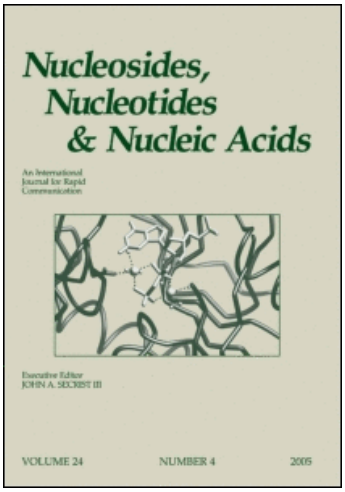

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Inhibition of Influenza Virus Replication by Phosphorothioate and Liposomally Endocapsulated Oligonucleotides

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INHIBITION OF INFLUENZA VIRUS REPLICATION BY PHOSPHOROTHIOATE AND LIPOSOMALLY ENDOCAPSULATED OLIGONUCLEOTIDES

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ABSTRACT: We have demonstrated that antisense phosphorothioate oligonucleotides (S-ODNs) inhibit influenza virus A replication in MDCK cells. Phosphorothioate and liposomally encapsulated oligonucleotides with two target sites (PB1 and PB2) were synthesized and tested for virus-induced cytopathogenicity effects by a MTT assay using MDCK cells. The liposomally encapsulated S-ODNs complementary to the sites of the PB2-AUG initiation codon showed highly inhibitory effects. On the other hand, the inhibitory effect of the liposomally encapsulated S-ODNs targeted to PB1 was considerably decreased in comparison with the PB2 target sites. The liposomally encapsulated oligonucleotides exhibited higher inhibitory activity than the free oligonucleotides. The activities of the modified oligonucleotides are effectively enhanced by using the liposomal carrier.

INTRODUCTION

Antisense oligonucleotides have become widely used research tools for the specific inhibition of gene expression. They are now under investigation as possible therapeutic agents.[1] Such oligonucleotides may be designed to complement a region of a particular gene or messenger RNA. Using this approach, oligonucleotides can serve as potential blockers of transcription or translation through sequence-specific hybridization with

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#This article is dedicated to the memory of Professor Tsujiaki Hata.

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targeted genetic segments. Antisense oligonucleotides have potential roles in the treatment of AIDS, cancer, influenza virus, herpes simplex virus, and other diseases.[2-13] Although viral replication could be inhibited by unmodified oligonucleotides, the problems of nuclease sensitivity and low cellular uptake limit *in vivo* applications.[14,15] Investigations have focused on designing more stable oligonucleotide derivatives, such as methylphosphonates[16], phosphorothioates[17], and phosphoramidites[18]. Several reports have described the development of phosphorothioate oligonucleotides as potential anti-viral therapeutic agents. Although extensive studies of their molecular mechanisms have highlighted the potential value of this novel therapeutic strategy, very little is known about the *in vivo* pharmacokinetics and metabolism of these compounds.

In this report, we present a detailed analysis of the inhibition of influenza virus RNA polymerase (PB1 and PB2) gene expression by antisense phosphorothioate oligonucleotides (S-ODNs), as determined by the virus-induced cytopathic effects in MDCK cells. We also describe the potential of liposome encapsulation.[19]

RESULTS AND DISCUSSION

Influenza virus is a negative strand RNA virus with a segmented genome. Essentially all studies of the transcription and the replication of influenza virus have been carried out with A strain viruses, which contain eight virion RNA (vRNA) segments. Three types of virus-specific RNAs are synthesized in infected cells: (1) viral messenger RNAs (mRNAs); (2) full-length copies of the v-RNAs, which serve as templates for vRNA replication; and (3) vRNAs. At present, there is more information available about the synthetic mechanism of the viral mRNAs than about the synthesis of the template RNAs and the vRNAs.

Viral mRNA synthesis is catalyzed by viral nucleocapsids[20], which consist of the individual vRNAs associated with four viral proteins, the nucleocapsid (NP) protein, and the three P (PB1, PB2, and PA) proteins.[21] The P proteins are responsible for viral mRNA synthesis, and some of their roles have been determined by analyses of *in vitro* reactions catalyzed by virion nucleocapsids.[21-23] The three P proteins form a complex that starts at the 3'-ends of the vRNA templates and moves down the templates in association with the elongating mRNA during transcription. The PB2 protein in this complex recognizes and binds to the cap of the primer RNA[21, 23] The PB1 protein, which was initially found at the first residue (a G residue) added onto the primer , moves

as part of the P protein complex on the 3'-ends of the growing viral mRNA chains, suggesting that it catalyzes nucleotide addition. In addition, the P protein(s) is an endonuclease and may function in viral mRNA synthesis.[22] On the other hand, the NP protein is the viral protein required for antitermination.[24] Consequently, NP acts by binding to the viral RNA transcript, rather than by binding to the P protein complex and catalyzing transcription. Viral mRNA synthesis is catalyzed by four viral proteins: the nucleocapsid (NP) protein and the three P (PB1, PB2, and PA) proteins. The viral RNA polymerase (PB1 and PB2) genes of influenza virus A are potential targets for antisense oligonucleotides.

The *in vitro* antiviral activities of the antisense oligonucleotides were assessed on the basis of their inhibitory effects on the cytopathogenicities of influenza virus A. To clarify the sequence specificity, we tested the phosphorothioate oligonucleotides containing sense- and antisense sequences as targets of PB1 and PB2, and random sequences with the same base composition as the two targets, on the expression of the RNA polymerase genes in MDCK cells (Fig. 1). We also evaluated the inhibitory effects of free and liposome-encapsulated phosphorothioate (S-ODN) oligonucleotides on influenza virus RNA polymerase gene expression.

We tested the endocapsulated antisense phosphorothioate oligonucleotides containing the AUG initiation codon and the loop-forming (splice donor site)[25] sequences as the targets of PB2 (S-ODN-PB2-AUG-as and -loop-as). The oligonucleotide targeted to the AUG initiation codon had the highest inhibitory effect, causing more than 50% inhibition at 0.15-10 μM concentrations (Table 1). However, the antisense oligonucleotide targeted to the loop-forming site (S-ODN-PB2-loop-as) did not lead to efficient inhibition (Table 1). In contrast, the antisense phosphorothioate oligonucleotides (S-ODN-PB1-AUG-as), containing the AUG initiation codon targeted to PB1, showed lower anti-viral-activity at 0.15-10 μM concentrations (Table 1). Furthermore, we could not detect any inhibitory effects on virus replication with the antisense phosphorothioate oligonucleotide (S-ODN-PB1-loop-as), containing the loop forming site sequences targeted to PB1 (Table 1).

For the control sequences, the sense- and random-oligonucleotides, we could not detect any inhibitory effects on the target AUG initiation codon and the loop forming sites. These results suggest that the endocapsulated antisense phosphorothioate oligonucleotide conferred sequence specific inhibition (Table 1). Our previous study of

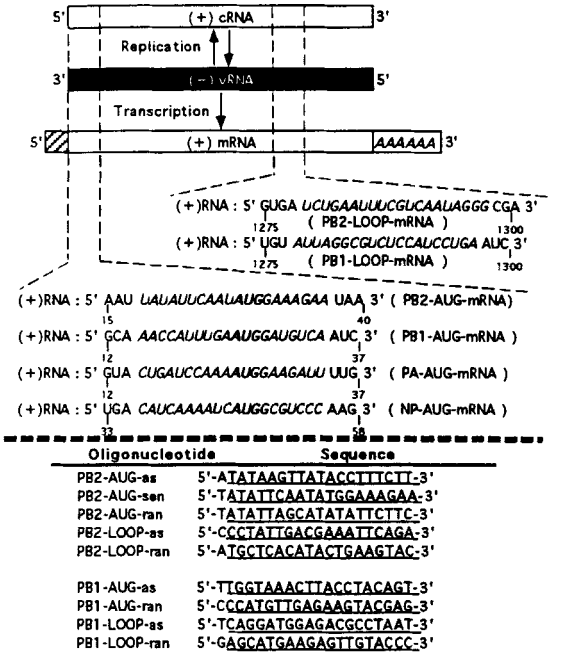

FIG. 1. Sequences of oligonucleotides tested for antiviral activity against influenza virus. Part of the RNA sequence of the PB2 and PB1 genes of influenza virus A/PR/8/34 is shown in the (+)-sense orientation. The oligonucleotide sequence corresponds to the 5'- end of the respective mRNA and includes twenty nucleotides present upstream of the initiation codon. The phosphorothioate derivatives are denoted by "S" (underlined). Targeted mRNA: 5' to 3' (*italics*). Abbreviations: as, antisense-sequence; sen, sense-sequence; ran, random-sequence.

the anti-HIV activity of antisense oligonucleotides indicated that a phosphorothioate oligonucleotide targeted to the HIV-1 gag gene was inhibitory, whereas no inhibition was observed for the sense and the random sequence oligomers.[26,27] This result showed the same order of anti-viral-activity as that of the antisense phosphorothioate oligonucleotide (S-ODN-PB2-AUG-as).

The S-ODN-PB2-AUG-as and S-ODN-PB1-AUG-as endocapsulated antisense phosphorothioate oligonucleotides showed increased inhibition efficiency with higher concentrations (Table 1), in contrast to the random sequence antisense phosphorothioate oligonucleotides (Table 1). These results also support the sequence specific inhibition.

Next, we investigated the inhibition of influenza virus A replication by free antisense phosphorothioate oligonucleotides on RNA polymerase gene expression in MDCK cells.

TABLE 1. Inhibition of influenza virus replication by liposomally endocapsulated antisense phosphorothioate oligonucleotides.

Oligonucleotides	Anti-Fluv activity (%)[a,c]								Cytotoxicity (%)[b,c]
	0.001	0.005	0.03	0.15	0.8	4.0	8.0	10.0 (μM)	10.0 (μM)
PB2-AUG-as	0	15	30	51	62	71	78	81	0
PB2-AUG-sen	0	0	5	10	7	10	8	6	0
PB2-AUG-ran	0	0	8	5	0	5	2	5	0
PB2-loop-as	0	0	5	7	4	8	3	4	0
PB2-loop-ran	0	0	5	5	3	9	6	7	0
PB1-AUG-as	0	0	15	14	15	31	38	34	0
PB1-AUG-ran	0	0	5	18	18	14	9	13	0
PB1-loop-as	0	3	5	5	5	5	7	5	0
PB1-loop-ran	0	0	3	0	0	4	0	3	0

a) Anti-Fluv activity was monitored as percent inhibition of Fluv-induced cytopathogenicity in MDCK cells.

b) Cytotoxicity was expressed as percent cell death of MDCK cells cultured with the test compound. The number of viable cells was determined by the MTT assay.

c) Data represent average values for at least three different experiments.

Surprisingly, the free antisense phosphorothioate oligonucleotides did not show any inhibitory effects at 0.15-10 μM concentrations in MDCK cells expressing the viral RNA polymerase genes (Table 2). This result suggests that the free antisense phosphorothioate oligonucleotide had very poor delivery efficiency into the MDCK cells. That is to say, the action of the antisense phosphorothioate oligonucleotides becomes exclusively sequence specific when directly delivered into the cell. It is worthwhile to note that the increased cell association of the oligonucleotides can explain the enhanced antisense oligonucleotide effect in the presence of DOTAP. Moreover, the activity of the antisense oligonucleotide may be improved by increasing the efficiency of intracellular liposome delivery and the rate of association for specific binding to mRNA, or by decreasing the rate of oligonucleotide degradation with a backbone modification. Akhtar *et al.* also reported that liposomes might be a good delivery system for antisense oligonucleotides, and this was demonstrated with the human ß-globin antisense S-oligomer.[28] Furthermore, these results suggested that the liposome enhanced the antisense activities. Although the main mechanisms of inhibiton by the endocapsulated antisense phosphorothioate oligonucleotide remain unclear, our results might suggest that the endocapsulated S-ODN-PB2-AUG-as inhibited gene expression by the viral RNA polymerases by replicational arrest in the nuclei of the MDCK cells.

TABLE 2. Inhibition of influenza virus replication by free antisense phosphorothioate oligonucleotides.

Oligonucleotides	Anti-Fluv activity (%)[a,c]								Cytotoxicity (%)[b,c]
	0.001	0.005	0.03	0.15	0.8	4.0	8.0	10.0 (μM)	10.0 (μM)
PB2-AUG-as	0	0	0	2	5	5	6	8	0
PB2-AUG-ran	0	0	0	0	3	4	4	6	0
PB2-loop-as	0	0	0	4	2	6	8	5	0
PB2-loop-ran	0	0	0	4	2	6	5	7	0
PB1-AUG-as	0	0	5	7	5	4	5	10	0
PB1-AUG-ran	0	0	5	0	3	6	7	9	0
PB1-loop-as	0	0	0	0	2	5	4	5	0

a) Anti-Fluv activity was monitored as percent inhibition of Fluv-induced cytopathogenicity in MDCK cells.

b) Cytotoxicity is expressed as percent cell death of MDCK cells cultured with the test compound. The number of viable cells was determined by the MTT assay.

c) Data represent average values for at least three different experiments.

In conclusion, the antisense oligonucleotides containing the AUG initiation codon targeted to the PB2 gene (the PB2 protein recognizes and binds to the cap-1 structure of the primer RNA) showed the greatest inhibition of influenza virus RNA polymerase gene expression in MDCK cells. In addition, the use of cationic lipids provides a simple and successful method for intracellular delivery of the oligonucleotides. We have observed a direct relationship between the intercellular stability of the oligonucleotides and their antiviral efficiency. Consequently, these specific antisense oligonucleotides would appear to be more favorable therapeutic agents for influenza virus infection.

EXPERIMENTAL

Oligonucleotide Synthesis. The oligonucleotides were synthesized by the phosphoramidite method using an Applied Biosystems Model 392 DNA/RNA synthesizer on the 1 μM scale, with controlled pore glass supports. For the oligonucleotides containing a phosphorothioate bond, the oxidation step was substituted with a sulfurization procedure using Beaucage's reagent.[29] The oligonucleotide derivatives were purified by reverse phase HPLC chromatography. Extinction coefficients of the oligonucleotides were determined by calculating the theoretical extinction coefficients as the sum of the nucleosides and multiplying with the experimentally determined enzymatic hypochromicity.[30]

Virus and Cells. Madin-Darby canine kidney (MDCK) cells were kindly provided by Dr. K. Nerome (National Institute of Health). The cells were grown as a monolayer stationary culture in Eagle's minimum essential medium (MEM) supplemented with 0.075 % sodium carbonate, 10% fetal calf serum, 100U of penicillin G per ml, and 100 μg of streptomycin per ml. Influenza A/PR/8/34 virus was passed in the allantoic cavity of 9 day-old fertilized eggs at low multiplicity. The allantoic fluid was clarified at 6000 x g for 15 min, and was stored at -80℃.

Antiviral activity in vitro. MDCK cells (1×10^6 ml^{-1}) were grown in a 96-well microtiter tray and were treated with MEM containing 10% FCS at 34℃. 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-trimethylammonium-methylsulfate; DOTAP, Boehringer Mannheim) in MEM (200 μl) was added, and the cells were incubated for 4 h at 34℃. The cells were washed with PBS, and each well was infected with 200 μl of virus suspension at a multiplicity of infection (MOI) of 0.01. All experiments were performed in triplicate. The cells were incubated for 4 days at 34℃ in a CO_2 incubator. The cell viability was quantified by a colorimetric assay monitoring the ability of viable cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) to a blue formazan product. Absorbances were read in a microcomputer-controlled photometer (Titertec Multican^R; Labsystem Oy, Helsinki, Finland) at two wavelengths (540 and 690 nm). The absorbance measured at 690 nm was automatically subtracted from that at 540 nm, to eliminate the effects of non-specific absorption. All data represent the mean values of triplicate wells. These values were then translated into percentages per well, cytotoxicity, and antiviral protection.

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