Inhibition of influenza virus replication in cultured cells by RNA-cleaving DNA enzyme

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Abstract Influenza virus replication has been effectively inhibited by antisense phosphothioate oligonucleotides targeting the AUG initiation codon of PB2 mRNA. We designed RNA-cleaving DNA enzymes from 10-23 catalytic motif to target PB2-AUG initiation codon and measured their RNA-cleaving activity in vitro. Although the RNA-cleaving activity was not optimal under physiological conditions, DNA enzymes inhibited viral replication in cultured cells more effectively than antisense phosphothioate oligonucleotides. Our data indicated that DNA enzymes could be useful for the control of viral infection. **© 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Influenza virus; RNA-cleaving DNA enzyme; Antisense oligonucleotide; PB2

1. Introduction

Antisense oligonucleotides have been widely used for the specific inhibition of gene expression. They were developed rapidly as therapeutic agents and soon moved from the laboratory into the clinical field [1–3]. When the administration of large amounts of antisense phosphothioate oligonucleotides is required, however, there is usually a toxicity problem [1].

Recently, general-purpose RNA-cleaving DNA enzymes have been developed and two catalytic motifs, 10-23 and 8-17, were reported [4,5]. These DNA enzymes are catalytic in nature and can be engineered to cleave a target gene in trans in a sequence-specific manner. The first antiviral application of DNA enzymes was in the inhibition of human immunodeficiency virus (HIV) [6–8]. Although DNA enzymes were shown to inhibit HIV infection, they have yet to be utilized to inhibit viral infections in general.

To date, the application of antisense phosphothioate oligonucleotides to inhibit the replication of various viruses has been of great success [1]. We have demonstrated that liposome-encapsulated antisense phosphothioate oligonucleotides complementary to PB2-AUG and PA-AUG initiation codons inhibited the influenza virus RNA polymerase activity [9–11], and influenza virus replication in MDCK cells [12–14] and in mice [15]. In this paper, we describe the inhibition of influenza virus replication in cultured cells by RNA-cleaving DNA enzyme which was designed from 10-23 catalytic motif targeting the PB2-AUG initiation codon, and compare the results with those for antisense phosphothioate oligonucleotides complementary to the same position [15].

2. Materials and methods

2.1. DNA enzymes targeting the influenza virus A/PR8/34 PB2 subunit

Based on Santoro and Joyce's reports [4,5], we designed RNA-cleaving DNA enzymes to target the translation initiation codon of PB2 mRNA (Table 1). 10-23 DNA enzyme sequence was flanked with two antisense sequences (7 or 8 nucleotides (nt)). As a control, a 16 nt random sequence was flanked with each 8 nt antisense sequence of PB2 mRNA.

2.2. Cells and viruses

MDBK and MDCK cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum. Influenza virus A/PR8/34 was grown in 11-day-old embryonated chicken eggs at 34°C for 48 h, and stored at −80°C until used.

2.3. In vitro RNA synthesis and cleavage of PB2 transcript by DNA enzyme

T7 promoter sequences were tagged at the 5′-termini of PB2 and PB1 cDNA, respectively. PB2 plasmid, pAPR102 [16], was PCR-amplified using KOD polymerase (Toyobo) and primer 5′-GTCGACTCTAGAGGATCCTAATACGACTCACTATAAGCGAAAGCAGGTCAATTAT-3′ and 5′-AGTAGAAACAAGGTCGTTTT-3′. PB1 plasmid, pAPR206 [16], was PCR-amplified using KOD polymerase (Toyobo) and primer 5′-GTCGACTCTAGAGGATCCTAATACGACTCACTATAAGCGAAAGCAGGCAAACCAT-3′ and 5′-AGTAGGAACAAGGCATTTTT-3′. The amplified fragments were digested with *Xba*I and inserted between *Xba*I and *Sma*I sites of pUC19, resulting in pT7cPB2 and pT7cPB1. [^{32}P]UMP-labeled positive sense RNA fragments of PB2 and PB1 were transcribed in vitro using T7 RNA polymerase (Toyobo, 15 U) and 1 μg of pT7cPB2 digested with *Nde*I (109 nt) and pT7cPB1 digested with *Nco*I (121 nt) as templates in 40 mM Tris–HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 0.5 mM each of ATP, CTP, GTP, 50 μM UTP and 50 μCi [α-^{32}P]UTP (Amersham). The transcribed ^{32}P-labeled RNA was purified by 6% polyacrylamide gel electrophoresis/7 M urea.

10 nmol of ^{32}P-labeled RNA substrate was incubated with 1 μM of DNA enzyme in various conditions as indicated in the text.

2.4. Inhibition of influenza virus replication by DNA enzyme

Subconfluent MDBK cells on 48-well plates were washed three times with phosphate-buffered saline and inoculated with influenza virus A/PR8/34 at a multiplicity of infection (moi) of 0.1 (unless otherwise indicated) at 4°C for 1 h, and then transfected with the indicated amount of oligonucleotides, DNA enzymes or antisense thio-oligonucleotides (ODN PB2as (20), ODN PB2ran (20), ODN PB2as (28) and ODN PB2ran (28)), in OPTI-MEM I (Lifetech Ori-

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ental) containing 2% lipofectin (Lifetech Oriental) at 34°C for 4 h [15]. After transfection, cells were further incubated in MEM containing 0.1% bovine serum albumin (BSA) and 2 μg/ml of trypsin (Difco) at 34°C for 20 h. The cells were harvested and the titer of the viruses was measured by plaque-forming assay on MDCK cells after three cycles of freezing and thawing. Confluent MDCK cells on 12-well plates were inoculated with serial dilutions of the viruses at 25°C for 1 h, and then overlaid with MEM containing 0.8% agarose, 5 μg/ml trypsin and 0.1% BSA [17]. After 2 days incubation at 34°C, the cells were fixed with ethanol:acetic acid (5:1) and stained with 0.1% amide black.

3. Results

3.1. Sequence-specific cleavage by DNA enzyme

First, the sequence-specific cleavage by DNA enzyme, DNzPB2 (14), DNzPB2 (16) and the control DNA enzyme which did not carry the catalytic sequence of 10-23, DNzPB2 (C), was examined using PB2 and PB1 transcripts. 10 nmol substrate RNA was incubated with 1 μM DNA enzyme or antisense phosphothioate oligonucleotides (Fig. 1). In these conditions, DNzPB2 (16) cleaved PB2 transcript more efficiently than DNzPB2 (14). Neither DNzPB2 (14) nor DNzPB2 (16) cleaved PB1 transcript at all. DNzPB2 (C) or antisense phosphothioate oligonucleotides (ODN PB2as (20) and ODN PB2as (28)) did not cleave any RNA substrates at all.

Next, the effect of concentration of $MgCl_2$ on RNA-cleaving activity was examined using DNA enzyme with a 16 nt complementary sequence to PB2 mRNA, DNzPB2 (16). ^{32}P-labeled PB2 transcript (10 nmol) and 1 μM DNzPB2 (16) were incubated in 0, 1, 2, 5, 10, 20, 50, 100, 200 or 500 M $MgCl_2$, 30 mM HEPES/NaOH, pH 7.6, and 150 mM NaCl at 37°C for 1 h (Fig. 2A). No cleavage occurred without $MgCl_2$. The most efficient cleavage was observed at 100 mM $MgCl_2$. The efficiency of cleavage did not increase when the $MgCl_2$ concentration was more than 200 mM.

However, the concentration of NaCl little affected the cleavage efficiency. PB2 transcript (10 nmol) and 1 μM DNzPB2 (16) were incubated in 0, 20, 100, 200, 300, 400, 500 or 1000 mM NaCl, 30 mM HEPES/NaOH, pH 7.6, and 20 mM $MgCl_2$ at 37°C for 1 h (Fig. 2B). The efficiency of cleavage was similar at NaCl concentrations less than 200 mM, and it increased according to the NaCl concentration at more than 300 mM.

The effect of pH was examined. PB2 transcript (10 nmol) and 1 μM DNzPB2 (16) were incubated in 50 mM sodium citrate (pH 4 and pH 5), 50 mM MES/NaOH (pH 6), 50 mM

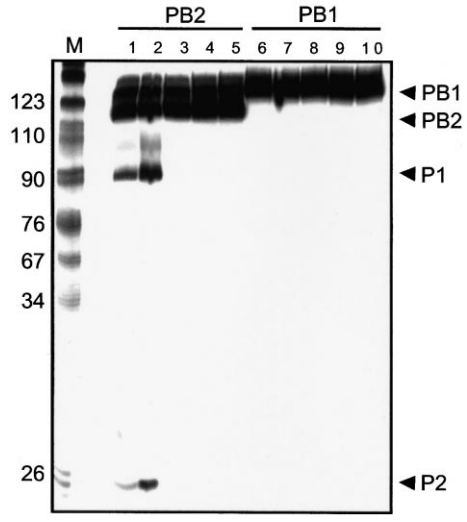

Fig. 1. Sequence-specific cleavage by DNA enzymes. The sequence-specific cleavage by DNA enzyme, DNzPB2 (14) (lanes 1 and 6), DNzPB2 (16) (lanes 2 and 7) and the control oligodeoxynucleotide DNzPB2 (C) (lanes 3 and 8) was examined using PB2 (lanes 1–5) and PB1 transcripts (lanes 6–10). 10 nmol substrate RNA was incubated with 1 μM DNA enzyme or antisense phosphothioate oligonucleotides (ODN PB2as (20), lanes 4 and 9; ODN PB2as (28), lanes 5 and 10). The positions of substrate RNAs (PB1 and PB2) and the cleaved products of PB2 (P1 and P2) are indicated by arrowheads.

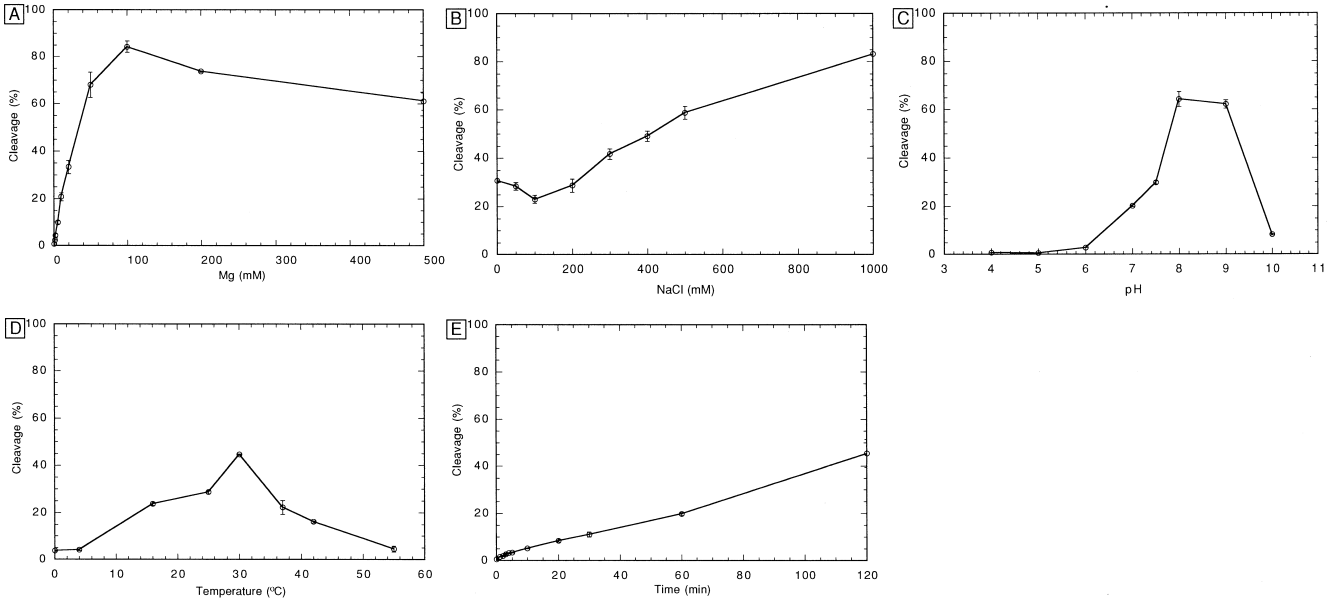

Fig. 2. In vitro cleavage by DNA enzymes. The effect of $MgCl_2$ concentration (A), NaCl concentration (B), pH (C), incubation temperature (D) and incubation time (E) was examined in vitro as described in Sections 2 and 3. The average % cleavage and the standard deviation were calculated from two independent experiments and plotted in the graph.

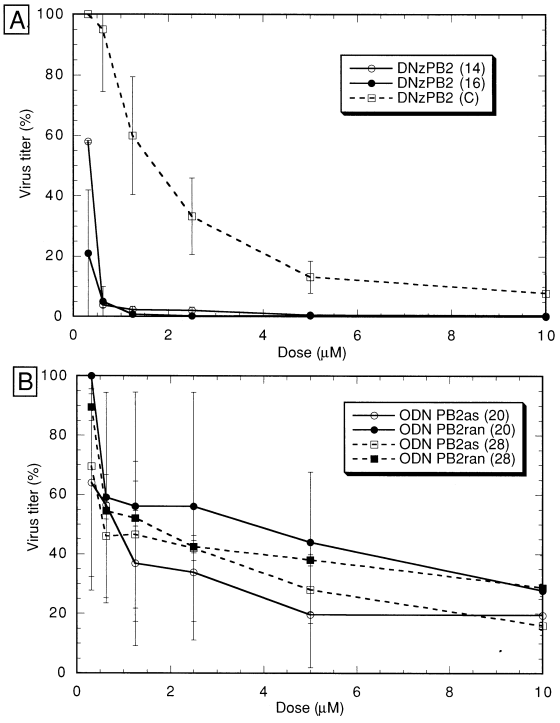

Fig. 3. Dose-dependent inhibition of influenza virus replication by DNA enzymes and antisense phosphothioate oligonucleotides. MDBK cells were infected with influenza virus A/PR8/34 at a moi of 0.1, and then transfected with the indicated concentration of oligonucleotides (DNA enzymes (A) or antisense thio-oligonucleotides (B)). The titer of the viruses was measured by plaque-forming assay on MDCK cells. The average and standard deviation of % virus yield were calculated from three independent experiments.

HEPES/NaOH (pH 7, pH 7.5 and pH 8), 50 mM CHES/NaOH (pH 9) and 50 mM CAPS/NaOH (pH 10) in the presence of 20 mM $MgCl_2$ and 150 mM NaCl, at 37°C for 1 h. The highest cleavage was achieved between pH 8 and 9 (Fig. 2C). At pH 10, the substrate RNA degraded.

The cleavage reaction was examined at various temperatures in 30 mM HEPES/NaOH, pH 7.5, 20 mM $MgCl_2$ and 150 mM NaCl for 1 h. The cleavage efficiency was best at 30°C (Fig. 2D). No specific cleavage was observed at 55°C. Below 4°C, however, about 20% cleavage was observed.

Finally, PB2 transcript (10 nmol) and 1 μM DNzPB2 (16) were incubated for various periods at 37°C in 30 mM HEPES/NaOH, pH 7.5, 20 mM $MgCl_2$ and 150 mM NaCl (Fig. 2E). Under the conditions, the cleavage reaction increased with incubation time.

3.2. Inhibition of influenza virus replication by DNA enzyme

Because our data indicated that antisense phosphothioate oligonucleotides targeting AUG initiation codon sequences of PB2 mRNA effectively inhibited the influenza virus RNA polymerase activity [9–11], and influenza virus replication in infected cells [12–14] and mice [15], we tested the DNA enzyme which carries 10-23 catalytic sequence flanked with complementary sequences around the AUG initiation codon of PB2 mRNA (Table 1).

First, the inhibitory effect on the concentration of oligonucleotides was tested. MDBK cells were inoculated with influenza virus A/PR8/34 (moi = 0.1) at 4°C for 1 h, and transfected with 10, 5, 2.5, 1.25, 0.625 and 0.3125 μM of

Table 1
Design of DNA enzyme

Oligonucleotides	Sequence[a]
DNzPB2 (14)	CTTTCCA**GGCTAGCTACAACGA**ATTGAAT
DNzPB2 (16)	TCTTTCCA**GGCTAGCTACAACGA**ATTGAATA
DNzPB2 (C)	TCTTTCCAAGTCAGTCAGTCAGTATTGAATA

[a]Sequence of 10-23 DNA enzyme is shown in bold.

oligonucleotides in 2% lipofectin at 34°C for 4 h. At 20 h after transfection (at 24 h after infection), the cells were harvested and the virus titer was measured by plaque-forming assay on MDCK cells. The average percent virus titer of control MDBK cells without oligonucleotides as well as the standard deviation was calculated from three independent experiments (Fig. 3). DNA enzyme, DNzPB2 (16), significantly inhibited viral replication at 0.3 μM. At more than 0.6 μM, however, both DNA enzymes, DNzPB2 (14) and DNzPB2 (16), significantly inhibited the replication of influenza virus. Even a control oligonucleotide, DNzPB2 (C), inhibited viral replication less than 50% at more than 2.5 μM. Antisense phosphothioate oligonucleotides 20 and 28 nt long inhibited viral replication less than 50% at more than 1 μM (Fig. 3B). ODN PB2as (28) was slightly stronger than ODN PB2as (20). At a high concentration (more than 5 μM), oligonucleotides inhibited viral replication.

Next, at 1 μM of DNz and antisense phosphothioate oligonucleotides, the inhibitory effect on the challenged viral dose was examined. MDBK cells were inoculated at a moi of 0.001, 0.01, 0.1, 1 and 10 and then treated as mentioned above (Fig. 4). When the moi was less than 1, 1 μM DNzPB2 (16) and ODN PB2as (28) significantly inhibited viral replication.

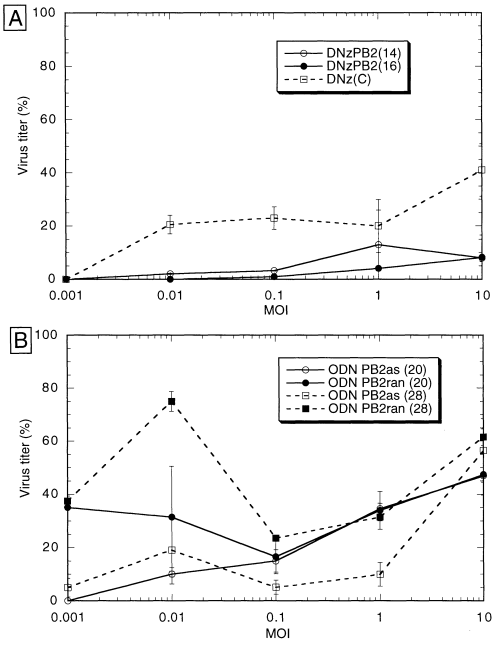

Fig. 4. The effect of virus amount on inhibition by DNA enzymes and antisense phosphothioate oligonucleotides. MDBK cells were infected with influenza virus A/PR8/34 at the indicated moi, and then transfected with 1 μM oligonucleotides (DNA enzymes (A) or antisense thio-oligonucleotides (B)). The titer of the viruses was measured by plaque-forming assay on MDCK cells. The average and standard deviation of % virus yield were calculated from three independent experiments.

When the moi was less than 0.1, 1 μM DNzPB2 (14) and ODN PB2as (20) significantly inhibited viral replication.

When MDBK cells were transfected with DNA enzymes first and then infected with virus, no inhibition was observed (data not shown). However, when the cells were transfected with ODN PB2as (20) and ODN PB2as (28) first and then infected with virus, viral replication was inhibited (data not shown), which agreed with the previous results [12–14]. Sometimes the inhibitory effect of oligonucleotides was affected by the lot of lipofectins (data not shown). Treatment of lipofectin alone decreased the viral titers (data not shown). When cells were incubated with 10 μM oligonucleotides without lipofectin, no significant inhibition was observed (data not shown).

4. Discussion

Influenza A virus contains eight single-stranded RNA segments of negative polarity as the genome and an RNA-dependent RNA polymerase as a virion component. Influenza A virus RNA polymerase purified from influenza virus consists of one part each of three subunits, PB1, PB2 and PA [19]. The RNA polymerase catalyzes both transcription (the synthesis of plus-strand mRNA containing the host cell-derived cap-I structure at the 5′-terminus and poly(A) tail at the 3′-terminus) and replication (the synthesis of full-length plus-strand complementary RNA (cRNA) and the cRNA-dependent synthesis of minus-strand viral RNA) [20,21]. In the infected cells, mRNAs of viral RNA polymerase, NP and NS1 were transcribed preferentially early on [20]. The amounts of mRNAs of viral RNA polymerase subunits were the smallest of all the viral mRNAs (T. Toyoda, unpublished data). This evidence indicated that mRNAs of influenza virus RNA polymerase subunits (PB2, PB1 and PA) would be the best target for inhibition of viral translation.

Antisense phosphothioate oligonucleotides with the target AUG initiation codon sequences of influenza virus A/PR8/34 PB2 mRNA were shown to inhibit effectively the viral replication in infected cells [12–14] and mice [15]. In order to test the inhibitory effect of DNA enzyme on the replication of influenza virus in a cell culture system, we have synthesized 10-23 DNA enzyme flanked with the same target sequence of PB2 mRNA.

DNA enzyme with longer arms (8-mer each) complementary to the target sequences cleaved the PB2 mRNA more efficiently than that with shorter arms (7-mer each) (Fig. 1). The longer one also inhibited viral replication more efficiently than the shorter one (Fig. 3). The cleavage reaction depended mainly on $MgCl_2$ concentration as previously reported [18]. However, DNzPB2 (16) showed maximum cleavage activity at 100 mM $MgCl_2$ and at higher concentrations, the activity slightly decreased (Fig. 2A). Not only $MgCl_2$, but also NaCl increased the activity (Fig. 2B). The optimal pH of the cleavage reaction was between 8 and 9 (Fig. 2C). The activity was lost at high temperature (Fig. 2D). All of these results indicate that the annealing condition of DNA enzyme and the substrate RNA affected the cleavage activity. A major concern with DNA enzyme is that at the physiological $MgCl_2$ concentration (0.5–3.5 mM), the cleavage activity is lower than at $MgCl_2$ concentrations above 10 mM. In cultured cells, DNzPB2 (16) inhibited influenza virus replication most effectively even at a high moi (Figs. 3 and 4).

From our results, one can conclude that RNA-cleaving DNA enzyme inhibited influenza virus infection more effectively than the same amount of antisense phosphothioate oligonucleotides (Figs. 3 and 4), although the cleaving activity was shown to be low in physiological conditions. The potential toxicity of phosphothioate oligonucleotides may stem from the release of sulfate [1]. Although we did not observe any difference in the cell numbers among the oligonucleotides 24 h after transfection (data not shown), DNA enzymes of phosphodiester oligonucleotides seem to be less toxic than phosphothioate oligonucleotides in vivo. Our data clearly indicated that DNA enzyme is another promising agent for control of viral infection.

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