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Inhibition of influenza virus replication by phosphorothioate oligodeoxynucleotides

(antisense DNA/antiviral/RNA virus)

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ABSTRACT **Oligodeoxynucleotides (ODNs) were synthesized and tested for their antiviral activity against influenza viruses. ODNs corresponded to the polymerase *PB1* gene of either influenza A/WSN/33 virus or influenza C/JJ/50 virus. All compounds were 20 nucleotides long, including control ODNs containing mismatches. The phosphodiester ODNs (O-ODNs) failed to inhibit replication of influenza A and influenza C viruses at concentrations up to 80 μM, possibly due to intracellular nuclease digestion of the unmodified oligomers. By contrast, the phosphorothioate derivatives (S-ODNs) were found to inhibit replication of both influenza A and influenza C virus. The antiviral effect of S-ODNs against influenza A virus was found at concentrations as low as 1.25 μM and was present with mismatched oligomers. In the case of influenza C virus, the S-ODN complementary to the 3′ end of the viral RNA of the *PB1* gene revealed a sequence-specific antiviral activity at a concentration of 20 μM. (At the same concentration, S-ODNs with one or three mismatches showed little or no antiviral activity.) Reduction in plaque number reached six logarithms when this sequence-specific S-ODN was used at a concentration of 80 μM.**

Influenza viruses may cause acute respiratory disease in humans and animals. A long-term vaccination approach has failed mainly because the surface proteins of the virus, hemagglutinin and neuraminidase, constantly undergo minor antigenic changes (antigenic "drift") and are periodically subjected to major rearrangements (antigenic "shift") (1). In general, specific immunity developed against a particular virus subtype does not establish protection against new variants. Hence, alternative antiviral strategies need to be found.

Oligodeoxynucleotides (ODNs) comprise a class of antiviral compounds found to be active against Rous sarcoma virus (2), human immunodeficiency virus (HIV) (3–10), vesicular stomatitis virus (11–13), influenza virus (14), and herpes simplex virus (15). In this report, influenza A virus, as well as influenza C virus, was used to test a panel of different phosphodiester ODNs (O-ODNs) and phosphorothioate ODNs (S-ODNs) for their antiviral activity, and we provide data showing inhibition of influenza virus replication by S-ODNs.

MATERIALS AND METHODS

Synthesis and Purification of ODNs and Their Phosphorothioate Analogues. Oligonucleotides were synthesized on an automated synthesizer (Biosearch 8700, Milligen, Bedford, MA). Both O-ODNs and S-ODNs were assembled by H-phosphonate chemistry. The synthesis and purification of O-ODNs and S-ODNs were carried out by the method previously reported (10). Concentrations of ODNs were determined by absorbance at 260 nm, taking into account the molar extinction coefficient of the nucleotides present in each sequence (16).

Viruses and Cells. Influenza A/WSN/33 (ts^+) virus was grown in Madin–Darby canine kidney (MDCK) cells as described (17). Influenza C/JJ/50 virus was grown in the amniotic sac of 11-day-old embryonated chicken eggs at 35°C.

Virus Yield Assay. MDCK cells (2×10^5 cells per well) were seeded into 24-well dishes (Nunc) and allowed to grow overnight. The next day, cells were washed with phosphate-buffered saline (PBS) and 200 μl of minimal essential medium containing 0.2% bovine serum albumin (MEM-BA) containing the ODN was added to each well. At various times after addition of compound, 50 μl of virus sample, containing 2×10^5 plaque-forming units (pfu) in MEM-BA, was added per well. In some experiments, only 200 pfu per well was used to allow for multicycle replication. After adsorption for 45 min at 37°C, cells were washed twice with PBS, and 1 ml of MEM-BA containing the ODN and trypsin (3 μg/ml) was added. Cells were incubated at either 37°C or 33°C and samples of medium were taken at various times after infection for hemagglutination assay and titration on MDCK cells.

Plaque Formation Assay. MDCK cells were grown in 35-mm dishes. Confluent cells were infected with serial 1:10 dilutions of virus (100 μl per dish). One hour after infection, the inoculum was aspirated and agar overlay (2.5 ml per dish) containing 3 μg of trypsin per ml and ODN at the indicated concentration was added. Cells were incubated at either 37°C for 2.5 days (influenza A virus) or 33°C for 4–5 days (influenza C virus).

Computer Sequence Analysis. Sequence analysis was performed on a Vax 11/780 computer running under the VMS 4.6 operating system. Version 6.1 of the sequence analysis software package by the Genetics Computer Group (Madison, WI) (18) was used. The WORDSEARCH program, which is based on the algorithm of Wilbur and Lipman (19), was used to identify regions in the influenza C virus genome that are identical with at least eight contiguous nucleotides of ODN sequences. This was done by setting the mask to "++++++++" and searching the data base with the query sequence ODN 1–9. The data base was comprised of the influenza C virus sequences found in GenBank release 60.0 and the sequences of the C virus polymerase genes (20).

RESULTS

ODNs Used in This Study. After infection of cells with influenza virus, the (−)-sense viral RNA segments were

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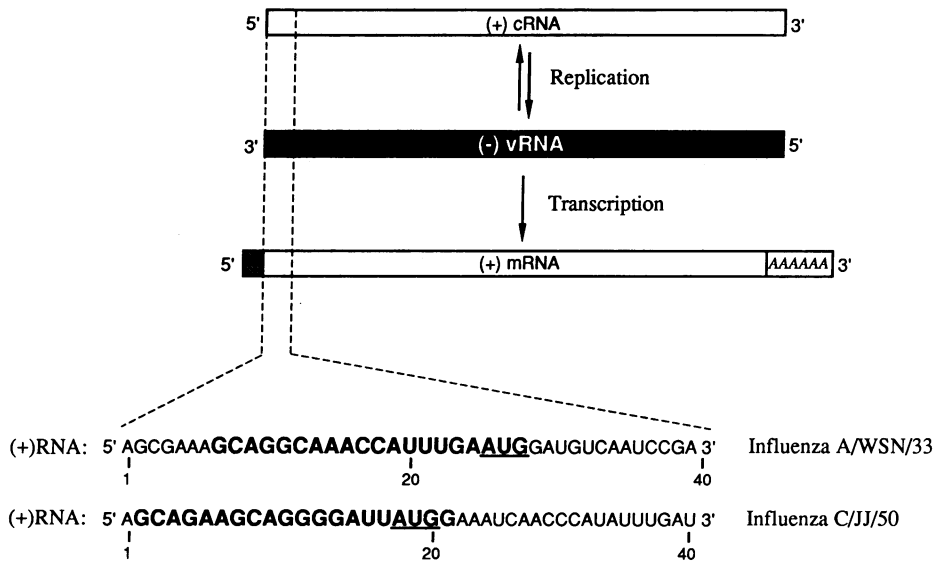
Abbreviations: HIV, human immunodeficiency virus; ODN, oligodeoxynucleotide; O-ODN, phosphodiester ODN; S-ODN, phosphorothioate ODN; pfu, plaque-forming units; moi, multiplicity of infection.

transcribed into mRNAs and replicated into full-length complementary RNAs (Fig. 1). All three forms of virus-specific RNA (viral RNA, mRNA, and complementary RNA) are potential targets for antisense ODNs. Therefore, O-ODNs and S-ODNs corresponding to viral sequences in the positive or negative orientation were synthesized and tested for antiviral activity. ODNs 1–2 and 3–4 are targeted against the polymerase PB1 RNA of influenza A/WSN/33 virus (21) and C/JJ/50 virus (20), respectively. ODNs 5–9 contain different numbers of mismatches relative to specific influenza virus sequences.

O-ODNs Show No Anti-Influenza Virus Activity. We first tested unmodified ODNs for antiviral activity using influenza A virus. O-ODN 1, O-ODN 2, and O-ODN 7 were synthesized and assayed for antiviral activity. Cells were infected at a low multiplicity of infection (moi) (0.001) to allow for multicycle replication of the virus. O-ODNs were added 30 min prior to infection and again 24 hr after infection. The concentrations in the medium were 80, 27, and 9 μM. No antiviral activity was observed at any concentration (Fig. 2*A*; data not shown). Similar results were obtained for influenza C virus (see below).

S-ODNs Are Active Against Influenza A Virus. We next synthesized phosphorothioate derivatives of ODN 1 (S-ODN 1) and ODN 2 (S-ODN 2). Two different control sequences, S-ODN 8 and S-ODN 9, were also made. S-ODN 8 and S-ODN 9 were examined by computer sequence analysis for their occurrence in the influenza A virus genome. They were found to share not more than 10 contiguous nucleotides with the (−)-strand viral RNA of influenza A virus (data not shown). Virus replication was tested under multicycle (moi 0.001) and single-cycle (moi 1) conditions. Most experiments were performed at 37°C but for some infections a temperature of 33°C was chosen. If the mechanism of action involves hybridization of S-ODNs to viral RNA, it was thought that this experimental condition might enhance the antiviral effect. The result of a typical experiment, performed at 33°C and a moi of 1, is shown in Fig. 2*B*. All sequences tested were found to inhibit the growth of influenza A virus when supplied in the phosphorothioate form. However, inhibition with mismatched oligomers was also observed. As expected, the antiviral effect was significantly stronger under multicycle replication conditions (Fig. 3*A*) and could be further enhanced by pretreating cells with S-ODN (Fig. 3 *B* and *C*). Under these conditions, the compounds were active at concentrations as low as 1.25 μM (Fig. 3; data not shown).

Sequence-Specific Inhibition of Influenza C Virus Using S-ODN 4. We then asked whether replication of influenza C virus could also be inhibited by S-ODNs. Influenza C virus grows more slowly than influenza A virus and the experimentation period is longer. We therefore decided to use a faster assay system. Briefly, S-ODNs were added to the agar overlay and plaques were counted at the end of the incubation period. In preliminary experiments, influenza A virus was assayed by this system and again inhibition with mismatched



ODN	Sequence		
1	5'-CATTCAAATGGTTTGCCTGC-3'	(−) Sense	A/WSN/33
2	5'-GCAGGCAAACCATTTGAATG-3'	(+) Sense	A/WSN/33
3	5'-CCATAATCCCCTGCTTCTGC-3'	(−) Sense	C/JJ/50
4	5'-GCAGAAGCAGGGGATTATGG-3'	(+) Sense	C/JJ/50
5	5'-GCAGAAGCAGAGGATTATGG-3'	ODN 4 with 1 mismatch	
6	5'-GCATAAGCAGAGGATCATGG-3'	ODN 4 with 3 mismatches	
7	5'-GGCAAGCTTTATTGAGGCTT-3'	Mismatched	
8	5'-ATCTTCATCATCTGAGAGAT-3'	Mismatched	
9	5'-CGTAAGCAACAGTAGTCCTA-3'	Mismatched	

FIG. 1. Sequences of ODNs tested for antiviral activity against influenza virus. (*Top*) Diagram depicts the three different types of RNA present in infected cells [complementary RNA (cRNA), viral RNA (vRNA), and mRNA]. The host cell-derived 5′ portion (hatched) and the polyadenylylated 3′ portion of the mRNA are indicated. (*Middle*) Part of the RNA sequence of the *PB1* gene of influenza A/WSN/33 virus (21) and C/JJ/50 (20) is shown in the (+)-sense orientation. The regions corresponding to the ODN are printed in boldface; initiation codons are underlined. (*Bottom*) ODNs 1–4 are specific for viral sequences. ODN 5 and ODN 6 are derived from ODN 4 by introducing point mutations at the indicated positions (underlined). ODNs 7–9 represent sequences not found in the influenza virus genome.

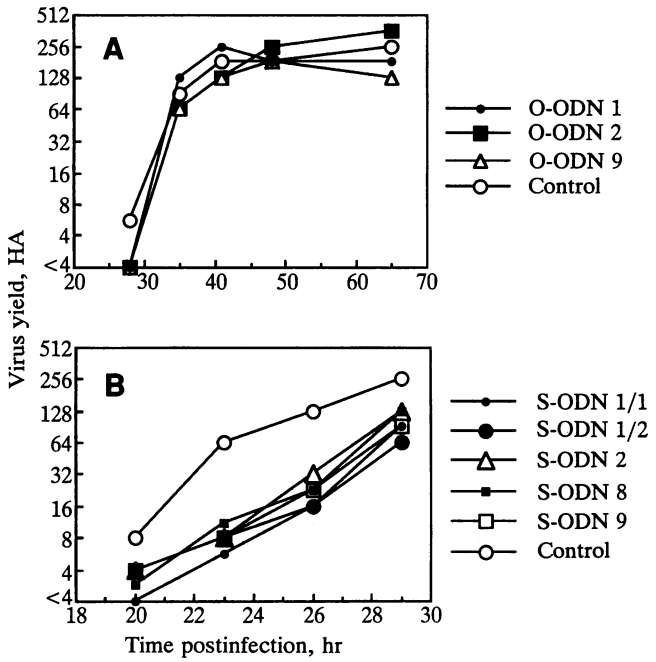


FIG. 2. Effect of O-ODN (*A*) and S-ODN (*B*) on influenza A virus replication. Cells were infected with influenza A virus as described in the text. Compounds were added to the cells 30 min prior to infection and were present throughout the experiment at a concentration of 80 μM. Infected control cells were not treated with compound. At the times indicated, samples of medium were taken for testing of hemagglutinin (HA) titers. (*A*) Infected cells (moi 0.001) were incubated at 37°C in the presence of O-ODN. (*B*) Infected cells (moi 1) were incubated at 33°C in the presence of S-ODN. S-ODN 1/1 and S-ODN 1/2 represent different batches of the same compound.

oligomers was obtained (data not shown). Sequences tested against influenza C virus included S-ODN 3 [complementary to (+)-RNA of C virus], S-ODN 4 [complementary to (−)-RNA of C virus], and S-ODN 7 [complementary to poly(A) signal of HIV-1]. In initial experiments, performed at a concentration of 80 μM, complete inhibition of influenza C virus plaque formation was observed when S-ODN 4 was used, but not with S-ODN 3 or S-ODN 7. To confirm this result, S-ODN 4 was synthesized in three different batches. Each batch was tested in two independent experiments and essentially the same results were obtained. Concentration dependence was then examined. A sequence-specific antiviral effect was observed at concentrations as low as 20 μM (Fig. 4). To define further the degree of specificity, we next used single (S-ODN 5) and triple (S-ODN 6) mismatched derivatives of S-ODN 4 (Fig. 1). S-ODN 5 was significantly less active than S-ODN 4 at a concentration of 20 μM (Fig. 5). S-ODN 6, as well as various other control sequences including the complementary sequence S-ODN 3, were found to be inactive at that concentration (Fig. 5). Completely inactive, at any concentration tested, was the phophodiester derivative O-ODN 4 (Fig. 5; data not shown).

DISCUSSION

In this paper, we report on the inhibition of influenza viruses using ODNs. For each sequence, up to eight different batches of oligomers were synthesized. Each batch was tested in at least two independent experiments, which yielded comparable results. Virus-specific ODNs were complementary to parts of the PB1 RNA (Fig. 1). This target was chosen for the following reasons: (*i*) the influenza virus protein PB1 is part of the polymerase complex that is required for transcription and replication of viral RNA (22–25). The PB1 protein is the most highly conserved protein of this complex (20) and is the

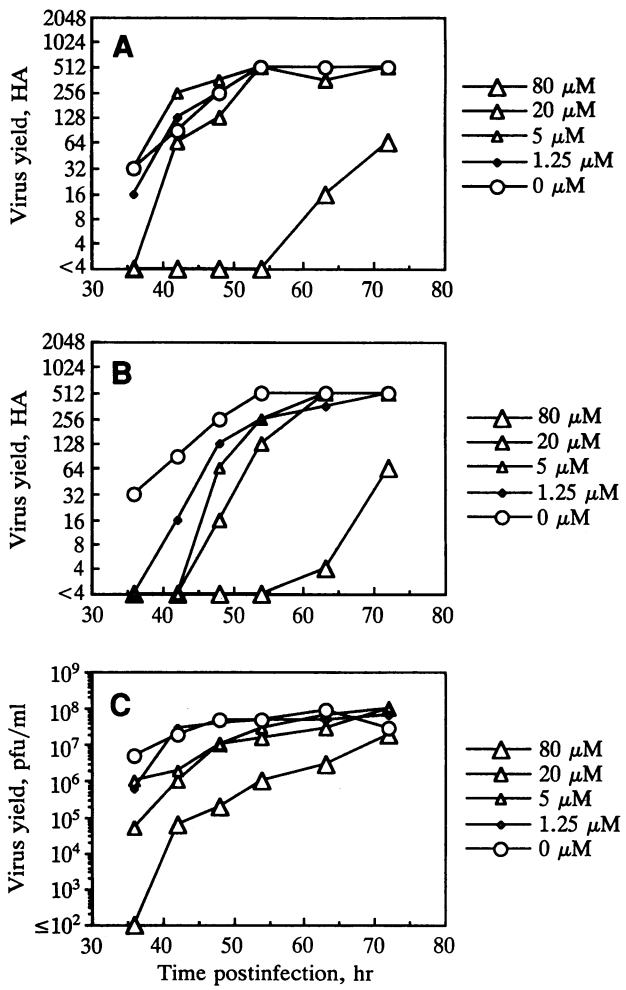


FIG. 3. Inhibition of influenza A virus replication by S-ODN. Cells were infected with influenza A virus (moi 0.001) and incubated at 37°C. S-ODN 2 was added to the cells at the concentrations indicated in the figure. (*A*) The compound was added 30 min prior to infection and was continuously present throughout the experiment. Virus yield was determined as described in Fig. 2. HA, hemagglutinin. (*B*) The compound was added 24 hr prior to infection and was continuously present throughout the experiment. Virus yield was determined as described in Fig. 2. (*C*) Same as *B* but virus yield was determined by titration of infectious virus on MDCK cells.

only one in which the Asp-Asp motif, which is found in many RNA polymerases (26), is conserved among influenza A, B, and C viruses (20). Taken together, this suggests an essential function of the *PB1* gene for virus growth and any interference with its expression is likely to result in decreased virus

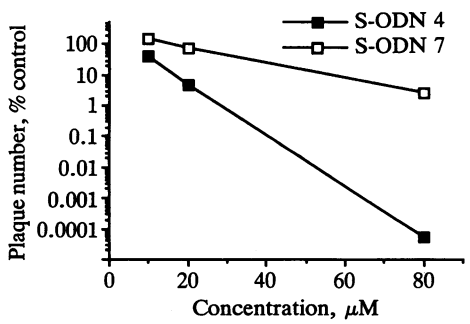


FIG. 4. Dose-dependent inhibition of influenza C virus by S-ODN 4. Cells were infected with influenza C virus and S-ODNs were added to the agar overlay 1 hr postinfection at the concentrations indicated. Control infected cells were not treated with compound.

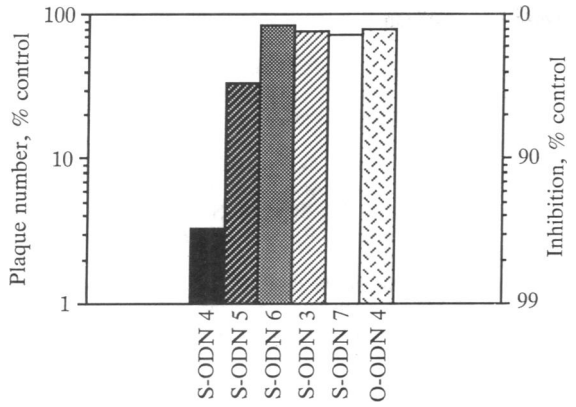


FIG. 5. Sequence-specific inhibition of influenza C virus replication by S-ODN 4. The plaque formation assay was performed as described in Fig. 4 with compounds at a concentration of 20 μM.

titers. (*ii*) Influenza virus polymerase mRNAs are expressed at relatively low levels in infected cells as compared to other viral mRNAs (27). Thus, a lower intracellular concentration of S-ODN should be required to block gene expression. (*iii*) The ends of the *PB1* gene were chosen as targets because a high degree of conservation was observed at the 3′ and 5′ ends among the genomic segments of influenza viruses (28). Therefore, if part of the ODN sequence is conserved among different RNA segments, this may result in the inhibition not only of the *PB1* gene but of several other genes as well. (The results of a computer analysis of ODN target sites in the influenza C virus genome are discussed below.)

O-ODNs were reported to inhibit Rous sarcoma virus (3), HIV (4), c-*myc* (29), and c-*myb* (30) oncogenes. Helene and coworkers (14) reported selective inhibition of the cytopathic effect of influenza A virus by O-ODNs that were covalently linked to an acridine derivative. In the present study, unmodified O-ODNs were not found to be active against either influenza A virus (Fig. 2*A*) or influenza C virus (Fig. 5). Based on the other experimental data (3, 29), we believe that the unmodified O-ODNs are ineffective because they are rapidly degraded extracellularly and/or intracellularly.

Phosphorothioate derivatives showed consistent inhibitory antiviral activity in multicycle and even single-cycle replication experiments using influenza A virus (Figs. 2*B* and 3). The effect was present with mismatched oligomers and most likely not the result of a cytotoxic effect of the compounds. This view is supported by several lines of evidence. First, MDCK cell monolayers were found to be intact throughout the experiment in uninfected control cells treated with 80 μM S-ODN compound (data not shown). Second, S-ODNs delayed influenza A virus replication rather than completely abolishing it (Fig. 3), which indicates that cells are able to support viral replication as late as 4 days after treatment with S-ODN. Third, these compounds were purified by the same method used for the O-ODNs, which showed no antiviral activity. Therefore, it is unlikely that the observed effect is due to impurities in the preparation. However, this effect could not be correlated with a perfectly matched sequence. S-ODNs are known to interact with reverse transcriptase (4) and presumably with other enzymes and proteins. Furthermore, effects of mismatched S-ODNs on viral replication have also been found in the case of HIV (4, 5, 7, 9, 10). Recent reports (8, 10) indicate, however, that in the case of cells already infected with HIV, complementary antisense S-ODNs are much superior to homooligomers or mismatched oligomers.

Interestingly, influenza C virus replication could be inhibited in a sequence-specific manner by using S-ODN 4. If present at 20 μM concentration in the medium, this compound was at least 10-fold more active than any of the other sequences tested (Fig. 5). The same sequence had no effect on influenza A virus replication using the same assay system (data not shown). At higher concentrations, an even more dramatic reduction of virus growth was observed (Fig. 4). The complementary sequence, S-ODN 3, was found to be inactive (Fig. 5). Computer analysis was performed to examine the degree of sequence identity between the ODN sequences tested and the influenza C virus genome. It was found that six of seven viral RNA segments are complementary to at least 13 nucleotides in ODN 4 (Fig. 6). This result was not unexpected since, as mentioned earlier, the ends of all influenza virus RNA segments are conserved. Introduction of a single mismatch in S-ODN 5 reduces the degree of sequence complementarity to 10 nucleotides, but six RNA segments still remain as potential targets. This may explain the remaining antiviral activity of this compound at a 20 μM concentration (Fig. 5). No other control, including the triple mismatched sequence of S-ODN 6, showed antiviral activity (Fig. 5) and none was complementary to more than eight contiguous nucleotides of the influenza C virus genome (Fig. 6). We have recently found (31) that in the case of O-ODNs, one or two mismatches between oligomer and RNA may result in loss of RNase H sensitivity of that complex. Taken together, we believe that the observed anti-influenza C virus effect requires an S-ODN sequence with more than 10 contiguous nucleotides complementary to the (−)-strand viral RNA. The effect of hybridization mismatches on viral inhibitions is variable and may depend on secondary structure and other features of the viral genome.

It is interesting that sequence-specific inhibition (no mismatches) could be obtained with influenza C virus but not with influenza A virus. S-ODN 4 is complementary to the influenza C virus (−)-strand viral RNA at positions 2–21, while S-ODN 2 is complementary to the influenza A virus (−)-strand viral RNA at positions 8–27 (Fig. 1). We therefore synthesized and tested a S-ODN complementary to the influenza A virus (−)-strand viral RNA at positions 2–21 (5′-GCGAAAGCAGGCAAACCATT-3′). Again, this compound was not found to be more active than mismatched control S-ODNs (data not shown). Therefore, the choice of the target site cannot explain the observed difference between these viruses. (However, we cannot exclude the possibility that other S-ODNs would show sequence-specific activity against influenza A virus.) Although this difference between influenza A and C viruses is puzzling, we should

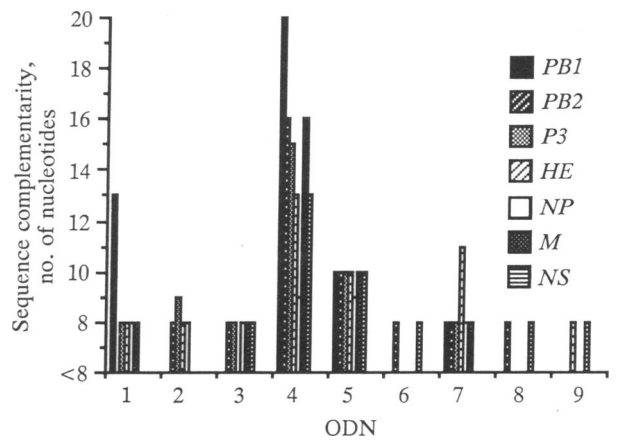


FIG. 6. Computer sequence analysis of ODNs 1–9. Complementarity of ODNs to the influenza C virus genome was determined. The WORDSEARCH program (17) was used to identify influenza C virus (−)-strand viral RNA segments (*PB1, PB2, P3, HE, NP, M,* and *NS* genes), which are complementary to eight or more nucleotides of the indicated ODNs.

take into consideration the very different replication rates of these viruses. A slowly replicating virus such as influenza C virus may be a much better target for antisense ODN inhibition than a fast-growing virus such as influenza A.

The term "antisense," in the case of a negative-stranded viral genome, deserves a little explanation. The viral replicase synthesizes a (+)-strand RNA, which can be converted into an mRNA, as illustrated in Fig. 1. An oligomer complementary to this mRNA can then be called an antisense RNA. An oligomer complementary to the viral RNA itself may be regarded as antisense to the viral RNA. The term "hybridon," suggested previously (2), avoids this semantic problem, being defined as "an oligonucleotide of specified sequence that acts in a metabolic sense by competitive hybridization."

Inhibition of influenza virus production in tissue culture by means of an ODN attached to a hydrophobic substituent has also recently been reported (32).

In summary, this report demonstrates two distinct antiviral activities of S-ODNs against influenza virus. One activity is present even with a high degree of mismatches and is found to be operative against influenza A virus and, at high concentrations (80 μM), against influenza C virus. The other effect is sequence specific and is observed when using influenza C virus and a (+)-sense S-ODN. Under these conditions, a reduction in the number of virus plaques of up to 10^6-fold could be observed.

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