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Phosphorothioate Oligonucleotides Derived from Human Immunodeficiency Virus Type 1 (HIV-1) Primer tRNA^{Lys3} Are Strong Inhibitors of HIV-1 Reverse Transcriptase and Arrest Viral Replication in Infected Cells

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Retroviral reverse transcriptase (RT) is involved in the selection of a specific tRNA primer which initiates proviral DNA minus-strand synthesis. Studies of the interactions between human immunodeficiency virus type 1 (HIV-1) RT and primer tRNA^{Lys3} have shown that the dihydrouridine (diHU), anticodon, and pseudouridine regions of tRNA are highly protected in the RT-tRNA complex. The CCA 3′ end of tRNA is also in close contact with the enzyme during the cDNA initiation step. Using synthetic oligoribonucleotides corresponding to the anticodon and diHU regions, we have previously shown a low but significant inhibition of HIV-1 RT activity (37). We extend this observation and show that primer tRNA-derived oligodeoxynucleotides (ODNs) carrying a phosphorothioate (PS) modification are strong inhibitors of HIV-1 RT. The affinity of PS-ODNs for the enzyme was monitored by gel mobility shift electrophoresis. Experiments with HIV-1-infected human cells (MT-2 cells) were performed with the latter ODNs. A PS-ODN corresponding to the 3′ end of tRNA^{Lys3} (acceptor stem [AS]) was able to inhibit HIV-1 replication. No effect of the other modified ODNs was observed in infected cells. The analysis of HIV-1 RNase H activity in a cell-free system strongly suggests that the inhibitory effect of the PS-AS may be mediated via both a sense and an antisense mechanism.

All retroviral reverse transcriptases (RTs) initiate proviral first-strand DNA synthesis from a tRNA hybridized to the primer binding site (PBS), a retroviral genomic region near the 5′ end which is complementary to the first 18 nucleotides of the 3′ end of primer tRNA. (For recent reviews on RT, see references 7, 25, 39, and 43). Determination of the genomic sequences of human immunodeficiency virus type 1 (HIV-1) and HIV-2 and further experimental approaches showed that tRNA^{Lys3} is their specific primer (45). A stable and specific complex between HIV-1 RT and tRNA^{Lys3} has been shown by different approaches in the absence of the PBS region. However, HIV-1 RT is also able to form a complex with other tRNAs (6, 35, 44). Using the footprinting technique, we have found that the anticodon and dihydrouridine (diHU) regions of tRNA^{Lys3} interact strongly with HIV-1 RT (37). This result agrees with those of Barat et al. (9), who showed that the anticodon region of primer tRNA was found to be preferentially linked to RT after UV irradiation. More recent results have shown that the TΨC region of tRNA^{Lys3} also interacts with HIV-1 RT (46). Using a 4-thiouridine UV-induced cross-linking strategy, Mishima and Steitz (29) localized the 5′ half of the primer tRNA at the interface between the two HIV-1 RT subunits, close to the RNase H domain of the viral polymerase. Mutations of the TΨC stem partially interfered with RT binding, while mutations in the D arm abolished the ability of tRNA^{Lys3} to bind HIV-1 RT. These results suggest that HIV-1 RT is able to recognize the central domain of the primer tRNA tertiary structure (30). Moreover, the same investigators (30) showed that HIV-1 RT facilitates the tRNA^{Lys3}-PBS annealing, confirming our previous results (37). RT is not the only factor involved in the annealing between the primer and the template since the *gag*-derived basic nucleocapsid protein has been reported to stimulate tRNA^{Lys3} annealing to the PBS region (see reference 18 for a review). Recently, it has been shown that the U-rich anticodon region of tRNA^{Lys3} facilitates the formation of the RNA template-tRNA primer duplex by interacting with an A-rich region localized downstream, near the PBS sequence (20).

Oligonucleotides have successfully been used to interfere with retrovirus replication. The most common approach is the antisense strategy which has been proposed for the potential treatment of AIDS, cancer, and other diseases (1). The antisense strategy has been used to arrest reverse transcription by murine and avian RTs in cell-free systems (12, 14) and by HIV-1 RT in vitro (13, 15), as well as HIV-1 replication in infected cells (16).

The decoy (or sense) strategy rests on the selective binding of an oligodeoxynucleotide (ODN) to a nucleic acid binding protein. The ODN mimics the natural ligand of the protein and therefore competes with it for complex formation with the protein. This association traps the protein in a nonfunctional complex. An example of the use of ODNs as decoys is the targetting of the NF-κB transcription factor involved in the initiation of eucaryotic transcription (42). Complex formation between primer tRNA and RT during the selection of the specific tRNA^{Lys3} primer and further positioning of the primer-template duplex on the PBS site are crucial steps in the initiation of retroviral replication and may be useful targets as decoy agents that can be used to block retroviral proliferation. The latter idea is supported by previous results showing the inhibition of HIV-1 RT DNA polymerase activity observed

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with oligoribonucleotides derived from the anticodon and diHU regions of $tRNA^{Lys3}$. Under the same conditions a random sequence did not affect the enzyme activity (37). The latter results showed that HIV RT was inhibited by phosphodiester (PO) oligoribonucleotides, but the in vivo application of unmodified ODNs is limited due to their sensitivities to nucleases and their relatively short half-lives. Therefore, novel ODNs with modifications to their phosphate backbones have been developed. Compared with other modified ODNs, the phosphorothioate (PS) derivatives (PS-ODNs) have several advantages: increased lifetime due to improved nuclease resistance, ability to elicit RNase H activity, and ease of synthesis. Many reports on the use of these compounds as potential anti-AIDS agents have been published (2, 8, 23).

In this article we describe the strong inhibition of HIV-1 RT activity by PS-ODNs derived from the $tRNA^{Lys3}$ sequence. The best inhibitory effect on DNA synthesis in a cell-free system was obtained with a PS-ODN corresponding to the 3′ end of the primer $tRNA^{Lys3}$ acceptor stem (AS) ($PS\text{-}AS^{Lys3}$), although other PS-ODNs also strongly inhibited cDNA synthesis. Interestingly, the $PS\text{-}AS^{Lys3}$ was able to inhibit the proliferation of HIV-1 in infected human cells. In contrast, the other $tRNA^{Lys3}$-derived PS-ODNs did not show any ex vivo effect. Long-term treatment (28 days) of the infected cells with the PS-AS agent showed that the inhibitory effect was maintained.

MATERIALS AND METHODS

Materials. Unlabeled nucleotides, unmodified oligonucleotides, and polynucleotides were purchased from Boehringer Mannheim, Sigma, and Pharmacia. Radioisotopes were purchased from Amersham. Calf intestinal alkaline phosphatase and polynucleotide kinase were from Boehringer Mannheim. T7 RNA polymerase, human placental RNase inhibitor, the *Sph*I restriction enzyme, and terminal deoxynucleotidyl transferase were from Bethesda Research Laboratories. Proteinase K was from Boehringer Mannheim, and pancreatic RNase A was from Sigma. 3′-Azido-3′-deoxythymidine (AZT) was purchased from Sigma. Activated calf thymus DNA was prepared as described before (5).

Enzyme purification. Recombinant HIV RT was purified from transformed yeast cells as described previously (11, 36).

Oligonucleotide synthesis. Oligonucleotides were synthesized on an Applied Biosystems automatic synthesizer by following the manufacturer's instructions. PS-ODN synthesis was performed on a Millipore Expedite synthesizer as described previously (33).

HIV-1 RNA synthesis in vitro. The plasmid pmCG6 containing the fragment corresponding to nucleotides 1 to 4005 of HIV-1 (Mal isolate) in pSP64, under the control of the bacteriophage T7 promoter, was a kind gift from J. L. Darlix (Institut National de la Santé et de la Recherche Médicale, Lyon, France). *Escherichia coli* HB101 (*recA* mutant) was used for plasmid amplification. After digestion of this clone with pSt 1 and in vitro transcription with T7 RNA polymerase, we obtained RNAs starting at position +50 of the *mal* sequence. The nucleotide sequence was determined and was shown to be the same as that of the original clone (3).

Viral RNA, corresponding to nucleotides 50 to 997 of the HIV-1 RNA (Mal isolate), was synthesized by in vitro transcription with T7 RNA polymerase. Three micrograms of linearized plasmid DNA was incubated for 2 h at 37°C in a total volume of 0.1 ml containing 40 mM Tris-HCl (pH 8.0), 8 mM $MgCl_2$, 10 mM spermidine, 25 mM NaCl, 10 mM dithiothreitol, 0.5 mM (each) ribonucleoside triphosphate, 100 U of T7 RNA polymerase, and 20 U of the placental RNase inhibitor RNasin. After treatment with 12 U of RNase-free DNase I (for 10 min at 37°C), RNA transcripts were extracted with 1 volume of a solution containing phenol-chloroform-isoamyl alcohol (24:24:1) and then with chloroform and were precipitated with 2.5 volumes of ethanol and 0.3 M ammonium acetate.

Purified enzyme assays in a cell-free system. (i) Reverse transcription with HIV-1 RNA template. Reverse transcription was performed with HIV-1 RNA template in a final volume of 50 μl. Hybridization was performed in the presence of 50 mM Tris-HCl (pH 8.0), 6 mM $MgCl_2$, 2 mM dithiothreitol, 30 mM NaCl, 150 nM HIV-1 RNA, and 1.6 μM synthetic deoxyoligonucleotide primer complementary to the PBS of HIV RNA for 30 min at 37°C. Then, 0.1 mM (each) deoxynucleoside triphosphate, 10 μCi of [α-^{32}P]dGTP (3,000 Ci/mmol), and 150 nM HIV-1 RT were added. The incubation was continued for 30 min at 37°C. The samples were extracted with phenol-chloroform and collected by ethanol precipitation at −20°C. Reaction products were analyzed by electrophoresis in 10% polyacrylamide–TBE (Tris-borate-EDTA)–7 M urea (urea-polyacrylamide gel electrophoresis [PAGE]) denaturing gels. The gels were autoradiographed and the films were analyzed by using the NIH Image Program (Macintosh).

(ii) Reverse transcription with activated DNA. For reverse transcription with activated DNA, the reaction mixture contained, in a final volume of 0.05 ml, 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 10 mM dithiothreitol, 50 mM KCl, 0.4 to 10 μg of activated DNA per ml, 50 μM (each) dATP, dCTP, and dGTP, 2 μM dTTP, 0.5 to 1 μCi of [^{3}H]dTTP (56 Ci/mmol), and recombinant RT. The reaction mixture was incubated for 15 min at 37°C, and the reaction was stopped by the addition of 1 ml of cold 10% trichloroacetic acid (TCA) plus 0.1 M sodium pyrophosphate. The precipitate was filtered through nitrocellulose membranes, washed with ice-cold 2% TCA, dried, and counted in a 2,5-diphenyloxazole–1,4-bis(5-phenyloxazolyl)benzene–toluene scintillation mixture.

Urea-PAGE analysis. Samples were extracted with phenol, precipitated with ethanol, dried, and resuspended in sample buffer (50% sucrose, 0.2% bromophenol blue, 0.2% xylene cyanol, and 8 M urea) and migration buffer 1× TBE (100 mM Tris-borate, 2 mM EDTA [pH 8.0]). The 8 M urea–10% polyacrylamide gels were prerun at 1,000 V for 1 h. Migration was for 3 to 4 h at 1,500 V. After electrophoresis, the gels were autoradiographed.

Gel mobility shift assays. HIV-1 RT and oligonucleotides were incubated at 37°C in 10-μl reaction mixtures containing 50 mM Tris-acetate buffer (pH 8.5) and 10 mM $MgCl_2$ for 10 min. Electrophoresis of the RT-oligonucleotide complexes was performed through a 6% polyacrylamide gel in 1× TBE buffer at 100 V for 3 to 4 h. The gels were dried and autoradiographed.

Cells, virus strains, and cell culture assays. MT-2 cells were chosen for their high degree of susceptibility to most laboratory strains of HIV-1. These cells were grown at 37°C in RPMI 1640 medium (Gibco-BRL) supplemented with 10% heat-inactivated fetal calf serum (Boehringer Mannheim), 2 mM L-glutamine, penicillin (100 IU/ml), streptomycin (0.1 ng/ml), and anti-interferon alpha (700 IU/ml; Boehringer Mannheim).

An HIV-1 stock (strain HTLV-IIIB) was obtained from the cell-free supernatant of HIV-1-infected H9 cells (H9/HTLV-IIIB cells). The 50% tissue culture infective dose ($TCID_{50}$) of cell-free virus stock was determined on day 4 by endpoint titration with MT-2 cells in 96-well microculture plates by the method of Reed and Muench (34). The titer of virus stock was about 10^6 $TCID_{50}$s/ml. In some experiments, viruses were pelleted by ultracentrifugation from clarified culture supernatants of H9/HTLV-IIIB cells, and the titer was calculated as 1.6×10^8 $TCID_{50}$s/ml, in duplicate, to allow for the simultaneous evaluation of their effects on HIV-infected cells. Uninfected or untreated samples (negative control) and infected and untreated samples (positive control) were included in each experiment. The inhibitory effect of AZT was tested in parallel.

Infection of MT-2 cells. (i) Posttreatment inhibitor experiments. A total of 6×10^4 MT-2 cells per well were exposed to HIV-1 for 2 h at 37°C. Infection was made at a multiplicity of infection of 1. After the unadsorbed viruses were washed off, cells were resuspended in fresh medium and were mixed with different concentrations of compounds in 96-well plates. The cultures were incubated at 37°C for 4 days.

(ii) Long-term effects of PS-ODNs on human cells infected with HIV-1. Untreated MT-2 cells were infected as described above and were incubated for 2 h at 37°C. The addition of different concentrations of ODNs was made as described above for the posttreatment inhibitor experiments, and the cells were incubated for 4 days. Fresh medium containing the same ODN concentrations was then added every 4 days for 28 days.

(iii) Virus adsorption inhibitor experiments. Compounds were added simultaneously to the mixture of HIV-1 and cells and were then washed off after 2 h of incubation at 37°C. Thereafter, the cultures were kept in drug-free medium for 4 days.

Antiviral assays in cell systems. The activities of the oligonucleotides against HIV-1/HTLV-IIIB strain replication were determined by following several criteria: appearance of cytopathic effect, protection of virus-induced cytopathogenicity in MT-2 cells, inhibition of RT activity, and specific antigen expression.

(i) Protection of virus-induced cytopathogenicity (MTT assay). The protective effects of the inhibitory agents were tested as follows. The viabilities of cells that had been infected or not infected with HIV-1 and then exposed to various compounds were measured spectrophotometrically via the reduction of 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) as described before (31).

(ii) Virion RT activity assay. The assay for RT activity was performed as described previously (38). Supernatants (50 μl) from each culture were incubated with 10 μl of virus-disrupting buffer (500 mM KCl, 50 mM dithiothreitol, 0.5% Triton X-100) for 15 min at 4°C. Thereafter, a solution (40 μl) containing 25 mM Tris-HCl (pH 7.8), 1.25 mM EGTA, 12.5 mM $MgCl_2$, 0.5 A_{260} units of poly(rA)-oligo(dT) per ml, and 3 μCi of [^{3}H]dTTP at 30 Ci/mmol was added, and the mixture was incubated for 1 h at 37°C. The reaction was stopped by the addition of 20 μl of 60% TCA containing 120 mM sodium pyrophosphate. The acid-insoluble nucleic acids were precipitated for 15 min at 4°C and were collected on glass fiber filters. The filters were washed with 5% TCA, dried, and placed in scintillation vials containing 1 ml of scintillation fluid, and the radioactivity was counted.

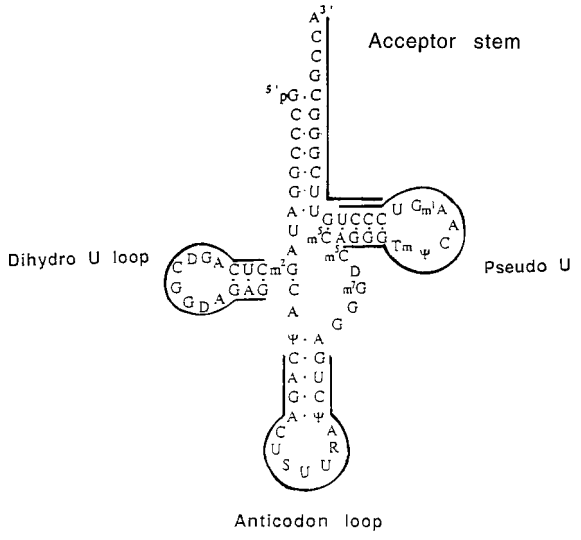

FIG. 1. Cloverleaf structure of $tRNA^{Lys3}$. Bold lines indicate the sequence of the different synthetic ODNs used in this work.

RESULTS AND DISCUSSION

We have focused our investigations on the search for agents able to interfere with the formation of the initiation complex of retroviral replication. RT and primer tRNA are key elements of the reverse transcription initiation complex. Our aim was to block the interaction between the retroviral HIV-1 RT and its primer tRNA to prevent initiation of cDNA synthesis by assuming that this enzyme or its in vivo precursor protein $p160^{gag\text{-}pol}$ is involved in tRNA selection (27). One way to inhibit the initiation of reverse transcription is via interference with the formation of the RT-tRNA complex by using tRNA-derived ODNs, which have previously been shown by footprinting, UV cross-linking analysis, etc., to be in close contact with the enzyme (10, 37, 46). These ODNs include anticodon, diHU, and TΨC. Another potential inhibitory ODN is the 3′ end of the CCA acceptor stem, a region of the tRNA primer involved in the RT-primer-template complex during initiation of cDNA synthesis. The 3′ AS region of primer tRNA interacts with the active site of the enzyme. Moreover, it is involved in the annealing to the retroviral PBS region. The scheme in Fig. 1 illustrates the cloverleaf configuration of rabbit liver $tRNA^{Lys3}$, whose sequence is assumed to be the same as that in its human counterpart (32).

Inhibition of HIV-1 RT by primer tRNA-derived PS-ODNs. The effect of the $tRNA^{Lys3}$-derived ODNs on DNA synthesis by HIV-1 RT was first studied with activated DNA as the template-primer. No inhibition was observed when PO-ODNs derived from the sequence of $tRNA^{Lys3}$ were used, even at concentrations as high as 40 μM. However, with the PS-ODNs the situation was drastically different. The PS-ODNs were strong inhibitors of HIV-1 RT: K_i values for the inhibition of DNA synthesis are presented in Table 1. All these PS derivatives interfered with DNA synthesis, with the most powerful inhibitors being the ODNs corresponding to the AS^{Lys3} and the anticodon (AC) stem-loop, with K_is of 14 and 22 nM, respectively. On the basis of the results showing a strong inhibition by the PS-AS^{Lys3}, we changed the sequence of the acceptor stem sequence (ODNs 101 and 103), mainly the G+C content. These bases may play an important role in the secondary structure of the AS-ODN since they are able to interact preferentially with the enzyme (19). Results of studies in which diHU, pseudouridine (pseudo-U), or different variants of the AS sequence of $tRNA^{Lys3}$ were used as inhibitors of the DNA polymerase activity of HIV-1 RT indicated that of all the PS-ODNs used, the best inhibitor of the retroviral DNA polymerase giving the lowest K_i value was the acceptor stem (Table 1).

The deletion of the CCA end, compound PS-ODN 104, led to a decrease in the level of inhibition which may be partly related to the shorter size of this ODN. Oligonucleotides 101, 103, and 106 (mismatched and scrambled sequences) were good inhibitors of HIV-1 RT but gave higher K_i values than those obtained with the AS^{Lys3} sequence. These results are in agreement with the strong interaction between HIV RT and the anticodon region, as well as the inhibitory effect observed with a dithioate derivative of the 3′ end of primer RNA (28).

In order to study the effects of several PS-ODN derivatives on the RNA-dependent DNA polymerase activity of RT, we used an HIV-1 RNA fragment corresponding to the 5′ long terminal repeat of the retroviral genome as the template and a synthetic 18-mer DNA primer complementary to the PBS region. As shown in Fig. 2, the expected full-length product (147 nucleotides) was obtained after in vitro cDNA synthesis, although some intermediate-size bands, which may correspond to pauses during reverse transcription, were also present. The

TABLE 1. Inhibition of HIV-1 reverse transcription by PS-ODNs[a]

PS-ODN	Length (no. of nucleotides)	Sequence (5′-3′)	K_i (nM)	IC_{50} (nM)
AS^{Lys}	16	CCC TGT TCG GGC GCC A	14 ± 1	40
Anticodon	15	CAG ACT TTT AAT CTG	22 ± 6	140
diHU	14	CTC AGT CGG TAG AG	37 ± 12	>280
Pseudo-U	15	AGG GTT CAA GTC CCT	35 ± 13	140
Mismatched AS^{Lys} (101)	16	CCC TGT TC**A AAC** GCC A	42 ± 2	75
Mismatched AS^{Lys} (103)	16	**TTT** TGT TCG GGC GCC A	32 ± 6	100
ΔCCA (104)	13	CCC TGT TCG GGC G	87 ± 13	150
INV-AS^{Lys}	16	A CCG CGG GCT TGT CCC	58 ± 6	ND[b]
Scrambled AS^{Lys} (106)	16	CTC GCT GCG ACC GTG C	45 ± 4	ND
AS^{Val}	16	CCG GGC GGA AAC ACC A	26 ± 3	140

[a] K_i values were obtained with PS-ODNs corresponding to different domains of primer $tRNA^{Lys3}$ and different sequences derived from the AS of $tRNA^{Lys3}$: oligonucleotides 101 and 103 are mismatched PS-ODNs, oligonucleotide 104 is a PS-ODN lacking the CCA at the 3′ end, INV-AS^{Lys} corresponds to the AS^{Lys3} inverted sequence, oligonucleotide 106 is a scrambled ODN, and AS^{Val} corresponds to the AS domain of bovine $tRNA^{Val}$. HIV-1 RT was assayed with activated DNA as described in the Materials and Methods. IC_{50}s were determined from densitometer readings from Fig. 2, 3, and 4. The nucleotides in boldface type are changes from the nucleotide sequence of AS^{Lys3}.

[b] ND, not determined.

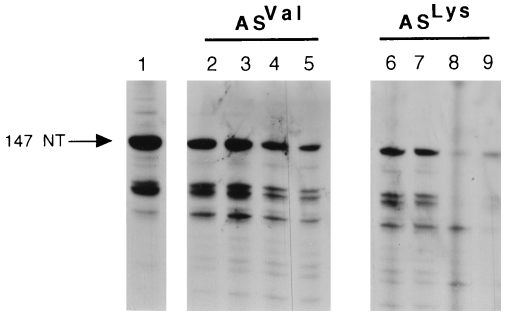

FIG. 2. Inhibition of reverse transcription by HIV-1 RT in the presence of the PS-AS ODNs derived from primer $tRNA^{Lys3}$ and from $tRNA^{Val}$. cDNA synthesis was performed as described in Materials and Methods in the absence of PS-ODN (lane 1) or in the presence of 50 nM (lanes 2 and 6), 100 nM (lanes 3 and 7), 150 nM (lanes 4 and 8), and 200 nM (lanes 5 and 9) concentrations of the corresponding PS-ODNs. NT, nucleotides.

PS-AS ODN derived from $tRNA^{Lys3}$ strongly inhibited the reaction, with a 50% inhibitory concentration (IC_{50}) of 40 nM, while inhibition by the PS-AS corresponding to $tRNA^{Val}$ was observed at significantly higher concentrations (IC_{50}, 140 nM; Table 1). Similar results were obtained when an RNA template-$tRNA^{Lys3}$ primer duplex was used (data not shown).

Figure 3 indicates the inhibitory effects of PS-ODNs corresponding to different regions of primer tRNA on cDNA synthesis. The IC_{50}s are presented in Table 1. The most important inhibitory effect was observed with PS-AS^{Lys3}. PS-ODNs corresponding to the anticodon and pseudo-U stem-loop regions gave similar levels of inhibition with IC_{50}s of 140 nM, while the diHU ODN barely affected DNA synthesis.

Using the same system, we evaluated the inhibitory effect of PS-ODN derivatives of PS-AS^{Lys3} (Fig. 4). A lower degree of inhibition was obtained with mismatched oligonucleotides 101 (IC_{50}, 75 nM) and 103 (IC_{50}, 100 nM). Oligonucleotide 104, from which the CCA end is deleted, was not inhibitory at these concentrations. Scrambled oligonucleotide 106 and the inverted ODN of the acceptor stem (INV-AS) gave weaker inhibitory values for cDNA synthesis (data not shown).

Binding of HIV-1 RT to PS and PO oligonucleotides monitored by gel mobility shift assay. An efficient approach to showing the formation of a complex and to measuring the affinity between nucleic acids and proteins or other ligands is the gel mobility shift or gel retardation technique. As described in the previous paragraph, the AS-PS corresponding to $tRNA^{Lys3}$ is a very strong inhibitor of cDNA synthesis by HIV-1 RT. The direct or decoy effect of ODNs implies the

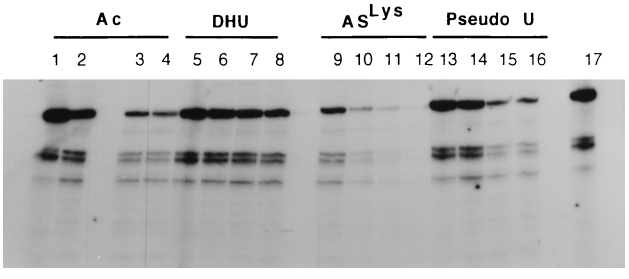

FIG. 3. Inhibition of HIV-1 reverse transcription by PS-ODNs corresponding to different regions of the $tRNA^{Lys3}$ sequence: AC, diHU (DHU), AS, and pseudo-U. Experiments were performed in the presence of 50 nM (lanes 1, 5, 9, and 13), 100 nM (lanes 2, 6, 10, and 14), 150 nM (lanes 3, 7, 11, and 15), and 200 nM (lanes 4, 8, 12, and 16) concentrations of each PS-ODN. Lane 17, control in the absence of PS-ODN.

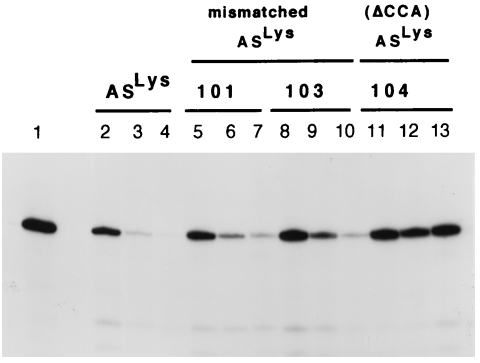

FIG. 4. Inhibition of HIV-1 reverse transcription by PS-ODNs derived from the AS domain of primer $tRNA^{Lys3}$. cDNA synthesis was performed as described in Materials and Methods in the presence of 50 nM (lanes 2, 5, 8, and 11), 100 nM (lanes 3, 6, 9, and 12), and 150 nM (lanes 4, 7, 10, and 13) concentrations of the PS-ODNs whose sequences are presented in Table 1. Lane 1, a control in the absence of PS-ODN.

formation of a stable complex between the inhibitory agent and the target protein. To check this point we performed gel mobility shift assays to follow complex formation between a radioactively labeled PS-ODN derived from the acceptor stem and HIV-1 RT. As indicated in Fig. 5 (lanes 1 to 4), with PS-ODNs, two retarded bands were observed. Two different hypotheses can explain this observation. (i) We may speculate that two kinds of complexes are evidenced by this method, one involving the heterodimeric form p66/p51 and the other involving a complex between the labeled ODN and other homodimeric recombinant forms of HIV-1 RT; or (ii) in the complex formed by HIV-1 RT and PS-ODN, the enzyme may bind to two molecules of ODNs with different affinities, leading

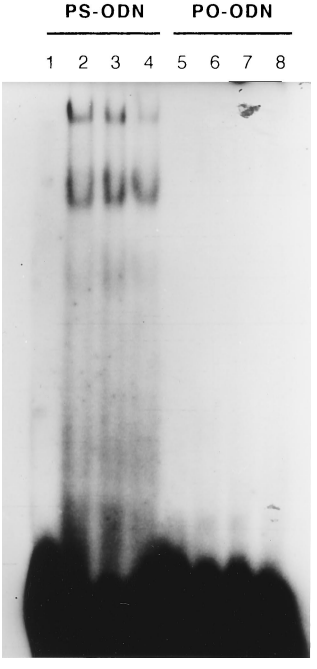

FIG. 5. Gel mobility shift of the complex between HIV-1 RT and the ODN (PS or PO) corresponding to the AS domain of $tRNA^{Lys3}$. Experiments were performed as described in Materials and Methods with 50 nM 5′[γ-^{32}P]ODNs and increasing concentrations of HIV-1 RT: no RT (lanes 1 and 5) and 75 nM (lanes 4 and 8), 150 nM (lanes 3 and 7), and 300 nM (lanes 2 and 6) RT.

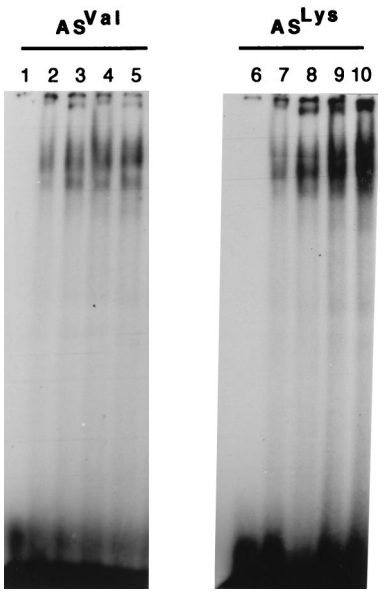

FIG. 6. Gel mobility shift of PS-AS corresponding to $tRNA^{Val}$ and $tRNA^{Lys3}$ in the presence of HIV-1 RT. Assays were done as described in the legend to Fig. 5 for labeled ODNs AS^{Val} (lanes 1 to 5) and AS^{Lys3} (lanes 6 to 10) with increasing concentrations of RT: no RT (lanes 1 and 6) and 50 nM (lanes 2 and 7), 100 nM (lanes 3 and 8), 200 nM (lanes 4 and 9), and 400 nM (lanes 5 and 10) RT.

to the observation of two retarded bands. Supporting this hypothesis, we have already shown by spectrofluorometric measurements that dimeric HIV-1 RT binds to two molecules of primer $tRNA^{Lys3}$ with different K_d values (4, 26).

When a PO-ODN with the same sequence was used, there was no complex formation (Fig. 5, lanes 5 to 8), which coincides with the lack of inhibition by these ODNs. These results indicate the importance of the thioate group on the stability of the enzyme-ODN complex. They are in agreement with the lower affinity of PO agents toward a protein compared with the affinity of PS-ODN. This has been evidenced by the lack of competition in gel retardation assays by unmodified PO-ODNs against PS-ODNs used as decoy agents against the NFκB transcription factor (17).

$PS\text{-}AS^{Val}$ may act as a good inhibitor of RT, even though its sequence is not related to that of the natural HIV-1 primer (Table 1). The interaction between HIV-1 RT and this PS-ODN was compared with that of HIV-1 RT and $PS\text{-}AS^{Lys3}$. Figure 6 shows that binding of $PS\text{-}AS^{Val}$ to HIV-1 RT is weaker compared with that of $PS\text{-}AS^{Lys3}$. A similar decreased interaction was observed with the PS-ODN derived from the acceptor stem lacking the terminal CCA (data not shown). These results are in agreement with the lower level of inhibition of in vitro DNA synthesis by HIV-1 RT in the presence of these PS-ODNs. To demonstrate that the retarded bands are the result of binding of the $PS\text{-}AS^{Lys3}$ to the tRNA binding region of HIV-1 RT, we performed competition experiments with $tRNA^{Lys3}$. A gel mobility shift assay was performed with labeled $tRNA^{Lys3}$ and RT in the presence of increasing amounts of either unlabeled $tRNA^{Lys3}$ or $PS\text{-}AS^{Lys3}$. The retarded labeled tRNA was displaced by $tRNA^{Lys3}$ as well as being displaced by $PS\text{-}AS^{Lys3}$, showing that it is able to compete for the tRNA site of HIV-1 RT.

The high affinity between $PS\text{-}AS^{Lys3}$ and HIV-1 RT was also observed by protein fluorescence quenching. Excitation was performed at 295 nm and emission was measured from 300 to 420 nm. By increasing the concentrations of $PS\text{-}AS^{Lys3}$, we showed an important quenching effect of the enzyme fluorescence, while the PO-ODN (at between 0 and 5 μM) with the same sequence gave no quenching effect under the same conditions (data not shown).

Effect of phosphorothioate oligonucleotides on cells chronically infected with HIV-1. The effects of PS-ODNs on the infection of MT-2 cells by HIV-1 were followed by using different criteria. The cytopathogenic effect was shown by the emergence of giant cells. The antiviral effect of PS agents was determined by their ability to inhibit the cytopathogenic effect in the MT-2 cells infected with the HIV-1/HTLV-IIIB strain as well as by the detection of RT activity. MT-2 cells were incubated with different amounts of the PS-ODN either after or during viral adsorption.

Antiviral activity and cytotoxicity with MT-2 cells. The effects of PS-ODNs on HIV-1 replication were determined by measuring the RT activity in the supernatants of infected cells. The only PS-ODN that showed an antiviral effect was the $PS\text{-}AS^{Lys3}$, with a 50% inhibitory dose of 400 to 750 nM in different experiments (Fig. 7A). PS oligonucleotides 106 and INV-AS gave 50% inhibitory doses four to five times higher, while PS-ODNs corresponding to other regions of primer tRNA, like AC, diHU, and pseudo-U stem-loops, did not show any effect under the same experimental conditions. AZT was used as a reference compound. No cytotoxic effect, determined by the MTT method, was observed with PS-ODN concentrations up to 10 μM (data not shown).

The latter results were obtained when ODNs were added after viral adsorption. Preliminary experiments performed by adding the inhibitory agents during viral adsorption led to a stronger inhibition by $PS\text{-}AS^{Lys3}$, since a 90% inhibition of RT activity was obtained when a concentration of about 100 nM was used (data not shown).

The viabilities of HIV-1-infected or -noninfected cells incubated with different concentrations of PS-ODN were determined spectrophotometrically as described by Pauwels et al. (31). Control experiments were done in parallel with AZT. As indicated in Fig. 7B, when compared with the value obtained with uninfected cells, $PS\text{-}AS^{Lys3}$ was able to inhibit HIV-1 proliferation, and this was followed by an increase in cell viability. At 0.5 μM there was a significant inhibition and at 1 μM the cytopathogenic effect was abolished to the same extent as it was with AZT. A very slight inhibitory effect was obtained with PS-AC at 1 μM. Other PS-ODNs (diHU, pseudo-U) showed no inhibitory effect under these conditions.

Although PS-ODNs are very effective in vitro inhibitors of reverse transcription, these compounds may exert their cytoprotective effects in part by interfering with the binding and adsorption of HIV-1 to the target cells (40, 41). Stein et al. (40) have shown that PS-ODNs may bind to the third variable loop domain (V3) of HIV gp120, competing with the binding of this domain to the CD4 receptor. It is important to point out that the latter results were obtained with an IC_{50} of approximately 30 μM, a concentration much higher than those used in this work (41). Although we cannot exclude the possibility that the strong inhibitory effect described in this report may be related in part to decreased viral adsorption, the lack of effect of several control PS-ODNs on HIV-1-infected human cell cultures and the specific effect of $PS\text{-}AS^{Lys3}$ point to an effect of the latter modified ODN on cDNA synthesis.

Long-term inhibition of HIV proliferation in infected human cells. Several anti-HIV drugs including some PS-ODNs have been shown to be effective in short-term infection assays but were not able to suppress virus replication in long-term cultures (23). However, a 25-mer antisense PS-ODN targeted

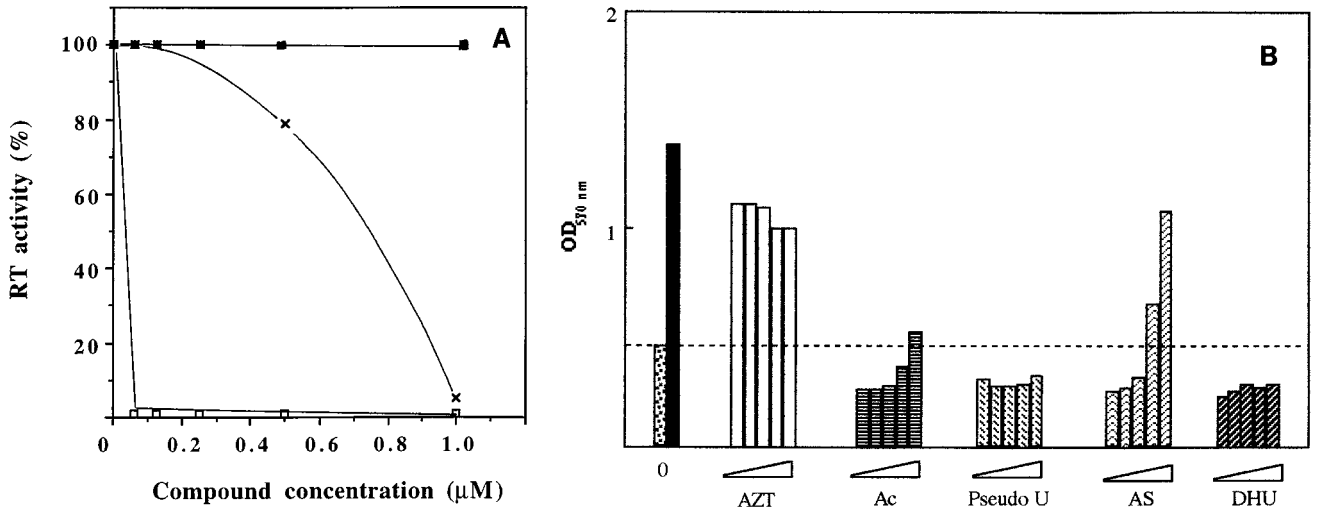

FIG. 7. Antiviral activities of PS-ODNs in MT-2-infected cells. Cells were infected with HIV-1/HTLV-IIIB strain and after 2 h of adsorption were incubated in the presence of serial dilutions of ODNs and AZT. (A) Virus production was determined by measuring RT activity in culture supernatants as a function of increasing concentrations of ODNs. ■, AC, pseudo-U, and diHU (DHU); ×, AS^{Lys3}; □, AZT. (B) The protective effect (or cell viability) produced by PS-ODNs was tested by the MTT assay as described in Materials and Methods. Increasing concentrations of each compound, including AZT, were used, as follows: 62.5, 125, 250, 500, and 1,000 nM. $OD_{530\ nm}$, optical density at 530 nm. For the bars labeled 0, results are for uninfected (■) and infected (▨) cells.

to the *gag* mRNA region of HIV-1 (GEM-91) inhibited HIV-1 replication in a sequence- and dose-dependent manner during a 28-day treatment of an HIV-1-infected T-cell line (24).

To evaluate the long-term efficacy of the $PS\text{-}AS^{Lys}$, infected MT-2 cells were maintained in the presence of either $PS\text{-}AS^{Lys}$, two control PS-ODNs (mismatched oligonucleotide 101 and scrambled oligonucleotide 106), or AZT for 28 days (Fig. 8). In the presence of PS-ODNs 101 and 106, a transient

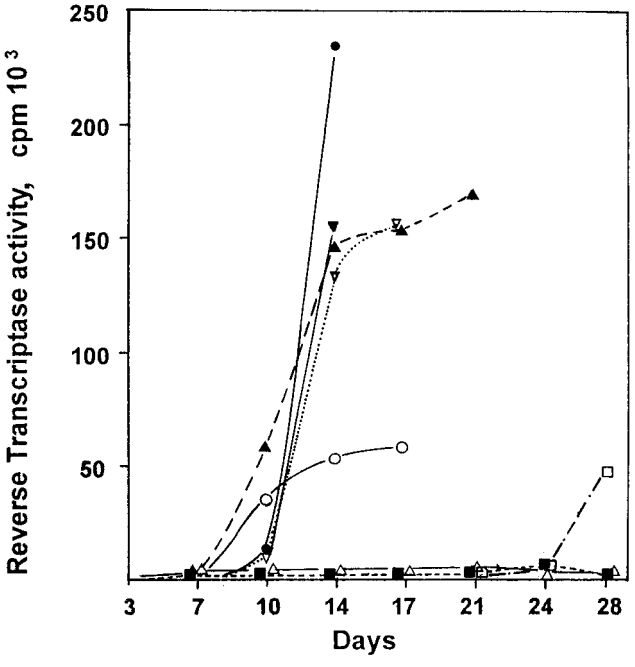

FIG. 8. Long-term inhibition of HIV-1/HTLV-IIIB strain replication (28 days) by PS-ODNs and AZT in MT-2 cells. Experimental conditions are described in Materials and Methods. △, AS, 2.5 μM; ▲, AS 1.25 μM; ○, oligonucleotide 101, 2.5 μM; ●, oligonucleotide 101, 1.25 μM; ▼, oligonucleotide 106, 2.5 μM; ▽, oligonucleotide 106, 1.25 μM; ■, AZT, 1 μM; □, AZT, 0.2 μM.

suppression of HIV replication was observed. Treatment with 1 μM AZT or 2.5 μM PS-AS showed similar efficiencies: both compounds completely blocked HIV-1 replication for up to 28 days. In contrast, lower doses (1.25 μM PS-AS or 0.2 μM AZT) failed to prevent virus replication after 7 and 24 days, respectively.

Inhibition by AS in infected cells may be explained by addition of a decoy and an antisense effect. The fact that the $PS\text{-}AS^{Lys3}$ was the only primer tRNA-derived fragment to inhibit HIV-1 proliferation in infected cells, while the other tRNA-derived PS-ODNs were strong inhibitors of cDNA synthesis in a cell-free system, was a surprising result. A possible explanation is that the $PS\text{-}AS^{Lys3}$ may act both directly on the viral polymerase (like other PS-ODNs) and by annealing to the PBS sequence to compete with the natural tRNA primer for the initiation of DNA synthesis. Moreover, the annealing of the $PS\text{-}AS^{Lys3}$ to the PBS region would provide a substrate for the retroviral RNase H activity. To address the question of whether a duplex formed by PS-ODN and HIV-1 RNA can elicit the RNase H activity associated with HIV-1 RT, different PS-ODNs were incubated with retroviral RNase H. Figure 9 presents the results obtained with two duplexes: $PS\text{-}AS^{Lys3}$ and mismatched PS-AS oligonucleotide 101. Only in the case of $PS\text{-}AS^{Lys3}$ did we observe RNA hydrolysis due to RNase H activity. After incubation of the duplex with the enzyme, two fragments with the expected lengths were obtained (Fig. 9, lanes 4 and 5). The other PS-ODNs tested, like PS-AS oligonucleotide 101, gave no digestion product, as indicated in Fig. 9, lanes 2 and 3. A control incubation was done in the presence of the labeled RNA and RT to show the absence of contaminating RNases that could degrade the RNA (Fig. 9, lane 1).

These results strongly suggest that at low ODN concentrations, viral proliferation may be selectively inhibited by the PS-AS, at least in part, by specific degradation of the viral RNA by the retroviral RNase H-associated activity. In addition to this antisense effect, the strong inhibition of cDNA synthesis by $PS\text{-}AS^{Lys}$ ODN when using templates devoid of the PBS sequence [poly(rA)-oligo(dT) or activated DNA] supports a direct inhibitory effect of the PS-ODN on the viral polymerase

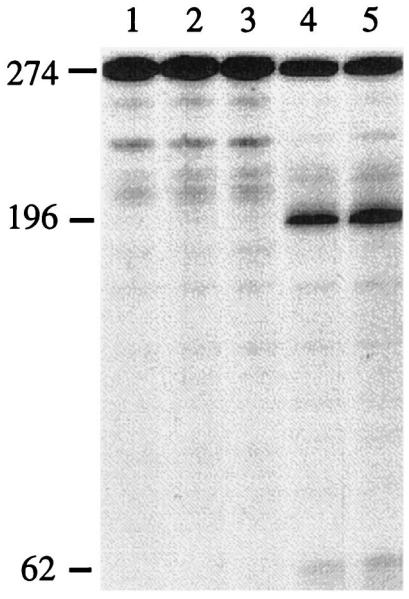

FIG. 9. RNase H activity. A fragment from HIV-1 RNA (274 nucleotides [indicated on the left]) containing the PBS sequence was uniformly labeled by in vitro transcription with the T7 RNA polymerase system. After extraction with phenol-chloroform and ethanol precipitation, RNA was purified by gel electrophoresis. In a final volume of 20 μl, labeled RNA (20 nM) was incubated in the presence of 50 mM Tris-HCl (pH 8.0), 2 mM dithiothreitol, 6 mM $MgCl_2$, 12 mM NaCl, 3 U of human placental RNase inhibitor, and the corresponding PS-ODN; after 10 min at 37°C, 50 nM HIV-1 RT was added and the incubation was carried out for another 30 min at 37°C. Lane 1, complete system including RT, but in the absence of PS-ODN; lane 2, 50 nM mismatched PS-AS (oligonucleotide 101); lane 3, 100 nM mismatched PS-AS (oligonucleotide 101); lane 4, 50 nM PS-AS^{Lys3}; lane 5, 100 nM PS-AS^{Lys3}. Samples were analyzed in a 6% polyacrylamide gel–7 M urea in TBE buffer. After electrophoresis, the gel was autoradiographed.

and correlates well with the formation of a complex between the primer binding domain of HIV-1 RT and the 3′ end of its specific primer tRNA.

Conclusions. The PS-modified oligonucleotide corresponding to the 3′ end of HIV-1 primer $tRNA^{Lys3}$ is a very good inhibitor of reverse transcription in a cell-free system. Other PS-ODNs showed inhibition, but with significantly higher K_i values. The situation in HIV-1-infected human cells is more specific toward the inhibition of viral replication by a defined PS-ODN. An inhibitory effect of viral replication was observed in HIV-1-infected MT-2 cells when the PS-AS^{Lys3} was present, while a very slight inhibition was observed at 1 μM with the anticodon sequence (Fig. 7B) and none was observed with the other PS-ODNs derived from primer $tRNA^{Lys3}$. This effect is even more interesting since no cytotoxic effects were observed at concentrations up to 10 μM. Moreover, the inhibitory effect was maintained during a long-term (28 days) exposure to the PS-AS^{Lys3}.

Why is there a difference between the results of experiments with a cell-free system and infected cells? HIV-1 RT forms a very stable complex with its specific $tRNA^{Lys3}$. The primer tRNA regions corresponding to anticodon, diHU, and pseudo-U have been shown to be in close contact with HIV-1 RT by nuclease footprinting or UV cross-linking, while the 3′ end of primer tRNA compulsorily interacts with the viral polymerase since it must be in close contact with the active site of HIV-1 RT to initiate cDNA synthesis. It can be speculated that the lack of effect of PS-ODNs, with the exception of PS-AS^{Lys3}, at low concentrations in infected cells may be due to the fact that even if these agents are bound to the RT in the virions of infected cells their inhibitory effects may go unnoticed either because of the low concentration of these internalized ODNs or because they may be outcompeted by other factors interacting with the viral polymerase. A somewhat similar situation was described in the case of AZT-resistant HIV-1 mutants in which the viral polymerase is resistant to the therapeutic agent in infected cell cultures, while the purified viral enzyme isolated from resistant strains is fully sensitive to the inhibitor in a cell-free system. No explanation has been found for this observation, but it has been suggested that the interaction of HIV-1 RT in the virion particle with other proteins or nucleic acids may produce a conformational change of the enzyme leading to the in vivo emergence of the resistance trait (22).

The specific inhibition by PS-AS^{Lys3} observed in HIV-1-infected MT-2 cells may correspond to the addition of two phenomena: a direct or decoy-like effect on RT and an antisense effect obtained by competition with the endogenous $tRNA^{Lys3}$ after annealing of PS-AS^{Lys3} to the PBS. Even if the internalized PS-AS^{Lys3} concentration is as low as those of the other inactive PS-ODNs mentioned above, the interaction of PS-AS^{Lys3} with a critical domain of the retroviral polymerase may lead to the arrest of HIV-1 proliferation in infected cells via its annealing to the PBS site. The efficient in vitro digestion of the duplex formed between the RNA-PBS region and PS-AS^{Lys3} supports the idea that an antisense effect may play a role in the inhibition mechanism. Moreover, an additional possibility that may explain the observed inhibition with PS-AS^{Lys3} emerges from results suggesting that an A-rich sequence downstream of the PBS region plays an important role in the initiation of reverse transcription by interaction with the U-rich anticodon loop of primer $tRNA^{Lys3}$ (21). Thus, once the PS-AS^{Lys3} is annealed to the PBS region of HIV-1 RNA, an abortive initiation complex is formed due to the high affinity of the PS-AS–HIV-1 RT complex, as well as the fact that other regions of primer tRNA that are absent from PS-AS are required for an efficient initiation of cDNA synthesis.

In summary, we have shown that interference with the formation of the complex between HIV-1 RT and its specific primer tRNA can be successful by using a modified ODN corresponding to a region of $tRNA^{Lys3}$, since important inhibition of HIV-1 replication was attained both in a cell-free system and in retrovirus-infected cells.

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