Efficient Target Site Selection for an RNA-cleaving DNAzyme through Combinatorial Library Screening

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Identification of accessible sites in targeted RNAs is a major limitation to the effectiveness of antisense oligonucleotides. A class of antisense oligodeoxynucleotides, known as the "10-23" DNA enzyme or DNAzyme, which is a small catalytic DNA, has been shown to efficiently cleave target RNA at purine-pyrimidine junctions *in vitro*. We have designed a strategy to identify accessible cleavage sites in the target RNA, which is hepatitis C virus nonstructural gene 3 (HCV NS3) RNA that encodes viral helicase and protease, from a pool of random DNAzyme library. A pool of DNAzymes of 58 nucleotides-length that possess randomized annealing arms, catalytic core sequence, and fixed 5'/3'-end flanking sequences was designed and screened for their ability to cleave the target RNA. The screening procedure, which includes binding of DNAzyme pool to the target RNA under inactive condition, selection and amplification of active DNAzymes, incubation of the selected DNAzymes with the target RNA, and target site identification on sequencing gels, identified 16 potential cleavage sites in the target RNA. Corresponding DNAzymes were constructed for the selected target sites and were tested for RNA-cleavage in terms of kinetics and accessibility. These selected DNAzymes were effective in cleaving the target RNA in the presence of Mg^{2+}. This strategy can be applicable to identify accessible sites in any target RNA for antisense oligonucleotides-based gene inactivation methods.

Key Words : Antisense oligonucleotide, 10-23 DNAzyme, DNAzyme library, Gene inactivation, RNA cleavage

Introduction

The RNA-cleaving short DNA molecules, named as DNA enzymes (DNAzymes), were derived by *in vitro* selection from a combinatorial library of DNA sequences that are capable of cleaving a short target RNA molecule in a sequence-specific manner.[1] DNAzyme possessing a catalytic motif of "10-23" have the potential to bind and cleave any target RNA that contains a purine-pyrimidine junction (Fig. 1A), thus, allowing far greater flexibility in choosing the target sites than hammerhead and hairpin ribozymes.[1] The Watson-Crick base pairing of binding arms confers substrate recognition and binding of the DNAzyme to any desired target site of RNA. Hence, DNAzyme is an attractive tool for gene inactivation strategies. A number of research groups have utilized DNAzymes to selectively cleave and disrupt the function of target genes.[2-5]

Despite the flexibility in potential cleavage sites for DNAzyme, the selection and identification of DNAzymes that might be effective and optimal in binding and cleavage of the target RNA has to be determined empirically. Efforts to predict RNA secondary structure and its influence on the binding of other nucleic acids through free-energy minimization algorithm were not accurate, particularly longer RNAs. Thus, finding accessible sites in longer targeted RNAs gives a major limitation to the effectiveness of DNAzymes. To date, there were attempts to screen RNA targets for

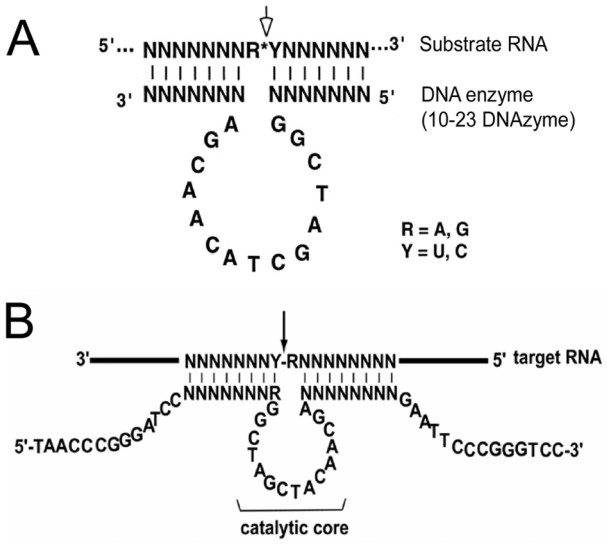

Figure 1. Schematic representation of the random DNAzyme library. (A) Diagram of a RNA-cleaving 10-23 DNAzyme, showing a central core 15-base sequence for a trans-acting RNA cleavage, flanked by 7-nt 5'/3' binding arms that form a DNAzyme-RNA substrate complex *via* Watson-Crick base pairing between generic deoxyribonucleotides (N) of the DNAzyme (bottom) and the corresponding ribonucleotides (N) in the target (top). Arrowhead indicates the site of cleavage. (B) Diagram of the DNAzyme oligodeoxynucleotide designed for a Dz-library. Catalytic core sequence is retained, and the 5'/3' binding arms are flanked with PCR-tails that contain a defined sequence.

accessible sites with a variety of strategies. RNaseH was used to probe the accessible sites by inducing site-specific hydrolysis where heteroduplexes are formed with conventional antisense oligodeoxynucleotides and target RNA.[6-9] Modified method for this technology was developed for the use of randomized and semi-randomized sequences, which allow for a universal approach to mapping accessibility in any target transcript.[10] Target site selection techniques based on the accessibility of transcripts produced *in vitro*, have also been attempted for ribozyme-based gene-suppression strategies. A combinatorial approach using libraries of molecules containing randomized binding domains or guide sequences have been used against target RNA to identify the most efficient cleavage sites for the hammerhead ribozyme, hairpin ribozyme, and group I intron.[11-13] Alternatively, a "mutiplex cleavage assay" was developed and shown to be useful for screening cleavage sites along the entire length of target mRNA, in which 10-23 DNAzymes that were constructed against entire potential cleavage sites in the target RNA were mixed and tested for accessibility and cleavage activity.[14] However, this method is more expensive and less flexible than the combinatorial approach using randomized sequences.

Here, we constructed random pool of 10-23 DNAzymes (Dz-library) that possess randomized annealing arms, catalytic core sequence, and fixed 5'/3'-end flanking sequences for recovery of bound species (Fig. 1B). After binding under inactive (magnesium-free) annealing conditions, the selected active Dz-library is incubated with target, and the sites of cleavage are identified on sequencing gels. Using full-length transcript of hepatitis C virus nonstructural gene 3 mRNA (HCV NS3 RNA, 1.54 kb in length) encoding the viral helicase and protease as a target mRNA, we demonstrate that active DNAzyme against HCV NS3 mRNA can be engineered using this selection protocol.

Materials and Methods

Construction of DNAzyme library and target RNA template. A single-stranded DNAzyme library containing 2.15×10^9 sequences (1.0 mg of DNA) was constructed by automated solid-phase synthesis (Bioneer primer synthesis service, Daejeon, Korea). Synthesized DNAzyme oligonucleotides (58 nts) were purified using 15% denaturing polyacrylamide gel electrophoresis (PAGE) containing 8 M urea. Linear double-stranded DNA template for the production of target RNA transcript was prepared by *Sal*I (Takara, Japan) digestion of the plasmid harboring HCV NS3 gene (kindly provided by Dr. S. K. Jang, POSTECH, Korea), 5' end of which the T7 RNA polymerase promoter was placed to. For the production of HCV NS3 RNA transcript, *in vitro* transcription was performed with the linear DNA template (30 nM) and T7 RNA polymerase (25 U/μL, Bioneer, Daejeon, Korea) at 37 °C. The transcription reaction mixture contained 15 mM $MgCl_2$, 2 mM spermidine, 5 mM dithiothreitol, 50 mM Tris (pH 7.5), and 2 mM each of the four NTPs. The reaction mixture was incubated at 37 °C for 2 hr, followed by a digestion with RNase-free DNase (10 U/μL) to destroy the template DNAs. The reaction was quenched by adding Na_2EDTA (15 mM final concentration) and an equal volume of gel-loading buffer (0.1% xylene cyanol FF and 0.1% bromophenol blue) containing 8 M urea. The HCV NS3 transcript (1543 nt) was purified by denaturing PAGE. The corresponding band was excised and homogenized in buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 20 mM EDTA), and then incubated for 5 h at room temperature with a shaking. Following centrifugation at 2000 g for 5 min the supernatant was recovered, and the RNA was precipitated with ethanol and then resuspended in 20 mM Tris-HCl (pH 7.4).

***In vitro* DNAzyme library screening.** *In vitro* selection was performed by annealing DNAzyme library with HCV NS3 RNA. In a total volume of 100 μL, 25 μM Dz-library DNA pool and 1 μM target RNA were mixed in 10 mM Tris-HCl (pH 7.4), heated to 85 °C for 3 min, and then cooled to 37 °C over a 30 min allowing RNA-DNA complex to form. One-fifth volume of loading buffer (20% glycerol and 0.05% bromophenol blue) was added, and the bound complexes were separated from the unbound Dz-library DNA pool in a nondenaturing (urea-free) 4% polyacrylamide gel at 4 °C. The RNA-DNA complexes containing the bound species from the Dz-library were isolated and purified as described above and resuspended in 20 mM Tris-HCl (pH 7.4). To remove the target RNA complexd with Dz pool, DNase-free RNase (0.04 U) was added to the resuspended solution, and the reaction (20 μL) was stopped by extraction in 100 μL of phenol/chloroform and recovered by ethanol precipitation.

The selected Dz-library DNAs were amplified through asymmetric PCR.[15] The PCR reaction was performed by using 3'-end primer (5'-GGACCCGGGAATTC-3') that was designed to anneal to the 3'-end tail of the DNAzyme and 5'-end primer that is same as 5'-end tail of the DNAzyme (5'-TAACCCGGGATCC-3') in 1 : 100 mixing ratio. In a total volume of 50 μL the selected Dz-library DNAs were mixed with each primers (100 pmol of 5'-end primer and 1 pmol of 3'-end primer) and subjected to PCR reaction using *Taq* DNA polymerase (0.03 U/μL, Sun Genetics, Daejeon, Korea) and 300 mM of each dNTP. After 30 temperature cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, the products were recovered by phenol/chloroform extraction followed by ethanol precipitation.

Primer extension and identification of cleavage sites. For a cleavage reaction with the selected Dz-library DNAzymes, full-length (1543 nts-long HCV NS3 RNA) substrate (0.1 μM) was incubated with the amplified DNAzyme pool (10 nM-0.3 μM) in the presence of 25 mM $MgCl_2$ in a buffer containing 20 mM Tris-HCl, pH 7.4 in a 20 μL-volume reaction. After 40 min at 37 °C, the reaction was stopped by emersion in ice and precipitated in ethanol. The quenched reaction that contains cleaved RNA fragments were used for primer extension assay.

Cleavage sites were mapped by primer extension of the cleavage products alongside a sequencing reaction generated

using the reverse transcriptase (AMV reverse transcriptase purchased from Roche, Germany). Each primer (1 μM) that anneals to designated sequences of the target RNA (underlined sequences in Fig. 3B) was radiolabeled at the 5'-terminal in 60 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 U polynucleotide kinase (New England Biolabs, MA, USA) and 10 μCi of [γ-^{32}P]ATP (Amersham Biosciences) at 37 °C for 30 min and 75 °C for 5 min. The primer extension reaction was initiated by combining 10 pmol labeled primer with the RNA fragments, which was obtained from the cleavage reaction. The reaction was denatured at 65 °C for 5 min and was allowed to anneal slowly between 42-65 °C before adding the first strand synthesis reaction buffer, dNTPs (final 160 μM), RNase inhibitor (50 U), and AMV reverse transcriptase (20 U) (according to the manufacturer's instructions). This mixture (20 μL) was incubated for 40 min at 42 °C before being transferred to ice. Sequencing fragments corresponding to each segment of the target were also generated by the same primer extension reaction on the cleaved RNA substrate stated as above in the presence of chain-terminating dideoxynucleotides (ddNTP). In these four reactions the dNTP concentration was reduced to 10 μM while being supplemented by 170 μM ddNTPs. The primer extended product and the sequencing reactions were analyzed on a sequencing gel simultaneously and radioactive bands were detected by autoradiography.

***In vitro* cleavage test of selected DNAzymes.** DNAzymes that targeted to the library-selected sites with a distinct cleavage product appearance on the sequencing gel were chosen and synthesized (sequences were shown in Table 1). For a test for RNA cleavage under single-turnover kinetics, a full-length HCV NS3 RNA transcript was incubated with a 20-fold excess of individual DNAzyme (1.0 μM) under the same condition as described above at 37 °C. The reaction was quenched at various time points, and the cleavage products were identified with the primer extension method as described above using the ^{32}P-labeled 3'-end terminal primer. Band intensity in the substrate and cleavage products was quantitated with a Cyclone and image software (PerkinElmer, Boston, MA). Fraction of substrate that was cleaved with DNAzyme was calculated and plotted against time. Each time-course was fit to a kinetic equation: fraction reacted = A ($1 - e^{-kt}$), where A and k is the amplitude and the first order rate constant, respectively.

Results and Discussion

The major obstacle for DNAzyme and other antisense oligonucleotides that directs their target by Watson-Crick base pairing is the underlying RNA secondary structure, which hampers accessibility of these antisense oligonucleotides. Because of unpredictable steric and topological constraints of long stretch of RNA, knowledge of the location of unpaired loop does not guarantee effective hybridization sites.[7,16] In this study we have demonstrated a method to identify accessible cleavage sites in hepatitis C virus NS3 RNA from a pool of random DNAzymes, and for isolation of DNAzymes. One study has shown that a "multiplex cleavage assay" is effective for screening cleavage sites along the entire length of target mRNA, in which DNAzymes that target numerous sites were generated prior to screening.[14] Despite its effectiveness in screening procedure, this approach requires *ad hoc* DNAzyme pool that is required to be prepared beforehand for each target RNA.

Design and *in vitro* selection of DNAzyme library that

Table 1. Summary of cleavage sites screened with the DNAzyme library

RT primer	RNA sequence	Cleavage site (base)	DNAzyme sequence[b]
NS3pr#1	5' UGGCAUGC***AU***GUCAGCU 3'	1488	–
	5' AUCAUGGC***AU***GCAUGUC 3'	1484	(Dz#1) 5' GACATGCAggctagctacaacgaGCCATGAT 3'
	5' CCAAAUUC***AU***CAUGGCA 3'	1476	–
	5' UAGGAGCC***GU***CCAAAAU 3'	1434	(Dz#2) 5' ATTTTGGAggctagctacaacgaGGCTCCTA 3'
	5' ACGGGCCA***AC***ACCCCUG 3'	1407	(Dz#3) 5' CAGGGGTGggctagctacaacgaTGGCCCGT 3'
NS3pr#1.5	5' GGUUGCCC***GU***CUGCCAG 3'	1191	–
	5' GCGGGCUU***AC***CUGAAUA 3'	1157	–
NS3pr#2	5' UGUAACAC***AU***GUGUCAC 3'	921	–
	5' ACUGUAAC***ACAU***GUGUC 3'	919	–
	5' GAUUGACU***GU***AACACAU 3'	914	(Dz#4) 5' ATGTGTTAggctagctacaacgaAGTCAATC 3'
NS3pr#2.5	5' GACAUCUC***AU***UUUCUGC 3'	727	–
	5' GCAAAGCC***AU***CCCCAUU 3'	681	–
	5' CUUCUACG***GC***AAAGCCA 3'	673	–
	5' CCCCUUCU***AC***GGCAAAG 3'	670	–
	5' ACCCCAAU***AU***CGAGGAG 3'	637	–
NS3pr#3	5' ACCCCAAC***AU***CAGAACU 3'	375	–

[a]The sequence, site of cleavage, and the corresponding RT-primer used in the primer extension are as shown in Figure 3B. [b]DNAzyme sequences (name in parenthesis) are designed to be complementary to sequences flanking the selected target cleavage site. The 10-23 catalytic motif is indicated in lower case and the sequence for the target binding domains are in uppercase.

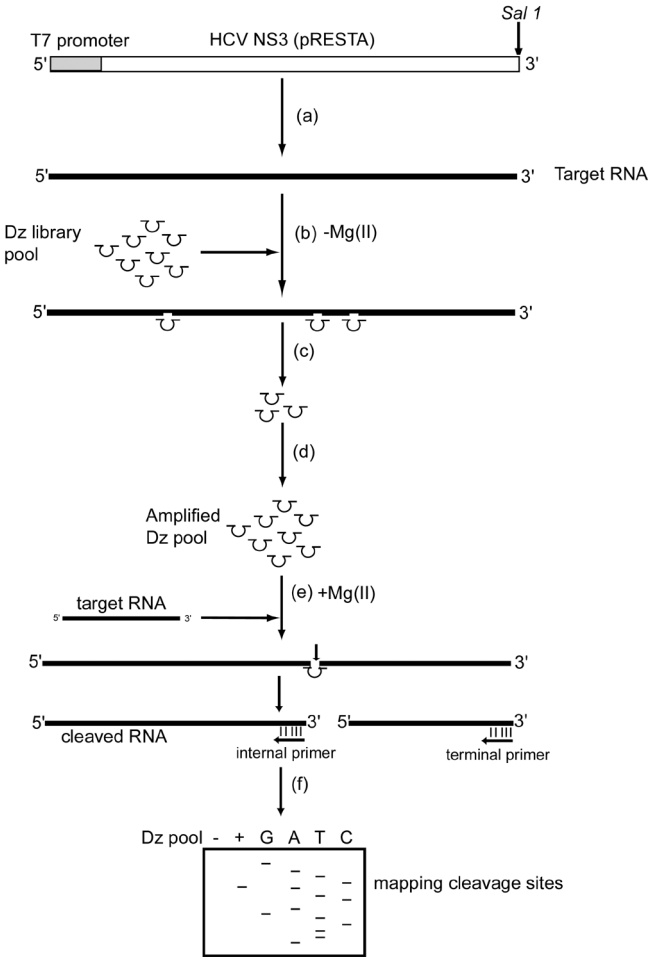

Figure 2. Schematic representation of the library selection procedure. The Dz-library DNA and target RNA are annealed to form DNA-RNA complex (step (b)). The complexes are then isolated (step (c)) and amplified by asymmetric PCR (step (d)). The amplified Dz-library DNA and the target RNA are mixed in the presence of Mg^{2+} to initiate cleavage reaction (step (e)). The cleaved RNA products are subjected to RT-primer extension reaction and the extended cDNAs corresponding to RNA cleavage products were separated on a sequencing gel (step (f)).

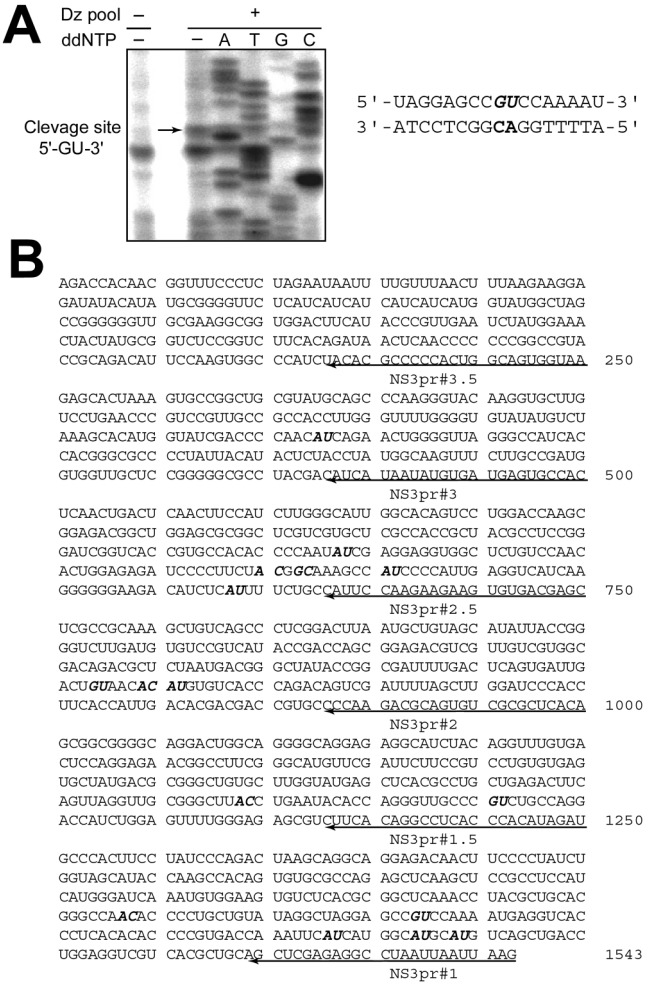

Figure 3. Identification of DNAzyme target sites. *In vitro* synthesized HCV NS3 RNA (1543 nts) and the random pool of DNAzymes were mixed and the cleaved RNA products were analyzed as described before. (A) shows a sequencing gel, where a site at position 1434 in the target RNA (shown as italicized bold characters in sequence) was identified by using the selected and amplified DNAzymes. Note that the primer-extended product band detected only in the presence of Dz-library pool (shown as arrow) was identified as a target site, whereas the bands appeared in the absence of Dz-pool show nonspecific cleavage of the target RNA. (B) The RNA sequence of the HCV NS3 transcript with 16 cleavable sites identified *via* Dz-library screening (shown as italicized bold characters in sequence). Arrows indicate the primer binding sites used for primer extension reactions.

cleaves target RNA. All DNAzyme oligonucleotides used in the library were 58-mers consisting of a central 10-23 catalytic core domain (15 nts) flanked by target-specific binding arms (8 nts) (Fig. 1B). The sequence diversity was created by randomizing sequences of two arms totaling 16 nt (8 Ns and 7 Ns plus 1 purine) and using fixed sequences for both 5'/3' ends. These fixed terminal sequences were designed to use PCR amplification of the DNAzymes that are bound to the target RNA substrate in the absence of magnesium. Target RNA was prepared by *in vitro* transcription, using T7 RNA polymerase, of the template DNA that harbors HCV NS 3 gene under T7 promoter (step (a) in Fig. 2).

The initial pool of Dz-library that was incubated with the target RNA did not yield cleavage products, due to the huge diversity of sequences in the library. To overcome this problem, we first subjected the Dz-library to selection under magnesium-free conditions. Under this condition, Dz-library is able to anneal to the corresponding target RNA but unable to cleave the target RNA (step (b) in Fig. 2). The DNAzyme molecules that were annealed to the target RNA were isolated by urea-free nondenaturing PAGE (step (c) in Fig. 2). The electrophoresis procedure was performed under low temperature (4 °C) to minimize possible dissociation of the RNA-DNAzyme complex during gel-running. The isolated annealed Dz-library DNA pool was subsequently amplified by asymmetric PCR (step (d) in Fig. 2). Upon amplification, this subset is enriched for DNAzymes targeted to accessible sites relative to the starting library, allowing cleavage

products to be identified. We used the amplified selected Dz-library DNA pool to cleave the target RNA in the presence of magnesium ion (step (e) in Fig. 2). The cleaved RNA products were subjected to primer extension and analyzed on sequencing gels in comparison with the corresponding dideoxy sequencing ladder (step (f) in Fig. 2), and the cleavage sites were precisely identified (Fig. 3A). The terminal and internal primers generated a sequence of ~300 nucleotides in length when analyzed on 6% sequencing gel. Sixteen cleavage sites were identified in the HCV NS3 target RNA (Fig. 3B and Table 1). The intensity of the cleaved products was assumed to be generally reflecting the catalytic activity of the corresponding DNAzymes. We have confirmed that the primer-extended products were observed only in the presence of magnesium ion (data not shown). Even in the absence of Dz-library pool, the target RNA was gradually degraded due to the presence of magnesium ion in the reaction. These nonspecific degradations were compared with the DNAzyme-specific RNA cleavage on the sequencing gel (Fig. 3A), and the cleavage products that were only observed in the DNAzyme reaction were identified as the cleavage sites. The positions of effective DNAzymes in the full-length target RNA tended to be clustered into groups separated by regions containing unreactive sites (Fig. 3B). In many cases, relatively active target sites such as cleavage sites 1434-1488, 914-921, and 670-681 nts (Table 1) were very close or even overlapping. This result indicates that these DNAzyme target sites may form an accessible single stranded loop in the RNA secondary structure.

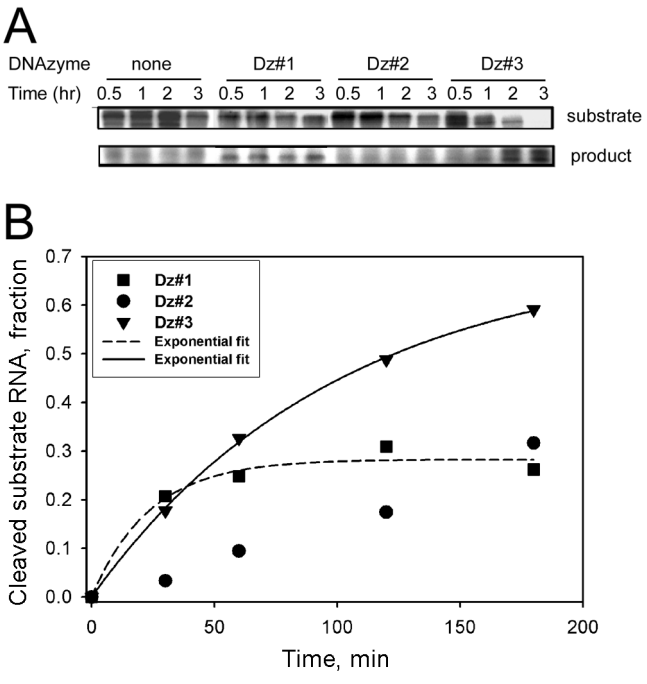

Figure 4. Kinetic analysis of the selected DNAzyme-mediated target RNA cleavage. (A) Selected DNAzymes cleavage of the target RNA over 3 hrs. The reaction time (hr) and bands of unreacted substrate and products are indicated at the top and right side of the gel, respectively. (B) Shown in graph is the reaction progress for DNAzymes used in the target RNA cleavage reaction appeared in the gel. Time courses for two DNAzymes were fit to the equation shown in the text (broken line for Dz#1 and continuous line for Dz#3), and fitting parameters were provided as follows; k = 0.042 min^{-1} and A = 0.28 for Dz#1; k = 0.010 min^{-1} and A = 0.71 for Dz#3.

Kinetic analysis of selected HCV NS3 RNA cleaving DNAzymes. 4 DNAzyme-working sites were chosen out of 16 identified cleavage sites based on the band intensity of cleavage products, and the corresponding DNAzymes were synthesized (Table 1). The reaction rate of these synthesized DNAzymes was examined individually under single-turnover conditions. This was achieved by incubating a full-length HCV NS3 RNA substrate for various time intervals up to 3 hrs with an excess amount of the DNAzyme. The progress of RNA cleavage reaction was monitored by densitometry and the fraction product plotted against time (Fig. 4). Two DNAzymes were turned out to be reactive with an exponential increase of RNA cleavage products, and these time-courses were fit to the exponential function (Fig. 4B), which provided the values of k_{obs} and reaction extent. The DNAzyme targeting the 1407th base of the substrate RNA (Dz#3) was the most effective in cleavage with k_{obs} of 0.01 min^{-1} and 70% of the substrate cleavage extent. Other selected DNAzymes (Dz#1 and #2) also performed well against the substrate, but the DNAzyme targeting the middle part of the substrate RNA (Dz#4) was failed to show its RNA cleavage activity (data not shown).

We modeled a part of HCV NS3 mRNA that is 3'-end portion after 1397th bases using the mFold program (found at http://www.bioinfo.rpi.edu/applications/mfold).[17,18] In the predicted secondary structure of 3'-end portion (147 bases)

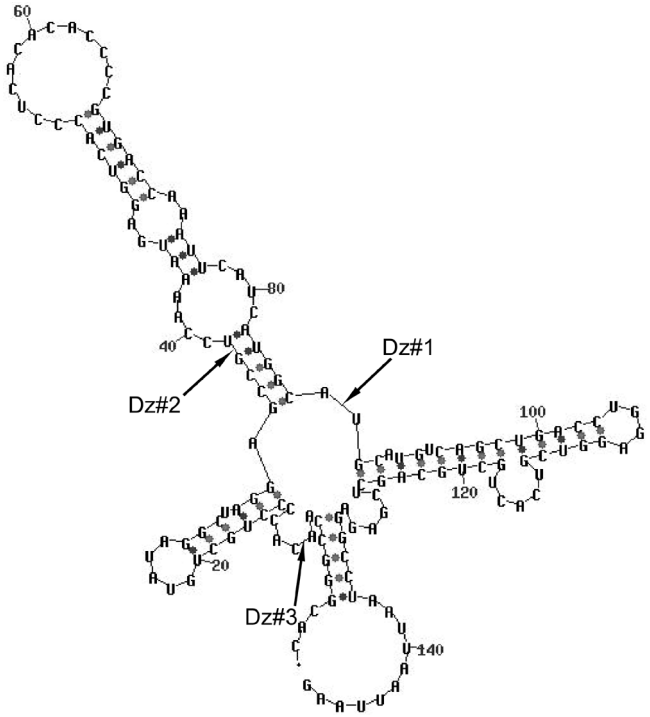

Figure 5. mFold plot of the HCV NS3 RNA of 3'-end portion after 1397th bases was modeled. Positions of the DNAzyme sites used in the RNA cleavage reaction (Fig. 4) are shown with arrows.

of the target RNA, 3 DNAzyme sites are denoted with arrows (Fig. 5). The cleavage sites for DNAzymes that showed moderate cleavage activity are found at a single-stranded region (Dz#1 and Dz#3) or at a transition site (Dz#2), where a double-stranded stem structure is flanked with a single-stranded loop structure. Although these sites might be readily accessible for the DNAzyme, a site for Dz#2 has a less advantage for access, resulting in a weaker cleavage activity of the DNAzyme. This result suggests that the local secondary structure of RNA affect the actual efficiency for oligonucleotide accession. In fact, many possible sites for RNA cleavage that are located at the predicted as single-stranded region are not selected after a screening. Instead, the potential cleavage sites for DNAzymes identified after a screening process mainly reside at a localized portion of the RNA stretch. Therefore, a strategy to identify DNAzyme target sites, which was used in this study, demonstrates its usefulness in design of RNA-cleaving oligonucleotides targeted to a long-stretched RNA.

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