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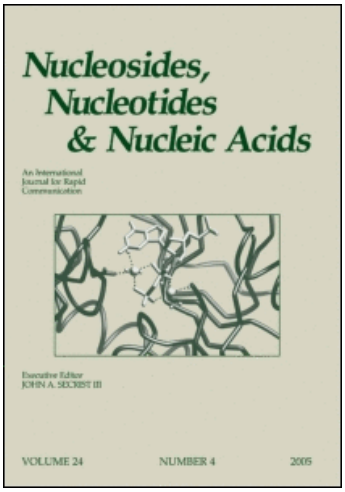

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ANTI-INFLUENZA VIRUS ACTIVITIES OF NICKED AND CIRCULAR DUMBBELL RNA/DNA CHIMERIC OLIGONUCLEOTIDES

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ABSTRACT: We have designed a new type of antisense oligonucleotide, containing two hairpin loop structures with RNA/DNA base pairs (sense (RNA) and antisense (DNA)) in the double helical stem (nicked and circular dumbbell DNA/RNA chimeric oligonucleotides). The reaction of the nicked and circular dumbbell DNA/RNA chimeric oligonucleotides with RNase H gave the corresponding anti-DNA together with the sense RNA cleavage products. These oligonucleotides were more resistant to exonuclease attack. We also describe the anti-Fluv activities of nicked and circular dumbbell DNA/RNA chimeric oligonucleotides.

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INTRODUCTION

Antisense oligonucleotides have been used to regulate the expression of genes.[1-3] Unmodified oligonucleotides, in particular, have limited survival *in vivo*. Antisense oligonucleotides with phosphorothioate backbones have increased nuclease resistance but hybridize more weakly with the complementary nucleic acids than the unmodified oligonucleotides and are eventually degraded, primarily from the 3' end. Recently, several stabilization methods have been proposed, such as the incorporation of various chemical substituents at the 3'-hydroxyl groups, the circularization of the oligonucleotides by joining the 3' and the 5' ends and the formation of a hairpin loop structure at the 3' end.[4-7]

We describe the design of a new class of oligonucleotides "dumbbell RNA/DNA chimeric oligonucleotides" consisting of a sense RNA sequence and its complementary antisense DNA sequence, with two hairpin loop structures. These oligonucleotides have increased nuclease resistance. Of particular interest, the antisense DNA is liberated by RNase H treatment of the dumbbell RNA/DNA chimeric oligonucleotides.

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EXPERIMENTAL

Oligonucleotide synthesis. The linear oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer, Model 392. The 5'-phosphorylated oligonucleotides were synthesized using 5'-phosphate on cyanoethyl phosphoramidite as the phosphorylating agent. The oligonucleotide derivatives were purified by polyacrylamide gel electrophoresis. Ligated oligonucleotide dumbbells (Fig. 1) were obtained by ligation of the corresponding 5'-phosphate oligonucleotides with T4 DNA ligase. The identity of each of the ligated dumbbells was verified by phosphodiesterase protection mapping.

RNase H activity. The 45 mer RNA (5'UGUUUCACAACAAAAGCCUUAGGCAU-CUCCAUGGCAGGAGAAG3') (1 pmole) was mixed with oligonucleotides (10 pmole) in 30 µl of 20 mM Tris/HCl (pH 7.5), 10 mM $MgSO_4$, 0.1 mM DTT; 100 mM KCl. The mixture was heated at 80°C for 3 min and then cooled to 5°C. RNasin (40 units) and *E.coili* RNase H (0.5 µl, 0.4 units) were added to the mixture which was incubated at 37°C. Aliquots were taken at 0, 15, and 30 minutes and were analyzed by PAGE (20 % polyacrylamide containing 8.3 M urea) followed by autoradiography (Fig. 2).

RNA Transcription of Clone 76 Cells. Clone 76 cells, grown in 60 mm dishes and approximately 50% confluent, were treated with 10^{-6} M dexamethasone in DMEM containing 10% FCS at 37°C. After 24 h, the cells were washed with PBS, a mixture of oligonucleotides and 5 µg of lipofection reagent (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammoniummethylsulfate; DOTAP, Boehringer Mannheim) in DMEM was added, and the cells were incubated for 4 h at 37°C. The cells were washed with PBS, 100 ng of RNA was mixed with 5 µg of DOTAP in DMEM, and the mixture was incubated for 4 h at 37°C. The medium was changed to fresh DMEM containing 10% FCS and the cells were incubated for a further 20 h. The amount of CAT protein in the cells was monitored by the CAT assay kit (anti-CAT-DIG, Boehringer Mannheim).[8]

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RESULTS AND DISCUSSION

The liberation of antisense DNA from the dumbbell RNA/DNA chimeric oligonucleotides by RNase H treatment was studied. The circularization of the 40 mer DNA/RNA chimeric oligonucleotide was carried out by enzymatic ligation with T4 ligase (Fig. 1). The RNase H activity assay was carried out in the presence of the 45 mer RNA and either the circular or nicked dumbbell RNA/DNA chimeric oligonucleotide (NDRD) with *E. coli* RNase H for up to 4 h (Fig. 2). The 45 mer RNA with the antisense ODN was completely cleaved by *E. coli* RNase H within 4 h (Fig. 2, lane 6). On the other hand, the reaction of the NDRD chimeric oligonucleotide with RNase H gave the corresponding anti-DNA together with the RNA cleavage product (Fig. 2 lane 8). The 45 mer RNA was then added to the above reaction, which produced the characteristically shortened RNA fragments (Fig. 2, lanes 9-11). Furthermore, when the circular dumbbell RNA/DNA chimeric oligonucleotide (CDRD) was used,in place of the NDRD, under the same conditions as described above, RNA template cleavage was observed (Fig. 2, lanes 14-16). The thermal stability of the base-stacked CDRD molecule was compared with that of the NDRD. The NDRD has a Tm= 55°C, whereas the CDRD has an estimated Tm=81°C, an increase of 26°C. Furthermore, the Tm of the circular oligonucleotide with the DNA-DNA base pairs (sense (DNA) and antisense (DNA)) in the double helical stem was 10°C lower than the

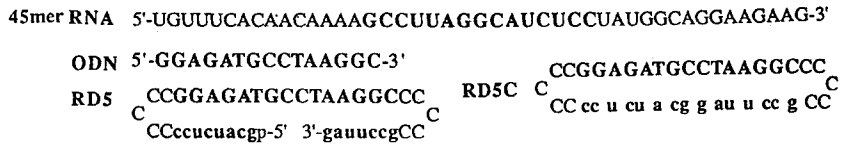

FIG. 1. The structures and sequences of the oligonucleotides used in this study and described in the text.

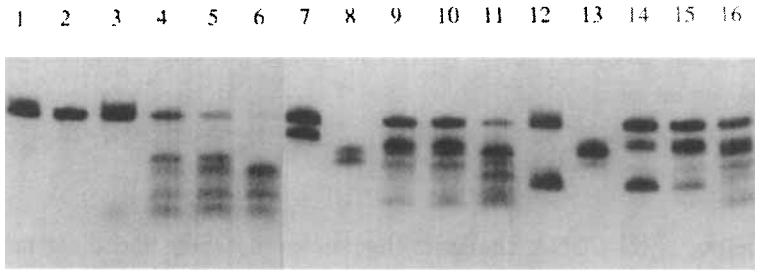

FIG. 2.Specific cleavage by RNase H of 45 mer RNA in the presence of the dumbbell RNA/DNA chimeric oligonucleotides (NDRD and CDRD) and the anti-ODN. lane 1, control:45 mer RNA; lane 2, control: 45 mer RNA/RNase H/4 h; lane 3, control:ODN/45 mer RNA; lane 4, ODN/45 mer RNA/RNase H/1 h; lane 5, ODN/45 mer RNA/RNase H/2 h; lane 6, ODN/45 mer RNA/RNase H/4 h; lane 7, control: NDRD/45 mer RNA; lane 8, control:NDRD/RNase H/4 h; lane 9, NDRD/45 mer RNA/RNase H/1 h; lane 10, NDRD/45 mer RNA/RNase H/2 h; lane 11, NDRD/45 mer RNA/RNase H/4 h; lane 12, control: CDRD/45 mer RNA; lane 13, control:CDRD/RNase H/4 h; lane 14, NDRD/45 mer RNA/RNase H/1 h; lane 15, NDRD/45 mer RNA/RNase H/2 h; lane 16, NDRD/45 mer RNA/RNase H/4 h.

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Tm of the CDRD. These results suggest that the increase in the stability depended on the strength of the base pairs in the double helical stem.

The stabilities of the oligonucleotides to exonuclease digestion were assessed by treatment with nuclease S1 and SVPD. Two enzymes, SVPD and nuclease S1, were used in comparative digestion studies of the NDRD and CDRD chimeric oligonucleotides. The anti-DNA was digested extensively by SVPD within 10 min, whereas, the NDRD and CDRD oligonucleotides were more resistant. The S1 endonuclease activity digested the anti-DNA to mononucleotides in 30 min, whereas the NDRD and CDRD oligonucleotides were very slowly digested. Similar results were obtained when the oligonucleotides anti-ODN, NDRD, and CDRD were studied for their nuclease sensitivities fetal bovine serum.

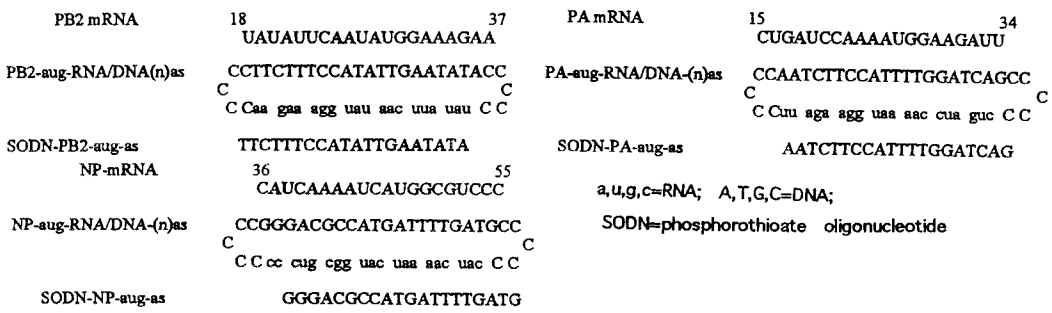

FIG. 3 The targeted FLuv A mRNA sequences and their complementary DNA sequences.

The dumbbell RNA/DNA chimeric oligonucleotides with the three target sites (influenza virus RNA polymerase PB2, PA, and nucleoprotein, NA) were tested for inhibitory effects by a CAT-ELISA assay using the clone 76 cells. The oligomer complementary to the site of the PB2-AUG initiation codon had high inhibitory effects, causing more than 80% inhibition at a 0.3 μM concentration. However, the oligomers complementary to the sites of the PA- and NA-AUG initiation codons showed lower anti-CAT activities at 0.3 μM concentrations. Of particular interest, the oligomer complementary to the site of the PB2-AUG showed increased antiviral activity as compared to the linear antisense phosphorothioate oligonucleotide (20 mer) (42% inhibition at 0.3 μM).

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