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# ssDNA–dsRNAs are cleaved at the next to its chimera-junction point by an unknown RNase activity

Shinichi Mochizuki [a], Sadaharu Higuchi [b], Kazuo Sakurai [a,c,*]

[a] Department of Chemistry and Biochemistry, The University of Kitakyushu, 1-1, Hibikino, Wakamatsu-ku, Kitakyushu, Fukuoka 808-0135, Japan
[b] Napa Jenomics Co., Ltd., CIIP 1304, 2-24-16 Nakamachi, Koganei, Tokyo 184-8588, Japan
[c] CREST, Japan Science and Technology Agency, 4-1-8, Honcho, Kawaguchi-shi, Saitama 332-0012, Japan

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ABSTRACT

We found that there is an unknown aspect in serum RNases that cleaves ssDNA–dsRNA and ssRNA–dsRNA. In the first step, RNase cleaves the phosphodiester linkage between the first and second RNA, where the first one is connected to the single stranded RNA or DNA. In the second step, the ssRNA overhang attached siRNA is cleaved. When the 2′ hydroxyl of the first RNA was replaced with methoxy, the cleavage did not occur. This RNase activity can be considered related to defense system against exogenous genetic materials.



## 1. Introduction

RNA interference (RNAi) is an RNA-dependent gene-silencing process that occurs in cytosol. RNAi is initiated by the cleavage of long double-stranded RNAs (dsRNAs) into short dsRNAs by an enzyme called Dicer [1,2]. The short dsRNAs, called small interfering RNAs (siRNAs) [1,3], are usually composed of 20–26 nucleotides with an overhang at the 3′ end. When exogenous dsRNAs enter the cytosol, RNAi plays an important role in the immune response [4]. For endogenous dsRNAs such as miRNAs, RNAi is related to gene silencing or downregulation [5]. The application of RNAi in biotechnology began with the groundbreaking discovery that artificially designed short dsRNAs (~21 nucleotides) can silence the expressions of certain endogenous genes homologous to the dsRNAs in mammalian cell lines [6]. This siRNA technology facilitated enormous progress in the study of gene functions. Although there is risk of activating the interferon response, many researchers are developing the therapeutic applications of siRNAs; some of them have reached clinical trials [7]. The development of a safe delivery method is critical for the clinical application of RNAi, because naked siRNAs are easily excreted via renal clearance and degraded in blood stream. Various delivery vehicles have been proposed in the last decades, including viral vectors, and cationic lipids and polymeric materials as non-viral vectors [8,9]. During our study of such vehicles [10,11], we found a strange phenomenon in which the junction point of single-stranded (ss) DNA–dsRNA was selectively cleaved in serum. This study describes this rather strange phenomenon and attempts to estimate its biological significance.

## 2. Materials and methods

### 2.1. Chimera composed of ssDNA and dsRNA

All oligonucleotides were synthesized by Hokkaido System Science Co., Ltd. (Hokkaido, Japan) and purified with high-performance liquid chromatography. All sequences used in this study are described in Supplementary Table 1.

### 2.2. Stability of chimeras to serum

After the chimeras were incubated in 10% human serum (HS), fetal bovine serum (FBS) or mouse serum (MS) at 20 μg/ml for 1 h at 37 °C, the they (50 ng) were analyzed by a 12% polyacrylamide gel (PAGE) for 1 h at 100 V. After the gel was stained with SYBR Gold (Invitrogen, Carlsbad, CA), the fluorescent image was obtained using a PharosFX (Bio-Rad, Richmond, CA).

## 3. Results

### 3.1. Cleavage of chimera in serum

We have been studying a series of ssDNA–dsRNA chimeras comprising oligo-deoxyadenine ($dA_{40}$ and $dA_{10}$) and one of siRNA sequences, denoted as siTNF [12] hereafter, in which the oligo-

* Corresponding author at: Department of Chemistry and Biochemistry, The University of Kitakyushu, 1-1, Hibikino, Wakamatsu-ku, Kitakyushu, Fukuoka 808-0135, Japan. Fax: +81 93 695 3390.
*E-mail address:* sakurai@kitakyu-u.ac.jp (K. Sakurai).

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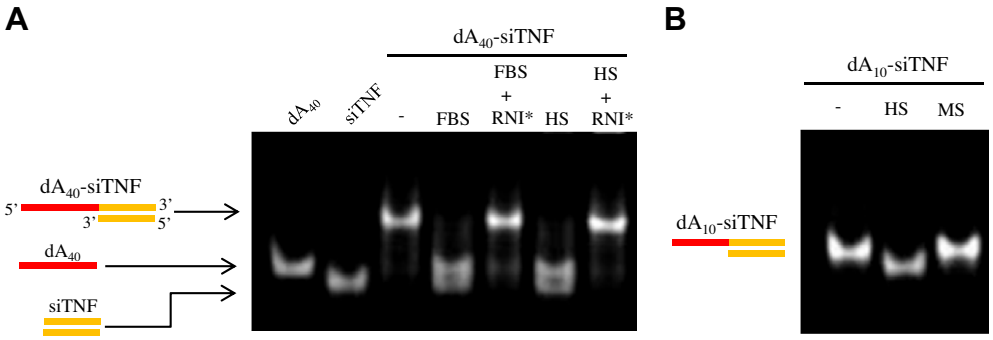


**Fig. 1.** (A) Hydrolyzed fragments from the ssDNA–dsRNA chimera ($dA_{40}$–siTNF see Supplementary Table 1 for sequence) in 10% FBS and HS for 1 h observed with 12% PAGE. –: without serum, RNI*: RNase inhibitor. (B) $dA_{10}$–siTNFs (see Supplementary Table 1) were treated in HS and MS and separated by PAGE. The diagrams illustrate each band.

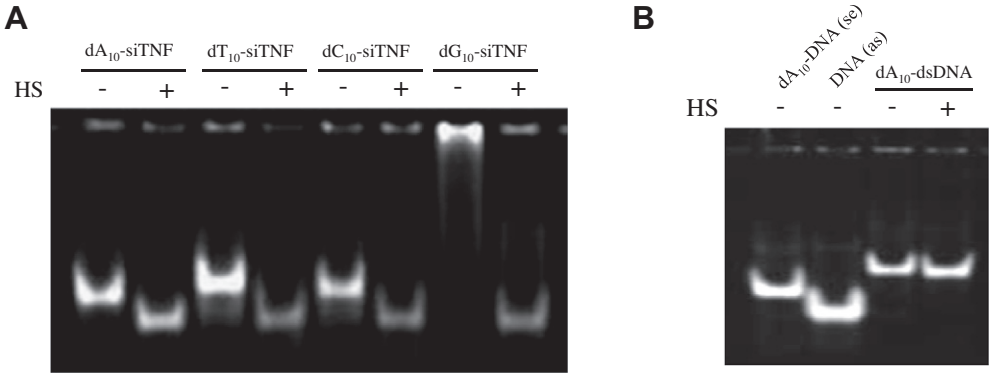


**Fig. 2.** (A) Stability of the chimera with different homo oligo-deoxynucleotides. The chimeras with dA, dT, dC or dG at ssDNA ($dA_{10}$–siTNF, $dT_{10}$–siTNF, $dC_{10}$–siTNF or $dG_{10}$–siTNF; Supplementary Table 1) were treated in HS and separated by PAGE. (B) The DNAs composed of $dA_{10}$–DNA(se) and DNA(as) (Supplementary Table 1) were treated in HS.

deoxyadenine is attached to the 5′ end of the sense strand of the siTNF sequence (Supplementary Table 1). Fig. 1A shows the extents of chimera degradation in FBS and HS. The chimera was cleaved into two fragments; its cleavage was suppressed by an RNase inhibitor RNaseOut™ (Invitrogen). Comparing the $dA_{40}$ and siTNF bands added as controls, the two cleaved fragments can be assigned to $dA_{40}$ and siTNF or to have more or less the same molecular weights with these two. When dsRNAs were degraded in FBS, more smear bands than those in Fig. 1A were generally observed due to random cleavage. However, the chimeras were cleaved noticeably earlier than the degradation of dsRNAs. The reproducibility of this result was checked with several different lots of FBS and HS and other siRNA sequences (data not shown). When the chimeras were exposed to mouse serum, the similar cleavage was not observed (Fig. 1B). After 4 h incubation, the half of chimera was cleaved, indicating that the chimeras are less stable in human serum compared to mouse serum. In previous report, 21 bp siRNAs with 3′-dTdT overhangs shows the different sensitivities against human and mouse serum; the siRNA is degraded rapidly in human serum, while it takes more time to be degraded in mouse serum [13]. These facts imply that systemic administration of siRNAs or chimeras in mouse may not reflect their stability in human blood stream. Thus, the therapeutic application of siRNAs in human can require a higher stability of the siRNAs than in mice, which are frequently used as experimental model to study systemic siRNA therapy. As long as the ssDNA was attached to siTNF at 5′ end, this occurred regardless of the DNA sequences (Fig. 2A). However, this cleavage phenomenon did not occur when the dsRNA part was replaced with DNA (Fig. 2B). To our knowledge, such DNA/RNA chimera-specific cleavage has not been reported.

### 3.2. Definition of the cleavage site

We examined this cleavage phenomenon more precisely with use of fluorescein isothiocyanate (FITC)-labeled chimeras in which FITC was attached at the 5′ or 3′ end of the antisense RNA strand (Fig. 3A, denoted by $dA_{40}$-5′F_siTNF and $dA_{40}$-3′F_siTNF, respectively). While $dA_{40}$-5′F_siTNF was cleaved into two fragments as expected from Fig. 1A, $dA_{40}$-3′F_siTNF was cleaved into three fragments. It should be noted that the most migrated band contained FITC but was not detected with SYBR Gold. Therefore, we presume this band corresponds to a mononucleotide fragment. The cleavage pattern of $dA_{40}$-3′F_siTNF was examined in more detail. The amount of the third band increased with increasing reaction time in HS. First cleavage proceeded so fast and completed within 5 min, while second reaction occurred with a half-life of 1 h (Fig. 3B). This means that, after the cleavage of $dA_{40}$-3′F_siTNF into $dA_{40}$ and siTNF, the FITC-labeled end of siTNF easily and rapidly underwent further hydration. This result can be explained by the fact that ssRNA is more fragile than dsRNA. Consequently, the FITC-labeled end of siTNF was present as a single strand (or overhang). Based on these findings, we propose the following stepwise degradation. The chimera is initially cleaved into $dA_{40}$ with a short ssRNA overhang and siTNF with an overhang complementary to the other (Fig. 3C, step 1). The overhang is then degraded by the same or other enzymes (Fig. 3C, step 2). From the data in Fig. 3, we conclude that the cleavage site is located at the phosphodiester linkage between RNAs in the vicinity of the DNA–RNA junction, probably one or two nucleotides away. We examined the cleavage pattern of the full RNA sequence in which the ssDNA was replaced with ssRNA ($A_{10}$) using FITC-conjugated siTNF. The non-specific degradation of ssRNA was observed. However, depending on the

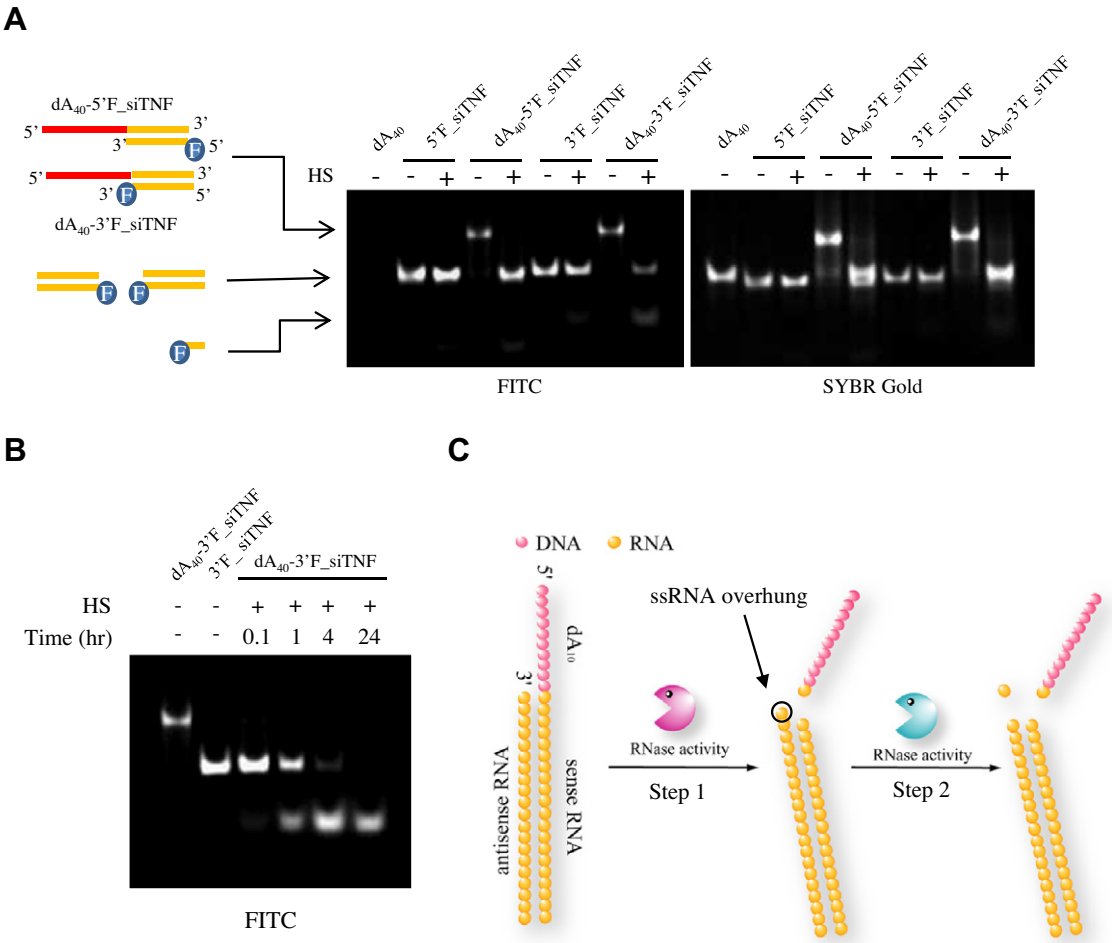


**Fig. 3.** Comparison of the hydration patterns of $dA_{40}$-5′F_siTNF and $dA_{40}$-3′F_siTNF in HS, where FITC was attached at 5′ or 3′ end of the antisense RNA strand and $dA_{40}$ was attached to 5′ end of the sense RNA strand. After FITC observation, the gel was stained with SYBR Gold. The diagrams illustrate each band. (B) Stepwise cleavage of chimera. $dA_{40}$-3'F_siTNFs were treated in HS for indicated times. (C) The stepwise and position-selective cleavage proposed on the basis of the present experimental results including those shown in Fig. 4. In the first step, RNase cleaves the phosphodiester linkage between the first and second RNA of the antisense RNA strand. In the second step, the ssRNA overhang attached siTNF is cleaved.

condition, we observed a cleavage pattern similar to that observed in Fig. 3A (Supplementary Fig. 1). These results suggest that the present findings can be generalized to ssDNA–dsRNA and ssRNA–dsRNA. However, due to experimental convenience and simplicity, we focus on ssDNA–dsRNA hereafter.

### 3.3. Prevention of cleavage

Since the RNase inhibitor suppressed the cleavage (Fig. 1A), it is reasonable to conclude that RNases are involved. Human pancreatic RNAase (hpRNAase), which is structurally and functionally similar to bovine RNase A, occupies approximately 70% of RNAse activity contained in human serum [14,15]. Thus, members of the RNase A family can be good candidates to mediate cleavage of the chimera in serum. Among the commercially available RNases, bovine RNase A and S exhibited the same activity mentioned above, whereas RNase H did not (data not shown). Bovine RNase A is one of the most common Rnases and specifically degrades ssRNAs at the 3′ end of unpaired C and U residues [16]. It also possesses helix-destabilizing activity toward dsRNA and digests dsRNA, but the activity is much lower rate than ssRNA [17]. The chimera was cleaved not only at the location mentioned above, but also at siTNF itself (Supplementary Fig. 2), suggesting that this activity is a previously unknown aspect of RNase A or other RNases.

The main RNA degradation mechanism is the nucleophile attack of the 2′ oxygen on the phosphate atom of the phosphodiester linkage [18]. One of the common chemical modifications for preventing this is the methylation of the 2′ oxygen of RNA. We introduced a methoxy group into the 2′ position of RNA riboses from the next base to the DNA up to the 4th riboses and examined the cleavage (Fig. 4). The methoxylation of the first RNA (i.e., next to the DNA) was sufficient to prevent the cleavage. This result indicates that the cleaved point is the phosphodiester connecting the first and second sense RNAs attached to DNA. This implies that after the first cleavage, the overhanging portion of the antisense sequence of siTNF is only one base (see Fig. 3C). Since we knew which position is cleaved, we examined the sequence specificity. Based on the siTNF sequence used above, the first sense base, C, was replaced with A, U, or G, and the cleavage was examined (Supplementary Fig. 3). The cleavage occurred when the sequence was C or U. This sequence specificity is same as that of bovine RNase A, although the C or U is not unpaired.

## 4. Discussion

The above results demonstrate that RNases have an unknown activity in HS and FBS, showing sequence- and position-selective cleavage of the chimera; this activity is shared with bovine RNase

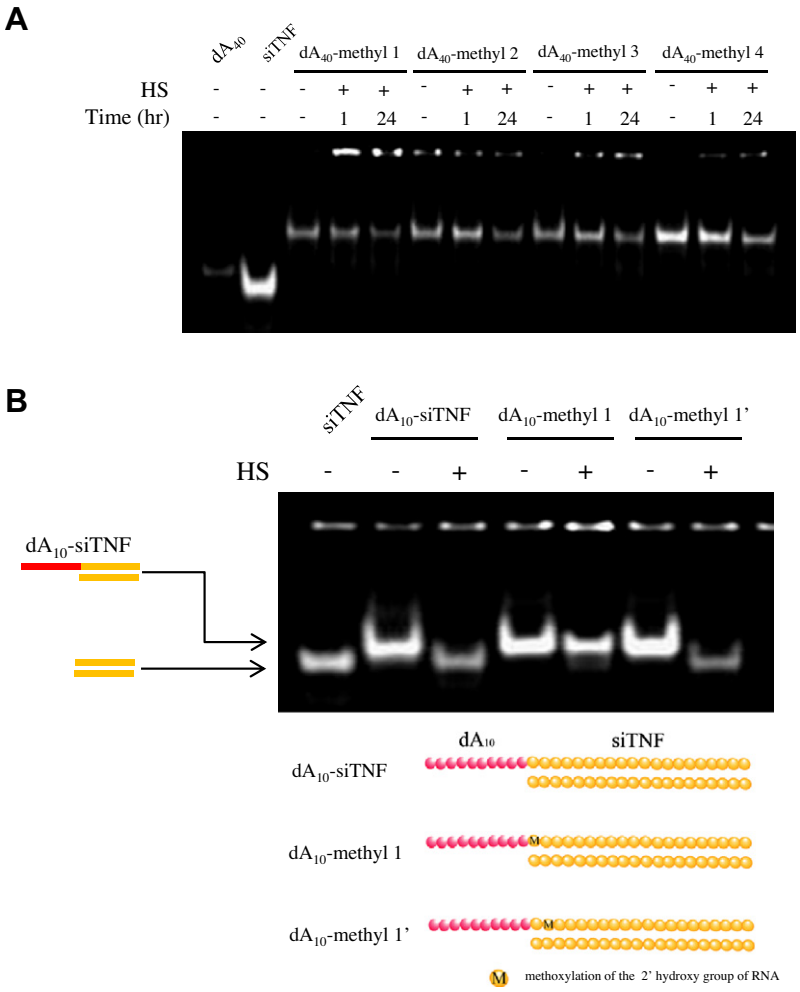


**Fig. 4.** (A) Protection of the cleavage by methoxy-modifying the 2′ hydroxyl group of RNA ribose of the RNA. The chimeras introduced methoxy groups into the 2′ position of RNA ($dA_{40}$-methyl 1, $dA_{40}$-methyl 2, $dA_{40}$-methyl 3 and $dA_{40}$-methyl 4; Supplementary Table 1) were treated in HS. (B) Protection of the cleavage caused by methoxy modifying the 2′ hydroxyl group of RNA ribose of the RNA and DNA junction point ($dA_{10}$-methyl 1 and $dA_{10}$-methyl 1′; Supplementary Table 1). The diagrams illustrate the position of methoxylation in the sequence.

A and S. However, it is unlikely that naked endogenous nucleotides are involved in any biological reaction in serum. By eliminating such impossibilities, we can speculate that this RNase activity may be related to a host defense mechanism. RNases are secreted from organs including the pancreas and kidneys and exist in body fluids [19,20]. Previous studies show that such RNases serve not only as cytotoxic agents in host defense, but also in angiogenic, immune-suppressive, and antitumor activities [21,22], supporting our speculation. Despite this, it remains difficult to conclude that ssDNA–dsRNA must be cleaved by RNases because no such chimera exists in nature. We should consider the possibility that this RNase activity is solely designed to cleave ssRNA–dsRNA into ssRNA and dsRNA instead of cleaving the chimera. This is because the same phenomenon was observed in ssRNA–dsRNA as mentioned above. Assuming this argument is correct, it is possible that this RNase activity is designed to defend against exogenous RNAs before recognizing them with Dicer. If there are long overhangs or other structures attached to the dsRNA end, the recognition with Dicer would be dramatically reduced. Therefore, these portions must be cleaved before dsRNA recognition with Dicer.

The recognition ability of ssDNA cannot be explained if the present RNase activity is designed to deal with only ssRNA–dsRNA. Although it is mere speculation, there is a possible explanation for this phenomenon. During the RNA world era, RNA molecules presumably underwent frequent mutations because of the lack of RNA-repairing enzymes; only RNA-dependent RNA polymerases and RNases were present at that time. Organisms acquired the ability to repair their genetic information when the role of information storage was transferred from RNA to DNA. During this transition, several RNA/DNA chimeras might have emerged as an evolutionary step. Moreover, RNA-dependent RNA polymerases or RNases had to acquire the ability to degrade RNA/DNA chimeras for repair. Some RNA polymerases and RNases may still retain this activity as a vestige, which may explain the observed cleavage. Recent studies report several RNA-dependent RNA polymerases in plants [23] and viruses as well as some mammals [24]. This is another possible biological explanation for the present finding.

In summary, RNases possess a previously unknown activity in FBS and HS that cleaves ssDNA–dsRNA and ssRNA–dsRNA at the phosphodiester linkage between the first and second RNA units, in which the first unit is connected to the ssRNA or ssDNA. However, the cleavage does not occur when the 2′ hydroxyl group in the first RNA is replaced with a methoxy group. The cleavage only occurs when the first base is U or C. To our knowledge, this is the first report of such an activity of RNase, which may be related to siRNA defense system.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.10.100.

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