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Mechanisms of the inhibition of reverse transcription by unmodified and modified antisense oligonucleotides

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We demonstrated that unmodified and modified (phosphorothioate) oligonucleotides prevent cDNA synthesis by AMV or 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 RT rather than to the template RNA, whereas there was no competitive binding of the phosphorothioate oligomer on the HIV RT during reverse transcription.

Reverse transcriptase; Antisense oligonucleotide; Rabbit β-globin; Retrovirus; Polyacrylamide gel

1. INTRODUCTION

Antisense RNA or 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 coded for by the gene. The addition of chemically modified antisense oligomers to culture medium and their 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 [8–10], vesicular stomatitis virus [11,12], herpes simplex virus [11,14], and influenza virus [14,15].

However, the mechanism by which the antisense oligonucleotide inhibited retroviral protein synthesis, syncytia formation, and reverse transcriptase activity has not been fully elucidated. Recently, Toulmé et al. have reported that unmodified oligonucleotides indeed arrested cDNA synthesis by AMV and MMLV RTs which have RNase H activity, but that α-oligonucleotide analogues did not [16,17].

We present here a detailed analysis of the effect of unmodified and modified (phosphorothioate) oligodeoxyribonucleotides [18] on cDNA synthesis by AMV and HIV [19] RTs. The phosphorothioate oligonucleotides that are nuclease-resistant analogues of oligodeoxyribonucleotides can be used to prevent reverse transcription.

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2. MATERIALS AND METHODS

2.1. *Oligonucleotides*

The unmodified oligonucleotide derivatives 5′-d[TTGTGTCAAAAGCAAGT][17 cap (n)], 5′-d[CACCAACTTCTTCCACA][17 sc (n)] and 5′-d[TGCCCAGGGCCTCAC] [15sc (n)], and modified (phosphorothioate) oligodeoxyribonucleotide derivatives 5′-d[TsTsGsTsGsTsCsAsAsAsAsGsCsAsAsGsT] [17 cap (s)] and 5′-d[CsAsCsCsAsAsCsTsTsCsTsTsCsCsAsCsA] [17 sc (s)] were synthesized on a Biosearch synthesizer. The oligonucleotide derivatives were purified by reverse-phase HPLC on an oligo-DNA column. Purified oligomers were evaluated by resolving ^{32}P-labeled by electrophoresis samples on 20% polyacrylamide/7 M urea gels.

2.2. *Enzymes and mRNAs*

Escherichia coli RNase H and RNasin were from Promega. T4 polynucleotide kinase was purchased from Toyo Boseki. AMV with RNase H as well as rabbit globin mRNA were purchased from Gibco/BRL and was used without treatment. HIV RT with RNase H was from Seikagaku Kogyo. 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 μg) was incubated with *E. coli* RNase H (10 U) in the presence of an oligonucleotide 15 (100 pmol) complementary to nucleotides 147–161 of the β-globin mRNA. The reaction mixture was incubated for 2 h at 37°C in 20 μl of a 20 mM Tris-HCl (pH 7.5) buffer containing 10 mM $MgCl_2$, 100 mM KCl and 0.1 mM dithiothreitol. After the reaction, the fragment, which was about a 150-nucleotide mRNA was phenol-extracted, ethanol-precipitated and dissolved in 25 μl of sterile water.

2.3. *cDNA synthesis*

Globin RNA (50 ng, containing 0.3 pmol of intact the β-globin), primer (50 pmol), and the desired amount of antisense oligonucleotides were preincubated for 30 min at 39°C. After adding 1 μl of

10 × RT buffer (1 M Tris-HCl, pH 8.3, 720 mM KCl, 100 mM $MgCl_2$, 100 mM dithiothreitol) containing 8 U of RNasin, 2 pmol of [α-^{32}P]dCTP (3000 Ci/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 U, i.e. 0.13–1.3 pmol) was then added. The reaction with HIV RT was allowed to proceed with 1 U, incubated for 1 h at 39°C. The cDNA was chloroform-extracted according to standard procedures, and loaded on a 10% PAGE. The results obtained for cDNA synthesis were corrected for the number of labels incorporated into each fragment.

3. RESULTS AND DISCUSSION

Reverse transcription of rabbit β-globin mRNA by AMV RT was primed with 17 sc, complementary to oligonucleotide 113–129 (Fig. 1), giving rise to the predicted cDNA fragment of about 130 nucleotides (Fig. 2a). In contrast, 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 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 transcription of this region. The inhibitory efficiency was dependent on the 17 cap (n) concentration. At concentrations as low as 1.0 μM, 17 cap (n), 98% inhibition of reverse transcription was observed. This resulted from competition between the 17 sc (n) primer and the 17 cap (n) antisense oligomer.

Next, we examined the cDNA synthesis by AMV reverse transcriptase using the 17 cap (s), phosphorothioate oligomer instead of 17 cap (n). Fig. 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 55%, 74% and 82%, respectively. However, these values are lower than that of the unmodified oligonucleotide at 1 μM. This suggested that part of the phosphorothioate oligomer binds to the RT enzyme. As a result, the synthesis of full-length

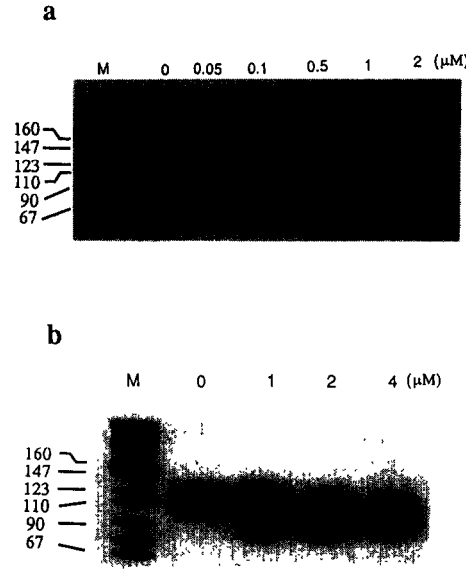

Fig. 2. Effect of the unmodified and modified antisense oligonucleotides on DNA synthesis. (a) Reverse transcription proceeded as indicated using 10 U of AMV RT primed by 5 μM oligomer 17 sc (n) without (0) or with various amounts (μM) of oligomer 17 cap (n). (b) cDNA analysis of the 17 cap (s) used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

cDNA can not be completely blocked by the decrease of the binding of the phosphorothioate oligomer to mRNA. This process was essentially sequence independent and was the result of the preferential binding of the modified oligomers to the RT enzyme compared with unmodified oligomers.

To characterize the inhibitory process of the phosphorothioate oligomers, we incubated 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

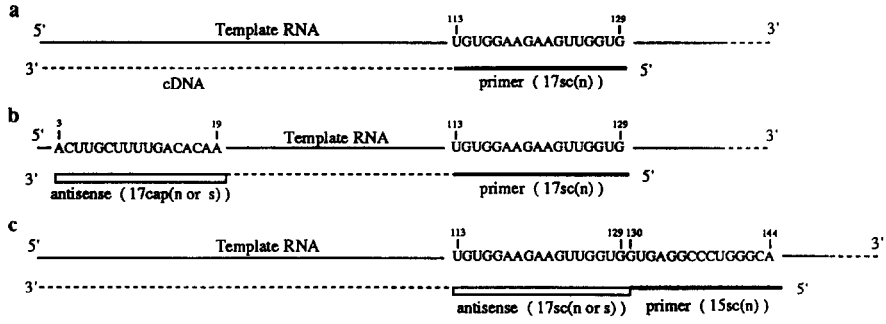

Fig. 1. Inhibition of reverse transcription by complementary oligonucleotides. The full length cDNA products derived from 17 sc (n) or 15 sc (n) (a) could be blocked by the antisense oligomers, 17 cap (n or s) and 17 sc (n or s) bound to a template sequence (b) or adjacent (c) to the primer-binding site.

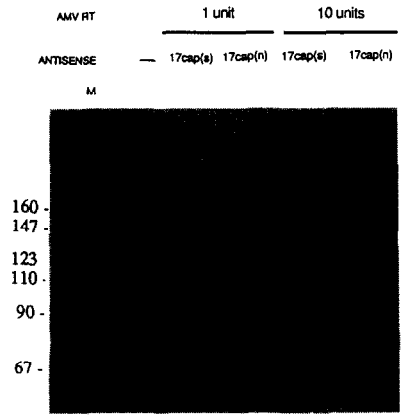

Fig. 3. The interaction between the antisense oligomers and AMV RT on the reverse transcript. Analysis of cDNA fragments synthesized by 1 U (left) or 10 U (right) of AMV RT. The reaction primed by a17 [17 sc (n) (5 μM)] complementary to the 113–129 oligonucleotide without (−) or with 2 μM antisense oligonucleotides, 17 cap (n) and 17 cap (s). DNA size markers were run in lane M.

(Fig. 3). It should be noted that the cDNA could not be synthesized by a fall of ability of the 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 phosphorothioate oligomer and its inhibitory efficiency [9,20]. It should be pointed out that the antisense phosphorothioate oligomer did not compete directly with the mRNA as the RT enzyme.

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. Fig. 4 shows that the antisense oligomers 17 cap (n) and 17 cap (s) induced the characteristic shortened cDNA fragments when the reaction proceeded with HIV RT. The

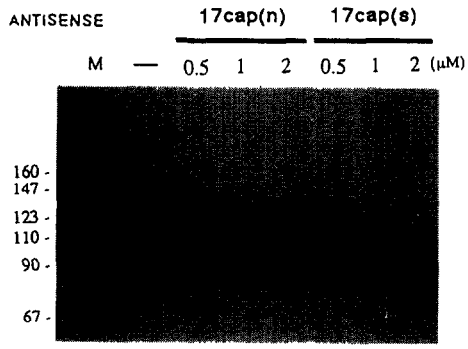

Fig. 4. Effect of antisense oligonucleotides on cDNA synthesis using HIV RT. Reverse transcription proceeded as indicated using 1 U of HIV RT primed by a 17mer [17 sc (n) (5 μM)] complementary to the 113–129 oligonucleotide without (−) or with various amounts (μM) of antisense oligonucleotides, 17 cap (n) (left) and 17 cap (s) (right). DNA markers were run in lane M.

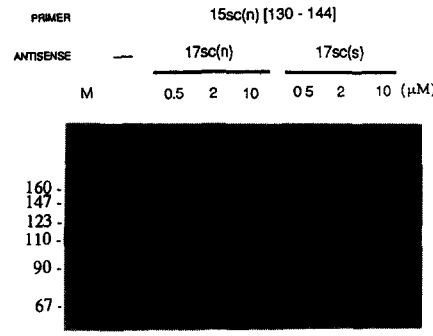

Fig. 5. Effect of the relative location of antisense and primer-binding sites. Analysis of cDNA fragments synthesized by 1 U of AMV RT. The reaction primed by a 15 complementary to the 130–144 oligonucleotide (5 μM) without (−) or with the antisense oligonucleotides, 17 sc (n) or 17 sc (s) at the concentrations (μM) indicated above the lanes. DNA size markers were run in lane M.

17 cap (s) 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 being a retroviral polymerase by bound to the RNA downstream from the primer. However, the inhibitory efficiency of phosphorothioate oligonucleotide is influenced by its relationship with RT.

We speculated that an oligomer adjacent to the primer (see Fig. 1) could function by a different mechanism: the primer-antisense tandem may be viewed as a single complementary sequence by the priming RT molecule. To test this mechanism, we synthesized the unmodified [17 sc (n)] and the modified [17 sc (s)] oligonucleotides complementary to nucleotides 113–129 of rabbit β-globin mRNA and unmodified 15,15 sc (n) complementary to nucleotides 130–144. Fig. 5 shows that 17 sc (n) or 17sc (s) reduced the synthesis of the 144 nucleotide long transcript; complete inhibition was achieved at < 2 μM. This suggests that the oligomer complementary to a site immediately adjacent to the primer binding site could then act as a primer which would be lengthened by polymerase. The inhibition involved degradation of the template by RNase H. However, the efficiency of an oligomer adjacent to the primer binding site was lower than that of an antisense oligomer bound to the 5′-end of mRNA. Furthermore, increased concentrations of the 17 sc (s) phosphorothioate oligomer in the reverse transcription mixture containing AMV RT (1 unit), led to the disappearance of the cDNA transcript. This result also suggested that the antisense phosphorothioate oligonucleotide inhibited polymerization by binding to the enzyme RT rather than to the template RNA.

It is notable that the antisense phosphorothioate oligonucleotide participates through a mechanisms of interaction different from those of the RT enzymes, in adherence and structural differences. Furthermore, the efficiency of an antisense oligomer bound to the 5′ end

of mRNA is higher than that of an oligomer adjacent to the primer. Therefore, the antisense oligonucleotides can be used with intact cells, and they prevent the development of retrovirus in culture.

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