Short Partially Double-Stranded Oligodeoxynucleotide Induces Reverse Transcriptase/RNase H-Mediated Cleavage of HIV RNA and Contributes to Abrogation of Infectivity of Virions

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ABSTRACT

We describe a novel mechanism of viral RNA eradication by an oligodeoxynucleotide A (ODN A) directly in HIV virions. The ODN A consists of an antisense and a passenger strand, and was designed to target the poly-purine tract (PPT) of HIV-1, a conserved region of the viral genome. It leads to HIV reverse transcriptase/ribonuclease H (RT/RNase H)-dependent degradation of the RNA in viral particles. Illimaquinone, a specific inhibitor of RNase H, activity of HIV RT/RNase H, prevents RNA cleavage. The effect of the ODN A is sequence-specific and the passenger strand is important, since a lack or alteration of this strand reduces the antiviral activity of the ODN. ODN A has a stronger antiviral effect compared to a control ODN CO, targeted to a site outside of the PPT. The pretreatment with ODN A strongly reduced the infectivity of virions in cell culture in the absence of any DNA carriers or detergents.

INTRODUCTION

HUMAN IMMUNODEFICIENCY VIRUS (HIV)-1 continues to be a global pandemic of enormous consequence to humanity. Current standard-of-care regimens recommended for the treatment of HIV infection include two or more nucleoside/nucleotide reverse transcriptase inhibitors (NRTI) in combination with nonnucleoside reverse transcriptase or protease inhibitors. Although success with these compounds has been demonstrated, toxicity and drug-resistance are the main problems.1 The discovery of RNAi has led many researchers to consider siRNA as a new antiviral tool. However, in long-term settings, HIV appears to escape from this therapeutic strategy through nucleotide mutations.2 In addition, HIV also appears to utilize a suppressor strategy to combat the cell’s RNAi defense. It has been shown that virus-encoded TAR RNA and Tat protein may at least partially block RNA silencing in human cells.3-5 Thus major focuses of the search for novel strategies to fight HIV-1 are to overcome these limitations and to develop agents with new targets and mechanisms of action.

Here we describe oligodeoxynucleotide A (ODN A), which exploits a unique mechanism of RNA degradation. Compared to other antisense oligonucleotides, ODN A exhibits a specific HIV reverse transcriptase/RNase H (RT/RNase H)-dependent antiviral effect. The principle for the design of ODN A was based on the general mechanism of reverse transcription of HIV, which involves two enzymatic functions of RT: the RNA- and DNA-dependent DNA polymerase and RNase H cleaving activities. During the course of reverse transcription HIV RT/RNase H initiates plus-strand DNA synthesis from a highly conserved, purine-rich RNA segment of the viral genome—the polypurine tract (PPT). This process occurs in several sequential steps including (1) minus-strand DNA synthesis over the PPT, (2) RNase H-mediated cleavage at the PPT 3’-terminus, (3) plus-strand DNA synthesis from the nascent PPT-containing RNA primer, and (4) primer removal. Both the polymerase and the RNase H activities are required for conversion of the viral RNA genome into DNA; mutations that inactivate either the polymerase or the RNase H activity block virus replication.6-8 The site of plus-strand initiation at the PPT is also important because it ultimately defines the end of the full-length double-stranded proviral DNA, which is recognized by the viral integrase.9 The PPT is highly conserved in most retroviruses and the mutagenesis rate for this region is minimal compared to other regions of the HIV RNA.10 Therefore, targeting the PPT with therapeutic drugs could potentially lead to reduced
or abolished virus replication. Based on this knowledge, we designed a partially double-stranded 54-mer ODN targeted to the extended PPT for intervention of replication. We show that the ODN A leads to RT/RNase H-dependent degradation of the RNA in intact virions, and abolishes infectivity of virus particles.

**MATERIALS AND METHODS**

**Oligodeoxynucleotides**

The ODN consists of a 25-mer antisense and a 25-mer passenger strand, connected by four thymidines (Fig. 1). The sequence of the PPT within the extended PPT. The sequences of ODN A and its variants are depicted in hypothetical partially self-complementary hairpin-loop structures. Curved lines symbolize the linker consisting of four phosphorothioated thymidines. ODN Sc has a randomized sequence of both strands and serves as a control for nonspecific action of phosphorothioated oligonucleotides. ODN CO targets a region on HIV RNA outside of the PPT. The other ODNs are designed to target the extended PPT. Sc, scrambled; B, one nucleotide removed from both 5' and 3' ends of ODN A; T, multiple substitutions in the passenger strand for complete complementarity to the antisense strand; H, triple substitution in the passenger strand for partial complementarity to the antisense strand; NT, triple substitution in the passenger strand in the site complementary to the CCCCCC site of the antisense strand; D, single substitution in the passenger strand in the site complementary to the AGT site of the antisense strand; CG, single substitution in the passenger strand in the site complementary to the TCT site of the antisense strand. The asEXT was used for a primer-extension experiment. Nucleotide changes made compared to ODN A are indicated in bold.

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**FIG. 1.** Sequences of ODNs. (A) The sequences of the extended polypurine tract, PPT, and a site downstream to the PPT of the viral RNA are shown in complex with ODN A and ODN CO accordingly. ODNs consist of an antisense strand and passenger strand linked by four thymidines. Watson–Crick bonds are shown by vertical bars. The sequence of the PPT within the extended PPT is indicated in bold. Antisense oligodeoxynucleotide asEXT targets sites outside of the extended PPT. Relative positioning of ODN A, ODN CO, and asEXT on synthetic RNA2 is schematically shown on the lower panel. The box represents the extended PPT and the white stripe symbolizes the cleavage site for the RT/RNase H at the 3' terminus of the PPT within the extended PPT. (B) The sequences of ODN A and its variants are depicted in hypothetical partially self-complementary hairpin-loop structures. Curved lines symbolize the linker consisting of four phosphorothioated thymidines. ODN Sc has a randomized sequence of both strands and serves as a control for nonspecific action of phosphorothioated oligonucleotides. ODN CO targets a region on HIV RNA outside of the PPT. All the other ODNs are designed to target the extended PPT. Sc, scrambled; B, one nucleotide removed from both 5' and 3' ends of ODN A; T, multiple substitutions in the passenger strand for complete complementarity to the antisense strand; H, triple substitution in the passenger strand for partial complementarity to the antisense strand; NT, triple substitution in the passenger strand in the site complementary to the CCCCCC site of the antisense strand; D, single substitution in the passenger strand in the site complementary to the AGT site of the antisense strand; CG, single substitution in the passenger strand in the site complementary to the TCT site of the antisense strand. The asEXT was used for a primer-extension experiment. Nucleotide changes made compared to ODN A are indicated in bold.
quence is partially complementary. ODN Sc served as a control for nonspecific activity of oligodeoxynucleotides. All the other oligodeoxynucleotides used were targeted to the extended PPT of HIV-1 with an exception of ODN CO, which had a secondary structure similar to ODN A but designed to target sequences downstream of the PPT (Fig. 1A).

The ODNs were phosphorothioated at each end (three bases) and in the T4 linker. The ODNs were purchased from MWG-Biotech, Ebersberg, Germany and Operon, Cologne, Germany.

Cells and viruses

The cellular assays were performed with the HTLV-1-transformed T cell line C81-66/45,11 kindly provided by R.C. Gallo (Institute of Human Virology, Baltimore, MD). The HIV-1IM viral strain was used. Syncytia formation was monitored as described previously.12

Determination of HIV RNA by RT-PCR and real-time RT-PCR

ODNs were incubated with intact HIV virions (107 virions per assay) at a concentration of 0.5 μM for 6 h at 37°C. For isolation of RNA QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA) and for reverse transcriptase polymerase chain reaction (RT-PCR) analysis Access RT-PCR Kit (Promega, Madison WI) were used according to the manufacturer’s instructions. RNA was reverse transcribed using High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) and the amount of undigested RNA was quantified by the real-time PCR assay using the ABI 7300 instrument (Applied Biosystems). Sequences of PCR primers (which cover a region close to the PPT) and probe were the following: For 1 (8536–8556): 5'-GAG-GAGGTTGGTTTTTCCTAGC-Rev1 (8641–8661): 5'-GGGAGGTTAGTTGATTGGATG- and probe (8565–8596): 5'-FAM-A-CTTTAAGAACCAYTAGCTTACAGGCAC-TAMRA. Coordinates for the primers refer to accession AF033819. A control for equal recovery of RNA in cellular assays was performed using GAPDH primers: Gapd-for1: 5'-GAAAAGGGGGGACUGGAAGGG- and probe (8565–8596): 5'-FAM-CAAGCTTCCC-GTTCACGCC-TAMRA.

Semiquantitative RT-PCR was performed using primers, which cover segments of HIV RNA close to the PPT (PT region): PU3-1 (8366–8385): 5'-GATAGTGTATTGGGATG-GCCT and PU3-4 (8694–8713): 5'-GTTGTTGAGATC-CACAGATC; and primers covering the HIV region upstream of PPT (Env-region): PU2.1 (5998–6020): 5'-ACAGACCCC-CAACCCCAAGAAG and PU2.2 (6442–6463): 5'-TTAGATCGCAAACACGCGC. A control for equal recovery of RNA in cellular assays was performed using GAPDH primers: GAPD-1: 5'-CCGTCATAGAAAAACCTGCCA and GAPD-2: 5'-GAGCTTGACAAAGTGGTGCGT. Primers and probes were purchased from Operon, Cologne, Germany. The synthesis of ODN A-primed cDNA by viral RT/RNase H in the presence of ODN A was analyzed by PCR using cDNA-specific primer FUGW (8566–8587): 5'-GGGGAGGCTTGTAGCCTTGC and ODN A-specific primer A(1): 5'-CTTCTGAGGTTGGTTGG-TTG yielding a product of 80 bp.

Inhibition of viral expression in HIV-infected cells after incubation of ODN A with virions

A concentrated stock of sucrose density gradient-purified HIV-1 virions was incubated with 0.5 μM or 5 μM ODNs for 1, 2, 4, or 8 h at 37°C in RPMI cell culture medium supplemented with 10% heat-inactivated fetal calf serum. C81-66/45 cells (2 × 105) were infected with preincubated virions at 0.01 or 1 MOL RNA was extracted from infected cells and HIV replication was analyzed by RT-PCR and real-time PCR as described above. p24 production in the supernatant of the infected cells was measured by an Alliance p24 ELISA kit (Perkin Elmer, Boston, MA) following the manufacturer’s instructions.

RT/RNase H inhibition

Purified HIV-1 (5 × 106 virions per assay) was permeabilized with 0.1% NP-40 and incubated with or without RT/RNase H (0.05 units/μl) (Amersham Bioscience, Piscataway, NJ). 0.1 μM ODN A and 150 μM Illimaquinone (Calbiochem, La Jolla, CA), a selective inhibitor of RNase H activity of HIV RT/RNase H13 in phosphate-buffered saline (PBS) (pH 6.8) for 30 min at 37°C. Then viral RNA was purified and real-time PCR analysis was performed.

Synthetic RNA production

A plasmid pUC18 harboring a T7 promoter and a PPT-containing HIV-1 segment (pUC18-PPT-long) was used to produce a synthetic RNA2. The RNA was in vitro transcribed from this plasmid and linearized by EcoRV using a T7-Megashortscript kit (Ambion, Austin, TX). The sequence of RNA2 is 5'-GGGAGGCGACUGUAGAUCUUAGCACCUCUUUUAAAGAAAAAGGGGGACUGGAAGGGCUGUAGAUCUUAGCACCUCUUUUAAAGAAAAAGGGGGACUGGAAGGGCUGUAGAUCUUAGCACCUCUUUUAAAGAAAAAGGGGGACUGGAAGGGCCGGCUAAUUCACUGCCCAAAGAAGCACAGCCACGCAUGCUUUCGGAAGCGC-3'. The binding sites for ODN A are indicated in bold, the sites for asEXT are in italic. A control RNA3 lacking PPT was obtained similar to RNA2.

RT/RNase H cleavage assay

Radioactively 5'-labeled RNA2 (10 nM) was mixed with 10 nM ODNs in hybridization buffer (50 mM NaCl, 10 mM MgCl2, 1 mM 2-mercaptoethanol, 0.4 mM spermine hydrochloride, 25 mM Tris-acetate, pH 6.8), heat-treated for 3 min at 90°C, slowly cooled down, and incubated at 37°C for 30 min. After annealing the samples were incubated with RT/RNase H (0.05 units/μl) for 30 min at 37°C. The cleavage reaction was stopped by adding formamide RNA sample buffer followed by incubation for 5 min at 90°C, cooled down on ice, and subsequently analyzed by denaturing 8 M urea/10% PAGE.

Primer extension assay

Unlabeled RNA2 (10 nM) was hybridized with either 10 nM ODN A, 1 μM external antisense primer (asEXT), or both primers combined in hybridization buffer (50 mM NaCl, 10 mM MgCl2, 1 mM 2-mercaptoethanol, 0.4 mM spermine hydrochloride, 25 mM Tris-acetate, pH 6.8) as described above. Primer extension was then initiated with 0.125 μCi/μl [α-35S]dATP and 1.56 units/μl of RT/RNase H, and the mixture
was incubated for 30 min at 37°C. The reaction was stopped by adding formamide sample buffer, and the products were analyzed by 8 M urea/10% PAGE. The sequence of asEXT is 5'-CATGCACGTTGACGTT (Microsynth, Balgach, Switzerland). To avoid displacement of annealed ODN A by the RT/RNase H during cDNA synthesis starting with asEXT we used only dATP to initiate very short primer extensions since in both cases the first nucleotides utilized by the RT/RNase H are dATPs. Signals were detected by phosphoimager (Storm 840, Molecular Dynamics).

RESULTS

In vitro analysis of ODNs

In this study ODN A, which consists of a 25-mer antisense and a 25-mer passenger strand connected by four thymidines (T4), was used. The sequences of the strands are partially complementary. The ODN A was phosphorothioated at each end (three bases) and in the T4 linker. ODN A was targeted to the extended PPT of HIV-1. We also used a few structural variants of ODN A: ODN T, which has a passenger strand fully complementary to the antisense strand; the single-stranded antisense PPT (asPPT), which lacks the passenger strand and the linker; ODN H and ODN NT, which both have three nucleotide changes at different positions of the passenger strand; ODN CG and ODN D, both having a single nucleotide change at different positions of the passenger strand; and ODN B, which has one nucleotide removed from both 5' and 3' ends. ODN Sc has the same length and nucleotide composition as ODN A but a randomized sequence of both strands. ODN CO has a secondary structure similar to ODN A but targets sequences downstream of the extended PPT. Antisense oligodeoxynucleotide asEXT, which targets sites outside of the extended PPT, was also used (Fig. 1).

We previously showed that the ODN A at high concentrations (1 μM or more) inhibits the RT/RNase H activity in vitro.12 To check the effect of ODN A at lower concentrations of RT/RNase H-dependent cleavage of RNA, we tested various concentrations of ODNs in an RNase H cleavage assay. We used synthetic 5'-end-labeled RNA2, which contains the extended PPT and sequences of the viral genome close to the PPT. As can be seen in Fig. 2A, RNA2 was indeed cleaved with 10 nM ODN A, but not with 1 μM ODN A, suggesting that at low concentration ODN A can mediate the cleavage of the RNA by RT/RNase H. The cleavage depended on the presence of the PPT on the RNA, since RNA3, which does not harbor the PPT sequence, was not affected by ODN A-mediated cleavage (Fig. 2A). For the present study we used low concentrations of the ODNs.

To test whether an ODN-mediated activity would preferentially occur at the PPT, we analyzed ODNs targeted to the extended PPT (ODN A) or to a region outside of the PPT (ODN CO and an external antisense oligodeoxynucleotide asEXT) in vitro. We used RNA2, which contains the PPT and the binding

FIG. 2. ODNs induce the RT/RNase H-dependent cleavage of the viral RNA in a sequence-specific manner. (A) Cleavage assay. In vitro transcribed 5'-labeled RNA (10 nM) was hybridized with 10, 100, and 1000 nM ODN A and incubated in the presence of RT/RNase H. The cleavage products were analyzed by denaturing PAGE as described in Materials and Methods. (B) Primer extension assay. Synthetic RNA2 was hybridized with ODN A, antisense primer (asEXT), or both primers combined, and the primer extension was performed as described in Materials and Methods. Elongation products were analyzed by 8 M urea/10% PAGE. (C) Cleavage assay. RNA/ODN hybrids, obtained using 10 nM RNA2 and 10 nM ODN A, asPPT, or ODN CO were incubated with RT/RNase H and cleavage products were analyzed by denaturing PAGE as described in Materials and Methods. Cleavage or extension products are presented schematically to the right of each blot. Cleavage sites are indicated by arrows and labeled cleavage products are shown by black lines. Dots on extension products indicate the incorporated labeled dATPs.
sites for ODN A, asEXT, and ODN CO. As shown in Fig. 2B, RT/RNase H initiated primer extension of the ODN A more efficiently than the antisense primer outside of the PPT (asEXT), demonstrating a preference for recognition of the PPT region. Indeed, in the reaction mixture containing both ODN A and asEXT primers, extension products, predominantly synthesized by the RT/RNase H, started with ODN A compared to the control ODN CO, which has a secondary structure similar to ODN A but is targeted to a site outside of the PPT (Fig. 2C, lanes ODN A and ODN CO). Thus, the ability of ODN A to mediate RT/RNase H-dependent cleavage of the viral RNA is PPT-dependent. Importantly, the effect of ODN A was slightly stronger compared to that of single-stranded antisense oligonucleotide asPPT, suggesting that the passenger strand of the ODN A is important for more efficient viral RNA cleavage (Fig. 2C, lanes ODN A and asPPT).

**ODN-mediated cleavage of RNA in HIV virions**

HIV replication is a complex process that includes reverse transcriptase processivity, RNase H activity for hydrolysis of the viral RNA, and requires the presence of the viral nucleocapsid protein and an appropriate secondary structure of the RNA. The optimization of all of these conditions may not be fully met in *in vitro* assays, however, more physiological conditions are encountered in HIV virions. Additionally, it has been shown that phosphorothioated oligodeoxynucleotides can be internalized in cells without any delivery systems. Therefore, in order to simulate the situation *in vivo*, we incubated intact virions with ODNs in cell culture medium without any detergents or ODN carriers. We used a higher concentration of ODNs than in *in vitro* studies, since the cellular uptake of oligonucleotides is low and the majority of ODNs may not penetrate through the cellular membrane. We assumed that in virions similar to cells only a small portion of ODNs could be internalized.

In our study we used ODN A, ODN CO, asPPT, and additional control oligodeoxynucleotides described in Fig. 1B: ODN T, ODN B, ODN NT, ODN, and ODN CG. HIV virions were incubated with ODNs, viral RNA was then purified, reverse transcribed, and the amount of undigested RNA was quantified by real-time PCR using a set of primers covering the PPT region of the HIV genome as indicated in Fig. 3A. We observed a profound difference in the antiviral activities between various structural variants of the ODNs. We could detect a significant decrease in the amount of amplification product in the case of ODN A compared to other control ODNs. Only ODN CG showed antiviral activity similar to ODN A. All other variants of ODN A showed less efficient cleavage of the viral RNA. These variants include deletions (ODN B), the scrambled nu-

![FIG. 3](image-url)
cletide sequence (ODN Sc), additional hydrogen bonds between antisense and passenger strands (ODN T and ODN H), alteration of poly(G)/poly(C) duplex (ODN NT), or removal of the passenger strand (asPPT) (Fig. 3B). Thus, the length and the sequences of both strands of the ODN proved to be important for the antiviral effect. The specificity of the ODNs is reminiscent of small interfering siRNAs.38,39

To test whether ODNs may interfere with the RT-PCR assay, used as a readout of RNA degradation, we purified the viral RNA preincubated with ODNs under conditions facilitating hybridization. Hybrid complexes were then repurified and RT-PCR analysis was performed as described in Materials and Methods. As shown in Fig. 3C binding of ODNs to RNA did not alter the accumulation of the amplification products, thus excluding the possibility that ODNs may interfere with the RT-PCR.

To prove that the reduction of viral RNA levels was indeed due to the enzymatic activity of the RNase H domain of the RT/RNase H, we used illimaquinone, previously used to specifically inhibit the RNase H activity of HIV RT/RNase H.13 We permeabilized virions with 0.1% NP-40 to facilitate the uptake of illimaquinone and measured the viral RNA degradation by real-time PCR. As shown in Fig. 4A, in the presence of illimaquinone the RT/RNase H failed to induce cleavage and the level of RNA remained as in the uncleaved control. Similar results were observed when the reaction mixture was supplemented with additional recombinant RT/RNase H to intensify the viral RNA degradation (Fig. 4B). To test the integrity of HIV RNA, we then analyzed the total viral RNA extracted from virions preincubated with ODNs by agarose gel electrophoresis in the absence of illimaquinone. As shown in Fig. 4C, a significant reduction of the viral RNA was observed when virions were incubated with ODN A in comparison to control ODNs. To learn more about RNA degradation, we performed RT-PCR using primers covering either the PPT region or primers covering a segment of the HIV genome 1924 nucleotides upstream of the PPT (Env region). In both cases a significant reduction in the amount of amplification products was observed in the case of ODN A compared to other ODNs (Fig. 4D).

![Figure 4](image-url)

**FIG. 4.** ODN A mediates RT/RNase H-dependent degradation of viral RNA. Permeabilized HIV virions were incubated without (A) or with (B) RT/RNase H (0.05 units/μl), 50 nM ODN A, and 150 μM illimaquinone, a selective inhibitor of RNase H activity of HIV RT/RNase H for 30 min at 37°C. Then viral RNA was purified and real-time PCR analysis was performed. Each bar represents the mean ± SD of three independent experiments. (C) Intact virions were incubated with 0.5 μM ODNs for 6 h in cell culture medium. Total viral RNA was then extracted and analyzed by agarose gel electrophoresis. (D) Virions were incubated with 0.5 μM ODNs for 6 h in cell culture medium. Viral nucleic acids were then extracted and RNase-treated, and PCR was performed using primers specific for viral cDNA and ODN A.
It has been previously shown that some reverse transcription can take place within extracellular HIV virions. Therefore, it is reasonable to assume that following ODN A-mediated cleavage by the RNase H activity of RT/RNase H, the polymerase activity extends the ODN A as an artificial primer and the RNase H activity degrades the viral RNA in a concerted action. Indeed, incubation of virions with ODN A led to cDNA production starting with ODN A as registered by PCR using ODN A- and viral cDNA-specific primers (Fig. 4E). This leads to increased RNA degradation and amplification of the efficiency of ODN A.

Preincubation of HIV virions suppresses the infectivity of viral particles in cell culture

So far we have demonstrated ODN A-mediated HIV RNA degradation in intact virions. As a next step we investigated the antiviral effect of ODNs by testing the infectivity of treated virions in cell culture studies. We used ODN A, ODN T, as PPT and ODN CO. C81-66/45 cells (2 × 10⁵) were infected with virions preincubated with different ODNs at 0.01 MOI for 1, 2, or 4 h prior to infection. RNA was extracted from infected cells and HIV replication was analyzed by real-time PCR 3 days postinfection. The time course analysis revealed that incubation of virions with ODN A for 4 h was sufficient for complete suppression of viral replication in infected cells (Fig. 5A); ODN CO was clearly less efficient, while ODN T and asPPT showed low but detectable levels in contrast to ODN A (Fig. 5B). Importantly, reduced viral replication in infected cells correlated with the kinetics of degradation of viral RNA within virions (Fig. 5C), attributing the antiviral effects of ODNs predominantly to virion-associated enzymes. The antiviral effect of ODN A appears to be dose-dependent, since the infection of C88-61/45 cells with ODN-pretreated virions at 100-fold higher MOI (MOI of 1) resulted in only partial suppression of HIV replication. However, the viral replication could be reduced again by an increase in ODN concentration, yet with reduced sequence specificity (Fig. 5D). This result demonstrates the concentration dependence of antiviral effect of ODN A.

Preincubation of virions with ODN A for 4 h and infection at low MOI (MOI of 0.1) did not significantly interfere with binding of virions to the host cells. Indeed, analysis of HIV RNA in infected cells 1 h postinfection showed that virions, pretreated with ODN A for 4 h, were taken up by cells only 14% less efficiently compared to virions treated with ODN Sc (Fig. 5E). However, viral RNA was completely degraded only in the presence of ODN A, as can be seen 24 h after infection (Fig. 5E). Importantly, virions pretreated with ODN B or ODN T showed uptake similar to ODN A, as demonstrated by analysis of HIV RNA in infected cells 1 h postinfection (Fig. 5F). Thus, ODN A-mediated abrogation of infectivity of pretreated virions may not exclusively depend on the degradation of RNA inside of the virions. The ODNs, including ODN A, may also bind to virions and mediate the cleavage of the viral RNA inside of infected cells, suggesting that intracellular factors could also play a role in this process.

To test the specificity of suppression of viral replication, the effect of ODN A on the infectivity of virions was compared with control ODNs. Virions were pretreated with the ODNs and HIV replication in infected cells was analyzed by real-time PCR, 5 days (Fig. 6A) or 20 days postinfection (Fig. 6B) and by measuring p24 production in the supernatant of the infected cells (Fig. 6C). These results were also confirmed by RT-PCR, 11 days postinfection (Fig. 6D), and by visual inspection of syncytia formation (Fig. 6E). Strikingly, cells that received virions pretreated with ODN A showed no sign of virus replication for at least 20 days after infection. Treatment of the virions with control ODNs also led to a suppression of HIV replication, but the effect was transient and 20 days postinfection the virus was able to propagate again and reach the level of the untreated control, which was not the case with ODN A.

DISCUSSION

We are describing the degradation of genomic HIV-1 RNA mediated by a highly specialized DNA oligodeoxynucleotide, ODN A, directly in HIV virions. We showed here and previously that at high concentrations (more than 1 μM) ODN A inhibited the RNase H activity of RT/RNaseH in vitro. The present data indicate that at low concentrations of ODN A, HIV RNA can be cleaved in virions before it is reverse transcribed into DNA. Furthermore, the ratio of ODNs to HIV virions is important. The design of ODN was originally based on rules for a triple helix. However, the three strands DNA–RNA–DNA instead of DNA–DNA–DNA with a parallel strand orientation for the passenger (putative “Hoogsteen”) strand are not optimal for a triple helix formation. Foldback triplex-forming oligonucleotides described by Hiratou et al. in vitro studies are much shorter in size and utilize different nucleotide modifications and therefore cannot be used for comparison with the data presented here. A typical DNA triple helix has been described previously in vivo for a subregion of the PPT of HIV, but only after cross-linking, or by an indirect effect where mutagenesis was measured. Nevertheless, the passenger strand is shown to be important in our studies since alteration or removal of the passenger strand reduced the antiviral effect of ODN A.

A variety of biochemical and structural studies by others indicate that the region of the PPT-containing RNA/DNA hybrid deviates from standard Watson–Crick geometry. It has been claimed that it is the structure of the PPT-containing segment of the RNA–DNA duplex that determines the specificity and efficiency of the cleavage. Kvaratskhelia et al recently showed that G-to-C mutations alter the structure of a PPT-containing RNA–DNA complex. ODN A used in the present study mimics a replication intermediate during the synthesis of the plus-strand DNA. Our in vitro data show that the modification of ODN structure impairs hydrolysis at the PPT region (Figs. 2C and D and 3B) suggesting that alterations of the ODN structure may affect the geometry of the RNA–ODN hybrid. Furthermore, the introduction of additional hydrogen bonds between antisense and passenger strands in ODN T and ODN H may reduce their binding to the target RNA.

It has also been demonstrated that changing either the nucleotides or the amino acids involved in contacts between RT/RNase H and the RNA–DNA duplex affects RNase H cleavage. The fact that modification or removal of the passenger strand affects the efficiency of RNA cleavage in our experiments suggests that nucleotides of this strand of ODN may be indeed in contact with the RT/RNase H. In addition, the passenger strand of ODN A may stabilize the RNA/ODN complex.
The reduced activity of oligonucleotide ODN B, shorter than ODN A, also suggests that the length of the ODN is important for correct positioning of the RNA/ODN complex at the active centers of RT/RNase H and thus may affect the efficiency of cleavage. Characterization of additional ODN A variants is required, and future studies on crystal analysis of the RT–ODN A–RNA complex will allow us to address this hypothesis accurately. The major focus of our work was to test the efficiency of ODNs in mediating cleavage of RNA in a cell-free system, thereby using conditions simulating the situation in vivo. Therefore we incu-
bated nonpermeabilized virions in regular cell culture medium, since uptake of phosphorothioated oligodeoxynucleotides without any delivery system was reported in several cellular systems.\textsuperscript{12,17} It has also been shown that a nonnucleoside reverse transcriptase inhibitor, Curie-pyridinone, was able to reach the reverse transcriptase inside of the nonpermeabilized extracellular virions.\textsuperscript{31} It is notoriously difficult to study uptake of ODNs directly in the virions, and we assumed that similar to cells, only small amounts of ODNs are expected to penetrate through the viral membrane. Yet, even in this case we could detect the effects of ODNs.

The action of ODN in virions is a complex process and it could take some time for ODN to be taken up and interact with RNA and thereby provide a substrate for the RT/RNase H. This may explain the relatively long incubation time required for the cleavage of RNA. The penetration of ODNs through the viral capsid appears to be a limiting factor, since permeabilization of the virions significantly accelerated ODN A-mediated RNA cleavage, observed just 30 min after the start of incubation (results not shown) in comparison to 6 h with nonpermeabilized native virions. However, the infectivity of ODN A-treated virions was already reduced after 1 h incubation (Fig. 5A). Therefore, ODN A can bind to virions and mediate the degradation of viral RNA not only in virions but also inside of infected cells.

Intracellularly, ODN A may activate not only the viral RT/RNase H but also the cellular RNase H1, an enzyme previously described as being responsible for most of the activity of antisense drugs.\textsuperscript{32} In addition, ODNs appear to affect the stability of the virions. We found that pretreatment of the virions for a long period of time (8 h or more) with ODN A, asPPT, ODN T, and ODN abolished the infectivity of the virions in all cases, while mock-treated virions remained infectious (results not shown). Other parameters, such as the intracellular stability of ODNs, could also be important and are currently under investigation in our laboratory. Thus, the antiviral action of ODN A is complex and does not solely rely on RNA cleavage within the virions.

In summary, we describe an ODN A that utilizes a unique mechanism for its action in virions. This mechanism is different from “classical” antisense, which is mediated by cellular RNase H. ODN A mimics a normal situation for reverse transcription, however, it recruits RT/RNase H for the cleavage of HIV RNA before viral replication is started, as was shown for virions. This should potentially allow ODN A to prevent “escape” mutations of HIV. Since retroviruses package two copies of viral RNA into each virion, reverse transcriptase can use portions of the genomes from each RNA as a template to generate a recombinant viral DNA. Recombination can also occur in virions.\textsuperscript{33} In this sense, ODN A could be attractive since it causes RNA degradation directly in virions before the reverse transcription reaction is completed. Besides, even in the case of coinfection with heterogeneous viruses, a pool of ODNs, targeting various strains of HIV, could be administered to HIV-infected patients. PPT is an extremely important region for viral replication and excessive mutations could be potentially lethal for the virus.

We have also detected antiviral effects of ODN A in HIV-infected cells.\textsuperscript{12,19,34} Phosphorothioated oligodeoxynucleotides
INHIBITION OF HIV BY DOUBLE-STRANDED OLIGODEOXYNUCLEOTIDE

have been used for more than a decade in various studies and have entered clinical trials proving the therapeutic safety of these compounds.35,36 The failure of the only single-stranded fully phosphorothioated oligonucleotides, GEM 91, targeted to a leader sequence of HIV in clinical trials shows that further modifications of ODN-based drugs are required. Some of these modifications we show here for ODN A. Furthermore, consistent problems with the vaccination approach and difficulties with siRNA-based strategies are observed. Thus, new approaches to prevent virus propagation are urgently needed. There are now promising results in several areas that suggest the potential of newer oligonucleotide-type materials and their derivatives as anti-HIV-1 therapeutics.37 Future studies to improve the uptake and stability of ODNs are required and will allow us to consider ODN A as a potentially interesting model for drug design.

Note added in proof: It should be noted that the retroviral RNase H is related to the enzyme Ago2, which cleaves small interfering siRNA. Similarities between the two systems suggest the name siDNA for the results described here.38,39

ACKNOWLEDGMENT

We thank all our laboratory colleagues for fruitful discussions.

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