Anti-HIV Activities and Mechanisms of Antisense Oligonucleotides

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1. Introduction

Antisense oligodeoxyribonucleotides within cells, targeted toward the RNA transcript of a specific gene, can inhibit the expression or promote the degradation of the transcript, resulting in suppression of the function encoded by the gene. The addition of chemically modified antisense oligomers to culture medium for uptake by cells has been used to inhibit the expression of specific target genes (1-4). These compounds have been used as antisense inhibitors of gene expression in various culture systems and are considered to be potential therapeutic agents (5,6).

Antisense oligonucleotides complementary to viral RNA inhibit viral replication in cells cultured with Rous sarcoma virus (7), human immunodeficiency virus (8-11), vesicular stomatitis virus (12,13), herpes simplex virus (12-14), and influenza virus (15,16). However, the mechanisms by which the antisense oligonucleotides inhibited retroviral protein synthesis, syncytia formation, and reverse transcriptase activity have not been fully elucidated. Recently, Toulm et al. reported that unmodified oligonucleotides indeed arrested cDNA synthesis by the AMV and MMLV RTs, which have RNase H activity, but that α-oligonucleotide analogues did not (17,18).

On the other hand, Matsukura et al. reported that the inhibition of *de novo* infection by S-ODNs is both composition- and length-dependent, for example, the homo-oligo S-dC^{28} is a better inhibitor than either the S-dC^{20} or S-ODNs (20mer, coding exon I of art/trs gene in HIV) (9). However, S-dC^{28} did not inhibit p24 *gag* expression in chronically infected T cells, whereas the

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S-ODNs complementary to the initiation sequence of HIV-*rev* inhibited the production of several viral proteins in chronically HIV infected T cells (19). On the other hand, Lisziewicz et al. (20) have reported that chemotherapy based on specifically targeted antisense oligonucleotide phosphorothioates is an effective means of reducing the viral burden in HIV-1 infected individuals, at clinically achievable oligonucleotide concentrations. However, despite advances in AIDS therapy, there still remains the issue of how to select an effective target sequence and length for the phosphorothioate oligonucleotides.

We present here a detailed analysis of the effect of unmodified and modified (phosphorothioate) oligodeoxyribonucleotides (21) on cDNA synthesis by the AMV, MMLV, and HIV RTs. We also describe the long-term treatment of human immunodeficiency virus-infected cells with antisense phosphorothioate oligonucleotides directed to three target sites (*gag*, *rev*, and *tat*) within the HIV gene. (see Fig. 8). The phosphorothioate oligonucleotides are nuclease-resistant analogues of oligodeoxyribonucleotides that can be used to prevent reverse transcription.

2. Experimental

2.1. Materials

The unmodified oligonucleotide derivatives 5'-d[TTGTGTCAAAAGCAAGT, 17 cap (n)], 5'-d[CACCAACTTCTTCCACA, 17 sc (n)], and 5'-d[TGCCCAGGGCCTCAC, 15sc (n)], and the modified (phosphorothioate) oligodeoxyribonucleotide derivatives, 5'-d[TsTsGsTsGsTsCsAsAsAsAsGsCsAsAsGsT, 17 cap (s)], 5'd[CsAs CsCsAsAsCsTsTsCsTsTsCsCsAsCsA, 17 sc (s)], (dC)20, (dA)20, (dT)20, and (dG)20, were synthesized on an Applied Biosystems 392A DNA synthesizer using the solid phase phosphoramidite method. The oligonucleotide derivatives were purified by reverse phase HPLC on an oligo-DNA column. Purified oligomers were evaluated by resolving. ^{32}P-labeled samples by electrophoresis on 20% polyacrylamide/7 M urea gels.

The phosphorothioate oligonucleotide analogues (S-ODNs) were prepared on a synthesizer, using our new phosphite approach, and were purified by HPLC according to published procedures (22). We used three genes [*gag*, *rev*, and *tat*] as targets for antisense interruption of viral gene expression.

A fragment, about 150 nucleotides long, was obtained by directed cleavage of rabbit β-globin mRNA by *E. coli* RNase H. β-globin mRNA (0.5 ng) was incubated with *E. coli* RNase H (10 units) in the presence of an oligonucleotide 15 (100 pmol), which is complementary to nucleotides 147-161 of the β-globin-mRNA. The reaction mixture was incubated for 2 h at 37°C in 20 ml of a buffer containing 20 mM Tris/HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM KCl, and 0.1 mM dithiothreitol. After the reaction, the fragment, which was an mRNA of about 150 nucleotides, was phenol-extracted, ethanol-precipitated, and dissolved in 25 µl of sterile water.

A 159 nucleotide transcription template containing a class III T7 promoter followed by the transcript was synthesized from DNA oligomers by standard ligation procedures (23). The DNA template was annealed in a one to one molar ratio in 0.8 mM HEPES-KOH (pH 7.5) by heating at 90°C for 3 min, followed by snap-cooling on ice. Non-radioactive transcription reactions contained 1 μg DNA template, 80 mM HEPES-KOH (pH 7.5)/12 mM $MgCl_2$, 2 mM spermidine, 20 mM DTT, 4 μM of each NTP, 20 μM GMP, 10 mM KCl, 10 mM $MgCl_2$, 20 μg/μl BSA, 40 U/l RNasin, and 7 ng/ml T7 RNA polymerase (10 μl). Radioactive transcription reactions differed only in having 4 μM CTP, GTP, UTP, 0.5 μM ATP, and [α-^{32}P]ATP (10 μl, 800 Ci/mmol, Ci=3.7GBq; NEN). The reaction mixture was incubated for 2 h at 37°C. Full-length transcripts (142 nucleotides) were purified by 7 M urea/10% polyacrylamide gel electrophoresis, elution, and ethanol precipitation in the presence of 3 M sodium acetate.

2.1.1. Primer extension

Rabbit β-globin RNA (50 ng, containing 0.3 pmol of the intact β-globin message), primer (50 pmol), and the desired amount of oligonucleotides were pre-incubated for 30 min at 39°C. After adding 1 μl of 10 X RT buffer (1M Tris/HCl, pH 8.3, 720 mM KCl, 100 mM $MgCl_2$, 100 mM dithiothreitol) containing 8 units of RNasin, 2 pmol of [α-^{32}P]dCTP (3000Ci/mmol; Ci = 37GBq; NEN), 5 nmol of the three dNTPs, and 2.5 nmol of dCTP, the volume of the mixture was adjusted to 10 μl with sterile water. AMV RT (1-10 units-ie., 0.13-1.3 pmol) or MMLV RT (50-200 units) was then added. The reaction with HIV RT was allowed to proceed with 1 unit, and was incubated for 1 h at 39°C. The cDNA was chloroform-extracted according to standard procedures, and electrophorezed on a 10% polyacrylamide gel (PAGE). The results obtained for cDNA synthesis were corrected for the amount of label incorporated into each fragment.

2.1.2. Analysis of the RNA template

The template RNA (142 nucleotides, corresponding to nucleotides 1-142 of the β-globin mRNA, 0.3 pmol), the primer (5 pmol/μl), and the antisense oligonucleotides (2 pmol/μl) were pre-incubated for 30 min at 39°C. After adding 1 μl of 10 X RT buffer (1 M Tris/HCl, pH 8.3, 720 mM KCl, 100 mM $MgCl_2$, 100 mM dithiothreitol) containing 8 units of RNasin, and 5nmol of the four dNTPs, the volume of the mixture was adjusted to 10 μl with sterile water. AMV RT (1-10 units-ie., 0.13-1.3 pmol) was then added. The reaction with HIV-1 RT was allowed to proceed with either 1 or 10 units, and was incubated for 15 min at 39°C. The RNA was chloroform-extracted according to standard procedures, and electrophoresed on a 10% polyacrylamide gel. Quantitative evaluation of cleavage was performed by densitometric analysis.

2.1.3. Cell line and virus strain

For the anti-HIV assay, the human T lymphotropic virus type-1 (HTLV-III)-positive human T cell line, MT-4, was subcultured twice a week at a concentration of $3x10^5$/mL in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 100 IU/mL penicillin, and 100 μg/mL streptomycin. The HIV-1 strain, HTLV-IIIB, was used for the anti-HIV assay. The virus was prepared from culture supernatants of MOLT-4/HTLV-IIIB cells, which were persistently infected with HTLV-IIIB. The HIV stock solution was titrated in MT-4 cells as determined by 50% tissue culture infectious doses ($TCID_{50}$) and plaque forming units, and was stored at -80°C until use.

2.1.4. Long-term experiment for inhibition of HIV-1 replication

To test whether antisense oligonucleotides inhibit viral gene expression, we used chronically HIV-1 infected MOLT-4 cells. Cells (30×10^4/ml) were infected with HIV-1 (IIIB isolate) for 1 h, and then washed to remove the previously produced viral particles from the media. After washing, 30×10^4 cells/ml were grown in the presence of phosphorothioate oligonucleotides at a 5 μM concentration, and were cultured in CO_2 incubators at 37°C. After 4 days, the culture supernatants were collected, the viable cells were counted by dye exclusion and split to 30×10^4 cells/ml, and the cultures were retreated with oligonucleotides (at 2.5 μM and 1 μM concentrations, as indicated). The number of viable cells and the amount of p24 *gag* protein in the culture supernatants were monitored by the indirect immunofluorescence (IF) method and the HIV-1 p24 assay kit of, (Abbott GmgH Diagnostika, Wiesbaden-Delkenheim, Germany), respectively, after 12, 20, 40, and 60 days of incubation.

2.1.5. Intracellular distribution of S-ODN-rev-sa in MOLT-4 and MOLT-4/HIV-1 cells

MOLT-4 and MOLT-4/HIV-$1_{HTLV-IIIB}$ cells were incubated with FITC-labeled S-ODNs-*rev* at 37°C for 10, 30, and 60 min. Cells were washed 3 times with PBS, resuspended in PBS containing 0.37% formaldehyde, and observed with a scanning confocal microscope (24).

3. Results and discussion

3.1. Mechanism of the inhibition of reverse transcription by antisense oligonucleotides

Reverse transcription of rabbit β-globin mRNA by AMV RT was primed with 17sc, complementary to nucleotides 113-129 (Fig. 1), and gave rise to the predicted cDNA fragment of about 130 nucleotides (Fig. 2a). In contrast,

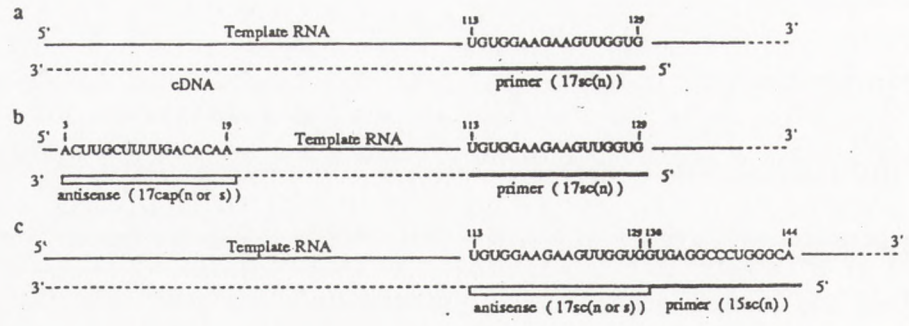

Fig. 1. Inhibition of reverse transcription by complementary oligonucleotides. The full length cDNA products derived from 17 sc (n) or 15 sc (n) (a) could be blocked by the antisense oligomers, 17 cap (n or s) and 17 sc (n or s) bound to a template sequence (b), or adjacent (c) to the primer-binding site.

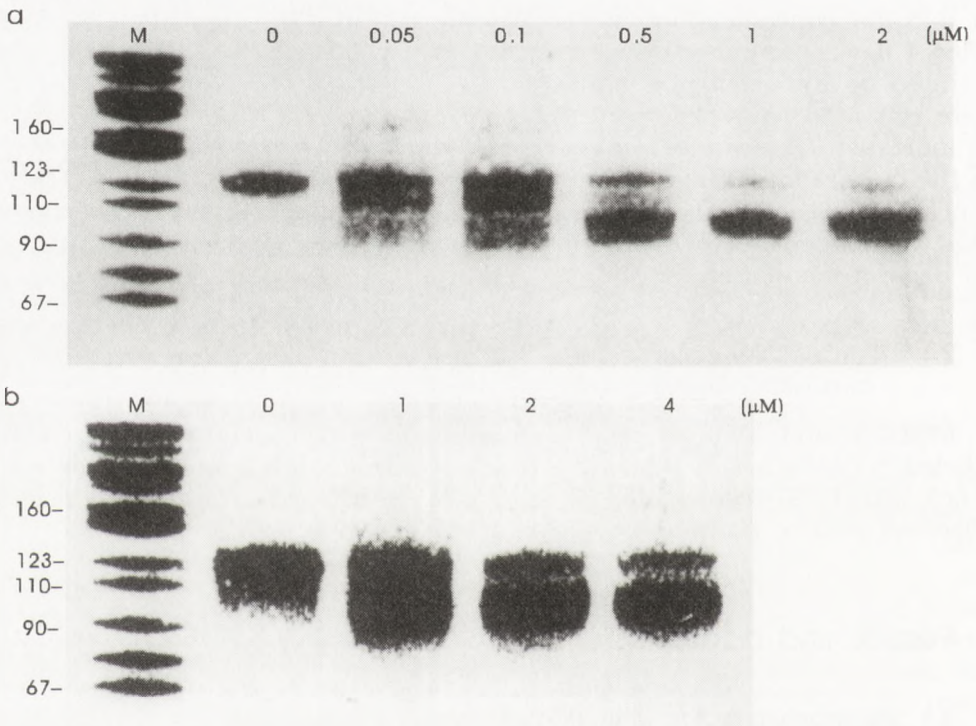

Fig. 2. Effect of the unmodified and modified antisense oligonucleotides on DNA synthesis. (a) Reverse transcription proceeded as indicated using 10 units of AMV RT primed by 5 μM oligomer 17 sc (n) without (0) or with various amounts (μM) of oligomer 17 cap (n). (b) cDNA analysis of the 17 cap (s) used in place of the 17 cap (n) in a. The first lanes on the left (M) in a and b correspond to the DNA size markers.

when the polymerization was performed in the presence of 17 cap (n) (0.05-2 μM), an oligonucleotide targeted to the cap region of the mRNA, a shortened DNA fragment was synthesized, at the expense of the full-length product. The size of the cDNA fragment corresponded to the distance between the primer and the binding site of the antisense oligonucleotides (Fig. 1). Therefore, the hybridization of this antisense oligonucleotide with the complementary sequence of the β-globin mRNA prevents the transcription of this region. The inhibitory efficiency was dependent on the 17 cap (n) concentration. At concentrations as low as 1.0 μM of 17 cap (n), 96% inhibition of reverse transcription was observed. This resulted from competition between the 17sc (n) primer and the 17 cap (n) antisense oligomer.

Next, we examined cDNA synthesis by the AMV reverse transcriptase using the 17 cap (s) phosphorothioate oligomer instead of 17 cap (n). Figure 2b shows that 17 cap (s) reduced the synthesis of the 110 nucleotide transcript; at concentrations of 1, 2, and 4 μM, the percentages of inhibition were, respectively, 55%, 74%, and 82%. However, these values are lower than that of the unmodified oligonucleotide at 1 M. This suggests that part of the phosphorothioate oligomer binds to the RT enzyme. As a result, the synthesis of the full length cDNA can not be completely blocked by the decrease of the binding of the phosphorothioate oligomer to the mRNA. This process was essentially sequence independent and was the result of the preferential binding of the modified oligomers to the RT enzyme, as compared with the unmodified oligomers.

To characterize the inhibitory process of the phosphorothioate oligomers, we incubated the antisense oligomers, 17 cap (n) or 17 cap (s) (2 μM), and the 17 sc (n) primer (5 μM) with AMV RT at concentrations of 1 or 10 units, under the same conditions as described above. Smaller amounts of AMV RT were used because the polymerase activity aborts synthesis. The synthesis of cDNA with 17 cap (n) was inhibited by 1 unit of AMV RT. However, cDNA synthesis was significantly decreased when the modified oligomer, 17 cap (s), was used (Fig. 3). It should be noted that the cDNA could not be synthesized because of a lock of polymerization, due to the competitive binding of phosphorothioate oligomers on the RT enzyme. On the other hand, when the AMV RT concentration was 10 units, the short 123 nucleotide transcript was detected in all cases (Fig. 3). This suggests a relationship between the RT enzyme and the phosphorothioate oligomer and its inhibitory efficiency (9,25). It should be pointed out that the antisense phosphorothioate oligomer did not compete directly with the mRNA, but it did with the RT enzyme.

Furthermore, we tested the ability of an antisense oligonucleotide to block the reverse transcription of rabbit β-globin mRNA by MMLV RT. The reverse transcription of rabbit β-globin mRNA was carried out by MMLV RT in the presence of 17sc as the primer and the antisense oligonucleotides (17cap (s) or 17cap (n)), respectively. Figure 4 shows that the oligomer 17cap (n) induced the characteristic shortened cDNA fragment, whereas 17cap(s) reduced the synthesis of the 110 nucleotide transcript; at a concentration of 1 μM, the percentage of cDNA synthesis was significantly decreased.

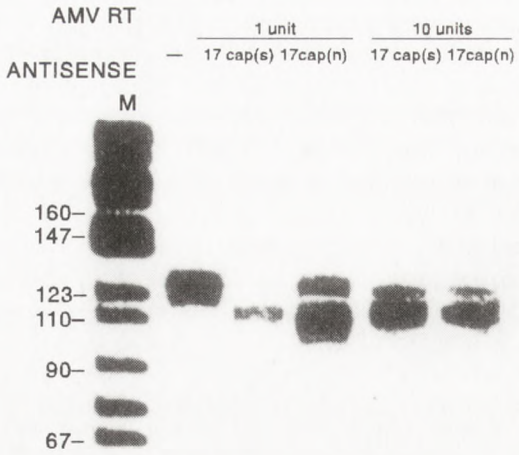

Fig. 3. The interaction between the antisense oligomers and AMV RT on the reverse transcript. Analysis of cDNA fragments synthesized by 1 unit (left) or 10 units (right) of AMV RT. The reaction primed by a 17 mer [17 sc (n) (5 μM)] complementary to the 113-129 oligonucleotide without (-) or with 2 μM antisense oligonucleotides, 17 cap (n) and 17 cap (s). DNA size markers are shown in lane M.

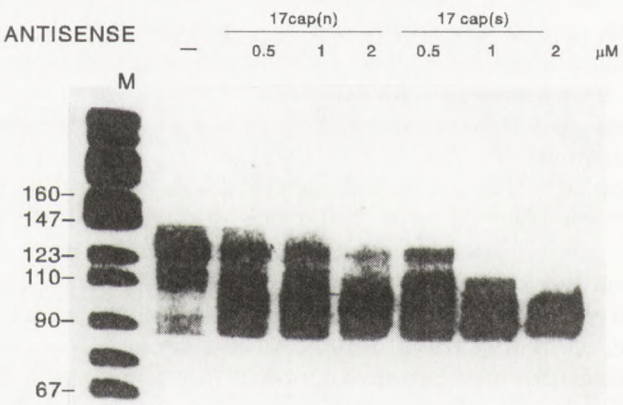

Fig. 4. Effect of antisense oligonucleotides on cDNA synthesis using MMLV RT. Reverse transcription proceeded as indicated using 50-200 units of MMLV RT, primed by a 17 mer [17 sc(n) (5 μM)] complementary to the 113-129 oligonucleotide, without (-) or with various amounts (μM) of antisense oligonucleotides, 17 cap(n) (left) and 17 cap(s) (right).

To further characterize the inhibitory process of the phosphorothioate oligomers, we examined how to prevent cDNA elongation by HIV RT instead of AMV RT, under the same conditions as described above. Figure 5 shows that the antisense oligomers 17 cap (n) and 17 cap (s) induced the characteristically, shortened cDNA fragments when the reaction proceeded with HIV RT. The 17 cap (s) oligomer, at a concentration as low as 1.0 μM, resulted in a shortened DNA fragment at the expense of the full-length product. This indicated that the phosphorothioate oligonucleotide inhibited the production of cDNA by the retroviral polymerase by binding to the RNA downstream from the primer. However, the inhibitory efficiency of the phosphorothioate oligonucleotide is influenced by its relationship with the RT.

The cleavage of an RNA template by the reverse transcriptase-associated RNase H activity is the major mechanism of inhibition in the antisense approach. We have demonstrated that the inhibitory process involves the attack of the antisense oligonucleotide-RNA hybrid by the RNase H activity associated with the reverse transcriptase enzyme. For this investigation, a 142-mer RNA fragment corresponding to the 5'-end of the β-globin mRNA was prepared by oligodeoxyribonucleotide directed transcription, using T7 RNA polymerase (23). Reverse transcription reactions of the rabbit β-globin mRNA by AMV RT (10 units) and HIV-1 RT (10 units) were primed with 17sc (n) and 17 cap (n) (2 μM), oligonucleotides targeted to the cap region of the mRNA, which is part of the 142 nucleotide RNA template, yielding the shortened RNA fragments (Fig. 6, lanes 2 and 4).

Next, we examined the RNA template cleavage by AMV RT and HIV-1 RT, using the 17 cap (s) phosphorothioate oligomer instead of 17 cap (n). Figure 6 (lane 2, AMV RT) shows that 17 cap (s) reduced the cleavage of the RNA template, because the 142 nucleotide RNA fragment was detected. This suggests that part of the phosphorothioate oligomer binds to the AMV RT enzyme. As a result, the 142 nucleotide RNA fragment cannot be completely cleaved, due to the decrease in the binding of the phosphorothioate oligomer to the RNA. This process was essentially sequence independent, and was the result of the preferential binding of the modified oligomers to the reverse transcriptase, as compared to the unmodified oligomers. However, the antisense oligonucleotides 17 cap (n) and 17 cap (s) produced the characteristically shortened RNA fragments when the reaction proceeded with the HIV-1 RT. The 17 cap (s) oligomer, with a 10 unit concentration of HIV-1 RT, cleaved the 142 nucleotide RNA template and produced the shortened fragments (Fig. 6, lane 6). This indicates that the phosphorothioate oligonucleotide leads to the cleavage of the RNA template by the RT-associated RNase H by binding to the RNA downstream from the primer. To characterize the inhibition of the RNase H activity by the phosphorothioate oligonucleotides, we incubated the antisense oligonucleotides, 17 cap (n) or 17 cap (s) (2 μM), and the 17 sc (n) primer (5 μM) with AMV RT (1 unit) and HIV-1 RT (1 unit) at low concentrations, under the same conditions as described above. The template RNA with 17 cap (n), was completely cleaved by 1 unit of either AMV RT or HIV-1 RT (Fig. 6, lanes 4 and 7). However, cleavage of

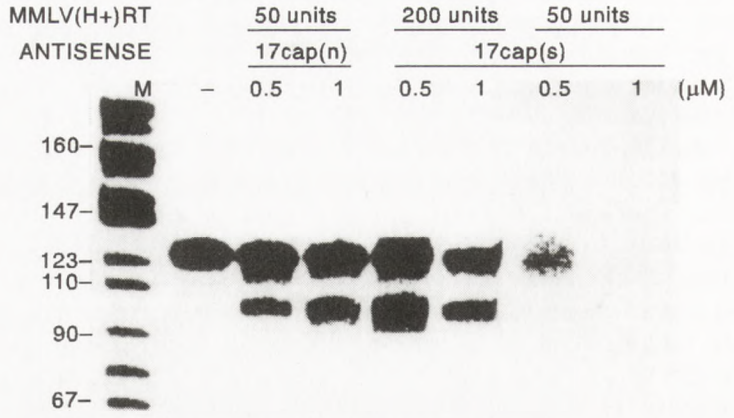

Fig. 5. Effect of antisense oligonucleotides on cDNA synthesis using HIV RT. Reverse transcription proceeded as indicated using 1 unit of HIV RT primed by a 17 mer [17 sc (n) (5 μM)] complementary to the 113-129 oligonucleotide without (-) or with various amounts (μM) of antisense oligonucleotides, 17 cap (n) (left) and 17 cap (s) (right). DNA size markers are shown in lane M.

Fig. 6. Analysis of an RNA template cleavage reaction of an internally labeled 142 nt fragment corresponding to the β-globin mRNA after reverse transcription by AMV RT and HIV-1 RT in the presence of the antisense oligonucleotides (17cap (n) or 17cap (s)). Lanes 1 and 2 (17cap (s) and 17cap (n), AMV RT, 10 units); lanes 3 and 4 (17cap (s) and 17cap (n), AMV RT, 1 units); lanes 5 and 6 (17cap (n) and 17cap (s), HIV RT, 10 units); lanes 7 and 8 (17cap (n) and 17cap (s), HIV RT, 1 units). Lanes 9 and 10 were reverse transcribed in the presence of dAMP (20 mM) and AMV RT (10 units). Lane C, the 142 mer RNA treated only with RNase H.

the RNA template by AMV RT at a 2 μM concentration of the phosphorothioate oligonucleotide, 17 cap (s), was strongly blocked (Fig. 6, lane 3). It should be noted that the inhibition of the RNA template cleavage was due to a decrease in the RNase H activity, by the competitive binding of the phosphorothioate oligonucleotides on the RT enzyme. On the other hand, when 1 unit of HIV-1 RT was used, an RNA cleavage product from the antisense oligonucleotide 17 cap (s) site was detected (Fig. 6, lane 8). The RNase H activity of the HIV-1 RT was not affected by adding an excess of the phosphorothioate oligonucleotide.

To further investigate the inhibition of RNase H activity of AMV RT by the phosphorothioate oligonucleotide, 17cap (s), we reverse-transcribed the RNA template with either 17cap (n) or (s) and the RNase H inhibitor, deoxyadenosine 5'-monophosphate (dAMP). dAMP can inhibit the RNase H activity of AMV RT without perturbing the polymerase function (26). The addition of 20 mM dAMP to the reaction with 17cap (n) decreased the yield of the RNA cleavage product (Fig. 6, lane 9). However, in the case of 17cap (s)-dAMP, the amount of the RNA cleavage product was the same as in the reaction in lane 2. These results suggest that the RNase H activity of AMV RT was already inhibited with the phosphorothioate oligonucleotide, before the inhibition of the RNase H activity by dAMP. The phosphorothioate oligonucleotides are better inhibitors for AMV RT, and they would act as competitive inhibitors of either a reverse transcriptase or a DNA polymerase. These studies raise interesting questions concerning the locus of the reaction and the mechanism by which the antisense phosphorothioate oligonucleotides inhibit the RNase H activity by binding to the AMV RT, rather than to the template RNA, whereas the RNase H activity of HIV RT is not affected by the antisense phosphorothioate oligonucleotides. At present, very little is understood about the functions of the different mechanisms or the factors that may determine which mechanisms are involved after the antisense oligonucleotides bind to their receptor sequences (27). Consequently, any discussion of mechanisms would remain largely theoretical. However, we have demonstrated that selective inhibition of HIV-1 gene expression involves the degradation of the template RNA bound to the antisense phosphorothioate oligonucleotide by the RNase H activity associated with the HIV-1 polymerase. The RNase H-mediated cleavage, which generated the shortened RNA fragments, was also demonstrated to be involved in the inhibition by unmodified antisense oligonucleotides. The antisense phosphorothioate oligonucleotide was complementary to the target, and was able to inhibit cDNA synthesis by a true antisense effect, i.e., it hybridized to the RNA template, rather than to the HIV-1 RT.

We tested the ability of the homooligonucleotides (dA20 (n or s), dG20 (n or s), dC20 (n or s), dT20 (n or s), and dT15 (n or s) to block the reverse transcription of β-globin mRNA by AMV RT and HIV RT. We incubated homooligonucleotide analogues (phosphodiester (n) or phosphorothioate (s)) (0.5-10 μM) with either AMV RT or HIV RT (1 unit) under the same conditions as described above. Figure 7 shows that the phosphodiester (n) homooligo-

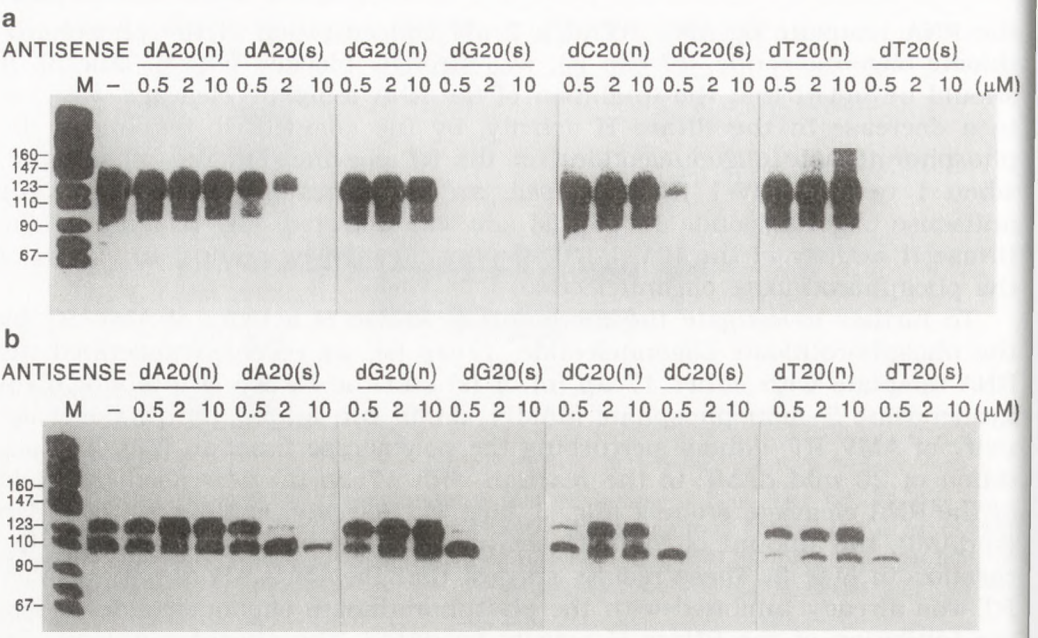

Fig. 7. Effect of the homooligonucleotides on DNA synthesis. (a) Reverse transcription proceeded as indicated using 1 unit of AMV RT primed by 5 μM oligomer 17 sc(n), without (-) or with various amounts (μM) of the unmodified homooligomers (n) and modified homooligomers (s). (b) cDNA analysis of HIV RT, used in place of AMV RT.

nucleotides did not inhibit cDNA synthesis *via* an antisense mechanism: only the full-length cDNA fragment was obtained (90%). In contrast, cDNA synthesis by the phosphorothioate oligonucleotides could not be carried out, because of the AMV RT and HIV RT enzymes. However, dA20(s) did not affect the synthesis of the full-length cDNA product any better than the other homooligomers (dG20(s), dT20(s), and dC20(s)), at a concentration of 0.5 μM. We examined how to prevent cDNA elongation by dC15(s) instead of dC20(s), using the same conditions described above. The homooligomer dC15(s) allowed the HIV RT-mediated synthesis of a full-length cDNA fragment at high concentrations (2-10 μM). We found similar results for the anti-HIV activity of the phosphorothioate homooligonucleotides, dC28(s) and dC15(s), in the cytopathic-effect inhibition assay using MOLT-4 cells (10). It is worthwhile to note that 0.02 μM dC28(s) showed more activity than 0.1μM dC15(s), and a 0.5 μM concentration of dC15(s) was relatively toxic. These results support the proposal that the phosphorothioate homooligonucleotides act as inhibitors of reverse transcription, essentially by a mechanism involving their binding to the reverse transcriptase enzyme, in a process that is mostly sequence-independent. On the other hand, in the case of HIV-1, the inhibition by antisense phosphorothioate oligonucleotides is length- and sequence-dependent: oligonucleotides complementary to different regions of HIV-1 mRNA block viral replication following binding to their target sequences.

4. Anti-HIV-1 effect of antisense phosphorothioate oligonucleotides

4.1. Long-term experiment for inhibition of HIV-1 replication

The phosphorothioate oligonucleotides complementary to the *tat* mRNA gene, the *gag* mRNA, and the *rev* mRNA were previously described as effective anti-HIV-1 agents (10,11,28,29). The *tat*, *gag*, and *rev* oligonucleotides showed concentration-dependent inhibition of HIV-1 replication in an acute infection assay. Furthermore, to examine the long-term antiviral activity of the phosphorothioate oligomers, we selected targets (S-ODNs-*gag*, *rev*, and *tat*) that were involved in the viral recognition step (Fig. 8). These include the AUG initiator codons and splice acceptor sites involved in RNA processing. Splice sites are good targets for HIV, as well as for herpes simplex virus (30). Sites involved in the translation and processing of RNA should have some homology with host sequences, and the oligonucleotides should bind to the corresponding viral sequences.

At a 2.5 μM concentration, the antisense oligonucleotide treatments with the *gag*-AUG-28 oligomer and the *gag*-(p24)-28 oligomer inhibited virus replication by 99%, as compared to the *tat*-SA-28 oligomer and the *rev*-trans-

6006 6033 (*rev*-trans-28)
5'-*CAUCUCCUAUGGCAGGAAGAAGCGGAGA*-3'
3'-GTAGAGGATACCGTCCTTCTTCGCCTCT-5'

5809 5836 (*tat*-SA-28)
5'-*UAUCCAUUUUCAGAAUUGGGUGUCGACA*-3'
3'-ATAGGTAAAAGTCTTAACCCACAGCTGT-5'

1190 1217 (*gag*-(p24)-28)
5'-*AUUACCUAUAGUGCAGAACAUCCAGGGG*-3'
3'-TAATGGATATCACGTCTTCTAGGTCCCC-5'

786 813 (*gag*-AUG-28)
5'-*UAGAAGGAGAGAGAUGGGUGCGAGAGCG*-3'
3'-ATCTTCCTCTCTCTACCCACGCTCTCGC-5'

Fig. 8. The targeted RNA sequences and thier complementary DNA sequences. The phosphorothioate portions are underlined.

28 oligomer (Fig. 9). When assayed 40-60 days after infection, the *tat*-SA-28 oligomer and the *rev*-trans-28 oligomer inhibited HIV-1 replication by 0%, and 63% and 70%, respectively, of these cells were positive by the IF method. This finding differs from those of Lisziewicz et al. (20). In contrast, 40-days

after infection, we did not detect any inhibitory effect of the *gag*-AUG-28 oligomer and the *gag*-(p24)-28 oligomer, as well as the *tat*-SA-28 oligomer and the *rev*-trans-28 oligomer, at 1 μM concentrations (Fig. 9). This result suggests that long-term treatment with the *gag*-AUG-28 oligomer and the *gag*-(p24)-28 oligomer kept virus expression at a very low level (p24 antigen in the supernatants was <1 ng/ml per 30 x 10^4 cells). In adittion, no IF-positive cells were observed, implying that the *gag*-28 oligomers can interfere with HIV-1 replication for a longer period of time.

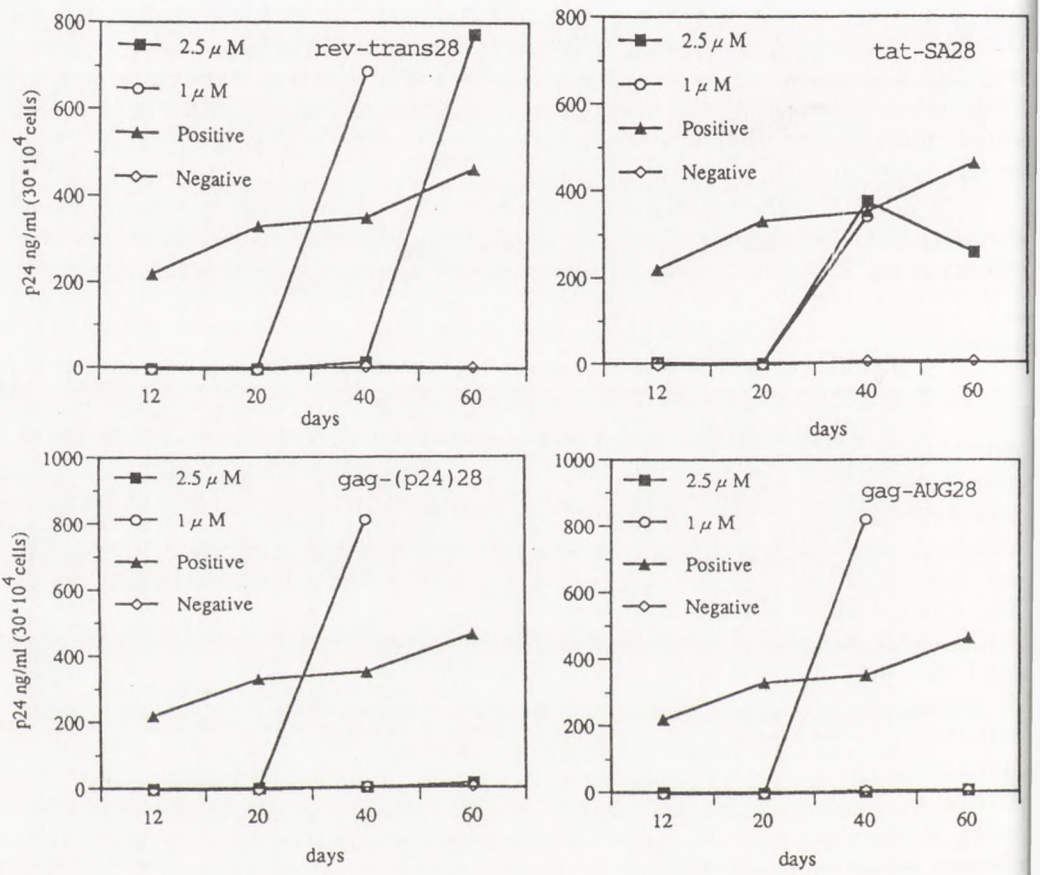

Fig. 9. Comparison of the antiviral activites of *gag*, *rev*, and *tat* oligonucleotides at different concentrations in long-term cultures.

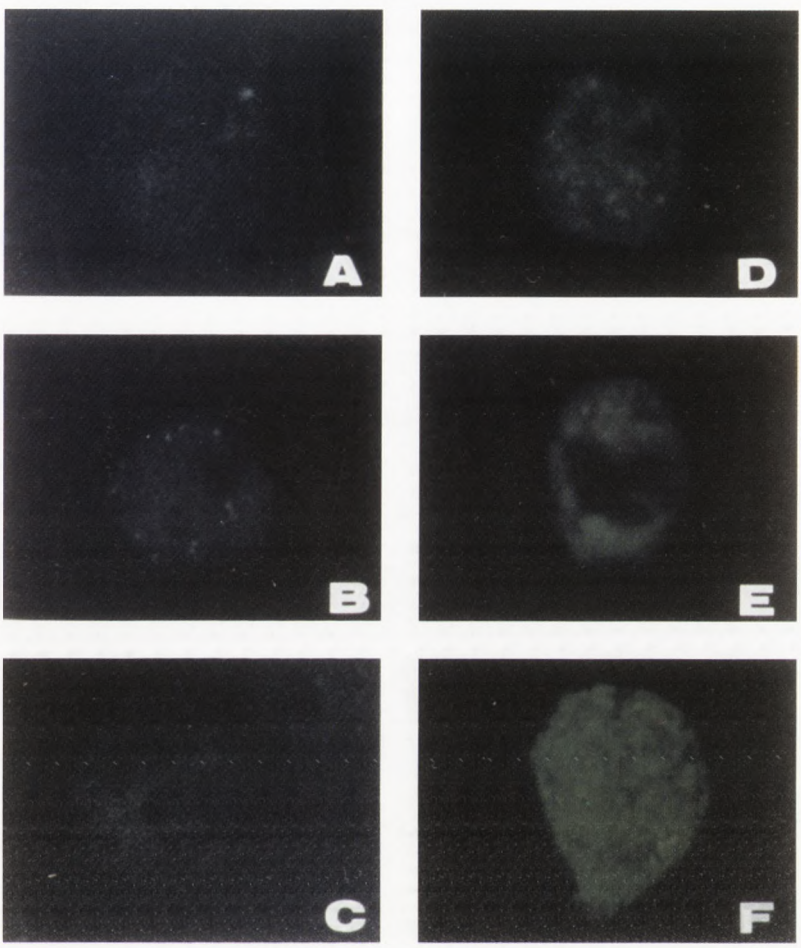

Fig. 10. Intracellular distribution of FITC-labeled S-ODN-*rev*. MOLT-4 (A, B, and C) or MOLT-4/HIV-1 cells (D, E, and F) were incubated with FITC-labeled S-ODN-*rev*. for 0 min (A), 10 min (D), 30 min (B and E), and 60 min (C and D). The fluorescence was observed with a scanning confocal microscope.

4.2. Localization of S-ODNs-*rev* in MOLT-4 and MOLT-4/HIV-1 cells

To shed some light on the antisense mechanism and function, possible sites for oligonucleotide localization within the cells were investigated. We incubated MOLT-4 and MOLT-4/HIV-1 cells in tissue culture chamber slides with medium containing 1 μM of FITC-labeled oligonucleotide. Following an incubation, the chambers were washed and removed, and the slide was prepared for microscopy. The FITC-labeled S-ODNs-rev was found in the cytoplasm of MOLT-4 cells after 30 and 60 min incubations (Fig. 10 B and C). On the other hand, the FITC-labeled S-ODNs-*rev* was diffusely located in the cytoplasm of MOLT-4/HIV-1 cells after 10 and 30 min incubations, and was localized within the cell nucleus after a 60 min incubation (Fig. 10 D, E, and F). Under microscopic observation, the fluorescence intensity of the FITC-labeled phosphorothioate oligonucleotides in MOLT-4 cells did not differ significantly between the 30 and 60 min incubations, whereas the fluorescence intensity increased in MOLT-4/HIV-1 cells with time (Fig. 10). This result suggest that the S-ODNs-*rev* could penetrate both MOLT-4 and MOLT-4/HIV-1 cells and that the S-ODNs-*rev* might hybridize to the target sequence of the viral mRNA, thereby becoming localized in the MOLT-4/HIV-1 cells, especially in the nuclear region. Since these target sequences do not exist in MOLT-4 cells, the S-ODNs-*rev* could neither hybridize nor remain in the cells for a long time. Therefore, the antisense phosphorothioate oligonucleotides may show antiviral activity when administered to HIV-infected individuals.

The phosphorothioate oligonucleotide inhibited cDNA production by the retroviral polymerase by binding to the RNA downstream from the primer. However, the inhibitory efficiency of the phosphorothioate oligonucleotide is influenced by its relationship with the RT. Selective inhibition of HIV-1 gene expression involves the degradation of the template RNA bound to the antisense phosphorothioate oligonucleotide by the RNase H activity associated with the HIV-1 polymerase.

A 2.5 μM concentration of the 28 mer phosphorothioate oligonucleotides directed to the *gag* mRNA (*gag*-AUG-28 oligomer and *gag*-(p24)-28 oligomer) can completely inhibit HIV-1 replication in a long-term (60 days) experiment. This treatment was not toxic to T cells (MOLT-4) in culture. Furthermore, we have demonstrated the intracellular distribution of antisense phosphorothioate oligonucleotides in chronically HIV-1 infected MOLT-4 cells. It appears that the majority of the oligonucleotide is localized within the cell nucleus. Our results suggest that chemotherapy based on specifically targeted antisense phosphorothioate oligonucleotides may be an effective method for reducing the viral burden in HIV-1 infected individuals.

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Anti-HIV Activities and Mechanisms of Antisense Oligonucleotides

Summary

We demonstrated that unmodified and modified (phosphorothioate) oligonucleotides prevent cDNA synthesis by the AMV, MMLV, and HIV reverse transcriptases. Antisense oligonucleotide/RNA hybrids specifically arrest primer extension. The blockage involves the degradation of the RNA fragment bound to the antisense oligonucleotide by the reverse transcriptase associated RNase H activity. However, the phosphorothioate oligomer inhibited polymerization by binding to the AMV and MMLV RTs, rather than to the template RNA, whereas there was no competitive binding of the phosphorothioate oligomer on the HIV RT during reverse transcription. Observation of FITC-S-ODN-*rev*-treated MOLT-4 cells with a confocal laser scanning microscope, revealed diffuse fluorescence, apparently within the cytoplasm. Interestingly, fluorescent signals were accumulated in the nuclear region of chronically infected MOLT-4/HIV-1 after a 60 min incubation. We also describe the long-term treatment of human immunodeficiency virus-infected cells with antisense phosphorothioate oligonucleotides.

Key words:
anitisense oligonucleotide, phosphorothioate, reverse transcriptase, AMV, MMLV, HIV.

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