, 40, or 80 nM for 37 NT SELEX and 0, 40, 80, 160, or 320 nM for 37 NT Random) of cold competitor were also included in the reactions. Samples were incubated at room temperature for 30 minutes. Reactions were initiated by the addition of 2 μ L dNTPs (100 μ M final in reactions) and heparin “trap” (1 μ g/ μ L final in reactions) in the same buffer as described above. After 2 minutes, reactions were terminated with 10 μ L of 2 $\times$ loading buffer and subjected to electrophoresis on 12% polyacrylamide denaturing gels as described below. The amount of extended product was determined with a Bio-Rad FX phosphorimager. A representative quantified gel is presented. The experiment was repeated with similar results.

Gel electrophoresis. Ten or twelve percent denaturing polyacrylamide (19:1 w/w acrylamide:bisacrylamide, 7 M urea) gels were prepared. Electrophoresis was carried out using standard Tris-borate-EDTA electrophoresis buffer (Sambrook and Russell, 2001).

Results

Determination of equilibrium dissociation constant (K_d) values for modified substrates

In the previous study, K_d values were determined for several substrates derived from the SELEX protocol. These consisted of two complementary strands with a 46-nt primer

FIG. 1. The primer-template selected by SELEX protocol. Shown is the sequence of a primer-template that was selected for high affinity to HIV-RT using a SELEX-based approach (**A**) and a representative sequence selected from the starting material (**B**) (DeStefano and Cristofaro, 2006). The sequence shown in (**A**) was very similar to several others that were present in the final SELEX pool and was chosen for further analysis. Nucleotides in boldface were derived from the randomized region of the starting material while those in normal case were within a region of a nonvariable sequence used for PCR amplification.

strand and 50-nt template bound such that a 4-nt 5' template overhang was present (Fig. 1). For use as an inhibitor, a single-stranded substrate would likely be more stable and easier to produce than a double-stranded substrate. In this study, we focused on single-stranded loop-back substrates only. Size may also be an important parameter for an inhibitor with smaller aptamers being potentially easier to deliver and less expensive to make. We first sought to determine how small the aptamers could be without losing their high-affinity nature. Shown in Figure 2 are loop-back nucleic acids of 60-, 37-, 27-, and 22-nt derived from a high-affinity SELEX primer-template (Fig. 1) or a random sequence from the starting pool in the previous report (Fig. 2A and 2C) (DeStefano and Cristofaro, 2006). The structures shown were predicted with the DNAfold program using default conditions with 80 mM monovalent cation and 6 mM Mg^{2+} at 37°C (Zuker, 2003). The SELEX aptamers all have 7 G residues at the 3' end and a predicted 3 nt or 4 nt loop made of A residues. The 5'-AATA-3' overhang is also identical in these SELEX aptamers. They were designed by progressively eliminating nucleotides adjacent to the loop from 60-nt SELEX., which had a 25-bp duplex region while the 37 (2F), 27 (2G), and 22 (2H) nt aptamers had 15, 10, and 7 nt duplexes, respectively. All aptamers were completely phosphorylated at the 5' end as the state of 5' phosphorylation was shown to affect binding (see below). Individual K_d values for each of these substrates were determined by RT extension assays as described in Materials and Methods. A representative experiment is shown in Figure 2P and 2Q where 2P shows a gel analysis using the substrates shown in Figure 2F and 2L, while 2Q shows a graphical quantification of K_d values. The calculated K_d values for the 60 (0.44 ± 0.23 nM) and 37 (0.66 ± 0.16 nM) NT SELEX aptamers were both below 1 nM and were nearly identical. In contrast, the K_d increased about 100-fold for the 27 NT SELEX aptamer (60 ± 1 nM), while a $K_d > 1$ μ M was measured for the 22 NT SELEX aptamer. The random sequence controls bound with much lower affinity and unlike the SELEX, a large ~8-fold decrease in affinity was observed when shortening the aptamer from 60 (2A) to 37 (2C) nt ($K_d = 20 \pm 4$ nM and 161 ± 18 nM, respectively). A 22-nt version of the random sequences showed no extension

even after prolonged incubation with RT (data not shown). The relatively low affinity of the tested random sequence was not due to a unique property as this sequence was comparable to the affinity measured for a random pool and other randomly selected sequences in the previous report (DeStefano and Cristofaro, 2006). Two additional control substrates were also tested (Fig. 2D and 2E). In 2D, the positions of the G and C runs in 37 NT SELEX (2F) were flipped without significant alterations to the ΔG values (~19 kcal/mol). This resulted in a ~80-fold decrease in affinity of RT for the substrate compared with 37 NT SELEX. Figure 2E represents a control in which the nucleotides in the stem portion of 37 NT SELEX were scrambled, while not changing the ΔG . An ~400-fold increase in K_d was observed for this control. Although the three controls varied in the degree of fold decrease in affinity, all showed weak binding compared with 37 NT SELEX. Thus, there was no correlation between structural stability (ΔG) and affinity, and the binding to RT was driven by the specific sequence.

We previously established that the G-tract was the key parameter responsible for tight binding of the aptamers (DeStefano and Cristofaro, 2006). The 37 NT SELEX aptamer from above was used to examine how small perturbations of this tract affected binding. Changing the 3' terminal G residue to a C (Fig. 2I) or A (Fig. 2J) (and the corresponding paired base to G or T, respectively) resulted in a small ~3-fold increase in the K_d (from 0.66 to 1.7 and 2.3 nM for the C and A change, respectively). In contrast, changing a G to C in the middle of the tract (Fig. 2K) resulted in ~12-fold increase (to 8.0 ± 1.7 nM). The greater sensitivity in the middle of the tract could result from greater structure perturbation as the tight binding conferred by the G run probably has a structural basis (Cote et al., 2003; Rausch and Le Grice, 2004; DeStefano and Cristofaro, 2006). A G to A change in the tract (2L) resulted in a smaller increase in K_d (4.4 ± 1.4 nM). This suggests some preference for purines in the run.

The length of the 5' overhang was also tested as a potential parameter for tight binding as it has been demonstrated that the overhang length is important for RT binding (Patel et al., 1995; Gorshkova et al., 2001). Two additional variants of the tight binding 37-mer with 3-nt or 5-nt 5' overhangs

FIG. 2. (A–Q) Sequence, structure, K_d , and ΔG values of the various single-stranded loop-back DNAs that were tested. The structures shown were predicted with the DNAfold program using default conditions with 80 mM monovalent cation and 6 mM Mg^{2+} at 37°C (Zuker, 2003). Names include the length in nucleotides of each DNA and whether it was derived from the selected high-affinity primer-template (SELEX) or a random sequence selected from the starting pool in the original paper (Random) (see Fig. 1). 2C, 2D, and 2E are controls (see text). Nucleotides in gray represent important changes to 37 NT SELEX (2F). The K_d values are averages (number of experiments in parentheses) from independent experiments $\pm$ standard deviation values. (P) A representative autoradiogram from an experiment using 37 NT SELEX (F) and 37 NT SELEX C to T pos. 8 and G to A pos. 34 (L) is shown. K_d determination assay was performed as described in Materials and Methods. Lane A, no enzyme; lane B, full extension (no trap); lane C, trap control (trap added prior to enzyme addition); other lanes left to right, 0.31, 0.63, 1.25, 2.5, 5, and 10 nM HIV-RT. (Q) A graph of extended substrate (nM) vs. [RT] for the experiment shown in (P). The calculated K_d (determined as described in Materials and Methods) and R values for each substrate are indicated next to the curve.

(Fig. 2M and 2N, respectively) were constructed. Shortening the overhang to 3 increased the K_d to about twice that observed with the normal 4 nt overhang while increasing it to 5 had essentially no effect. This suggested that a nt overhang of 4 or more was necessary for optimal binding.

As was noted in the Introduction, a potential problem with nucleic acid inhibitors as therapeutics is their susceptibility to nuclease attack in the cell. One approach to minimizing this problem is including modified nucleotides that are less susceptible to cellular nucleases. Phosphothioate nucleosides in which the α -phosphate is replaced by a sulfur atom have been used successfully by others with DNA oligonucleotides designed as HIV inhibitors (Lavigne et al., 2001; Ferguson et al., 2006; Moelling et al., 2006; Matzen et al., 2007). As single-stranded regions of nucleic acid are particularly susceptible to nucleases, we designed a 38-nt aptamer with its three 5'-nt and three loop nucleotides replaced by phosphothionucleosides (Fig. 2O). The double-stranded loop portion of this aptamer is identical to the 37 NT SELEX aptamer (Fig. 2F), while the loop is composed of phosphothioate Ts rather than A residues. An additional phosphothioate T was also added to the 5' end. The K_d value (0.28 ± 0.14 nM) indicated very tight binding to this aptamer. The fact that it was slightly lower than for the 37 or 38 NT SELEX aptamers was probably not significant but the results clearly indicate that the presence of the phosphothioate residues does not significantly affect binding to RT.

Competition assays indicate that phosphorylation of the 5' end is required for optimal binding of aptamers with 3-nt or 4-nt 5' overhangs, addition of ddNTPs to the 3' end does not affect binding, and a blunt-end aptamer does not bind well.

The above assay uses extension from the 3' end to measure binding affinity. Therefore, nonextendable aptamers, similar to those terminated with dideoxy nucleosides or blunt-ended cannot be directly tested in the assay. A competition assay was used in the previous study to show that dideoxy termination of the tight binding primer-templates did not significantly affect binding (DeStefano and Cristofaro, 2006). Several versions of the 37 NT SELEX aptamer were designed and tested in a similar assay. A 33-nt blunt-ended version was constructed in which the 4 nt of the 5' overhang were deleted. Also constructed was a version with the 3' G residue replaced by ddG. In the competition assay, extension of 5' 32 P-labeled 37-mer (without ddG) is measured after incubation in the presence of different amounts of cold competitor. The assay is designed such that only a single round of primer extension occurs (see Materials and Methods). In the control, increasing amounts of cold 37 NT SELEX aptamer are added and extension is measured. As expected adding an equal amount of cold aptamer resulted in a ~50% reduction in extension and the level of extension decreases with increasing competitor (Fig. 3). The ddG-terminated aptamer was essentially equivalent to the normal aptamer. In contrast, the 37-mer without phosphate at the 5' terminus was significantly less competitive and a 50% reduction required ~8-fold excess competitor. The 37 NT Random sequence (Fig. 2C) and 33-nt blunt-ended version of the 37 NT SELEX aptamer produced no significant reduction in extension even

FIG. 3. Competition assay showing that phosphorylation of the 5' end is required for optimal binding of 37 NT SELEX, while a 3' dideoxy residue does not affect binding. Shown is a graph of relative extension of 37 NT SELEX vs. fold excess competitor for the various competitors indicated. The assay was designed to monitor the extension of 5' γ - 32 P end-labeled 37 NT SELEX in the presence of various amounts of cold competitor. Inclusion of a heparin "trap" at the start of the reaction limits extension to a single round of binding between the enzyme and substrate. Asterisk represents that relative values were derived by setting the level of extension in the absence of added competitor to 1. See Materials and Methods for details.

at 16-fold excess indicating that they bind relatively poorly to RT. Similar to the 37-mer, 5' phosphorylation of the 36-mer (Fig. 2M) also enhanced its competitiveness in the competition assay. In contrast, the 38-mer (Fig. 2N) was equally competitive with or without phosphorylation (data not shown). This suggests that a phosphate group following the fourth nucleotide of the 5' overhang is required for maximal binding, although a 5' terminal phosphate is not.

To examine the 5' phosphate effect more directly, the 60 NT SELEX (Fig. 2B), in phosphorylated or unphosphorylated form, was tested using internal incorporation (see Materials and Methods). The phosphorylated and unphosphorylated forms had K_d s of 0.29 ± 0.08 nM and 2.2 ± 0.1 nM (average of two experiments $\pm$ SD), respectively. The first value is close to what was measured in the assays with 5' end-labeled aptamer (0.44 ± 0.23 nM, Fig. 2B), while the latter was the same as the measurement for this substrate as reported previously (2.2 ± 0.4 nM) (DeStefano and Cristofaro, 2006). Overall, the results confirm the strong effect of phosphorylation on binding for the substrates with 4 base 5' overhangs.

The 37-nt SELEX aptamer is a potent inhibitor of HIV-RT in vitro

A primer extension assay was performed to determine whether the 37 NT SELEX aptamer could effectively inhibit

FIG. 4. (A and B) Inhibition assay showing that 37 NT SELEX is a potent inhibitor of HIV-RT primer extension. Shown is a schematic representation of the primer-template used in the assays (A) along with a graph (B) of extended primer vs. time in the presence of various amounts of 37 NT SELEX or 37 NT Random aptamers (as indicated). Each aptamer contained a 3' terminal dideoxy G residue in place of the normal G. The assay was conducted by incubating HIV-RT (0.25 nM) with the primer-template (5' γ - 32 P end-labeled primer, 50 nM) and aptamers, then initiating reactions with dNTPs and removing aliquots at the indicated times. The samples were run on a gel and quantified as described in Materials and Methods.

HIV-RT *in vitro*. A 33-nt 5' end-labeled DNA primer was hybridized to a 50 nt template (50 nM final concentration, Fig. 4A) and primer extension in the presence or absence of aptamer was performed using 0.25 nM HIV-RT. In these experiments, both the 37 NT SELEX (Fig. 2F) and 37 NT Random (Fig. 2C) aptamers had the terminal 3' G residue replaced with ddG and were therefore nonextendable. The SELEX aptamer was a potent inhibitor with the reaction rate decreasing by ~50% in the presence of 2.5 nM aptamer (Fig. 4B). In contrast, 50 nM of 37 NT Random was required to yield approximately the same decrease. At 50 nM, the SELEX aptamer completely blocked extension. Although 37 NT Random was severalfold less effective, it was somewhat surprising given its low K_d that inhibition was observed at the levels used in the assay, although this may have resulted from the terminal ddG residue creating a "dead-end complex" that can bind RT more tightly ((Tong et al., 1997), see Discussion).

The 37-nt SELEX aptamer strongly inhibits primer-template binding

The most likely mode of inhibition for the SELEX aptamer is through competitive binding where the aptamer occupies RT's nucleic acid binding site blocking binding of

the substrate. To test this directly, the substrate used above (33-nt primer [5' end-labeled] and 50-nt template, 10 nM final concentration) was incubated with increasing amounts of aptamer (not terminated with a ddG) and 4 nM RT in the absence of dNTPs (Fig. 5). After 30 minutes, primer extension was initiated by adding dNTPs along with a heparin "trap" to sequester unbound enzyme and prevent rebinding after dissociation. Extension of the 33-nt primer is directly proportional to the amount of enzyme bound to the substrate which is determined by RT's affinity for the substrate vs. the aptamer. The SELEX aptamer was strongly competitive with <10% extension observed relative to the reaction without added aptamer when the primer-template and aptamer were used at equal concentrations (10 nM). This indicates that RT binds the SELEX aptamer more strongly than the substrate, even though the substrate is a "more ideal" primer template with a longer double-stranded DNA region and 5' overhang. In contrast, a 32-fold excess of the 37 NT Random sequence (320 nM) was required to get ~50% inhibition, indicating that this aptamer binds much more poorly than the substrate.

Discussion

Development of aptamers for diagnostics, therapeutics, and as general molecular biology tools is an emerging area of science (see Introduction). In this study, we set out to optimize as potential HIV-RT inhibitors, double-stranded primer-template based aptamers that were isolated using a unique SELEX approach (DeStefano and Cristofaro, 2006). Although it is unclear what properties may lead to higher efficacy *in vivo* or even in cell culture, these initial experiments aimed to produce single-stranded inhibitors that were as small as possible for cost and delivery purposes, yet retained tight binding. The most "optimal" aptamers were 37–38 nt in length, and their affinity for RT was unaffected by replacement of specific nucleotides in single-stranded regions with phosphothioate nucleotides. This change could be advantageous in cells (see Results).

Shortening the 60 NT SELEX aptamer to 37 nt (cf. Fig. 2B and Fig. 2F) did not significantly alter binding. In contrast, a similarly designed random sequence showed ~8-fold decrease in binding when shortened from 60 (Fig. 2A) to 37 (Fig. 2C). Each 37-mer contains a 15-bp duplex region, which is too short to span the polymerase and RNase H domains (Wöhrle and Moelling, 1990; DeStefano et al., 1991; Furfine and Reardon, 1991; Arnold et al., 1992; Fu and Taylor, 1992; Gopalakrishnan et al., 1992; Kohlstaedt et al., 1992). The strong binding of 37 NT SELEX may reflect the contribution to binding of the 3' G run, through its interaction with the RT polymerase domain. This effect may allow tight binding despite a suboptimal length. Still, there is a clear threshold as 27 NT SELEX (Fig. 2G), which retains the G-run but has only a 10-bp duplex region, bound much more poorly.

The length and phosphorylation state of the 5' overhang was an important determinant of binding affinity. Affinity decreased when the overhang was shortened from 4 to 3 (Fig. 2M) or with the 4 base overhang without a 5' phosphate group (Fig. 3). A blunt-ended aptamer with no 5' overhang bound very poorly (Fig. 3). Addition of a fifth nucleotide to the overhang (Fig. 2N) did not enhance binding and RT's affinity

FIG. 5. (A and B) Binding inhibition assay shows that 37 NT SELEX but not 37 NT Random strongly blocks binding of the primer-template. Autoradiograms showing extension of 5' γ - 32 P end-labeled 33 nt primer on the template shown in Figure 4A are depicted. The assay was conducted essentially as described in Figure 3 except extension of the primer-template rather than radiolabeled 37 NT SELEX was monitored in the presence of various amounts of cold 37 NT SELEX (A) or 37 NT Random aptamer (B). The 3' terminal nucleoside of each aptamer was normal deoxy G in this assay. Primer-template was at 10 nM and cold aptamers were included at 0, 2.5, 5, 10, 20, 40, and 80 nM for 37 NT SELEX or 0, 40, 80, 160, and 320 nM for 37 NT Random. The level of relative extension in comparison to the assay without competitor ("0") is indicated above each lane as are positions of the primer and extended primers. Other lanes: -E, no enzyme included in reaction; FE, full extension control with enzyme incubated with primer-template and dNTPs in the absence of aptamer or heparin trap for 10 minutes in order to determine the maximum level of primer extension; TC, trap control to determine the effectiveness of the heparin trap in sequestering the enzyme. Enzyme was mixed with trap, dNTPs and primer-template added in succession, and incubations continued for 2 minutes before reaction termination.

for this aptamer (38 NT SELEX) was not strongly sensitive to the phosphorylation state of the 5' end (data not shown). The results showed that at least a 4-nt overhang, including a 5' phosphate are required for maximal binding. The 60-nt loop-back aptamer (60 NT SELEX, Fig. 2B) bound $\sim$ 7–8-fold more tightly when phosphorylated at the 5' end as judged by internal incorporation assays (0.29 ± 0.08 nM vs. 2.2 ± 0.1 nM). Interestingly, the value for the unphosphorylated aptamer was the same as the value measured for this aptamer previously (2.2 ± 0.4 nM) (DeStefano and Cristofaro, 2006). In the earlier report, although a 5' end-labeling assay was used, material was not completely phosphorylated so it is not surprising that the values would be different. We presume that the increase in binding affinity between 3 and 4 nt overhangs results from additional amino acid contacts between RT and the 5' template overhang. Binding of HIV-RT to primer-templates is known to be sensitive to the length of the 5' overhang on both DNA-DNA (Patel et al., 1995) and RNA (template)-DNA (primer) (Gorshkova et al., 2001) with relatively large changes occurring between 3 and 6 nt. As for the phosphorylation effect, this probably also results from additional contact with amino acids, however, what those contacts may be is unclear. Amino acid contacts with the +1 to +3 nt of the template overhang have been confirmed by both crystallographic and biochemical studies (Huang et al., 1998; Dash et al., 2006). Although it is postulated that contacts as far as +7 could affect binding or catalysis (Wöhrle et al., 1995), crystal structures are disordered beyond +3 so direct contacts have not been mapped (Huang et al., 1998; Dash et al., 2006).

Alterations in the G tract decreased binding affinity especially those within the central portion (see Fig. 2I, 2J, 2K, and 2L). Changing the fourth G in the 7 G run to A or C resulted

in about 7-fold and 12-fold, respectively, increases in K_d values. Changes at the 3' terminus had more modest effects. In addition to denoting the importance of the G-tract for binding, the results also further support a structural contribution for binding as changes in the center of the run are more likely to perturb the structure than those at the terminus. The importance of the G-tract being located in the "primer" strand is clearly illustrated by the 37 NT SELEX G-C flip control (Fig. 2D), which demonstrated that moving the G-tract to the "template" strand resulted in a $\sim$ 80-fold decrease in binding. It was still interesting that this control bound RT considerably better than the other two controls (Fig. 2C and 2E). This was not due to a difference in structural stability as the 37 NT SELEX Δ G control (2E) was as stable as the G-C flip aptamer but bound less tightly. We suggest that the structure of the G-run is an important determinant for high-affinity binding to HIV-RT (for a detailed discussion, see DeStefano and Cristofaro, 2006). This could be true irrespective of whether the hybrid is RNA-DNA (similar to the HIV ppt) or DNA-DNA (as in the substrates tested here), as reports have shown that runs of nucleotides in either nucleic acid can form similar structures (Cote et al., 2003).

Competitive binding assays showed that the dideoxy G terminated version of the 37 NT SELEX aptamer bound RT with essentially the same affinity as the version terminated with deoxy G, indicated no role for the 3'-OH in tight binding (Fig. 3). This same aptamer was a potent inhibitor of RT in inhibition assays (Fig. 4B). In the cell, an aptamer with a 3' recessed terminus is likely to be extended in the presence of RT and dNTPs, generating a blunt-end. This can be prevented by including a chain terminating base at the terminus. Although ddG was used in our experiments, other

nonextendable moieties such as 3'-3' terminal nucleotide junctions may also be successfully employed in its place. This may be important in the development of primer-template-based aptamers as HIV therapeutics. The nature of the chain terminator may be especially important as HIV can develop resistance to chain terminating inhibitors by producing RT mutants with enhanced ability to excise the terminator (Smith and Scott, 2006). Reports in the literature suggest that ddG may be a more effective terminator than other chain terminators although extensive studies with ddG have not been performed. The closely related chain terminator ddI (dideoxy inosine) is excised much more slowly by drug resistant RTs than several others that are being used in HIV therapy (Naeger et al., 2002). Also, the excision of 3'-azido-2', 3'-ddG (AZddG) by an AZT resistant HIV-RT mutant was relatively slow (Sluis-Cremer et al., 2005). Specific studies of ddI or ddG resistant RT mutants would be more informative in evaluating the efficacy of ddG termination.

It was interesting that a ddG-terminated version of the 37 NT Random sequence was a modest inhibitor of RT in the inhibition assay (Fig. 4B), yet the dG terminated form bound RT with low affinity (Fig. 2C) and was a very weak competitor in both types of competition binding assays (Figs. 3 and 5B). A possible reason for this is the formation of a "dead-end complex" when the chain terminator is present. Dideoxy terminated primer-templates bind with a very long half-life to HIV-RT in the presence of the template-directed dNTP that follows the dideoxy moiety (Tong et al., 1997). This effect may be observed in the inhibition assays where dNTPs are present during multiple rounds of extension. Clearly, however, a "dead-end complex" alone cannot explain the strong inhibition of 37 NT SELEX as it still shows severalfold more potent inhibition than the 37 NT Random sequence. Approximately 50% inhibition required 50 nM 37 NT Random and only about 2.5 nM 37 NT SELEX.

Other single-stranded DNA aptamers have also been selected for high affinity binding to HIV-RT using SELEX (Schneider et al., 1995; Mosing et al., 2005). In one set of experiments the selected ligands included several that were predicted to fold and form 3' recessed termini that contained a short run of G residues. This was not surprising as the invariant region of these molecules contained a run of 4 G's at the 3' terminus (Schneider et al., 1995). These aptamers typically contained an unhybridized "bulge" region just upstream of the 3' terminal G run and extension assays indicated that RT was bound to the aptamers with the 3' terminus positioned for productive nucleotide incorporation. Our procedure did not select any tight binding primer-template sequences with short (3-4 nt) runs of G at the 3' end (DeStefano and Cristofaro, 2006). This suggests that a short run by itself is unlikely to result in high-affinity binding. One possibility is that bulges or other structures preceding the short G run allowed the 3' terminal region of these aptamers to conform to a shape similar to a run of several G's. The authors suggested that the bulges in these aptamers could attain a "bent" structure allowing tighter binding to the RT substrate binding cleft (Schneider et al., 1995). It would also be interesting to see if the G run in these aptamers was important to tight binding. As the run was derived from the invariant rather than variable region of the starting material,

it is unclear whether any base changes in this region would have influenced RT affinity. Other DNA aptamers have been isolated that bear no obvious resemblance to the primer-template forms selected by us and others, suggesting that there are additional structural or sequence motifs that generate tight binding (Schneider et al., 1995; Mosing et al., 2005; Held et al., 2006a; Kissel et al., 2007a, 2007b).

In conclusion, this work shows that small primer-template-based nucleic acids can bind with high affinity to RT and inhibit its activity. As these aptamers are essentially optimized versions of RT's natural ligand (primer-template), they may be more difficult to develop resistance to. Tests for virus inhibition in cells are ongoing and should help determine if this new class of aptamers could potentially be used as therapeutics.

Acknowledgment

This work was supported by National Institute of General Medicine grant number GM051140.

References

- ARNOLD, E., JACOBO-MOLINA, A., NANNI, R.G., WILLIAMS, R.L., LU, X., DING, J., CLARK, A.D., JR., ZHANG, A., FERRIS, A.L., CLARK, P., ET AL. (1992). Structure of HIV-1 reverse transcriptase/DNA complex at 7 Å resolution showing active site locations. *Nature* **357**, 85-89.
- BRODY, E.N., and GOLD, L. (2000). Aptamers as therapeutic and diagnostic agents. *J. Biotechnol.* **74**, 5-13.
- CHEN, H., and GOLD, L. (1994). Selection of high-affinity RNA ligands to reverse transcriptase: inhibition of cDNA synthesis and RNase H activity. *Biochemistry* **33**, 8746-8756.
- COTE, M.L., PFLOMM, M., and GEORGIADIS, M.M. (2003). Staying straight with A-tracts: a DNA analog of the HIV-1 polypurine tract. *J. Mol. Biol.* **330**, 57-74.
- DASH, C., FISHER, T.S., PRASAD, V.R., and LE GRICE, S.F. (2006). Examining interactions of HIV-1 reverse transcriptase with single-stranded template nucleotides by nucleoside analog interference. *J. Biol. Chem.* **281**, 27873-27881.
- DEISINGH, A.K. (2006). Aptamer-based biosensors: biomedical applications. *Handb. Exp. Pharmacol.* 341-357.
- DESTEFANO, J.J., and CRISTOFARO, J.V. (2006). Selection of primer-template sequences that bind human immunodeficiency virus reverse transcriptase with high affinity. *Nucleic Acids Res.* **34**, 130-139.
- DESTEFANO, J.J., BUISER, R.G., MALLABER, L.M., BAMBARA, R.A., and FAY, P.J. (1991). Human immunodeficiency virus reverse transcriptase displays a partially processive 3' to 5' endonuclease activity. *J. Biol. Chem.* **266**, 24295-24301.
- ELLINGTON, A.D., and SZOSTAK, J.W. (1990). *In vitro* selection of RNA molecules that bind specific ligands. *Nature* **346**, 818-822.
- FERGUSON, M.R., ROJO, D.R., SOMASUNDERAM, A., THIVIANATHAN, V., RIDLEY, B.D., YANG, X., and GORENSTEIN, D.G. (2006). Delivery of double-stranded DNA thioaptamers into HIV-1 infected cells for antiviral activity. *Biochem. Biophys. Res. Commun.* **344**, 792-797.
- FISHER, T.S., JOSHI, P., and PRASAD, V.R. (2002). Mutations that confer resistance to template-analog inhibitors of human immunodeficiency virus (HIV) type 1 reverse transcriptase lead to severe defects in HIV replication. *J. Virol.* **76**, 4068-4072.
- FU, T.B., and TAYLOR, J. (1992). When retroviral reverse transcriptases reach the end of their RNA templates. *J. Virol.* **66**, 4271-4278.

- FURFINE, E.S., and REARDON, J.E. (1991). Reverse transcriptase RNase H from the human immunodeficiency virus. Relationship of the DNA polymerase and RNA hydrolysis activities. *J. Biol. Chem.* **266**, 406–412.
- GOLD, L. (1995). The SELEX process: a surprising source of therapeutic and diagnostic compounds. *Harvey Lect.* **91**, 47–57.
- GOPALAKRISHNAN, V., PELISKA, J.A., and BENKOVIC, S.J. (1992). Human immunodeficiency virus type 1 reverse transcriptase: spatial and temporal relationship between the polymerase and RNase H activities. *Proc. Natl. Acad. Sci. USA* **89**, 10763–10767.
- GORSHKOVA, I.I., RAUSCH, J.W., LE GRICE, S.F., and CROUCH, R.J. (2001). HIV-1 reverse transcriptase interaction with model RNA-DNA duplexes. *Anal. Biochem.* **291**, 198–206.
- HELD, D.M., KISSEL, J.D., PATTERSON, J.T., NICKENS, D.G., and BURKE, D.H. (2006a). HIV-1 inactivation by nucleic acid aptamers. *Front. Biosci.* **11**, 89–112.
- HELD, D.M., KISSEL, J.D., SARAN, D., MICHALOWSKI, D., and BURKE, D.H. (2006b). Differential susceptibility of HIV-1 reverse transcriptase to inhibition by RNA aptamers in enzymatic reactions monitoring specific steps during genome replication. *J. Biol. Chem.* **281**, 25712–25722.
- HSIEH, J.C., ZINNEN, S., and MODRICH, P. (1993). Kinetic mechanism of the DNA-dependent DNA polymerase activity of human immunodeficiency virus reverse transcriptase. *J. Biol. Chem.* **268**, 24607–24613.
- HUANG, H., CHOPRA, R., VERDINE, G.L., and HARRISON, S.C. (1998). Structure of a covalently trapped catalytic complex of HIV-1 reverse transcriptase: implications for drug resistance [see comments]. *Science* **282**, 1669–1675.
- JAEGER, J., RESTLE, T., and STEITZ, T.A. (1998). The structure of HIV-1 reverse transcriptase complexed with an RNA pseudoknot inhibitor. *EMBO J.* **17**, 4535–4542.
- JAMES, W. (2007). Aptamers in the virologists' toolkit. *J. Gen. Virol.* **88**, 351–364.
- JOSHI, P., and PRASAD, V.R. (2002). Potent inhibition of human immunodeficiency virus type 1 replication by template analog reverse transcriptase inhibitors derived by SELEX (systematic evolution of ligands by exponential enrichment). *J. Virol.* **76**, 6545–6557.
- JOSHI, P.J., FISHER, T.S., and PRASAD, V.R. (2003). Anti-HIV inhibitors based on nucleic acids: emergence of aptamers as potent antivirals. *Curr. Drug Targets Infect. Disord.* **3**, 383–400.
- JOSHI, P.J., NORTH, T.W., and PRASAD, V.R. (2005). Aptamers directed to HIV-1 reverse transcriptase display greater efficacy over small hairpin RNAs targeted to viral RNA in blocking HIV-1 replication. *Mol. Ther.* **11**, 677–686.
- JOYCE, G.F. (1989). Amplification, mutation and selection of catalytic RNA. *Gene* **82**, 83–87.
- KENSCH, O., CONNOLLY, B.A., STEINHOFF, H.J., MCGREGOR, A., GOODY, R.S., and RESTLE, T. (2000). HIV-1 reverse transcriptase-pseudoknot RNA aptamer interaction has a binding affinity in the low picomolar range coupled with high specificity. *J. Biol. Chem.* **275**, 18271–18278.
- KISSEL, J.D., HELD, D.M., HARDY, R.W., and BURKE, D.H. (2007a). Active site binding and sequence requirements for inhibition of HIV-1 reverse transcriptase by the RT1 family of single-stranded DNA aptamers. *Nucleic Acids Res.* **35**, 5039–5050.
- KISSEL, J.D., HELD, D.M., HARDY, R.W., and BURKE, D.H. (2007b). Single-stranded DNA aptamer RT149 inhibits RT polymerase and RNase H functions of HIV type 1, HIV type 2, and SIVCPZ RTs. *AIDS Res. Hum. Retroviruses* **23**, 699–708.
- KOHLSTAEDT, L.A., WANG, J., FRIEDMAN, J.M., RICE, P.A., and STEITZ, T.A. (1992). Crystal structure at 3.5 Å resolution of HIV-1 reverse transcriptase complexed with an inhibitor. *Science* **256**, 1783–1790.
- KOLB, G., REIGADAS, S., CASTANOTTO, D., FAURE, A., VENTURA, M., ROSSI, J.J., and TOULME, J.J. (2006). Endogenous expression of an anti-TAR aptamer reduces HIV-1 replication. *RNA Biol.* **3**, 150–156.
- LAUVIGNE, C., YELLE, J., SAUVE, G., and THIERRY, A.G. (2001). Lipid-based delivery of combinations of antisense oligodeoxynucleotides for the *in vitro* inhibition of HIV-1 replication. *AAPS PharmSci.* **3**, E7.
- MATSUGAMI, A., TOCHIO, H., NIYADA, E., TAMURA, Y., KUDO, M., MISONO, T.S., KUMAR, P., and KATAHIRA, M. (2005). Structure of RNA aptamer complexed with an RNA-binding peptide of Tat with aid of residue-specific ¹³C, ¹⁵N labeling. *Nucleic Acids Symp. Ser. (Oxf.)* **69–70**.
- MATZEN, K., ELZAOUK, L., MATSKEVICH, A.A., NITZSCHE, A., HEINRICH, J., and MOELLING, K. (2007). RNase H-mediated retrovirus destruction *in vivo* triggered by oligodeoxynucleotides. *Nat. Biotechnol.* **25**, 669–674.
- METIFIOT, M., LEON, O., TARRAGO-LITVAK, L., LITVAK, S., and ANDREOLA, M.L. (2005). Targeting HIV-1 integrase with aptamers selected against the purified RNase H domain of HIV-1 RT. *Biochimie* **87**, 911–919.
- METIFIOT, M., FAURE, A., GUYONNET-DUPERAT, V., BELLECAVE, P., LITVAK, S., VENTURA, M., and ANDREOLA, M.L. (2007). Cellular uptake of ODNs in HIV-1 human-infected cells: a role for viral particles in DNA delivery? *Oligonucleotides* **17**, 151–165.
- MOELLING, K., ABELS, S., JENDIS, J., MATSKEVICH, A., and HEINRICH, J. (2006). Silencing of HIV by hairpin-loop-structured DNA oligonucleotide. *FEBS Lett.* **580**, 3545–3550.
- MOSING, R.K., MENDONSA, S.D., and BOWSER, M.T. (2005). Capillary electrophoresis-SELEX selection of aptamers with affinity for HIV-1 reverse transcriptase. *Anal. Chem.* **77**, 6107–6112.
- NAEGER, L.K., MARGOT, N.A., and MILLER, M.D. (2002). ATP-dependent removal of nucleoside reverse transcriptase inhibitors by human immunodeficiency virus type 1 reverse transcriptase. *Antimicrob. Agents Chemother.* **46**, 2179–2184.
- NIMJEE, S.M., RUSCONI, C.P., and SULLENGER, B.A. (2005). Aptamers: an emerging class of therapeutics. *Annu. Rev. Med.* **56**, 555–583.
- PATEL, P.H., JACOBO-MOLINA, A., DING, J., TANTILLO, C., CLARK, A.D., JR., RAAG, R., NANNI, R.G., HUGHES, S.H., and ARNOLD, E. (1995). Insights into DNA polymerization mechanisms from structure and function analysis of HIV-1 reverse transcriptase. *Biochemistry* **34**, 5351–5363.
- PELISKA, J.A., and BENKOVIC, S.J. (1992). Mechanism of DNA strand transfer reactions catalyzed by HIV-1 reverse transcriptase. *Science* **258**, 1112–1118.
- PORSCHESKI, P., GRATTINGER, M.A., KLENZKE, K., ERPENBACH, A., BLIND, M.R., and SCHAFER, F. (2006). Using aptamers as capture reagents in bead-based assay systems for diagnostics and hit identification. *J. Biomol. Screen.* **11**, 773–781.
- RAUSCH, J.W., and LE GRICE, S.F. (2004). 'Binding, bending and bonding': polypurine tract-primed initiation of plus-strand DNA synthesis in human immunodeficiency virus. *Int. J. Biochem. Cell Biol.* **36**, 1752–1766.
- SAMBROOK, J. and RUSSELL, D.W. (2001). *Molecular cloning: A Laboratory Manual*, 3rd ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY) pp 1274–1280.
- SCHNEIDER, D.J., FEIGON, J., HOSTOMSKY, Z., and GOLD, L. (1995). High-affinity ssDNA inhibitors of the reverse transcriptase of type 1 human immunodeficiency virus. *Biochemistry* **34**, 9599–9610.
- SLUIS-CREMER, N., ARION, D., PARIKH, U., KOONTZ, D., SCHINAZI, R.F., MELLORS, J.W., and PARNIAK, M.A. (2005). The 3'-azido group is not the primary determinant of 3'-azido-3'-deoxythymidine (AZT) responsible for the excision phenotype of AZT-resistant HIV-1. *J. Biol. Chem.* **280**, 29047–29052.

- SMITH, A.J., and SCOTT, W.A. (2006). The influence of natural substrates and inhibitors on the nucleotide-dependent excision activity of HIV-1 reverse transcriptase in the infected cell. *Curr. Pharm. Des.* **12**, 1827–1841.
- TONG, W., LU, C.-D., SHARMA, S.K., MATSUURA, S., SO, A.G., and SCOTT, W.A. (1997). Nucleotide-induced stable complex formation by HIV-1 reverse transcriptase. *Biochemistry* **36**, 5749–5757.
- TRIPATHI, S., CHAUBEY, B., BARTON, B.E., and PANDEY, V.N. (2007). Anti HIV-1 virucidal activity of polyamide nucleic acid-membrane transducing peptide conjugates targeted to primer binding site of HIV-1 genome. *Virology* **363**, 91–103.
- TUERK, C., and GOLD, L. (1990). Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science* **249**, 505–510.
- TUERK, C., MACDOUGAL, S., and GOLD, L. (1992). RNA pseudoknots that inhibit human immunodeficiency virus type 1 reverse transcriptase. *Proc. Natl. Acad. Sci. USA* **89**, 6988–6992.
- WÖHRL, B.M., and MOELLING, K. (1990). Interaction of HIV-1 ribonuclease H with polypurine tract containing RNA-DNA hybrids. *Biochemistry* **29**, 10141–10147.
- WÖHRL, B.M., TANTILLO, C., ARNOLD, E., and LE GRICE, S.F. (1995). An expanded model of replicating human immunodeficiency virus reverse transcriptase. *Biochemistry* **34**, 5343–5356.
- ZHANG, Z., BLANK, M., and SCHLUESENER, H.J. (2004). Nucleic acid aptamers in human viral disease. *Arch. Immunol. Ther. Exp. (Warsz)* **52**, 307–315.
- ZUKER, M. (2003). Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res.* **31**, 3406–3415.

Address reprint request to:

Prof. Jeffrey J. Destefano

Department of Cell Biology and Molecular Genetics

University of Maryland

Biosciences Building

College Park, MD 20742

E-mail: jdestefa@umd.edu

Received August 01, 2007; accepted in revised form

January 18, 2008.