A new modified DNA enzyme that targets influenza virus A mRNA inhibits viral infection in cultured cells

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Abstract DNA enzymes are RNA-cleaving single-stranded DNA molecules. We designed DNA enzymes targeting the PB2 mRNA translation initiation (AUG) region of the influenza A virus (A/PR/8/34). The modified DNA enzymes have one or two N3′-P5′ phosphoramidate bonds at both the 3′- and 5′-termini of the oligonucleotides, which significantly enhanced their nuclease resistance. These modified DNA enzymes had the same cleavage activity as the unmodified DNA enzymes, determined by kinetic analyses, and reduced influenza A virus replication by more than 99%, determined by plaque formation. These DNA enzymes are highly specific; their protective effect was not observed in influenza B virus (B/Ibaraki)-infected Madin–Darby canine kidney cells.

Key words: Modified DNA enzyme; N3′-P5′ phosphoramidate oligonucleotide; Influenza A virus; Kinetic analysis; Nuclease resistance; Plaque formation assay

1. Introduction

Influenza viruses cause severe colds with generalized symptoms. Influenza is a very infectious disease, and lethal pneumonia often develops, particularly in aged patients or high-risk patients with chronic respiratory disorders or heart disease. The influenza viruses are classified into three types, A, B, and C, on the basis of differences in the serotypes of the nucleoproteins and the membrane proteins. Influenza virus is a negative-strand RNA virus with a segmented genome. Essentially all of the transcription and replication studies of influenza virus have been performed with A viruses, which contain eight virion RNA segments. Viral mRNA synthesis is catalyzed by four viral proteins [1]: the nucleocapsid protein and the three P (PB1, PB2, and PA) proteins [2–4]. The PB1, PB2, PA, and NP genes are potential targets for antisense oligonucleotides.

We previously demonstrated that an antisense phosphorothioate oligonucleotide (S-ODN-PB2-as), containing AUG initiation codon sequences and targeted to PB2 of the influenza A virus RNA polymerases, has highly specific suppressive activity against influenza A virus growth. Its activity was superior to the antisense phosphorothioate oligonucleotides targeted to the PA RNA polymerases in mice infected with the influenza A virus [5].

Selective inactivation of a target gene by antisense mechanisms is an important biologic tool to delineate the specific functions of the gene product [6–10]. Approaches mediated by RNA-cleaving ribozymes are attractive, because of their ability to catalytically cleave the target RNA [11–14]. These molecules (both as native nucleic acids and in modified forms), however, are highly susceptible to enzymatic hydrolysis, and have the potential for side effects in the cellular environment, thus limiting their pharmaceutical applications in a direct delivery mode. Recently, a new class of catalytic molecules, made of single-stranded DNA (deoxyribozyme or DNA enzyme), was obtained through in vitro selection [15–20]. One type, the deoxyribozyme, is especially useful because of its ability to bind and cleave any single-stranded RNA at purine/pyrimidine junctions [18]. The sequence-specific cleavage activities of short DNA molecules possessing two previously identified catalytic motifs (10–23 and 8–17) [18] were recently recognized as powerful biologic tools to interfere with gene expression. The performance of DNA enzymes bearing the 10–23 motifs has been demonstrated in a number of in vivo biologic systems [20–25]. These RNA-cleaving DNA enzymes (DNA enzymes) are expected to be more stable than short catalytic RNAs (ribozymes), which are inherently less stable. DNA enzymes with natural linkages, however, are degraded by nucleases present in serum and cells [26]. One approach to this problem has been the development of protective modifications of the 3′- and/or the 5′-ends of the chain, because the primary degradation enzymes present in cells are of the 3′-exonuclease type [27,28]. Recently, several stabilization methods for phosphodiester oligonucleotides were proposed, such as the incorporation of various chemical substituents at the 3′-hydroxyl groups [27,28], the circularization of the oligonucleotides by joining of the 3′- and 5′-ends [29,30], and the formation of a hairpin loop structure at the 3′-end [31,32]. More recently, uniformly modified oligonucleotide N3′-P5′ phosphoramidates, in which every 3′-oxygen is replaced by

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Abbreviations: NP, nucleoprotein; PB, basic polymerase; Dz, DNA enzyme; N, oligonucleotide N3′-P5′ phosphoramidate; M, 2′-O-methyl-nucleoside; I, inactive DNA enzyme; PR/8, influenza virus A/Puerto Rico/8/34 (H1N1); DOTAP, {*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate; MDCK cell, Madin–Darby canine kidney cell; moi, multiplicity of infection; FITC, fluorescein isothiocyanate

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a 3′-amino group, were synthesized by Gryaznov et al. [33,34]. These compounds have very high affinity for single-stranded RNA, and thus have potential utility as antisense agents.

In this paper, we describe the design of partially modified DNA enzymes with N3′-P5′ phosphoramidates [33] at both the 3′- and 5′-ends. These modified DNA enzymes might have enhanced stability against intracellular degradation by nucleases [35]. We also investigated the cleavage potentials of these novel DNA enzymes and compared their abilities to inhibit the expression of influenza virus A.

2. Materials and methods

2.1. Oligonucleotides

Unmodified DNA enzymes were synthesized by Hokkaido System Science (Sapporo, Japan). N3′-P5′ Phosphoramidate-modified DNA enzymes were synthesized by Transgenomic (South Plainfield, NJ, USA). The DNA sequences of these oligonucleotides are as follows: PB2Dz-9, 5′-TTCTTTCCAGGCTAGCTACAACGAATTGAATAT-3′, and PB2Dz-9-I: 5′-TTCTTTCCAGGCAACATCGATCGAATT-GAATAT-3′.

2.2. Virus and cells

Madin–Darby canine kidney (MDCK) cells were kindly provided by K. Nerome (National Institute of Health, Tokyo, Japan). The cells were grown as a monolayer stationary culture in modified Eagle's medium (MEM) supplemented with 7.5% sodium hydrogen carbonate, 10% calf serum, and 20 mg/ml fungizone. Influenza A/PR/8/34 (H1N1) virus was grown in the allantoic cavity of 10 day old embryonated chicken eggs for 48 h at 35°C. The allantoic fluid was clarified by centrifugation at $6000\times g$ for 15 min and stored at −80°C.

2.3. Stability assay of N3′-P5′ phosphoramidate DNA enzymes

Briefly, each unlabeled DNA enzyme (6.6 μg) was incubated in 90 μl of 10% fetal bovine serum (FBS) at 37°C, and duplicate 5 μl samples were removed at 0, 1, 2, 4, 8, 12, and 24 h. Immediately upon sampling, a 295 μl aliquot of 50 mM Tris/EDTA was added to the 5 μl aliquot, and a phenol/chloroform extraction was performed. All of the samples from each time point were run directly on 20% polyacrylamide gels without further purification or precipitation, thus revealing all of the intact oligonucleotides and degradation products. Densitometric analysis of gels stained with silver nitrate was performed on an FLA-2000G image (Fuji Photo Film, Tokyo, Japan).

2.4. In vitro cleavage and kinetic analyses

To determine the cleavage activity of the DNA enzymes, a substrate RNA (PB2 mRNA) was synthesized by Genset (France), and labeled with fluorescein isothiocyanate (FITC) at the 5′-terminus. The sequence of this RNA is as follows: PB2 mRNA, 5′-FITC-AUUAU-AUUCAAUAUGGAAAGAAUAA-3′. Cleavage was performed with 10 μM DNA enzyme and 1 μM synthetic PB2 mRNA, in 25 mM $MgCl_2$ and 50 mM Tris–HCl (pH 7.5), with the reactions incubated at 37°C for 10, 20, 30, 60, or 120 min. The reactions were stopped with 50 mM EDTA, 9 M urea, and 0.1% xylene cyanol, denatured for 1 min at 95°C, and cooled on ice. The product fragments and the unreacted substrate in these samples were resolved by electrophoresis on a 20% denaturing polyacrylamide gel. The extent of the reaction at each time point was determined by densitometry of the gel image produced through a FLA-2000G image (Fuji Photo Film).

The efficacy of the DNA enzyme in vitro was determined by measuring the rate of RNA cleavage under multiple turnover conditions. Reactions were performed with 0.02 μM DNA enzyme and five different concentrations of a FITC-labeled synthetic RNA substrate (0.2, 0.4, 1.0, 1.6, or 3.2 μM) with 25 mM $MgCl_2$ in 50 mM Tris–HCl (pH 7.5), with the reactions incubated at 37°C for 5, 10, 20, 30, 60, and 120 min. The values for k_{obs} (derived from the slope of these time course experiments) were used to generate a best fit line in a modified Eadie–Hofstee plot (k_{obs} versus k_{obs}/[S]). In this expression, the values for K_M and K_{cat} are derived from the negative slope of the regression line and the intercept, respectively.

2.5. Inhibition of influenza virus replication by modified DNA enzymes

The inhibitory effect on influenza virus replication by the DNA enzyme was determined with a plaque-forming assay. MDCK cells were cultured in Dulbecco's minimal essential medium (MEM; Sigma, St. Louis, MO, USA) supplemented with 5% heat-inactivated FBS at 37°C in 5% CO_2. MDCK cells were plated at 2×10^5 cells/well in a 6 well plate and cultured for 24 h. Each DNA enzyme (final concentration 1, 5, and 10 μM) was transfected with the {*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate (DOTAP) transfection reagent (Roche, Indianapolis, IN, USA). After 4 h, the MDCK cells were washed with phosphate-buffered saline (PBS) and infected with influenza virus A/Puerto Rico/8/34 (H1N1) or influenza virus B (B/Ibaraki) at a multiplicity of infection (moi) of 0.01 at 37°C for 40 min. After infection, the cells were washed with PBS and cultured in MEM containing 0.1% heat-inactivated FBS and 2 μg/ml of trypsin (Denka Seiken, Tokyo, Japan) for 3 days. The cells were harvested, and the titer of the viruses was measured by a plaque formation assay on MDCK cells after three cycles of freezing and thawing. Confluent MDCK cells on 6 well plates were inoculated with serial dilutions of the viruses at 37°C for 40 min, and then overlaid with MEM containing 0.8% agarose, 5 μg/ml trypsin, and 0.1% FBS. After a 3 day incubation at 37°C, the virus titer was calculated from the plaques.

3. Results

3.1. Design and stability assay of N3′-P5′ phosphoramidate DNA enzymes

For efficient catalysis in vivo, the length of the antisense arms that recognize the target sequence and the use of modified oligonucleotides to enhance nuclease resistance in vivo are both important. The degradation of unmodified phosphodiester DNA enzymes in cultured cells, culture media, and serum occurs primarily as a result of the 3′-exonuclease activity. Investigations have focused on designing more stable DNA enzymes with phosphorothioate [25,26] or 2′-O-methyl groups [26] at both the 5′- and 3′-ends, and with 3′,3′-linked inverted thymidines [23,24]. In this paper, we studied the nuclease sensitivities of modified and unmodified DNA enzymes. To cleave the PB2 mRNA, we targeted the translation initiation region (AUG) of the influenza A virus (A/PR/8/34). To confer serum nuclease resistance to the DNA enzymes, we designed native DNA enzymes (PB2Dz-7, PB2Dz-8, and PB2Dz-9), and modified DNA enzymes with different arm lengths [PB2Dz-9-N and PB2Dz-9-N(*2)] with one or two N3′-P5′ phosphoramidate modifications at both the 3′- and 5′-ends (Fig. 1). We previously reported that antisense phosphorothioate oligonucleotides targeting the AUG initiation codon sequences of PB2 mRNA effectively inhibited the influenza virus RNA polymerase activity and influenza virus replication in infected cells [36,37] and mice [5,38]. For comparison with the active DNA enzyme, we also designed inactive DNA enzymes, PB2Dz-9-I, PB2Dz-9-IN, and PB2Dz-9-IN(*2) (same arm sequences as PB2Dz-9, PB2Dz-9-N, and PB2Dz-9-N(*2) with an inverted catalytic core sequence) [24]. We therefore prepared PB2Dz-9-M(*2), corresponding to PB2Dz-9-N(*2), as a control modified DNA enzyme with two 2′-O-methyl-ribonucleosides introduced at both the 3′- and 5′-ends, according to the procedure of Warashina et al. [26] (Fig. 1).

We analyzed the stability of the modified DNA enzymes, PB2Dz-9-N and PB2Dz-9-N(*2), and the control modified DNA enzyme, PB2Dz-9-M(*2), in 10% FBS (Fig. 2). The synthetic oligonucleotides were incubated in 10% FBS for 1, 2, 4, 8, 12, and 24 h at 37°C, and the products were analyzed by electrophoresis on a 20% polyacrylamide gel. Significant portions of the unmodified PB2Dz-9 were completely de-

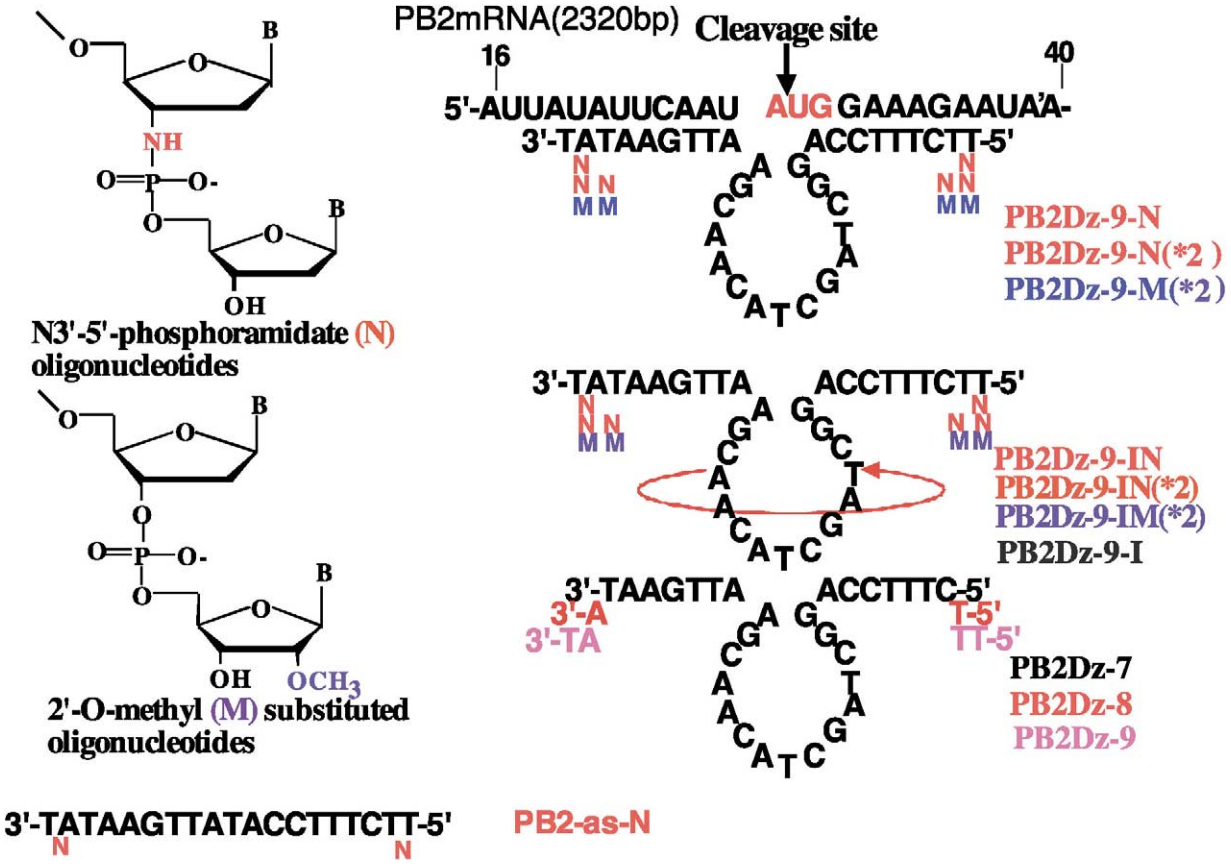

Fig. 1. A schematic representation of DNA enzymes with 9×9 arms, modified at both the 5′- and 3′-ends. A DNA enzyme can be targeted to cleave the phosphodiester bonds between a selected unpaired purine and a paired pyrimidine by virtue of the nucleotide sequences in its 5′ and 3′ arms. The figure shows the DNA enzyme targeting the translation start site of the influenza A virus, PB2 mRNA. The antisense deoxyribooligonucleotide (PB2-as-N) was targeted to the translation start site of the influenza A virus, PB2 mRNA. Its N3′-P5′ phosphoramidate-modified derivatives include: N, N3′-P5′ phosphoramidate-substituted residues, and N(*2), two N3′-P5′ phosphoramidate-substituted residues; M(*2), two 2′-O-methyl-substituted residues; I, inverted catalytic domain; as, antisense oligonucleotide.

graded within 8 h (Fig. 2). In contrast, the modified PB2Dz-9-N and PB2Dz-9-N(*2), and the control modified oligonucleotide, PB2Dz-9-M(*2), remained even after a 24 h incubation. PB2Dz-9-M(*2), however, was significantly less stable than the N3′-P5′ phosphoramidate-modified DNA enzymes. The PB2Dz-9-N and PB2Dz-9-N(*2) enzymes had substantially greater stability in serum as compared with the unmodified DNA enzyme, PB2Dz-9.

3.2. *Cleavage activity of DNA enzymes for the PB2 mRNA substrate*

Before examining the DNA enzymes in cells, we investigated their cleavage activity against the PB2 mRNA substrate in vitro. To determine whether the DNA enzyme cleaved RNA, we synthesized a substrate composed of 25 nucleotides, including the PB2 mRNA translation initiation region. Reactions were performed in 50 mM Tris–HCl (pH 7.5) and 25 mM $MgCl_2$ under single turnover conditions at 37°C. The unmodified DNA enzymes [PB2Dz-7 (7-mer), PB2Dz-8 (8-mer), and PB2Dz-9 (9-mer)] and the modified DNA enzymes [PB2Dz-9-N, PB2Dz-9-N(*2), and PB2Dz-9-M(*2)] cleaved the 25 nucleotide RNA (labeled with FITC at the 5′-end) (Fig. 3A,B). The 13 nucleotide product was consistent with the distance between the A28–U29 junction and the 5′- (FITC-labeled) end of the substrate. The longer-armed DNA enzyme (PB2Dz-9) had more powerful cleavage activity than the corresponding shorter-armed DNA enzymes (PB2Dz-7 and 8) (Fig. 3A). Furthermore, the modified DNA enzymes [PB2Dz-9-N, PB2Dz-9-N(*2), and PB2Dz-9-M(*2)] also exhibited strong cleavage activity (Fig. 3B). In contrast, the inactive DNA enzymes [PB2Dz-9-I, PB2Dz-9-IN, and PB2Dz-9-IM(*2)] did not catalyze the release of the

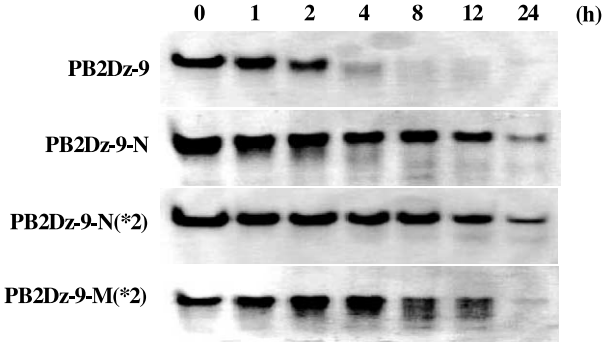

Fig. 2. Stability assay of the modified DNA enzymes in FBS. DNA enzymes were incubated in 10% FBS. Samples were collected at different time points as indicated, and the products were analyzed by electrophoresis on a 20% polyacrylamide gel. Densitometric analysis of gels stained with silver nitrate was performed on a FLA-2000G image (Fuji Photo Film).

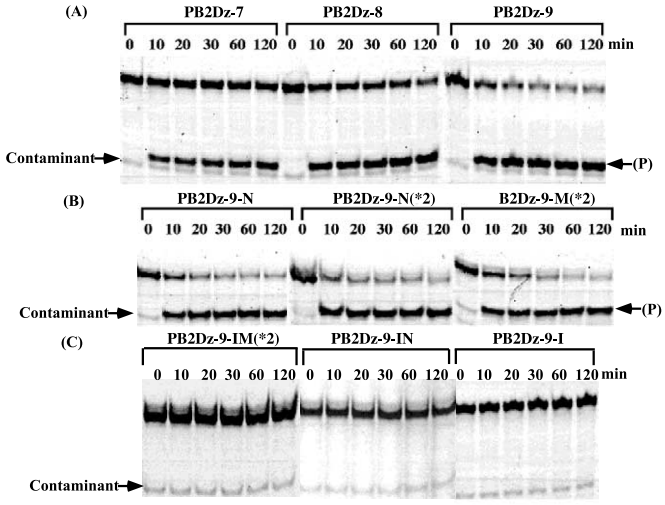

Fig. 3. Cleavage activity and specificity of the DNA enzymes in vitro. The unmodified (A) and modified (B) DNA enzymes. Inactive DNA enzymes (C). The substrate RNA, with the sequence 5′-FITC-AUUAUAUUCAAUAUGGAAAGAAUAA-3′, was cleaved by all three DNA enzymes (PB2Dz-7, 8, and 9) to generate a single product (P). The DNA enzyme (1 μM) and the synthetic PB2 mRNA (0.1 μM) were incubated at 37°C for the indicated times in 25 mM $MgCl_2$ and 50 mM Tris–HCl (pH 7.5). The reactions were stopped with 50 mM EDTA, 9 M urea, and 0.1% xylene cyanol, denatured for 1 min at 95°C, and cooled on ice. Each reaction mixture was then subjected to electrophoresis on an 18% polyacrylamide/7 M urea gel.

cleavage product (P) (Fig. 3C). To further characterize the modified DNA enzymes, we determined their kinetic parameters for cleavage of the PB2 mRNA substrate under multiple turnover conditions.

The overall catalytic efficiency of each deoxyribozyme, as measured by the K_{cat}/K_M values, varied significantly between the modified and unmodified species. The activities of the unmodified DNA enzymes (PB2Dz-7, PB2Dz-8, and PB2Dz-9) were remarkably sensitive to the length of the substrate-binding arms (Table 1) [26,39]. In the case of the longer-armed DNA enzymes (PB2Dz-9), which have two additional nucleotides in their binding arms as compared with the shorter variants, the longer binding arms yielded higher K_{cat}/K_M values [39]. On the other hand, a comparison of the kinetic parameters revealed no significant differences in the K_{cat}/K_M values between the modified and unmodified DNA enzymes (Table 1). Therefore, we performed an in vivo assay using the modified DNA enzymes [PB2Dz-9-N and PB2Dz-9-N(*2), and PB2Dz-9-M(*2)], because of their similar kinetic parameters to those of the unmodified DNA enzyme (PB2Dz-9) and their nuclease resistance in the subsequent anti-influenza activity studies.

Table 1
Kinetic parameters of PB2 mRNA substrate cleavage

DNA enzyme	K_{cat} (min^{-1})	K_M (μM)	K_{cat}/K_M (μM^{-1} min^{-1})
PB2Dz-7	1.5×10^{-1}	8.5×10^{-1}	1.8×10^{-1} (±0.021)
PB2Dz-8	3.2×10^{-1}	6.0×10^{-1}	5.3×10^{-1} (±0.049)
PB2Dz-9	1.1	1.2	9.2×10^{-1} (±0.41)
PB2Dz-9-N	6.9×10^{-1}	4.2×10^{-1}	1.6 (±0.21)
PB2Dz-9-N(*2)	7.0×10^{-1}	6.7×10^{-1}	1.0 (±0.62)
PB2Dz-9-M(*2)	2.9×10^{-1}	3.4×10^{-1}	8.5×10^{-1} (±0.33)

Rate constants were measured with 25 mM $MgCl_2$ and 50 mM Tris–HCl (pH 7.5) under enzyme-saturating (multiple turnover) conditions at 37°C. Rate constants are averages of results from two sets of experiments.

3.3. Comparison of the anti-influenza activities and specificities of modified DNA enzymes in influenza virus A-infected MDCK cells

The in vivo antiviral activities of the unmodified and modified DNA enzymes were assessed on the basis of their inhibitory effects on the influenza virus A titer. We compared the efficacies of PB2Dz-9-N, PB2Dz-9-N(*2), and PB2Dz-9-M(*2), as representative DNA enzymes, together with the inactive DNA enzymes with inverted catalytic domains [PB2Dz-9-IN and PB2Dz-9-IM(*2)] and the antisense oligonucleotide (PB2-as-N) with one N3′-P5′ phosphoramidate modification at both the 3′- and 5′-ends, by examining the virus titer of A/PR/8/34-infected MDCK cells. In the reactions with the inactive DNA enzymes, PB2Dz-9-I, PB2Dz-9-IN, and PB2Dz-9-IM(*2), there was no cleavage activity, even when the enzymes were incubated with the substrate for 120 min (Fig. 3C). We used these as the inactive DNA enzymes in subsequent studies. The inhibition of influenza virus replication (virus titer) by DNA enzymes was determined with a plaque-forming assay on MDCK cells.

Inhibition experiments were performed with two different DNA enzymes, and the results of one set of experiments are summarized in Fig. 4. The concentration of DOTAP used in these experiments had no cellular toxicity. The DOTAP/oligonucleotide complex (67 μg/ml/10 μM oligo) was delivered daily for 10 consecutive days, and produced no toxicity. The DOTAP-encapsulated PB2Dz-9-N and PB2Dz-9-N(*2) oligonucleotides both suppressed influenza virus (A/PR/8/34) expression in MDCK cells with extremely high efficacy (>99% inhibition). The control modified DNA enzyme, PB2Dz-9-M(*2), had a slightly lower inhibitory effect (84%) than PB2Dz-9-N and PB2Dz-9-N(*2) in the influenza virus (A/PR/8/34)-infected cells. When the controls, including the modified inactive DNA enzymes with one or two N3′-P5′ phosphoramidates at both the 3′- and 5′-ends [PB2Dz-9-IN and PB2Dz-9-IN(*2)] and the 2′-O-methyl-substituted ribonucleoside derivative DNA enzyme, PB2Dz-IM(*2), were trans-

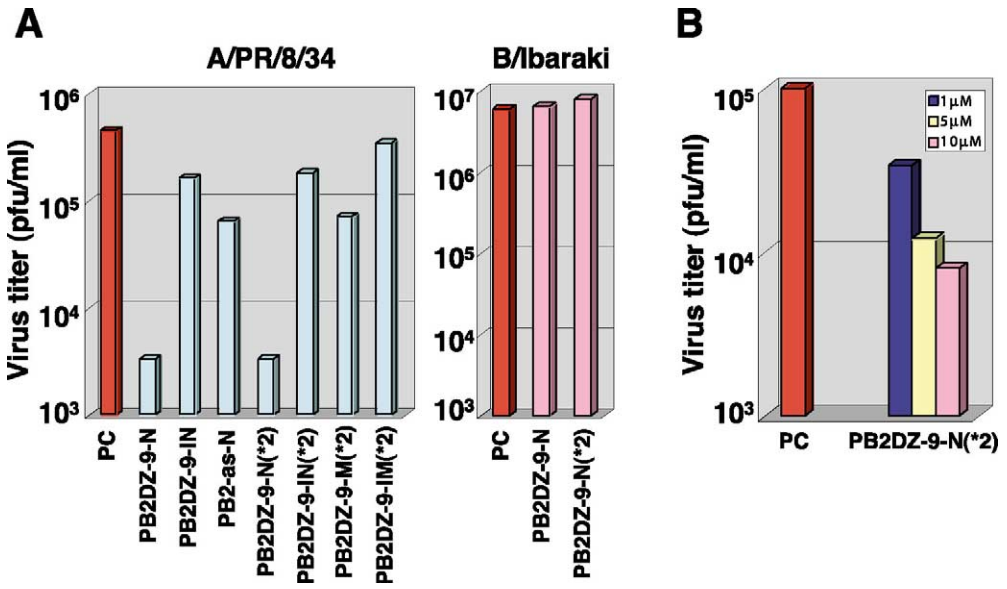

Fig. 4. Inhibition of influenza virus replication by modified DNA enzymes and antisense oligonucleotides. A: MDCK cells were transfected with a 10 μM concentration of either the DOTAP-encapsulated modified DNA enzymes or the antisense oligonucleotide (PB2-as-N), and infected with influenza virus A (A/PR/8/34) and influenza virus B (B/Ibaraki) at an moi of 0.01. B: The influenza virus (A/PR/8/34)-infected MDCK cells were treated with 1 μM, 5 μM, and 10 μM each of the DOTAP-encapsulated PB2Dz-9-N(*2). The inhibitory effect (virus titer) on influenza virus replication by the DNA enzyme was determined with a plaque-forming assay on MDCK cells.

fected with the DOTAP transfection reagent, the inactive DNA enzymes also had inhibitory effects (58%, 49%, and 22%) on the replication of influenza virus (A/PR/8/34). The protective effect of the modified DNA enzymes [PB2Dz-9-N and PB2Dz-9-N(*2)], however, was not observed in the influenza B virus, B/Ibaraki-infected MDCK cells (Fig. 4A). The nucleotide sequence identity between the PB2 mRNAs of the influenza A and B viruses is very low. Furthermore, the influenza virus (A/PR/8/34)-infected MDCK cells were treated with 1 μM, 5 μM, and 10 μM each of PB2Dz-9-N(*2) for 3 days (Fig. 4B). As expected, the N3′-P5′ phosphoramidate-modified DNA enzymes inhibited replication of the influenza virus (A/PR/8/34) in a dose-responsive manner.

We also examined the effect of the modified antisense oligonucleotide (PB2-as-N) with one N3′-P5′ phosphoramidate at both the 3′- and 5′-ends. The PB2-as-N had highly inhibitory effects, similar to those of the S-ODN-PB2 in the influenza virus (A/PR/8/34)-infected MDCK cells (Fig. 4A) [36,37]. The inhibitory effects of these modified DNA enzymes [PB2Dz-9-N and PB2Dz-9-N(*2)], however, were higher than that of the antisense oligonucleotide (PB2-as-N) (Fig. 4A).

4. Discussion

Influenza virus infection has the potential to become a much more dangerous disease than at present, because of easy transmission, antigenic shift, and viral drift, as well as the limited efficacy of current vaccines and therapies [40]. We demonstrated that modified DNA enzymes possess potent anti-influenza activity and confer significant protection to host cells challenged with influenza virus.

The in vivo antiviral activities of the new modified DNA enzymes were assessed on the basis of their inhibitory effects on the replication of influenza virus A. We designed DNA enzymes that have one or two N3′-P5′ phosphoramidate modifications at both the 3′- and 5′-ends [33] (Fig. 1). We also designed DNA enzymes with inverted catalytic domains [PB2Dz-9-IN and PB2Dz-9-IN(*2)] [33], and with two 2′-O-methyl-ribonucleosides [PB2Dz-9-M(*2)] at both the 3′- and 5′-ends [26] (Fig. 1).

We first studied the nuclease sensitivities of the modified and unmodified DNA enzymes. The use of one or two N3′-P5′ phosphoramidate [PB2Dz-9-N and PB2Dz-9-N(*2)], or 2′-O-methyl-substituted residues [PB2Dz-9-M(*2)] at the 5′- and 3′-ends significantly enhanced the nuclease resistance. Consistent with an earlier report [26], the modified DNA enzyme with the 2′-O-methyl-substitution [PB2Dz-9-M(*2)] was less nuclease-resistant than the modified DNA enzyme with the N3′-P5′ phosphoramidate.

We then investigated the cleavage activity against the PB2 mRNA substrate in vitro. The cleavage activity of the unmodified DNA enzymes was dependent on the length of the substrate-binding arms (Fig. 3A). Furthermore, the modified DNA enzymes [PB2Dz-9-N, PB2Dz-9-N(*2), and PB2Dz-9-M(*2)] also cleaved the 25 nucleotide substrate with a PB2 mRNA translation initiation region (Fig. 3B). On the other hand, the inactive DNA enzymes [PB2Dz-9-I, PB2Dz-9-IN, and PB2Dz-9-IM(*2)] had no cleavage activity (Fig. 3C). Furthermore, we determined the kinetic parameters for the cleavage of the PB2 mRNA substrate under multiple turnover conditions. The activities of the unmodified DNA enzymes (PB2Dz-7, PB2Dz-8, and PB2Dz-9) were remarkably sensitive to the length of the substrate-binding arms (Table 1). Comparisons of the kinetic parameters revealed no significant differences in the K_{cat}/K_M values between the unmodified and modified DNA enzymes (Table 1). From the above results, we selected the modified DNA enzymes [PB2Dz-9-N and PB2Dz-9-N(*2), and PB2Dz-9-M(*2)], with higher cleavage activity and nuclease resistance, for the subsequent anti-influenza activity studies [38]. As the control oligonucleotides, we used the inactive DNA enzymes with inverted catalytic do-

mains [PB2Dz-9-IN, PB2Dz-9-IN(*2), and PB2Dz-9-IM(*2)] and the antisense oligonucleotide (PB2-as-N) with one N3′-P5′ phosphoramidate modification at both the 3′- and 5′-ends. The modified PB2Dz-9-N and PB2Dz-9-N(*2) enzymes suppressed the influenza virus expression (A/PR/8/34) in MDCK cells with extremely high efficacy (>99% inhibition) (Fig. 4A). Although the control modified DNA enzyme, PB2Dz-9-M(*2), was also very active, its activity was lower than that of PB2Dz-9-N and PB2Dz-9-N(*2) (Fig. 4A). The protective effect of these modified DNA enzymes, however, was not observed in influenza B virus, B/Ibaraki-infected MDCK cells (Fig. 4A). On the other hand, the control modified inactive DNA enzymes [PB2Dz-9-IN and PB2Dz-9-IN(*2), and PB2Dz-9-IM(*2)] also had inhibitory effects (22–58%) (Fig. 4A). This is because these DNA enzymes include antisense sequences that bind to their target mRNA. Therefore, we also examined the effect of the modified antisense oligonucleotide (PB2-as-N) with one N3′-P5′ phosphoramidate at both the 3′- and 5′-ends. The modified PB2-as-N inhibited influenza virus A replication in the influenza virus (A/PR/8/34)-infected MDCK cells (Fig. 4A). These results suggest that the modified DNA enzymes might be capable of inducing a conventional antisense effect.

In conclusion, influenza virus A PB2 mRNA can be inhibited by partially modified DNA enzymes. These DNA enzymes exhibited higher efficacy than the antisense oligonucleotides. Among the limited modifications that we introduced, the N3′-P5′ phosphoramidates appeared to be the most effective, because the introduction of one or two such modifications at each end of the DNA enzyme (PB2Dz-9-N) resulted in significant nuclease resistance without loss of specificity. In contrast, the 2′-O-methyl-substituted version of the same DNA enzyme [PB2Dz-9-M(*2)] had no inhibitory effect, despite the increased nuclease resistance. The DNA enzymes are easier to synthesize and are more stable in vivo than ribozymes. The appropriately modified DNA enzymes possess potent anti-influenza activity and confer significant protection to host cells challenged with influenza virus.

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