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Sequence-specific inhibition of human immunodeficiency virus (HIV) reverse transcription by antisense oligonucleotides: Comparative study in cell-free assays and in HIV-infected cells

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ABSTRACT **We have investigated two regions of the viral RNA of human immunodeficiency virus type 1 (HIV-1) as potential targets for antisense oligonucleotides. An oligodeoxynucleotide targeted to the U_5 region of the viral genome was shown to block the elongation of cDNA synthesized by HIV-1 reverse transcriptase *in vitro*. This arrest of reverse transcription was independent of the presence of RNase H activity associated with the reverse transcriptase enzyme. A second oligodeoxynucleotide targeted to a site adjacent to the primer binding site inhibited reverse transcription in an RNase H-dependent manner. These two oligonucleotides were covalently linked to a poly(L-lysine) carrier and tested for their ability to inhibit HIV-1 infection in cell cultures. Both oligonucleotides inhibited virus production in a sequence- and dose-dependent manner. PCR analysis showed that they inhibited proviral DNA synthesis in infected cells. In contrast, an antisense oligonucleotide targeted to the *tat* sequence did not inhibit proviral DNA synthesis but inhibited viral production at a later step of virus development. These experiments show that antisense oligonucleotides targeted to two regions of HIV-1 viral RNA can inhibit the first step of viral infection—i.e., reverse transcription—and prevent the synthesis of proviral DNA in cell cultures.**

Oligonucleotides have been used to control the expression of viral genomes (see ref. 1 for a review). Two approaches can be developed to inhibit viral replication with synthetic oligonucleotides: the oligonucleotide can be designed either to interact with proteins involved in the biosynthetic process (sense or "decoy" approach) or to bind to a complementary sequence of the viral genome, which is used as a template by the viral polymerase—e.g., retroviral reverse transcriptases (RTs) (antisense approach). Formation of the double-stranded oligonucleotide L-RNA structure may block elongation of the newly synthesized strand. Alternatively, the RNase H activity associated with retroviral RTs may digest the RNA genome when oligodeoxynucleotides are used. Antisense oligonucleotides can also be targeted to the viral mRNAs in order to block the synthesis of viral proteins. Any oligonucleotide targeted to the viral RNA downstream of the transcription start site is also complementary to viral mRNA(s).

An antisense approach to inhibit reverse transcription *in vitro* has been recently developed (2, 3). In the present study, we have evaluated the inhibitory activity of several antisense oligonucleotides targeted to sequences internal to the U_5 region (anti-U_5 oligonucleotides) or adjacent to the primer binding site (PBS) (anti-PrePBS oligonucleotides). Previous *in vitro* studies (3) have shown that these regions of the viral genome are potential targets for antisense oligonucleotides. Anti-U_5 and anti-PrePBS oligonucleotides were tested in an *in vitro* reverse transcription system. Two oligonucleotides were selected for further studies in cell cultures. They were conjugated to poly(L-lysine) (PLL) to enhance cell uptake and tested for their ability to inhibit viral development in human immunodeficiency virus (HIV)-infected cells. Sequence-specific antiviral effects have been previously observed in *de novo* infected cells with PLL-conjugated oligonucleotides directed against the *tat* mRNA (4). These conjugates did not inhibit synthesis and integration of viral DNA and thus the reverse transcription step. Here we show that anti-U_5 and anti-PrePBS oligonucleotide–PLL conjugates prevent the synthesis of proviral DNA in a sequence-specific manner.

MATERIALS AND METHODS

Materials. Unlabeled nucleotides, oligonucleotides, or polynucleotides were obtained from Sigma or Pharmacia. Radioisotopes were purchased from Amersham and New England Nuclear. Calf intestinal alkaline phosphatase and polynucleotide kinase were from Boehringer Mannheim. The RNase H^- and RNase H^+ forms of HIV RT were isolated and purified as described (3, 5). T7 RNA polymerase, RNasin, *Sph* I restriction enzyme, and terminal deoxynucleotidyltransferase were purchased from BRL; proteinase K was from Boehringer Mannheim and pancreatic RNase A was from Sigma. DNA molecular weight markers were from Boehringer Mannheim.

RT Assays. HIV RT (100–200 nM) was added to a mixture containing 50 mM Tris·HCl (pH 8.0), 6 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 30 mM NaCl, 8 nM PBS RNA, 1.5 μM anti-PBS oligonucleotide, 30 μCi of [α-^{32}P]dGTP (3000 Ci/mmol; 1 Ci = 37 GBq), 100 μM dNTP. The mixture (50 μl) was preincubated for 30 min at 37°C in the absence of enzyme. After addition of the enzyme, incubation was continued for 30 min at 37°C. Antisense oligonucleotides were added to the preincubation mixture as indicated in the figure legends. To determine the exact location of the blocking sites, sequencing of HIV RNA was performed by adding 5 μM ddCTP or ddTTP to the incubation mixture.

Samples were extracted with phenol, precipitated with ethanol, dried, and resuspended in sample buffer (50% sucrose/0.2% bromophenol blue/0.2% xylene cyanol/8 M urea) and migration buffer (1× TBE; 100 mM Tris borate/1 mM EDTA, pH 8.0). The 8 M urea/10% polyacrylamide gels were prerun at 1000 V for 1 h. Migration occurred for 3–4 h at 1500 V. Gels were subjected to autoradiography.

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Abbreviations: RT, reverse transcriptase; PBS, primer binding site; PLL, poly(L-lysine); HIV, human immunodeficiency virus.

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A 1100-bp DNA fragment containing R, U_5, and PBS HIV sequences (a gift from J. L. Darlix, Institut National de la Santé et de la Recherche Médicale, Lyon) was cloned into the *Pst* I site of a pBluescript plasmid. The insert was transcribed by T7 RNA polymerase after vector linearization by *Sph* I to give a 1033-nt RNA as described (3).

Oligonucleotide Synthesis. Oligodeoxynucleotides were synthesized on an Applied Biosystems automatic synthesizer according to the manufacturer's instructions.

Antisense Modification. Oligodeoxynucleotides (150 μM) were resuspended in a final vol of 0.2 ml in the presence of 5× tailing buffer (0.5 M potassium cacodylate, pH 7.2/10 mM $CoCl_2$/1 mM DTT)/1 mM ddNTP complementary to the nucleotide immediately 5′ to the region covered by the antisense oligonucleotides on the viral sequence. The mixture was incubated for 2 h in the presence of 56 units of terminal transferase and the operation was repeated under the same conditions. The reaction products were purified on Sep-Pak minicolumns and precipitated with ethanol.

Assays for Inhibition of HIV by Oligonucleotides in Infected Cells. For these assays, oligonucleotides were covalently linked to PLL. Synthesis of the antisense oligonucleotides was started on the 3′ side by a ribonucleotide complementary to the viral RNA. The ribose was used to establish a covalent linkage with PLL, as described by Leonetti *et al.* (6). The antiviral activity in *de novo* infected cells was established as described by Degols *et al.* (7). In brief, MT4 cells (8) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and 2 mM glutamine. MT4 cells were harvested during the exponential growth phase, concentrated to 3×10^6 cells per ml, infected with an equal volume of virus-containing medium ($TCID_{50}$, 1000), and incubated for 30 min at 4°C. Cells were washed, diluted to 3×10^5 cells per ml, and incubated at 37°C in the presence of the oligonucleotide–PLL conjugates. Four days after infection, culture samples were removed to measure RT activity (9). Data shown in Fig. 4 are averages of three independent experiments.

PCR Analysis of HIV-1 DNA in Infected Cells. MT4 cells were infected in the presence of PLL conjugates as described above. Three hours after infection, total HIV-1 DNA production was evaluated by PCR as described (4). Briefly, HIV fragment amplification was performed on 5×10^5 cells with oligomer primers (5′-CGTTTCAGACCCACCTCCCAATCCC-3′, nt 7947–7971; 5′-GGGTTTTCTTTTAAAAAGTGGCTAAGATC-3′; nt 8628–8656). As a control, a c-*myc* fragment was amplified in the same reaction mixture with primer oligomers 5′-CGAGTTAGATAAAGCCCCGAAAACC-3′ (nt 513–537) and 5′-TCCCTGGCTCCCCTCCTGC-3′ (nt 417–435). The amplified products (a 709-bp HIV fragment and a 120-bp c-*myc* fragment) were analyzed by electrophoresis on a 1.5% (wt/vol) agarose gel and visualized by ethidium bromide staining.

RESULTS

Antisense Oligonucleotides Targeted to the U_5 Region. Previous *in vitro* results have shown that a 26-mer oligodeoxynucleotide targeted to the U_5 region (anti-U_5; Fig. 1) could block reverse transcription on a viral RNA fragment (3). A series of oligonucleotides targeted to the same RNA region but shortened at their 5′ or 3′ ends were synthesized in order to determine the minimal length required to block reverse tran-

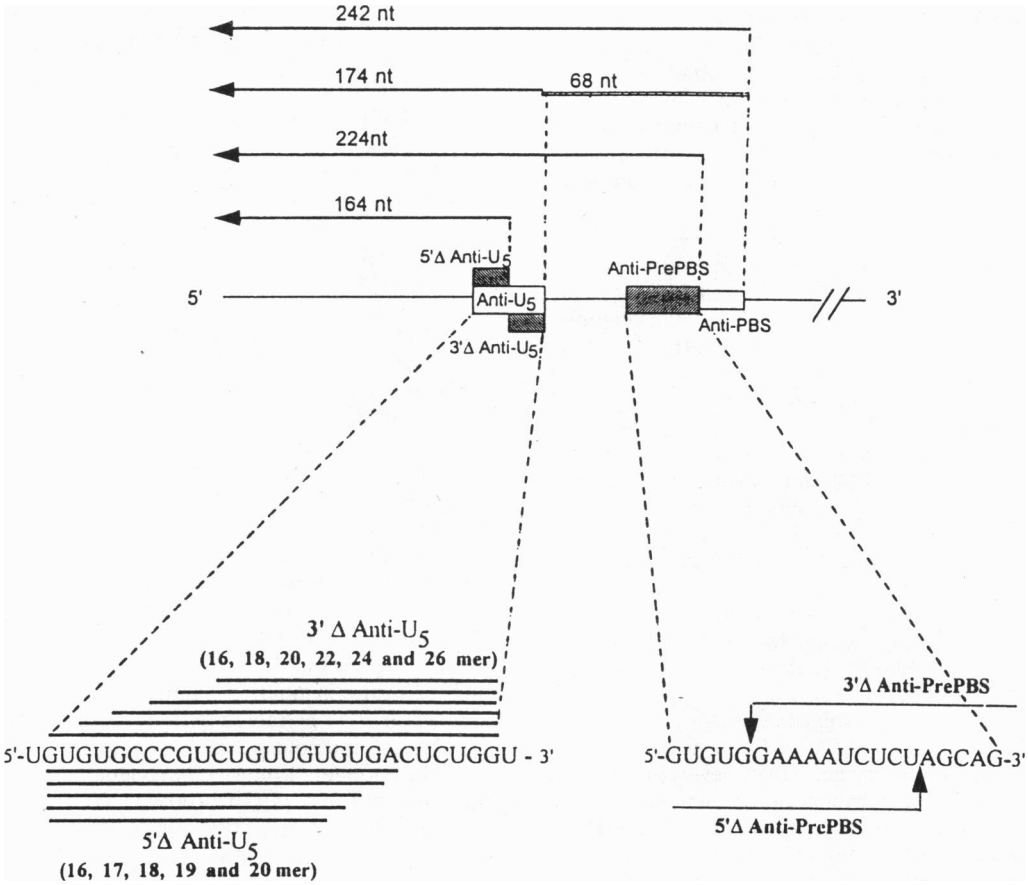

FIG. 1. Target sequences on HIV viral RNA with the DNA fragments generated when antisense oligonucleotides are used as primers for reverse transcription. The 18-mer anti-PBS oligonucleotide was used as primer (free 3′-OH group) to investigate the efficacy of 3′-dideoxynucleotide-substituted antisense oligonucleotides to arrest HIV RT. Sequences of the oligonucleotides targeted to the U_5 and PrePBS regions are indicated above and below the viral RNA sequence (bottom part).

scription (Fig. 1). This study was important before undertaking studies on cell cultures infected by HIV-1 using oligonucleotide–PLL conjugates; better results were previously obtained in other systems when the oligonucleotide attached to PLL was <20 nt (6). In addition, shorter oligonucleotides should exhibit less of the nonspecific effects that result from formation of partial hybrids with cellular mRNAs.

Anti-U_5 oligonucleotides (16–26 nt) could be used as primers by HIV RT, showing that they could hybridize to their complementary sequence on the viral RNA (results not shown). They were substituted at their 3′ ends with a dideoxynucleotide complementary to the viral sequence in order to avoid antisense oligonucleotide elongation by RT and to allow us to investigate their true antisense activity. An 18-mer oligodeoxynucleotide (5′-GTCCCTGTTCGGGCGCCA-3′) binding to the PBS was used as a primer to evaluate the effect of the 3′-blocked antisense oligonucleotides on reverse transcription. Using this primer, the DNA fragment synthesized by RT on the viral RNA template was 242 nt (see Fig. 1). The results are shown in Fig. 2 for oligonucleotides shortened at the 5′ end (5′Δanti-U_5) and in Fig. 3 for an oligonucleotide shortened at the 3′ end (3′Δanti-U_5). The length of the DNA transcripts was measured starting from position 1 at the 5′ end

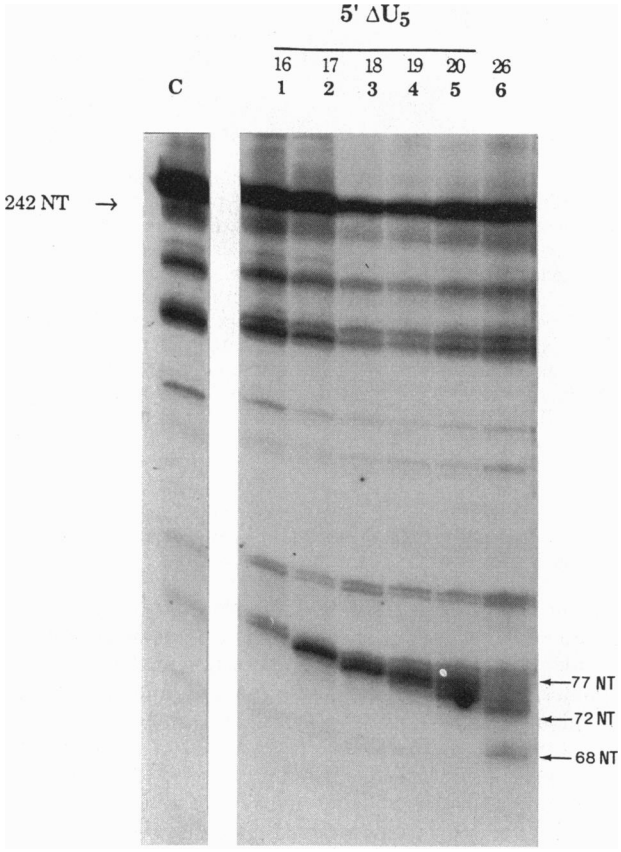

FIG. 2. Effect of length and hybridization site of the different 5′Δanti-U_5 oligonucleotides on HIV reverse transcription. cDNA synthesis assays were performed as described. The preincubation mixture contained the 18-mer anti-PBS primer and 1 μM 5′Δanti-U_5 16-mer (lane 1), 17-mer (lane 2), 18-mer (lane 3), 19-mer (lane 4), 20-mer (lane 5), and 26-mer (lane 6). After preincubation, HIV RT was added for a 30-min incubation at 37°C. The HIV-1 RT exhibits numerous pause sites, which are observed both in the absence (lane C) and in the presence of oligonucleotides. Nucleotide U at the 3′ end of the template RNA sequence in Fig. 1 is 68 nt from the 3′ end of the PBS sequence. Note that all oligonucleotides used in this study were substituted at their 3′ ends with a dideoxynucleotide complementary to the viral sequence. This dideoxynucleotide is not included in the oligonucleotide length indicated on the figure.

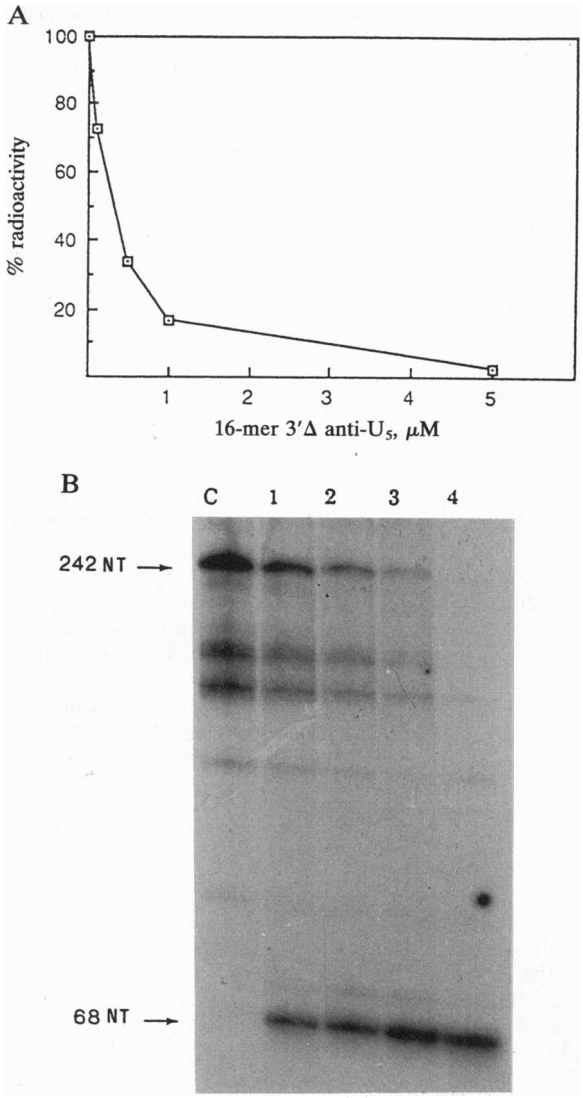

FIG. 3. (*A*) Effect of increasing concentrations of 3′Δanti-U_5 16-mer on viral reverse transcription. cDNA synthesis assays were performed as described. (*B*) Preincubation mixture contained viral RNA, anti-PBS 18-mer primer, and 0 μM (lane C), 0.1 μM (lane 1), 0.5 μM (lane 2), 1 μM (lane 3), or 5 μM (lane 4) 3′Δanti-U_5 16-mer substituted with a dideoxynucleotide at the 3′ end.

of the 18-mer anti-PBS primer (Fig. 1). An arrest of RT at the uracil on the 3′ side of the target sequence shown in Fig. 1 should give a DNA fragment of 68 nt. All 3′Δanti-U_5 oligonucleotides cover target sequences starting at the guanine residue preceding this uracil residue. All 5′Δanti-U_5 oligomers cover target sequences with the same 5′ end—i.e., guanine at position 95 (taking into account the dideoxynucleotide added at the 3′ end of the oligonucleotides). As shown in Fig. 2, the 16-mer 5′Δanti-U_5 was inefficient at inhibiting reverse transcription. The 17-mer oligonucleotide arrested RT at position 77, and the site of reverse transcription arrest moved downstream on the RNA template when the antisense oligonucleotide was elongated at its 5′ end from 17 to 20 nt. The main arrest site did not move when the oligonucleotide was further elongated to 26 nt even though a truncated fragment of 68 nt could be detected with the 26-mer.

For the 3′Δanti-U_5 series the 16-mer was the most active at inhibiting reverse transcription. A 68-nt fragment was obtained (Fig. 3). The 16-mer 3′Δanti-U_5 oligonucleotide inhibited full-length cDNA synthesis with an IC_{50} of ≈0.2 μM and was more efficient than the 26-mer anti-U_5 (3). It should be noted

that the same results were obtained with the RNase H^+ and RNase H^- RT, indicating that the antisense effect of the anti-U_5 oligonucleotides, which resulted in the arrest of RT elongation, did not require cleavage of the viral RNA template by RNase H activity.

Antisense Targeted to the pre-PBS Region. Previous results (3) have shown that a 20-mer oligodeoxynucleotide targeted to a site adjacent to the 18-nt PBS sequence (on its 5′ side) was able to block initiation of reverse transcription. Two 15-mer oligonucleotides targeted to the same region were used in the present study (Fig. 1) and tested for their efficacy to inhibit the RNase H^- and RNase H^+ forms of HIV RT. In the absence of the 18-mer anti-PBS, none of them could act as primer for RT, in agreement with the proposed stable secondary structure surrounding the PBS on the viral RNA (10). When the 15-mer 3′Δanti-PrePBS oligonucleotide was used together with the 18-mer anti-PBS primer, two transcripts were observed whose lengths (242 and 224 nt) corresponded to initiation of reverse transcription by both the 18-mer and the 15-mer (Fig. 1). However, when the 15-mer 5′Δanti-PrePBS was used together with the 18-mer anti-PBS primer, only the transcript initiated at the 18-mer (242 nt) was observed. These results show that only the oligonucleotide that is complementary to a site adjacent to the PBS can bind the viral RNA cooperatively with the adjacent 18-mer, in agreement with previous results (3).

The two anti-PrePBS 15-mer oligonucleotides were substituted at their 3′ ends with a dideoxynucleotide complementary to the viral sequence. The 5′Δanti-PrePBS oligonucleotide did not block reverse transcription initiated by the 18-mer anti-PBS oligonucleotide, as expected from the absence of binding to a site that is not adjacent to the PBS. In contrast, the 3′Δanti-PrePBS oligomer blocked reverse transcription at 1 μM when the RNase H^+ RT was used. The inhibition was higher with the antisense oligonucleotide substituted at its 3′ end with a dideoxynucleotide. No inhibition was observed when the RNase H^- RT was used, indicating that a large part of the effect of the 3′Δanti-PrePBS was mediated through RNase H cleavage of the template RNA.

Inhibition of HIV-1 *de Novo* Infected Cells with Anti-U_5 and Anti-PrePBS Oligonucleotides Conjugated to PLL. Sequence-specific effects of antisense oligonucleotides have rarely been observed in acute HIV infection assays when either unmodified oligonucleotides or phosphorothioate analogues have been directly added to the cell culture medium. However, we have previously demonstrated that covalent attachment of oligonucleotides to a PLL carrier confers upon the oligonucleotide a sequence-specific antisense activity in this experimental model (4). PLL conjugation most likely eliminates oligonucleotide binding to either the CD4 receptor or the gp120 viral protein, which inhibits virus entry into cells in culture as demonstrated in the case of phosphorothioate oligomers.

Two oligonucleotides (3′Δanti-U_5 16-mer and 3′Δanti-PrePBS 15-mer), which exhibited the strongest *in vitro* inhibitory effect, were selected for cell culture experiments with acute HIV-1 infection. Control experiments were carried out with an anti-tat oligonucleotide and a random oligonucleotide having the same base composition as the anti-U_5 oligomer. In a previous study (7), the anti-tat oligonucleotide was shown to act primarily at the level of *tat* mRNA translation and did not interfere with provirus integration. All oligonucleotides were covalently linked to PLL and their antiviral activity was measured in dose–response experiments. Fig. 4*A* shows the mean values of three experiments performed with oligonucleotide–PLL conjugates obtained from independent reactions. Similar efficiencies were obtained with the 3′Δanti-U_5 and 3′Δanti-PrePBS oligonucleotides ($EC_{50} \approx 0.2$ μM). These conjugates exhibited antiviral effects in the same concentration range as an oligonucleotide complementary to the region

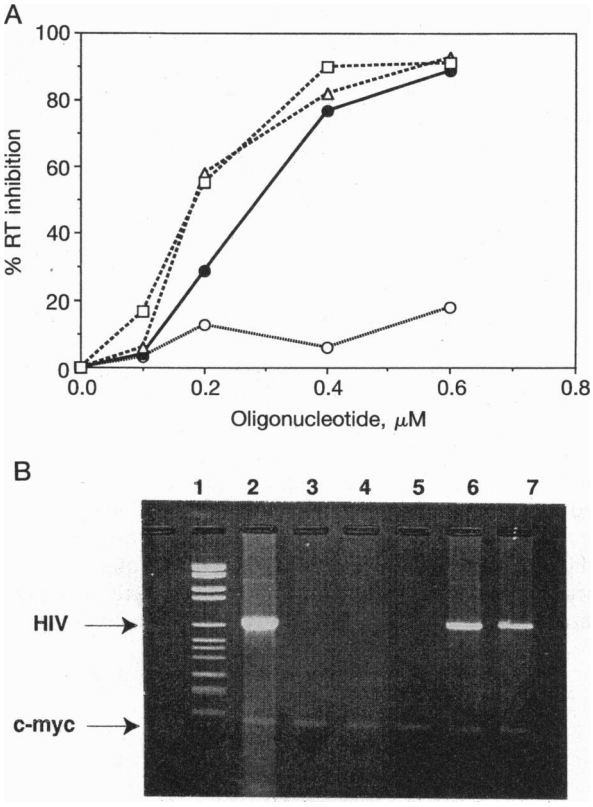

FIG. 4. (*A*) Antiviral activity of PLL-conjugated oligonucleotides. MT4 cells were infected as described and treated with the various conjugates at the indicated concentrations. RT activities were measured 4 days after infection and results are expressed as % inhibition of RT activity in control infected cells. ●, Anti-tat; △, 3′Δanti-U_5 16-mer; ○, random anti-U_5; □, 3′Δanti-PrePBS 15-mer. Each experimental point is the average of three independent experiments. (*B*) PCR amplification of HIV DNA in cells treated with oligonucleotide–PLL conjugates. A 709-bp fragment of the viral DNA and a 120-bp fragment of the c-*myc* oncogene were coamplified 3 h after infection. Lanes: 1, DNA molecular weight marker VI (Boehringer); 2, infected cells; 3, uninfected cells; 4, infected cells treated with the 3′Δanti-U_5 oligonucleotide–PLL 16-mer; 5, infected cells treated with the 3′Δanti-PrePBS oligonucleotide–PLL 15-mer; 6, infected cells treated with the anti-tat oligonucleotide–PLL; 7, cells treated with the random anti-U_5 oligonucleotide–PLL. Oligonucleotide–PLL conjugates were used at a final concentration of 0.5 μM.

adjacent to the translation initiation codon of *tat* mRNA. Inhibition was sequence specific since a random U_5 oligonucleotide, having the same base composition as the 3′Δanti-U_5 oligomer, did not promote any significant antiviral activity. PLL alone had no antiviral effect in the same concentration range (results not shown).

Synthesis of viral DNA was quantitated by PCR amplification in order to ascertain the mechanism of action of these oligonucleotides in acutely infected cells (Fig. 4*B*). Three hours after infection, synthesis of viral DNA was completely inhibited in cells treated with 3′Δanti-U_5 or 3′Δanti-PrePBS oligonucleotides conjugated to PLL. However, no reduction in the amount of amplified DNA could be detected in cells treated with the random anti-U_5 or with anti-tat oligonucleotides, showing that the oligonucleotides did not prevent virus entry into cells in culture in a nonspecific way.

DISCUSSION

We have investigated the effect on reverse transcription of antisense oligonucleotides targeted to two regions of HIV-1 RNA. Internal sequences within the U_5 region were originally

selected on the basis of a computer-generated model of HIV secondary structure (11), which suggested the presence of a single-stranded region surrounded by two highly structured regions. Secondary structures of HIV-1 RNA have been refined through the use of chemical and enzymatic probes (10). These recent studies have led to a model in which the U_5 region is engaged in formation of two branches and one hairpin. In spite of the high degree of structure, reverse transcription can be initiated by antisense oligonucleotides targeted to this region. This result suggests that secondary structures can be displaced by complementary oligonucleotides at 37°C under the ionic conditions used in the *in vitro* reaction in the presence of RT.

Antisense oligonucleotides targeted to the U_5 region of the viral RNA are good inhibitors of elongation by RT. Similar results were obtained with an RNase H^+ and an RNase H^- RT, indicating that RNA cleavage by RT-associated RNase H activity was not a prerequisite to observe the antisense effect. The positions of some of the arrest sites suggested that the active species might not be solely the hybridized oligodeoxynucleotide but also an oligodeoxynucleotide–RNA–RT ternary complex involving a RT molecule bound to the hybrid. Oligodeoxynucleotide–RNA hybrids can serve as substrates for the RT-associated RNase H even when the 3′ end of the oligonucleotide is blocked by a dideoxynucleotide (3). Therefore, the complex of a dideoxynucleotide-substituted oligomer with RNA constitutes a binding site for HIV RT. The size of the hybrid region covered by one RT molecule can be estimated to be 15–20 nt from the available RT crystal structure (12) and hydroxyl radical footprinting (13). This might explain why the major arrest sites of RT did not change when the 5′Δanti-U_5 oligonucleotides increased in length from 20 to 26 nt even though some arrest of RT was also observed at the 5′ end of the hybridized oligonucleotide.

Anti-PrePBS oligonucleotides do not bind to the viral RNA, probably as a result of a stable secondary structure upstream of the PBS. They could not act as primers for RT. However, in the presence of an 18-mer oligonucleotide complementary to the PBS sequence, an adjacent 15-mer could bind and block initiation of reverse transcription. In contrast to the anti-U_5 oligonucleotides, the RNase H activity of HIV RT was required to observe inhibition of reverse transcription by anti-PrePBS oligonucleotides in the submicromolar concentration range.

The selected 16-mer anti-U_5 and 15-mer anti-PrePBS oligonucleotides inhibited HIV infection of MT4 cells in culture in the submicromolar concentration range when conjugated to PLL. PCR analysis of infected cells showed that there was no proviral DNA synthesized and integrated into the host cell genome. Therefore, the inhibitory effect is occurring at or before the reverse transcription step. In contrast, an anti-tat antisense oligonucleotide did not prevent proviral DNA integration but inhibited HIV development at a later stage (7). The inhibitory effect of the anti-U_5 and anti-PrePBS oligonucleotides was sequence specific, as shown by the absence of inhibition when a control oligonucleotide was used with the same base composition as the anti-U_5 oligomer but of a different sequence. Based on the *in vitro* results, it seems likely that the biological effect of the anti-U_5 and anti-PrePBS oligonucleotide–PLL conjugates in cell cultures is due to the inhibition of reverse transcription. Any oligonucleotide complementary to the genomic viral RNA is also complementary to viral mRNA(s). An inhibition of reverse transcription requires that the viral RNA sequence should be accessible to the oligonucleotide within the virus capsid after infection. A difference in accessibility might explain the different behavior of the anti-U_5 or anti-prePBS oligonucleotides, which block reverse transcription, and the anti-tat oligomer, which acts at the level of mRNA translation. Phosphorothioate analogues of oligodeoxynucleotides have been previously shown to inhibit HIV production in acutely or chronically infected cells (9, 14). These oligonucleotides act at several steps, including virus binding to cell membranes, and interact with RT and mRNAs. The results presented here show that antisense oligonucleotides could also be targeted to the viral RNA and prevent synthesis of proviral DNA through inhibition of reverse transcription. This inhibition of an early step of viral infection might be of interest in the development of antisense agents against HIV infection.

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