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Kinetic analyses of DNA-linked ribonucleases H with different sizes of DNA

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Abstract A series of DNA-linked ribonucleases H with DNA adducts varying in size and sequence, ranging from heptamer to nonamer, were constructed and examined for their ability to cleave the 12-base RNA (5′-CGGAGAUGACGG-3′) site-specifically. The DNA-linked RNase H with the 9-base DNA (5′-GTCATCTCC-3′) cleaved the 12-base RNA specifically at A6-U7. Kinetic studies revealed that the DNA-linked RNase H with the 8-base DNA (5′-TCATCTCC-3′) cleaved it slightly more effectively than that with the 9-base DNA. Factors that may affect the specificity and catalytic efficiency of a DNA-linked RNase H are described.

Key words: RNase H; DNA-linked enzyme; Kinetic study; Site specificity

1. Introduction

The development of a method to cleave RNA site-specifically is expected to facilitate structural and functional studies of RNA. Several DNA-linked enzymes, in which a 14–22-base DNA is covalently linked to non-specific nucleases, such as RNase A and staphylococcal nuclease, through a flexible tether, have been constructed for this purpose [1–3]. We have constructed the DNA-linked RNase H, d9-C135/RNase H, to improve the catalytic efficiency and specificity of these DNA-linked proteins [4,5]. In this DNA-linked RNase H, the d9-mer of 5′-GTCATCTCC-3′ is covalently attached through a 21 Å linker to the *E. coli* RNase HI variant, C-135/RNase H, in which all three free Cys residues, at positions 13, 63, and 133, are replaced by Ala, and a unique Cys residue is introduced at position 135.

RNases H hydrolyze the RNA strand specifically in a DNA/RNA hybrid, and do not recognize either single- or double-stranded RNA as a substrate [6]. This unique substrate specificity makes the enzyme ideal for targeting specific RNA sequences by the attachment of DNA oligomers. A DNA-linked RNase H cleaves the RNA only within the target sequence complementary to a DNA adduct, which directs the RNase H portion to the RNA. In addition, the destabilization of the DNA/RNA hybrid formed between the DNA adduct in the DNA-linked enzyme and the substrate RNA upon the cleavage of the RNA allows the DNA-linked enzyme to perform multiple turnovers. Among the various RNase H enzymes, *E. coli* RNase HI was chosen as a catalytic component of the DNA-linked enzyme, because its structure and function have been most extensively studied [7].

The sequence and size of the DNA oligomer attached to *E. coli* RNase HI, as well as the species and size of the linker between them, affect the specificity and catalytic efficiency of the DNA-linked RNase H. The effect of alterations in the size of the linker on the specificity and catalytic efficiency of the DNA-linked enzyme has already been examined [8]. Among the various DNA-linked RNases H with different sizes of linkers, ranging from 18 Å to 27 Å, that with a 27 Å linker most effectively cleaved synthetic RNA oligomers site-specifically. However, it remains to be determined whether the enzymatic efficiency of the DNA-linked RNase H is improved by modulating the size of the DNA adduct. DNA-linked RNases H with shorter DNAs may be more useful as RNA restriction enzymes than those with longer ones, because the frequencies of the appearances of the target sequences to be cleaved in the RNA would increase. It is therefore important to examine whether the DNA-linked RNases H, with DNA adducts that are shorter than a 9-mer in size, can cleave the RNA efficiently and site-specifically.

In this report, we have examined the effect of a reduction in the size of the DNA adduct on the specificity and catalytic efficiency of the d9-C135/RNase H. We found that truncation of one residue from the 5′-terminus of the d9-mer resulted in a slight increase in the k_{cat} value, but further truncations, as well as those from the 3′-terminus, resulted in an increase in the K_m value along with a decrease in the k_{cat} value. These results suggest that an octamer is the minimum size of the DNA adduct that is required to keep the efficiency of the DNA-linked RNase H high. We also provide evidence that shows that the covalent attachment of the DNA oligomers to *E. coli* RNase HI quite effectively restricts the cleavage site.

2. Materials and methods

2.1. Materials

[γ^{32}P]ATP (>5,000 Ci/mmol) was purchased from Amersham. The r12-mer (5′-CGGAGAUGACGG-3′) was synthesized by Toray Research Center Co. Ltd. (Tokyo, Japan). An amino hexyl phosphate linker with a methoxy phosphate protecting group (Aminolink 2) was purchased from Applied Biosystems. Acetylated bovine serum albumin was from Bethesda Research Laboratories, and *Crotalus durissus*

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Abbreviations: RNase H, ribonuclease H; dX-mer, X-base DNA oligomer; rX-mer, X-base RNA oligomer.

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phosphodiesterase was from Boehringer Mannheim. Other chemicals were of reagent grade. The *E. coli* RNase HI variant, C135/RNase H, was prepared previously [4].

2.2. Constructions of DNA-linked RNases H

Derivatives of DNA oligomers, in which a maleimide group is attached to the 5′-terminus of the DNA oligomers, were synthesized as described for the 5′-maleimide-d9-mer [4], except that aminolink 2, instead of amino modifier II, was used to incorporate a primary amino group into the 5′-terminus of the DNA oligomers. Coupling reactions between the C135/RNase H and the 5′-maleimide-DNA oligomers, and the purifications of the resultant DNA-linked RNases H, were carried out as described for d9-C135/RNase H [4].

2.3. Time course for the cleavage reaction of the d090-mer/r12-mer hybrid with C135/RNase H

The cleavage reaction of the r12mer (0.1 μM) hybridized to the d090-mer with 0.8 nM of the C135/RNase H was carried out at 37°C in 20 μl of 10 mM Tris-HCl buffer, pH 8.0, containing 10 mM $MgCl_2$, 50 mM NaCl, 1 mM 2-mercaptorthanol, and 0.01% acetylated bovine serum albumin. An aliquot (2 μl) of the reaction mixture was removed at 0 min, 3 min, 10 min, 30 min, and 120 min, and the reaction was stopped by heating at 90°C for 2 min.

2.4. Assay

For the assay of the DNA-linked RNases H, the dodeca-ribonucleotide with a sequence of 5′-CGGAGAUGACGG-3′ (r12-mer) was ^{32}P-labeled at the 5′-end and used as a substrate. For the assay of the free enzyme without DNA adducts (C135/RNase H), the oligomeric DNA/RNA hybrids were prepared by hybridizing the ^{32}P-labeled-r12-mer with 3 molar equivalents of various complementary DNA oligomers, ranging in size from heptamer to nonamer, and were used as substrates. Hydrolysis of the substrate with the enzyme was carried out at 37°C or 30°C for 10 min in 10 mM Tris-HCl buffer, pH 8.0, containing 10 mM $MgCl_2$, 50 mM NaCl, 1 mM 2-mercaptoethanol, and 0.01% acetylated bovine serum albumin. After the reaction was stopped by heating the reaction mixture at 90°C for 2 min, the hydrolysates were fractionated on 20% polyacrylamide/7 M urea sequencing gels (0.3 mm × 60 cm). They were identified by comparing their migrations on the gel with those of the oligonucleotides generated by the partial digestion of the ^{32}P-labeled r12-mer with snake venom phosphodiesterase [9]. The amount of each hydrolysate was directly quantitated using a Fujix BA100 Bioimage Analyzer. To determine the kinetic parameters, the substrate concentrations were varied from 0.1 to 10.0 mM.

2.5. Protein and substrate concentrations

The concentration of the r12-mer was determined from UV absorption, with a molar coefficient at 260 nm of 1.1×10^5. The concentrations of the DNA-linked RNases H were also determined from the UV absorption at 280 nm, assuming that their molar absorption coefficients are 9.2×10^4 for d090-RNase H, 8.5×10^4 for d081-RNase H, 7.8×10^4 for d072-RNase H, 8.5×10^4 for d180-RNase H, and 7.9×10^4 for d270-RNase H, the sum of those for the C135/RNase H and the DNA oligomers.

2.6. Designations of DNA Oligomers and DNA-linked RNases H

DNA oligomers, with truncations at either the 5′- or 3′-terminus of the nonadeoxyribonucleotide with a sequence of 5′-GTCATCTCC-3′ (d9-mer), were used as DNA adducts to construct the DNA-linked RNases H. They are designated as dXYZ-mer, in which X and Z represent the number of nucleic acid residues truncated at the 5′- and 3′-termini of the d9-mer, respectively, and Y represents the size of the DNA oligomer. For example, the d090-mer is identical with the d9-mer, and the d270-mer is the d7-mer with a sequence of 5′-CATCTCC-3′. DNA-linked RNases H are designated as dXYZ-RNase H, in which the dXYZ-mer is covalently attached to the C135/RNase H.

3. Results

Five DNA-linked RNases H, with a series of DNA adducts varying in size and sequence, were constructed (Fig. 1). Among them, the d090-RNase H has the largest DNA adduct (d090-mer) in size. Others have DNA adducts with systematic truncations at either the 5′- or 3′-end of this d090-mer. A 27 Å linker [maleimide-$(CH_2)_5$-CONH-$(CH_2)_6$-], instead of a 21 Å linker [maleimide-$(CH_2)_5$-CONH-CH_2-$CH(CH_2OH)$-], was used to construct these DNA-linked RNases H, because the catalytic efficiency and specificity of the DNA-linked enzyme were shown to be improved by replacing the 21 Å linker with the 27 Å linker [8].

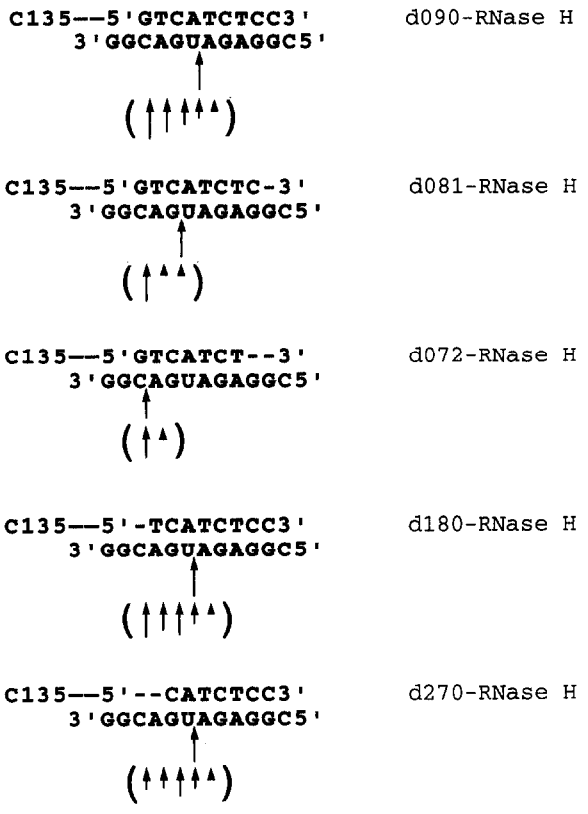

Fig. 1. Histograms of the preferential cleavage sites. The DNA–RNA hybrids formed between the DNAs in DNA-linked RNases H and the r12-mer are shown. The primary cleavage sites in the r12-mer with the DNA-linked RNases H are shown by arrows, and those in the r12-mer hybridized to the DNA oligomers with the C135/RNase H are shown by arrows in parentheses. Difference in the size of arrows reflects the relative cleavage efficiencies at the indicated positions.

3.1. Cleavage of r12-mer with DNA-linked RNase H

The r12-mer was used as a substrate representing a single-stranded RNA, instead of the r9-mer that was previously used as a substrate for the assay of the DNA-linked RNase H, d9-C135/RNase H [4]. Because the number of the scissile bond increases as the size of the RNA increases, the r12-mer is a better substrate than the r9-mer for the evaluation of the substrate specificities of the DNA-linked enzymes. Time courses for the cleavage reactions of the r12-mer with all the DNA-linked RNases H were analyzed (data not shown). When that with the d090-RNase H was examined, only the r6-mer, 5′-CGGAGA-3′, accumulates in parallel with a decrease of the r12-mer. It is therefore clear that the d090-RNase H cleaves the r12-mer exclusively at A6-U7. This result is in good agreement with the previous finding that the Hybrid Enz-4, which is identical with the d090-RNase H, specifically cleaves the r9-mer and r22-mer at the site corresponding to A6-U7 in the r12-mer [8]. The r12-mer cleavage sites with all the DNA-linked RNases H

are summarized in Fig. 1. The d081- and d072-RNases H cleave the r12-mer almost exclusively at U7-G8 and A9-C10, respectively. Thus, truncations at the 3'-end of the DNA adduct in the d090-RNase H force the resultant DNA-linked enzymes to cleave the r12-mer at the phosphodiester bonds located downstream of that between A6 and U7. In contrast, truncations at the 5'-end of the DNA in the d090-RNase H do not affect the specificity of the resultant DNA-linked enzymes.

The kinetic parameters of these DNA-linked RNases H are summarized in Table 1. Truncations at the 3'-end of the DNA adduct resulted in a sequential increase in the K_m value, along with a sequential decrease in the k_{cat} value. In contrast, truncations at the 5'-end of the DNA adduct did not seriously affect the k_{cat} value of the DNA-linked enzyme. In addition, the K_m value of the DNA-linked RNase H considerably increased only when two residues were truncated from the 5'-terminus. Consequently, the k_{cat}/K_m values of all the DNA-linked RNases H with the truncated DNAs, except for that of the d180-RNase H, considerably decreased as that of the d090-RNase H. The d180-RNase H is apparently the most efficient DNA-linked enzyme among those examined, with the highest k_{cat}/K_m value, 45 $mM^{-1}min^{-1}$.

3.2. Cleavage of r12-mer hybridized to DNA oligomers with C135/RNase H

For the purpose of evaluating the effect of the cross-linking between the DNA and the protein on the substrate specificity and catalytic efficiency, the cleavages of the r12-mer hybridized to various DNA oligomers with the C135/RNase H were examined. Time courses for the cleavage reactions of the r12-mer hybridized to all DNA oligomers with the C135/RNase H were analyzed, and that of the r12-mer hybridized to the d090-mer is shown as a representative in Fig. 2. The r12-mer hybridized to the d090-mer was cleaved by the C135/RNase H at five positions. The most preferential site was the phosphodiester bond between the 9th A and the 10th C, because the r9-mer is the major product in the beginning of the cleavage reaction. However, the amount of the r9-mer in the reaction mixture gradually decreased as the cleavage reaction proceeded, probably because this r9-mer was further cleaved by the enzyme in the presence of the d090-mer. As a result, the r6-mer and r7-mer, which were produced by the cleavages at A6-U7 and U7-G8, respectively, became the major products when the r12-mer was almost completely digested.

The primary cleavage sites in the r12-mer hybridized to various DNA oligomers with the C135/RNase H are summarized in Fig. 1. Like the r12-mer hybridized to the d090-mer, both the r12-mer hybridized to the d081-mer and that hybridized to the d072-mer were preferentially cleaved by the C135/RNase H at A9-C10. In these cases, however, the final products were different from one another. After the complete digestions of the r12-mers hybridized to the d081-mer and the d072-mer, the r9-mer and the r7-mer, and the r9-mer, respectively, were the major products. These results indicate that a reduction in the size of the RNA between the cleavage site and the 5'-end of the DNA/RNA hybrid limits the interaction between the enzyme and the substrate. In contrast to the truncations at the 3'-end of the d090-mer, those at the 5'-end made the cleavage of the r12-mer at A6-U7 by the C135/RNase H less preferential. As a result, the C135/RNase H cleaved the r12-mer at multiple sites simultaneously, even in the beginning of the cleavage reaction, although the r6-mer and r7-mer were the major products when the r12-mer was almost completely digested.

Table 1
Kinetic parameters of DNA-linked RNases H

Enzyme	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1}\cdot\mu M^{-1}$)
d090-RNase H	0.6	25	42
d081-RNase H	1.1	9	8
d072-RNase H	2.8	3	1
d180-RNase H	0.6	27	45
d270-RNase H	2.9	23	8

Hydrolyses of the r12-mer with the DNA-linked RNases H were carried out at 37°C for 10 min in 10 mM Tris-HCl (pH 8.0) containing 10 mM $MgCl_2$, 50 mM NaCl, 1 mM 2-mercaptoethanol, and 0.1 mg/ml acetylated bovine serum albumin. Errors, which represent the 65% confidence limits, are all at or below 30%.

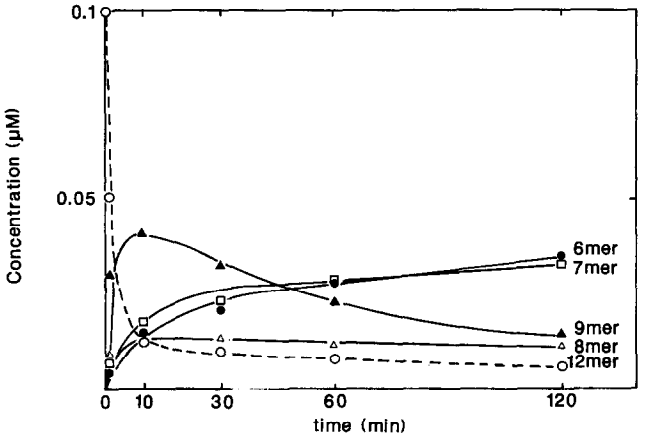

Fig. 2. Time course for the cleavage reaction of the d090-mer/r12-mer hybrid with C135/RNase H. The cleavage reaction of the r12-mer (0.1 μM) hybridized to the d090-mer with 0.8 nM of the C135/RNase H was carried out as described in section 2. The amounts of the r12-mer (-○-), r9-mer (5'-CGGAGAUGA-3') (-▲-), r8-mer (5'-CGGAGAUG-3') (-△-), r7-mer (5'-CGGAGAU-3') (-□-), and r6-mer (5'CGGAGA-3') (-●-) in the reaction mixture are shown.

Because the r12-mer was cleaved preferentially at A9-C10 when it was hybridized to the d090-mer, d081-mer, and d072-mer, the kinetic parameters could be determined for the cleavage of the r12-mer hybridized to these DNA oligomers (Table 2). The K_m value increases and the k_{cat} value decreases, as the size of the DNA oligomer decreases. When these values are compared with those determined for the hydrolysis of the r12-mer with the DNA-linked RNases H, the K_m and k_{cat} values increased by 2–2.5 times and 10–40 times, respectively. These results suggest that the affinity of the C135/RNase H to the substrate is improved, whereas its turnover rate is decreased, by cross-linking the DNA oligomer to the protein.

3.3. Effect of temperature on kinetic parameters

All the kinetic parameters presented in this report were determined at 37°C. To examine whether the stability of the d090-mer/r12-mer hybrid is affected by the truncation of the d090-mer at 37°C, the kinetic parameters of the C135/RNase H for the hydrolysis of the d090-mer/r12-mer, d081-mer/r12-mer, and d072-mer/r12-mer duplexes were also determined at 30°C (data not shown). For these different substrates, both the K_m and k_{cat} values of the C135/RNase H determined at 30°C were equally

reduced, by nearly 50%, as compared to those determined at 37°C. These results suggest that all the oligomeric DNA/RNA hybrids used in this experiment are stable at 37°C. The thermal melting temperature (T_m) of the d9-mer (identical to the d090-mer)/r9-mer hybrid was reported as 49°C [4]. Only a limited amount of the r12-mer or r9-mer was available, however, and the T_m values of the oligomeric DNA/RNA hybrids used in this experiment were not determined.

4. Discussion

In the DNA-linked RNases H, the DNA oligomers attached to the RNase H portion direct the catalytic site of RNase H to the RNA sequence targeted for cleavage. The d090-RNase H cleaves the r12-mer specifically at A6-U7, indicating that the cleavage site in the RNA is located 5 residues downstream from the 5′-end of the DNA/RNA hybrid region formed between the d090-mer and the r12-mer. The cleavage site in the r12-mer was sequentially shifted by one or two residues in the 3′-direction when the DNA in the d090-RNase H was truncated at the 3′-terminus by single, sequential deletions. Thus, the cleavage of the r12-mer always occurs 5–6 residues downstream of the 5′-end of the DNA/RNA hybrid region. These results are consistent with the previous ones [10–14], as well as a model proposed for the complex between the enzyme and the substrate [15,16], which suggests that *E. coli* RNase HI recognizes a tetra-or penta-nucleotide hybrid preceding the cleavage site in the RNA. In contrast to the distance between the cleavage site and the 5′-ends of the DNA/RNA hybrid regions, the distances between the cleavage sites and the 3′-ends of the DNA/RNA hybrid regions are variable. The d090-, d180-, and d270-RNases H cleave the RNA 4, 3, and 2 residues upstream from the 3′-end of the DNA/RNA hybrid regions, respectively.

The 081- and d072-RNase H cleaved the r12mer at U7-G8 and A9-C10, respectively. In contrast, the d090-RNase H with a longer DNA adduct than that of the d081- or d072-RNase H could not cleave the r12mer at these sites (Fig. 1). The side-chain of Cys^{135} in the C135/RNase H faces away from the active site of this protein. Therefore, the linker between the DNA and the protein must form a loop in order to bring the DNA/RNA hybrid in contact with the active-site of the protein. This loop might prevent the cleavage of the r12-mer at U7-G8 or A9-C10, because of a steric constraint. The d081- or d072-RNase H cleaves the r12-mer at these sites, probably because the binding force between the active-site of the protein and the pentanucleotide hybrid preceding the cleavage site dominates this constraint.

Kinetic analyses of the DNA-linked RNases H showed that the d090-RNase H and the d180-RNase H are the best DNA-linked enzymes in the catalytic efficiency (k_{cat}/K_m) among those examined. The K_m and k_{cat} values of the d090-RNase H are 0.6 mM and 25 min^{-1} at 37°C, which are comparable to those previously reported for the hydrolysis of the r9-mer with the d9-C135/RNase H at 30°C [4]. The truncation at the 3′-terminus of the DNA in the d090-RNase H, and that of the 5′-terminus of the DNA in the d180-RNase H, resulted in dramatic decreases in the k_{cat}/K_m values (Table 1). These results suggest that an appropriate size for the DNA in a DNA-linked RNase H is an octamer or nonamer. The RNA containing a sequence complementary to this DNA octamer or nonamer would be cleaved by the DNA-linked RNase H at the site that is located 5 residues downstream from the 5′-end of the DNA/RNA hybrid. It should be noted, however, that the catalytic efficiency of the DNA-linked RNase H is dependent on the sequence of its DNA adduct. For example, the k_{cat}/K_m value of the d180-RNase H is 6 times higher than that of the d081-RNase H. Both of these DNA-linked enzymes cleave the r12-mer at the site that is located 5 residues downstream from the 5′-end of the DNA/RNA hybrid. The d180-RNase H cleaves the r12-mer at A6-U7 and the d081-RNase H cleaves it at U7-G8. The sensitivities of these sites to cleavage with RNase H are probably different from each other.

It has previously been shown, using the r9-mer, which is complementary to the d090mer, as a substrate, that the cross-linking of the d090-mer to the C135/RNase H limits the interaction between the enzyme and the substrate [4]. The r9-mer hybridized to the d090-mer was cleaved by the C135/RNase H at A5-U6 and U6-G7 (corresponding to A6-U7 and U7-G8 in the r12-mer) almost equally, whereas the r9-mer was specifically cleaved by the d9-C135/RNase H at A5-U6 (corresponding to A6-U7 in the r12-mer). This effect of the cross-linking was much more clearly shown using the r12-mer, instead of the r9-mer, as a substrate, because the r12-mer hybridized to the d090-mer was preferentially cleaved by the C135/RNase H at A9-C10, whereas the r12-mer was specifically cleaved by the d090-RNase H at A6-U7.

E. coli RNase HI shows no base specificity, but slightly prefers to cleave phosphodiester bonds adjacent to pyrimidine residues [6]. This preference also was observed when the r12-mer was used as a substrate. The phosphodiester bonds in the r12-mer, which are not adjacent to pyrimidine residues (G5-A6 and G8-A9), were less preferentially cleaved by the C135/RNase H than others (A6-U7, U7-G8, and A9-C10) in the presence of the d090-mer. Reid et al. have shown that the substrate recognition site of RNase H fits into the minor groove of the DNA–RNA hybrid, which assumes neither an A-form nor a B-form conformation [17,18], and proposed that sequence-dependent groove modulation, which was shown in DNA duplexes [19], is responsible for the slight sequence preference of RNase H. For the construction of DNA-linked RNases H with high catalytic efficiencies, it would be important to design a DNA adduct so that the RNase H portion in the DNA-linked RNase H can cleave the RNA at the most preferential site. In this report, we have shown that a DNA-linked RNase H, in which a DNA octamer or nonamer is attached to the protein through a 27 Å linker, cleaves the RNA at the site located 5 residues downstream from the 5′-end of the DNA/RNA hybrid. However, it seems difficult to predict the most preferred cleavage site for RNase H in a given RNA sequence.

Table 2
Kinetic parameters of the C135/RNase H for different substrates

DNA oligomer	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1} \cdot \mu M^{-1}$)
d090-mer	1.2	305	254
d081-mer	3.4	234	70
d072-mer	5.6	69	12

Hydrolyses of the r12-mer hybridized to various DNA oligomers with the C135/RNase H were carried out as described in the legend for Table 1. Prior to the addition of the enzyme, which initiated the cleavage reaction, the r12-mer was incubated with the DNA oligomers at 37°C for 15 min.

Further investigations, including crystallographic analyses of the RNase H protein complexed with a DNA/RNA hybrid, will be helpful to understand the reason why the enzyme has a sequence or base preference, and thereby will allow precise prediction of the preferred RNA cleavage site for the enzyme.

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