Specific inhibition of influenza virus RNA polymerase and nucleoprotein gene expression by circular dumbbell RNA/DNA chimeric oligonucleotides containing antisense phosphodiester oligonucleotides

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Abstract We have designed 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. The reaction of the nicked (NDRDON) and circular (CDRDON) dumbbell RNA/DNA chimeric oligonucleotides with RNase H gave the corresponding antisense phosphodiester oligodeoxynucleotide together with the sense RNA cleavage products. The liberated antisense phosphodiester oligodeoxynucleotide was bound to the target RNA, which gave RNA cleavage products by treatment with RNase H. The circular dumbbell RNA/DNA chimeric oligonucleotide showed more nuclease resistance than the linear antisense phosphodiester oligonucleotide (anti-ODN) and the nicked dumbbell RNA/DNA chimeric oligonucleotide. The CDRDON with four target sites (influenza virus A RNA polymerases (PB1, PB2, PA) and nucleoprotein (NP)) was synthesized and tested for inhibitory effects by a CAT-ELISA assay using the clone 76 cell line. The circular dumbbell DNA/RNA chimeric oligonucleotide (CDRDON-PB2-as) containing an AUG initiation codon sequence as the target of PB2 showed highly inhibitory effects.

Key words: Circular dumbbell RNA/DNA chimeric oligonucleotide; Antisense phosphodiester oligonucleotide; Melting temperature; RNase H; Exonuclease resistance; Influenza virus; RNA polymerase; Nucleoprotein; CAT assay

1. Introduction

Antisense oligonucleotides have been used to regulate gene expression [1–4]. Unmodified (phosphodiester) antisense oligonucleotides have been reported to have an inhibitory effect against HIV-1 [5,6]. Unmodified oligonucleotides, in particular, have limited survival in vitro and in vivo. These oligonucleotides are degraded by nucleases present in serum and in cells [7,8]. Antisense oligonucleotides with phosphorothioate backbones exhibit several advantages over the other forms, including relatively high nuclease resistance and the capacity to induce the degradation of the target sequence by RNase H [9,10]. However, phosphorothioate oligonucleotides hybridize more weakly with the complementary nucleic acids than the unmodified oligonucleotides and are eventually degraded, primarily from the 3′ end. Phosphorothioate oligonucleotides have also been shown to block the proliferation of HIV-1 in acutely infected cells in a non-sequence-specific manner [11], probably by the inhibition of reverse transcriptase [12,13] and/or the viral entry process [14,15]. Another problem in the use of antisense phosphorothioate oligonucleotide is their inefficient cellular uptake. One approach to these problems has been the development of modified antisense oligonucleotides with P=O groups in the internucleotide bonds. In addition, the degradation of phosphodiester oligonucleotides can be slowed considerably by blocking 3′ or 3′ and 5′ ends of the chain, because the primary degrading enzymes present in cells are the 3′-exonuclease type [16,17]. Several stabilization methods for the phosphodiester oligonucleotides have been proposed, such as the incorporation of various chemical substituents at the 3′-hydroxyl groups [16–18], the circularization of the oligonucleotides by joining the 3′ and the 5′ ends [19–21], and the formation of a hairpin loop structure at the 3′ end [22,23]. The circular dumbbell DNA oligonucleotides have biological relevance as aptamers or decoys for hybridizing proteins such as transcription factors [24,25].

In this paper, 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 and cellular uptake. The antisense phosphodiester deoxyoligonucleotide is liberated by RNase H treatment of the dumbbell RNA/DNA chimeric oligonucleotides. We have also tested the inhibition of influenza virus RNA polymerase and nucleoprotein gene expression by dumbbell RNA/DNA chimeric phosphodiester as determined by CAT protein expression (CAT activity), in the clone 76 cell line [26,27].

2. Materials and methods

2.1. Oligonucleotide synthesis

The oligonucleotides were synthesized by means of the phosphoramidite approach using an Applied Biosystems DNA synthesizer, Model 392. The 5′-phosphorylated oligonucleotides were synthesized using a dimethoxytrityl-hexa-ethyloxy-glycol-2-cyanoethyl-*N*,*N*-diisopropyl phosphoramidite as the phosphorylating agent. The oligonucleotide derivatives were purified by polyacrylamide gel electrophoresis or by reverse phase HPLC chromatography. The 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. The 5′-phosphorylated nicked dumbbell RNA/DNA chimeric oligonucleotide (0.2 A_{260}) was incubated in 10 μl of 50 mM Tris-HCl, pH 7.5, and 10 mM $MgCl_2$ at 90°C for 5 min, and then at 15°C for 2 min. The solution was incubated with 1 μl of 100 mM dATP, 1 μl of 25 μg BSA per ml, and 40 units of T4 DNA ligase at 37°C for 30 min. The reaction mixture was extracted with an equal

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volume of water-saturated chloroform/phenol (1:1, v/v), and was precipitated with ethanol. The circular oligonucleotides were purified by denaturing PAGE (20% polyacrylamide containing 7 M urea). The band corresponding to the desired oligonucleotide was eluted from the gels with water, and was precipitated with ethanol. The identity of each of the ligated dumbbells was verified by phosphodiesterase protection mapping.

2.2. Nuclease stability of nicked and circular dumbbell RNA/DNA chimeric oligonucleotides

To determine the resistance of the oligonucleotides to nucleases in fetal bovine serum, the oligonucleotides (0.2 A_{260}) were incubated with 200 μl of culture medium containing 10% fetal bovine serum for 16 h at 37°C. Aliquots were taken at 0, 3, 6, 12, and 24 h and were analyzed by PAGE (20% polyacrylamide containing 7 m urea). Densitometric analysis of gels stained with silver nitrate was performed on a Millipore BioImage 60 S.

2.3. RNase H activity

The 45 mer RNA (5′-UGUUUCACAACAAAAGCCUUAGGCAUCUCCAUGGCAGGAGAAG-3′) (1 pmol) was mixed with oligonucleotides (10 pmol) in 30 μl of 20 mM Tris-HCl (pH 7.5), 10 mM $MgSO_4$, 0.1 mM DTT, and 100 mM KCl. RNasin (40 units) and *Escherichia coli* RNase H (0.5 μl, 0.4 units) were added to the mixture, which was incubated at 37°C. Aliquots were taken at 0, 1, 2, and 4 h, and were analyzed by PAGE (20% polyacrylamide containing 8.3 M urea). Densitometric analysis of gels stained with silver nitrate was performed on a Millipore BioImage 60 S.

2.4. Cellular uptake of ^{32}P-labeled nicked and circular dumbbell RNA/DNA chimeric oligonucleotides

The 5′ ends of the nicked dumbbell RNA/DNA chimeric oligonucleotide were labeled with [γ-^{32}P]dATP and T4 polynucleotide kinase in buffer containing 100 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 10 mM DTT, 0.2 mM spermidine, and 0.2 mM EDTA at 37°C for 30 min. The labeled oligonucleotides were purified by denaturing PAGE (20% polyacrylamide containing 7 M urea). The band corresponding to the desired oligonucleotide was eluted from the gels with water, and precipitated with ethanol. The circularization of the 5′-phosphorylated nicked dumbbell RNA/DNA chimeric oligonucleotide to give the circular dumbbell RNA/DNA chimeric oligonucleotide was carried out as described above, and then the oligonucleotide was purified using a Quick spin column (G-25). MDCK cells were diluted to 1×10^5 cells/ml in RPMI 1640 medium containing 10% heat-inactivated FCS and were dispensed in 24 multi-well plates. After a 48 h incubation at 37°C in a CO_2 incubator, the RPMI 1640 medium was changed. The cells were further incubated with the 3′ end-labeled oligomers for the stated periods. After washing the cells four times with chilled phosphate-buffered saline (PBS), the cells were pelleted by centrifugation and were lysed in 0.5 ml of 1% sodium dodecyl sulfate (SDS). The cell associated radioactivity was determined by liquid scintillation counting and is expressed as pmol/10^5 cells.

2.5. Cells

The murine C127 derivative cell line, clone 76 [26,27], was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS).

2.6. In vitro RNA synthesis

The plasmid pOUMS101 contains the CAT gene sandwiched between the 5′- and 3′-terminal sequences of the cDNA encoding RNA segment 8 of influenza virus A/PR/8/34 in the antisense orientation of the T7 RNA polymerase [28]. Transcription of *Mbo*II-digested pOUMS101 by T7 RNA polymerase results in the synthesis of the antisense CAT RNA. The template DNA was removed by treatment with DNase I. The RNA transcript was purified by TBE/7 M urea, 5% polyacrylamide gel electrophoresis, elution, and ethanol precipitation in the presence of 3 M sodium acetate.

2.7. 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*-trimethylammonium-methylsulfate; 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).

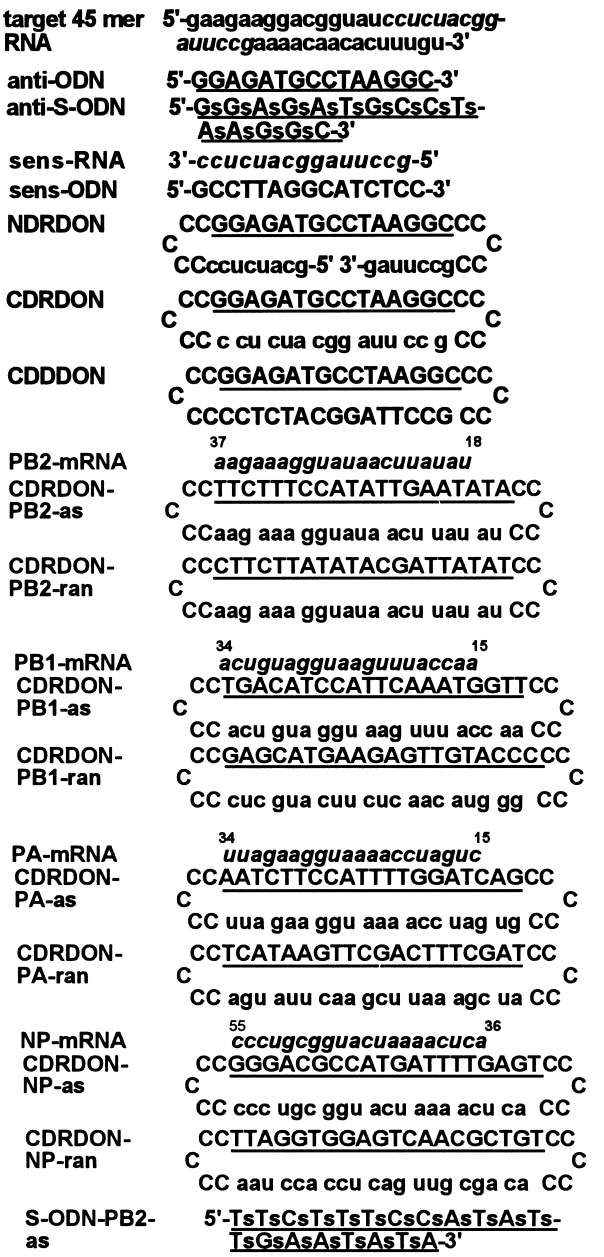

Fig. 1. The structures and sequences of the oligonucleotides used in this study and described in the text. Capital letters: DNA; small letters: RNA. The antisense sequences are underlined. Targeted RNA: 3′ to 5′ (italics); synthetic oligonucleotides: 5′ to 3′. Abbreviations: as, antisense sequence; ran, random sequence.

3. Results and discussion

3.1. Nuclease sensitivities of nicked and circular dumbbell RNA/DNA chimeric oligonucleotides

The nuclease sensitivities of the nicked (NDRDON) and the

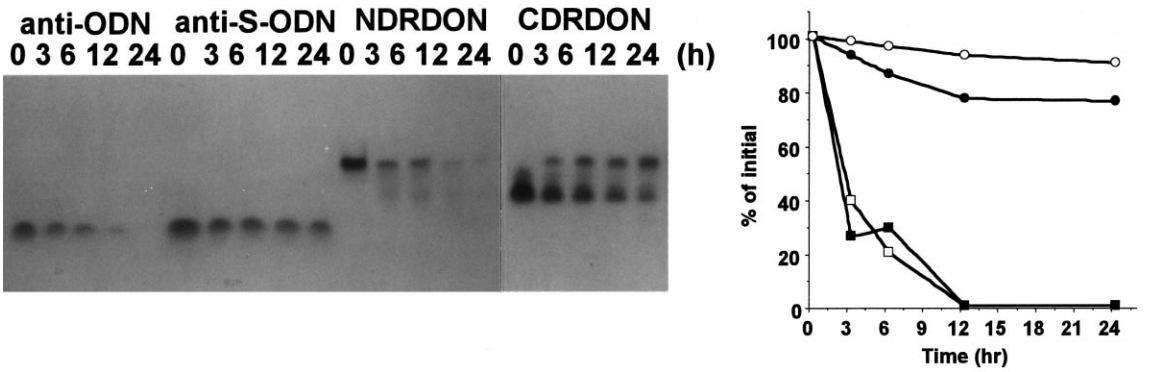

Fig. 2. Digestion of the antisense oligonucleotide and nicked, and circular dumbbell RNA/DNA chimeric oligonucleotides in the presence of 10% calf serum at 37°C for 0–24 h. Percentages of the anti-ODN, anti-S-ODN, NDRDON, and CDRDON are shown as open squares, solid circles, solid squares, and open circles, respectively. Data represent average values of at least three different experiments.

circular (CDRDON) dumbbell RNA/DNA chimeric oligonucleotides with the RNA-DNA base pairs (sense (RNA) and antisense (DNA)) in the double helical stem were studied in fetal bovine serum (Fig. 2). For comparison, the anti-ODN and the anti-S-ODN were chosen as controls. The results obtained by incubating the circular dumbbell oligonucleotides in fetal bovine serum provided additional evidence of their relative stability (Fig. 2). The CDRDON was stable (90%) after 24 h of incubation, whereas the anti-S-ODN was stable (76%) after 24 h. The NDRDON and the anti-ODN were completely degraded (100%) after 12 h. In the fetal bovine serum assay, the circular dumbbell oligonucleotide was converted to an open circular oligonucleotide by running on denaturing PAGE (20% polyacrylamide containing 8.3 M urea), and it remained stable after 24 h when the circular dumbbell oligonucleotide was converted to the nicked dumbbell oligonucleotide, it was quickly digested. The stability of the oligonucleotides was increased with two hairpin loop structures with RNA-DNA base pairs (sense-RNA and antisense-DNA) in the stem at the 3′ and 5′ ends.

3.2. Duplex hybridization

The hybridization between antisense-DNA and sense-RNA in the dumbbell RNA/DNA chimeric phosphodiester oligonucleotides is the key to the nuclease resistance in vitro as well as in vivo. We measured the melting temperature of oligonucleotides with either antisense phosphodiester (anti-ODN) or phosphorothioate (anti-S-ODN) bonds with the complementary sense phosphodiester oligodeoxyribonucleotide (sens-ODN) and oligoribonucleotide (sens-RNA) (Fig. 1). The T_m values of the duplexes of the phosphodiester anti-ODN with the phosphodiester sens-ODN and the sens-RNA were 40°C and 41°C, respectively (Table 1). The T_m values of the duplexes of the phosphorothioate anti-S-ODN with the phosphodiester sens-ODN and the sens-RNA were 33°C and 28°C, respectively (Table 1). The antisense oligonucleotides (anti-ODN) containing the phosphorothioate bonds hybridized more weakly with the complementary nucleic acids than the unmodified oligonucleotides. Next, we measured the melting temperatures of the nicked (NDRDON) and the circular (CDRDON) dumbbell RNA/DNA chimeric oligonucleotides. The T_m values of the NDRDON and the CDRDON were 47°C and 81°C, respectively. The nicked (NDRDON) and the circular dumbbell (CDRDON) DNA/RNA chimeric oligonucleotides had higher T_ms than the DNA-DNA and DNA-RNA duplexes. Furthermore, the circular oligonucleotide (CDDDON) with the DNA-DNA base pairs (sense (DNA) and antisense (DNA)) in the double helical stem has an estimated T_m of 71°C, which is 10°C less than the T_m of the CDRDON. These results suggest that the stability of the oligonucleotides also increased with the introduction of the two hairpin loop structures to the RNA-DNA base pairs (sens (RNA) and antisense (DNA)) in the double helical stem.

Next, we measured the melting temperatures of the oligonucleotides bound with the complementary 45-mer RNAs (5′-UGUUUCACAACAAAAGCCUUAGGCAUCUCCAUGGCAGGAGAAG-3′) (Table 1, Fig. 1). The T_m value of the duplex between the anti-ODN and the 45-mer RNA was 55°C. On the other hand, when the double-stranded DNA (anti-ODN/sens-ODN) was mixed with the 45-mer RNA, two transitions were observed: one, at 53°C, which was typical of the duplex between the anti-ODN and the 45-mer DNA, and one, at 41°C, which coincided with the double-stranded DNA. The T_ms of the duplexes between the nicked dumbbell RNA/DNA chimeric oligonucleotide (NDRDON) and the 45-mer RNA approached the T_m values of the duplex between the anti-ODN and the 45-mer RNA (51°C). In contrast, the circular (CDRDON) dumbbell RNA/DNA chimeric oligonucleotides did not undergo two transitions (Table 1). Furthermore, when the amount of the 45-mer RNA was in-

Table 1
Melting temperatures of oligonucleotides

Sequence	T_m (°C)[a,c]
anti-ODN/sens-ODN	40
anti-ODN/sens-RNA	41
anti-S-ODN/sens-ODN	33
anti-S-ODN/sens-RNA	28
NDRNON	47
CDRNON	81
CDDDON	71
anti-ODN/45-mer RNA	55
anti-ODN/sens-ODN/45-mer RNA	41, 53
CDRNON/45-mer RNA	80
NDRNON/45-mer RNA	51
CDRNON/45-mer RNA[b]	81
NDRNON/45-mer RNA[b]	52

[a]Values were obtained in 10 mM sodium phosphate buffer and 10 mM NaCl at pH 7.0.
[b]This reaction was carried out in the presence of 5 molar equivalent of the target 45-mer RNA.
[c]Data represent average values of at least three different experiments.

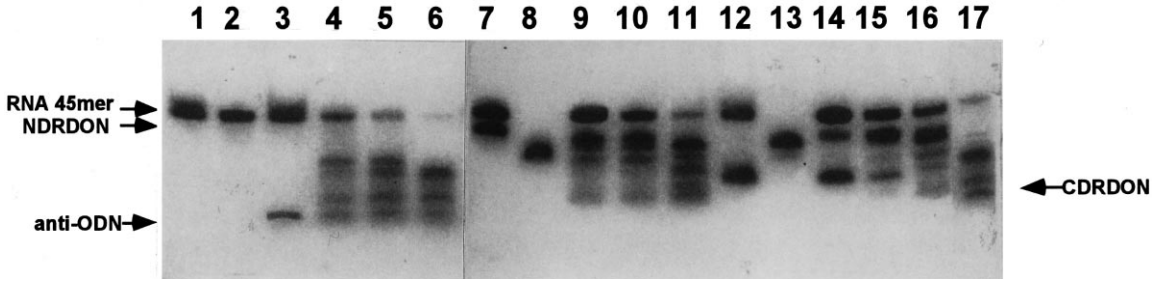

Fig. 3. Specific cleavage by RNase H of the target 45-mer RNA in the presence of the dumbbell RNA/DNA chimeric oligonucleotides (NDRDON and CDRDON), and the anti-ODN. Lane 1, control: 45-mer RNA; lane 2, control: 45-mer RNA/RNase H/4 h; lane 3, control: anti-ODN/45-mer RNA; lane 4, anti-ODN/45-mer RNA/RNase H/1 h; lane 5, anti-ODN/45-mer RNA/RNase H/2 h; lane 6, anti-ODN/45-mer RNA/RNase H/4 h; lane 7, control: NDRDON/45-mer RNA (faster run: NDRDON; slower run: 45-mer RNA); lane 8, control: NDRDON/RNase H/4 h (liberated anti-ODN from NDRDON); lane 9, NDRDON/45-mer RNA/RNase H/1 h (faster migrating band: liberated anti-ODN; slower migrating band: NDRDON; ladder bands: RNA cleavage product); lane 10, NDRDON/45-mer RNA/RNase H/2 h; lane 11, NDRDON/45-mer RNA/RNase H/4 h; lane 12, control: CDRDON/45-mer RNA; lane 13, control: CDRDON/RNase H/4 h (liberated anti-ODN from CDRDON); lane 14, CDRDON/45-mer RNA/RNase H/1 h (faster migrating band: CDRDON; intermediate migrating band: liberated anti-ODN; slower migrating band: 45-mer RNA); lane 15, CDRDON/45-mer RNA/RNase H/2 h; lane 16, CDRDON/45-mer RNA/RNase H/4 h (faster migrating band: liberated anti-ODN; slower migrating band: 45-mer RNA; ladder bands: RNA cleavage product); lane 17, CDRDON/45-mer RNA/RNase H/6 h.

creased up to five molar equivalent under the above conditions, the T_ms of the duplexes between the nicked dumbbell RNA/DNA chimeric oligonucleotide (NDRDON) and the 45-mer RNA approached the T_m value of the duplex between the anti-ODN and the 45-mer RNA (52°C). These results suggest that the nicked dumbbell oligonucleotides were only partially bound to the complementary RNA. However, the T_m value of the duplex between the circular dumbbell RNA/DNA chimeric oligonucleotide (CDRDON) and the 45-mer RNA is 81°C, which is typical of the CDRDON. In particular, the circular dumbbell RNA/DNA chimeric oligonucleotide with the higher T_m value showed more nuclease resistance than the linear antisense phosphodiester oligonucleotide and the nicked RNA/DNA chimeric oligonucleotide (Fig. 1 and Table 1).

3.3. RNase H treatment of the dumbbell RNA/DNA chimeric oligonucleotides

The RNase H-mediated liberation of antisense phosphodiester oligodeoxynucleotides from the dumbbell RNA/DNA chimeric oligonucleotides was studied. The RNase H activity assay was carried out with the target 45-mer RNA and either the circular (CDRDON) or the nicked (NDRDON) dumbbell RNA/DNA chimeric oligonucleotides in the presence of *E. coli* RNase H for up to 4 h (Fig. 3). The antisense phosphodiester oligodeoxynucleotide (anti-ODN) was used for comparison, as the control oligonucleotide (Fig. 3, lanes 3–6). The target 45-mer RNA with the anti-ODN was completely cleaved by *E. coli* RNase H within 4 h (Fig. 3, lane 6). On the other hand, the reaction of the NDRDON with RNase H gave the corresponding antisense phosphodiester oligodeoxynucleotide together with the RNA cleavage product (Fig. 3, lane 8). The target 45-mer RNA was then added to the above reaction, which produced the characteristically shortened RNA fragments (Fig. 3, lanes 9–11). After 4 h, the target 45-mer RNA was almost completely cleaved (90%) to the shortened RNA fragments (Fig. 3, lane 11). Furthermore, when the circular dumbbell RNA/DNA chimeric oligonucleotide (CDRDON) was used in place of the NDRDON, under the same conditions as described above, RNA template cleavage was observed (62%) (Fig. 3, lanes 14–16). In the case of the CDRDON, the reaction of the CDRDON with RNase H gave 100% of the corresponding antisense phosphodiester oligodeoxynucleotide together with the RNA cleavage product within 4 h (Fig. 3, lane 13). The RNase H cleavage rate for the target 45-mer RNA with the circular dumbbell RNA/DNA chimeric oligonucleotide (CDRDON) is slower than those for the nicked dumbbell RNA/DNA chimeric oligonucleotide (NDRDON) and the linear antisense oligonucleotides (anti-ODN and anti-S-ODN). However, after 6 h, the target 45-mer RNA with the circular dumbbell RNA/DNA chimeric oligonucleotide (CDRDON) was completely degraded by *E. coli* RNase H (Fig. 3, lane 17). The liberated antisense phosphodiester oligonucleotide is bound to the target mRNA; that is, the inhibition of viral replication occurs in a sequence-specific manner without the inhibition of RNase H activity. In contrast, the antiviral activity of the phosphorothioate oligonucleotide (S-ODN) is due to a direct effect of reverse transcriptase [12,13] and/or the viral entry process [14,15].

3.4. Cellular uptake of dumbbell RNA/DNA chimeric oligonucleotides

Oligonucleotides have been used as antisense inhibitors of gene expression in various culture systems and are considered

Table 2
Inhibition of influenza virus RNA polymerase and nucleoprotein gene expression by the circular dumbbell RNA/DNA chimeric oligonucleotides

Oligomer[a]	Inhibitory effect (%)[b,c]		
	0.03 μM	0.3 μM	1.0 μM
CDRDON-PB2-as	36	60	79
S-ODN-PB2-as	33	42	
CDRDON-PB2-ran	< 5	19	
CDRDON-PB1-as	< 5	15	21
CDRDON-PB1-ran	< 5	< 5	
CDRDON-PA-as	18	26	39
CDRDON-PA-ran	< 5	10	
CDRDON-NP-as	17	30	35
CDRDON-NP-ran	< 5	9	

[a]The antisense sequences are described in Fig. 1.
[b]The inhibitory effects are given as the percentage inhibtion of CAT protein expression, and are compared and normalized to 100% CAT protein expression.
[c]Data represent average values of at least three different experiments.

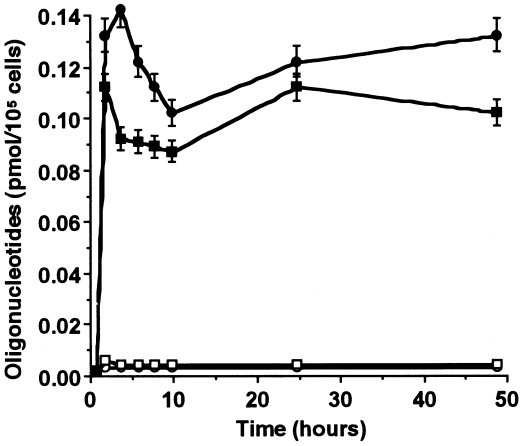

Fig. 4. Cellular uptake of the ^{32}P-labeled-CDRDON and NDRDON. The MOLT-4 cells were incubated with the ^{32}P-labeled anti-ODN (○), the ^{32}P-labeled NDRDON (□), the ^{32}P-labeled CDRDON (■), and the ^{32}P-labeled anti-S-ODN (●) at 37°C for various times. Cell-associated radioactivity was determined as described in Section 2. Experiments were carried out in triplicate and the data represent average values and standard deviations.

to be potential therapeutic agents against cancer and viral infections. In order to exert any of these effects, the oligonucleotides must enter the cytoplasmic and nuclear compartments of the cells. The problem in the use of antisense oligonucleotides is that the cellular uptake of oligonucleotides is inefficient [29,30]. Immunoliposomes have been studied as a transport system to deliver both membrane-permeable and impermeable molecules into cells [31,32]. However, in contrast to the use of endogenously transcribed or microinjected antisense oligonucleotides, regulatory activity of exogenous oligonucleotides appears to depend upon the uptake of sufficient amounts by cells. As shown in Fig. 4, the ^{32}P-labeled CDRDON strongly associated with the MDCK cells, but the ^{32}P-labeled NDRDON did not. The cell-associated radioactivity was negligible in the MDCK cells treated with the ^{32}P-labeled anti-ODN. On the other hand, the ^{32}P-labeled anti-S-ODN also strongly associated with the MDCK-4 cells. The cell-associated radioactivity in the MDCK cells treated with the ^{32}P-labeled CDRDON increased rapidly and reached a plateau after 60 min of treatment, and the NDRDON and anti-ODN oligomers yielded about 12-fold lower cell-associated counts than the CDRDON. A high level of cell-associated anti-S-ODN was observed. The circularization resulting from the joining of the 3′ and the 5′ ends of the RNA/DNA chimeric oligonucleotides containing two hairpin loop structures increases the cellular uptake, as compared with the nicked dumbbell RNA/DNA chimeric oligonucleotide (NDRDON) and the linear antisense phosphodiester oligonucleotides (anti-ODN).

3.5. *Inhibition of influenza virus RNA polymerase and nucleoprotein gene expression by circular dumbbell RNA/DNA chimeric oligonucleotides*

We have also tested the inhibition of influenza virus RNA polymerase and nucleoprotein gene expression by circular dumbbell RNA/DNA chimeric oligonucleotides as determined by CAT protein expression (CAT activity), in the clone 76 cell line [26,27]. The CDRDON containing antisense sequences as targets of PB1, PB2, PA, and NP, and random sequences with the same base composition as the four targets are illustrated in Fig. 1. The in vitro activities of these oligonucleotides on the expression of the influenza virus RNA polymerase and nucleoprotein genes were assessed on the basis of their inhibition of CAT protein expression with the CAT-ELISA method. The circular dumbbell RNA/DNA chimeric oligonucleotides were introduced into the clone 76 cells by liposome-mediated transfection.

The CDRDON containing an AUG initiation codon sequence as the target of PB2 (CDRDON-PB2-as) had the highest inhibitory effect, causing more than 50% inhibition at a 0.3 μM concentration (Table 2). The CDRDON-PA-as and NP-as, containing the AUG initiation codon sequence targeted to PA and NP, showed 26% and 30% inhibition of CAT protein expression at a 0.3 μM concentration. In contrast, the CDRDON-PB1-as, containing the AUG initiation codon targeted to PB1, showed lower anti-CAT activity at a 0.3 μM concentration (Table 2). As control sequences, the random oligonucleotides showed no inhibitory effects on the target AUG initiation codon. Furthermore, the circular dumbbell RNA/DNA chimeric oligonucleotides, CDRDON-PB2-as, CDRDON-PB1-as, CDRDON-PA-as, and CDRDON-NP-as, showed increased inhibition efficiency with an increase in the dumbbell RNA/DNA chimeric oligonucleotides (Table 2). These results suggest that the circular dumbbell RNA/DNA chimeric oligonucleotides conferred sequence-specific inhibition (Table 2). On the other hand, the phosphorothioate oligodeoxynucleotide (S-ODN-PB2-as) containing an AUG initiation codon sequence as the target of PBs was chosen for comparison with the CDRDON-PB2-as. The CDRDON-PB2-as showed higher inhibitory activity than S-ODN-PB2-as (Table 2) [33]. The phosphorothioate analogues have also been studied for their antiviral properties against influenza virus A [34]. The phosphorothioate analogues inhibited the replication of influenza virus A in a dose-dependent manner, and had no sequence-specific inhibition. In our experiment, sequence-specific inhibition could be obtained with influenza virus A by the circular dumbbell RNA/DNA chimeric oligonucleotides.

The circular dumbbell RNA/DNA chimeric oligonucleotide showed increased nuclease resistance and oligonucleotide uptake into cells, as compared with the nicked dumbbell RNA/DNA chimeric oligonucleotide and the linear antisense phosphodiester. Of particular interest, the antisense phosphodiester oligodeoxynucleotide is liberated together with the RNA cleavage product by RNase H treatment of the dumbbell RNA/DNA chimeric oligonucleotides. The liberated antisense phosphodiester deoxyoligonucleotide was bound to the target mRNA, which gave mRNA cleavage products upon the treatment with RNase H. In fact, circular dumbbell RNA/DNA chimeric oligonucleotides with four target sites (influenza virus A RNA polymerases (PB1, PB2, PA) and nucleoprotein (NP)) were synthesized and tested for inhibitory effects by a CAT-ELISA assay using the clone 76 cell line. The CDRDON-PB2-as containing an AUG initiation codon sequence as the target of PB2 showed highly inhibitory effects. That is to say, when the circular dumbbell RNA/DNA chimeric oligonucleotide is directly delivered into animal cells or virus-infected cells, its antisense phosphodiester oligodeoxynucleotide function appears. The limited toxicity of unmodified phosphodiester oligonucleotides and the sequence-specific binding to target mRNA indicate that circular dumbbell

RNA/DNA chimeric phosphodiester oligonucleotide can be used with intact cells, and may prevent viral development in culture.

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