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Site-specific cleavage of MS2 RNA by a thermostable DNA-linked RNase H

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A series of DNA-linked RNases H, in which the 15-mer DNA is cross-linked to the *Thermus thermophilus* RNase HI (TRNH) variants at positions 135, 136, 137 and 138, were constructed and analyzed for their abilities to cleave the complementary 15-mer RNA. Of these, that with the DNA adduct at position 135 most efficiently cleaved the RNA substrate, indicating that position 135 is the most appropriate cross-linking site among those examined. To examine whether DNA-linked RNase H also site-specifically cleaves a highly structured natural RNA, DNA-linked TRNHs with a series of DNA adducts varying in size at position 135 were constructed and analyzed for their abilities to cleave MS2 RNA. These DNA adducts were designed such that DNA-linked enzymes cleave MS2 RNA at a loop around residue 2790. Of the four DNA-linked TRNHs with the 8-, 12-, 16- and 20-mer DNA adducts, only that with the 16-mer DNA adduct efficiently and site-specifically cleaved MS2 RNA. Primer extension revealed that this DNA-linked TRNH cleaved MS2 RNA within the target sequence.

Keywords: MS2 RNA/primer extension/ribonuclease H/site-specific cleavage/*Thermus thermophilus*

Introduction

Development of a method to cleave RNA site-specifically is important, because this method can be used as a tool not only for probing structures and functions of RNA but also for selective inactivation of a target RNA *in vivo*. Several methods are available to cleave RNA site-specifically. They include enzymatic cleavages with ribozymes (Symons, 1992) and DNA-linked nucleases (Zuckermann and Schultz, 1988, 1989; Zuckermann *et al.*, 1988) and chemical cleavages with Fe(II)–bleomycin (Carter *et al.*, 1990) and DNA-linked heavy metal ions (Komiyama, 1995). However, none of these methods satisfy all the requirements to be able to be used practically, especially in site specificity and efficiency.

Ribonuclease H (RNase H) endonucleolytically hydrolyzes RNA of RNA–DNA hybrids (Crouch and Dirksen, 1982). We previously showed that DNA-linked ERNH, in which 9-mer DNA is covalently attached to the *Escherichia coli* RNase HI variant at position 135 through a flexible linker with a calculated length of 21 Å, cleaved an oligomeric RNA substrate in a sequence-specific manner (Kanaya *et al.*, 1992). We later showed that the site specificity and catalytic efficiency of a DNA-linked ERNH at 30°C can be improved by increasing the size of the linker to 27 Å (Uchiyama *et al.*, 1994) and decreasing the size of the DNA adduct to 8-mer (Kanaya *et al.*, 1994). In addition, we showed that a DNA-linked TRNH, which was constructed by using RNase HI from *Thermus thermophilus* HB8, instead of *E.coli* RNase HI, cleaved an oligomeric RNA substrate more efficiently than the DNA-linked ERNH at temperatures higher than 50°C (Haruki *et al.*, 2000). However, it remained to be determined whether position 135 is the most appropriate cross-linking site and whether a natural RNA molecule with highly ordered structure can be site-specifically cleaved by DNA-linked RNase H. It has been shown previously that DNA-linked ERNH site-specifically cleaved 132- and 534-mer RNAs, which were prepared by run-off transcriptions (Nakai *et al.*, 1994). However, it is unlikely that these RNA molecules are highly structured. Therefore, we decided to examine whether alteration of the cross-linking site affects the site specificity and catalytic efficiency of DNA-linked RNase H and whether DNA-linked RNase H can site-specifically cleave MS2 RNA.

MS2 RNA is RNA of bacteriophage MS2 and is composed of 3569 bases (Fiers *et al.*, 1976). This RNA is characterized by a relatively high melting temperature (58°C in 10 mM potassium phosphate buffer, pH 7.0, containing 0.1 M KCl) and high hyperchromicity (~30%) (Mitra *et al.*, 1963). Based on the enzymatic and chemical sensitivities of this RNA, it has been proposed that this RNA assumes a highly ordered structure, in which more than 70% of the nucleotides take part in base pairing and are condensed by long-distance interactions (Min Jou *et al.*, 1972; Fiers *et al.*, 1975, 1976; Skripkin *et al.*, 1990).

In this work, we showed that, of the DNA-linked TRNHs with the DNA adduct at positions 135, 136, 137 and 138, that with the DNA adduct at position 135 most efficiently cleaved the complementary RNA. We also showed that DNA-linked TRNH, in which the 16-mer DNA is cross-linked at position 135 of *T.thermophilus* RNase HI through a 27 Å linker, site-specifically cleaved MS2 RNA within the sequence complementary to its DNA adduct.

Materials and methods

Materials

[γ-^{32}P]ATP (>5000 Ci/mmol) was obtained from Amersham, reverse transcriptase (RAV-2) from Takara Shuzo, *Crotalus durissus* phosphodiesterase and bacteriophage MS2 RNA from Boehringer Mannheim and *N*-(ε-maleimidocaproyloxy)-succinimide from Dojindo Laboratories. The 15-mer RNA with a polypurine-tract sequence of HIV-1 RNA (PPT-RNA) and the complementary 15-mer DNA with aminohexyl linker at the 5′-terminus (5′-aminohexyl-d15-mer) were synthesized previously (Haruki *et al.*, 2000). The 5′-aminohexyl-d20-mer (5′-CATATAATATCGTCCCCGTA-3′) and the 5′-aminohexyl-d16-mer, 5′-aminohexyl-d12-mer and 5′-aminohexyl-d8-mer, which represent 3′-truncated forms of the 5′-aminohexyl-d20-mer, were synthesized at Kurabou.

Preparation of T.thermophilus RNase HI variants

The *T.thermophilus* RNase HI variant C135/TRNH with the Cys13→Ser, Cys63→Ala and Arg135→Cys mutations was constructed previously (Haruki *et al.*, 2000). The genes encoding *T.thermophilus* RNase HI variants C136/TRNH, C137/TRNH and C138/TRNH were constructed by polymerase chain reaction (PCR) using the gene encoding the C135/TRNH as a template, as described previously (Haruki *et al.*, 2000). The mutagenic primers were designed so that the codon for Cys135 (TGC) is changed to CGG for Arg and the codon for Glu136 (GAG), Ala137 (GCG) or Arg138 (AGG) is changed to TGT for Cys. The nucleotide sequences of these genes were confirmed by the dideoxy chain termination method (Sanger *et al.*, 1977). Overproduction and purification of the mutant proteins were carried out as described previously (Kanaya and Itaya, 1992; Haruki *et al.*, 2000). The purities of the mutant proteins were analyzed by SDS–PAGE (Laemmli, 1970).

Construction of DNA-linked RNases H

Synthesis of the 5′-maleimide-DNAs from the 5′-aminohexyl-DNAs and *N*-(ε-maleimidocaproyloxy)succinimide, coupling reactions between the *T.thermophilus* RNase HI variants and the 5′-maleimide-DNAs and the purification of the resultant DNA-linked RNases H (d15-C135/TRNH, d15-C136/TRNH, d15-C137/TRNH, d15-C138/TRNH, d8-C135/TRNH, d12-C135/TRNH, d16-C135/TRNH and d20-C135/TRNH) were carried out as described previously (Kanaya *et al.*, 1992). These DNA-linked RNases H have a 27 Å linker [maleimide–$(CH_2)_5$–CONH–$(CH_2)_6$–].

Enzymatic activity

The enzymatic activities of the C135–C138/TRNHs were determined at 30°C for 15 min in 20 µl of 10 mM Tris–HCl (pH 9.0) containing 1 mM $MgCl_2$ and 50 µg/ml acetylated bovine serum albumin by measuring the radioactivity of the acid-soluble digestion product from the substrate, ^{3}H-labeled M13 DNA–RNA hybrid, as described previously (Kanaya *et al.*, 1991a). One unit is defined as the amount of enzyme producing 1 µmol/min of acid-soluble material. The specific activity was defined as the enzymatic activity/mg of protein. The concentrations of the mutant proteins were determined from the UV absorption at 280 nm assuming that they have the same $A_{280}^{0.1\%}$ value of 1.6 as that of the wild-type protein.

Cleavage of PPT-RNA

Hydrolyses of the ^{32}P-labeled PPT-RNA with DNA-linked TRNHs at 65°C for 15 min and separation of the hydrolyzates on a 20% polyacrylamide gel containing 7 M urea were carried out as described previously (Haruki *et al.*, 2000). The reaction buffer was the same as that for the hydrolysis of M13 DNA–RNA hybrid. One unit is defined as the amount of enzyme cleaving 1 µmol/min of the substrate. The specific activity was defined as the enzymatic activity/mg of protein. The concentrations of the DNA-linked RNases H were determined from the UV absorption at 280 nm assuming that the molar absorption coefficients of the d15-TRNH, d8-TRNH, d12-TRNH, d16-TRNH and d20-TRNH are 1.47×10^5, 0.76×10^5, 0.97×10^5, 1.95×10^5 and 2.38×10^5, respectively.

Cleavage of bacteriophage MS2 RNA

Bacteriophage MS2 RNA (7 pmol) was digested with DNA-linked enzymes at 65°C for 15 min in 10 µl of the same buffer as for the hydrolysis of M13 DNA/RNH hybrid. Prior to the reaction, the enzyme solution was incubated for 15 min. The hydrolyzates were separated on a 3.5% polyacrylamide gel containing 7 M urea and were visualized with ethidium bromide staining.

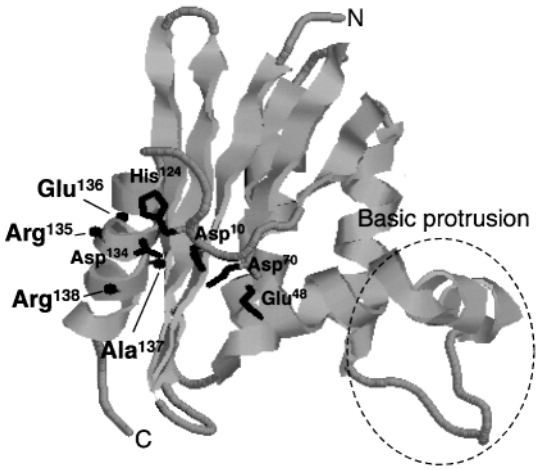


Fig. 1. Three-dimensional structure of *T.thermophilus* RNase HI. The backbone structure (Ishikawa *et al.*, 1993) was drawn with the program RasMol. N and C represent the N- and C-termini of this structure. The β-carbons of Arg135, Glu136, Ala137 and Arg138, and also the side chains of the active-site residues (Asp10, Glu48, Asp70, His124 and Asp134), are indicated. The basic protrusion which is important for substrate binding (Kanaya *et al.*, 1991b) is also indicated. This crystal structure has been deposited at the Brookhaven Protein Data Bank under accession number 1RIL.

Primer extension

The 16-mer DNA (5′-TGCAATCTCACTGGGA-3′) was labeled at the 5′-end with [γ-^{32}P]ATP and used as a primer. This primer (0.14 pmol) was hybridized to RNA in 20 µl of 50 mM Tris–HCl (pH 8.3) containing 75 mM KCl, 8 mM $MgCl_2$, 10 mM dithiothreitol and 1 mM each of dNTPs at 65°C for 30 min and then slowly cooled to room temperature. The reaction was started by addition of 25 U of RAV-2 reverse transcriptase, allowed to proceed at 42°C for 60 min and terminated by addition of formamide/dye loading buffer. The products were separated on a 20% polyacrylamide gel containing 7 M urea and were analyzed with Instant Imager (Packard). These products were identified by comparing their migrations on the gel with those of the oligonucleotides generated by the partial digestion of the ^{32}P-labeled 32-mer DNA with snake venom phosphodiesterase (Jay *et al.*, 1974). This 32-mer DNA shares the sequence with the 16-mer DNA used as a primer, but has a 16-base extension (5′-CATATAATATCGTCCC-3′) at the 3′-terminus.

Results

Mutant design

For the construction of DNA-linked *E.coli* RNase HI (ERNH) and *T.thermophilus* RNase HI (TRNH), position 135 has been chosen as the site to which a DNA oligomer is cross-linked at its 5′-terminus through a flexible linker (Kanaya *et al.*, 1992, 1994; Nakai *et al.*, 1994; Uchiyama *et al.*, 1994; Haruki *et al.*, 2000). This site was chosen because the orientation of the cross-linked DNA permits the interaction of a DNA–RNA substrate with the enzyme (Nakamura *et al.*, 1991). We previously showed that position 13 is not an appropriate site for cross-linking, because the d15-C13/TRNH, in which the 15-mer DNA is cross-linked at Cys13 of the TRNH variant, cleaved the PPT-RNA much less efficiently than did the d15-C135/TRNH, in which the 15-mer DNA is cross-linked at

Table I. Specific activities of the mutant proteins for the hydrolysis of the M13 DNA–RNA hybrid

Protein	Mutation	Specific activity[a] (units/mg)	Relative activity[b] (%)
Wild-type TRNH	–	1.6	100
C135/TRNH	$C^{13}\rightarrow S$, $C^{63}\rightarrow A$, $R^{135}\rightarrow C$	0.11	6.9
C136/TRNH	$C^{13}\rightarrow S$, $C^{63}\rightarrow A$, $E^{136}\rightarrow C$	0.12	7.5
C137/TRNH	$C^{13}\rightarrow S$, $C^{63}\rightarrow A$, $A^{137}\rightarrow C$	0.25	16
C138/TRNH	$C^{13}\rightarrow S$, $C^{63}\rightarrow A$, $R^{138}\rightarrow C$	0.02	1.3

[a]Errors, which represent the 67% confidence limits, are all at or below ±30% of the values reported.
[b]The relative activity was calculated by dividing the specific activity of the mutant protein by that of the wild-type protein.

Cys135 of the TRNH variant (Haruki *et al.*, 2000). Position 13 is not an appropriate cross-linking site, probably because it is located too close to the active site to permit an efficient interaction of the cross-linked DNA–RNA substrate with the enzyme. However, position 135 was arbitrarily chosen as a cross-linking site from several potential neighboring sites. Therefore, we decided to construct three mutant proteins, C136/TRNH, C137/TRNH and C138/TRNH, in which the single free cysteine residues are located at positions 136, 137 and 138, respectively, to examine whether the catalytic efficiency and site-selectivity of a DNA-linked TRNH can be altered by changing a cross-linking site. For the construction of DNA-linked RNase H, TRNH, instead of ERNH, was used, because DNA-linked TRNH is much more stable than DNA-linked ERNH and its maximum activity is higher than that of the latter (Haruki *et al.*, 2000). The localizations of Arg135, Glu136, Ala137 and Arg138 in the crystal structure of TRNH, which were individually replaced by Cys to construct the mutant proteins, are shown in Figure 1.

Enzymatic activities of the mutant proteins

The enzymatic activities of the mutant proteins determined by using M13 DNA–RNA hybrid as a substrate are summarized in Table I. All mutant proteins contain triple mutations. However, It has been reported previously that the Cys13→Ser mutation reduced the enzymatic activity by 84% whereas the Cys63→Ala mutation did not seriously affect it (Haruki *et al.*, 2000). Therefore, on the assumption that effect of each mutation on the enzymatic activity of TRNH is independent and cumulative, we could estimate that the Glu136→Cys mutation reduced the enzymatic activity of TRNH by ~50% as did the Arg135→Cys mutation, the Ala137→Cys mutation did not seriously affect it and the Arg138→Cys mutation greatly reduced it to 8% of that of the wild-type protein. The Arg138→Cys mutation seriously affects the enzymatic activity, probably because Arg138 faces the active site and therefore engages in substrate binding through electrostatic interactions.

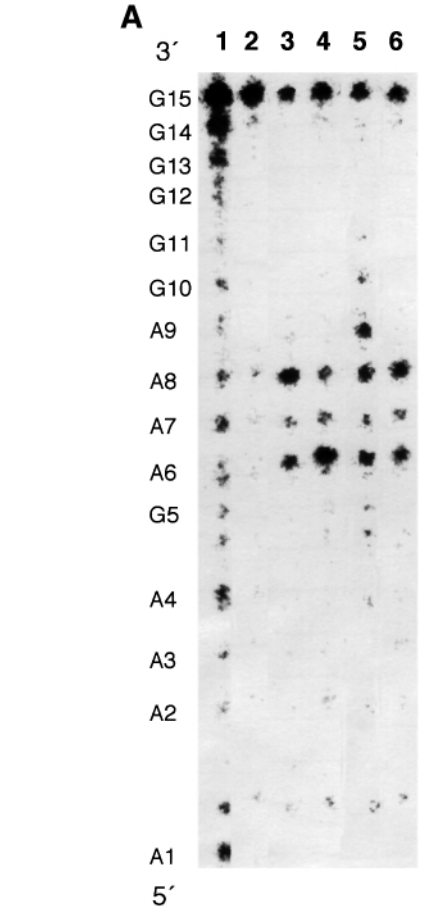
Cleavage of PPT-RNA with DNA-linked TRNHs

It was shown previously that the d15-C135/TRNH effectively cleaves the 15-mer RNA with a polypurine-tract sequence of HIV-1 (PPT-RNA) at high temperatures (most optimally at 65°C) (Haruki *et al.*, 2000). This DNA-linked TRNH was constructed by cross-linking the 15-mer DNA complementary to the PPT-RNA to Cys135 of the C135/TRNH. Therefore, the d15-C136/TRNH, d15-C137/TRNH and d15-C138/TRNH were constructed and examined to see whether and how these DNA-linked TRNHs cleave the PPT-RNA at 65°C. The results are shown in Figure 2A and summarized in Figure 2B. The d15-C135/TRNH, d15-C136/TRNH and d15-C138/TRNH cleaved the PPT-RNA at three positions, A6–A7, A7–A8 and A8–A9. However, d15-C136/TRNH differs from other two DNA-linked TRNHs in site preference. The former cleaved the PPT-RNA most preferentially at A6–A7, whereas the latter cleaved it most preferentially at A8–A9. Of these DNA-linked TRNHs, the d15-C135/TRNH most efficiently cleaved the PPT-RNA, because its specific activity was roughly double those of the d15-C136/TRNH and d15-C138/TRNH. The d15-C137/TRNH exhibited low enzymatic activity compared with those of other DNA-linked TRNHs. In addition, it cleaved the PPT-RNA at multiple positions in a less strict manner. These results indicate that position 135 is the most appropriate cross-linking site among those examined.

Cleavage of MS2 RNA with DNA-linked TRNHs

MS2 phage RNA consists of 3569 bases. Because any DNA oligomer can be cross-linked to C135/TRNH, any RNA sequence can be chosen as a target site of DNA-linked TRNH. However, DNA-linked TRNH can cleave RNA only when its DNA adduct forms a DNA–RNA hybrid. Because MS2 RNA is highly structured and consists of many stems and loops according to the secondary structure model (Fiers *et al.*, 1976), DNA-linked TRNH may not be able to cleave MS2 RNA at a stem region or a region which is buried inside the molecule. Therefore, we chose a loop around residue 2790 as a target site (Figure 3). This loop has been shown to be highly susceptible to RNase T_1 digestion (Fiers *et al.*, 1976) and is therefore expected to expose to the solvent.

Four DNA-linked TRNHs with a series of DNA adducts varying in size were constructed. They are d8, d12, d16 and d20-C135/TRNHs, in which the 8-, 12-, 16- and 20-mer DNAs are cross-linked to Cys135 of the TRNH variant, respectively. The 20-mer DNA is complementary to MS2 RNA at 2775–2794, in which residues 2783–2794 form a loop (Figure 3). Other DNA oligomers represent truncated forms of the 20-mer DNA at the 3′-terminus. These four DNA-linked TRNHs were constructed because the optimum size of the DNA adduct for efficient cleavage was not predictable. When these DNA-linked TRNHs were examined for the hydrolysis of MS2 RNA at 65°C for 15 min, only the d16-C135/TRNH hydrolyzed it to produce a single RNA fragment with a size of roughly 800 bases (Figure 4). This RNA fragment is equivalent to the 3′-terminal fragment of MS2 RNA in size, which is produced by the cleavage at the target site, suggesting that the d16-C135/TRNH site-specifically cleaves MS2 RNA within its recognition sequence. The d20-C135/TRNH hydrolyzed MS2 RNA much more efficiently than the d16-C135/TRNH, but produced multiple RNA fragments, in addition to the ~800 base RNA fragment (Figure 4). The d8- and d12-C135/TRNHs only poorly hydrolyzed MS2 RNA. These results suggest that the d16-C135/TRNH cleaves MS2 RNA most efficiently and



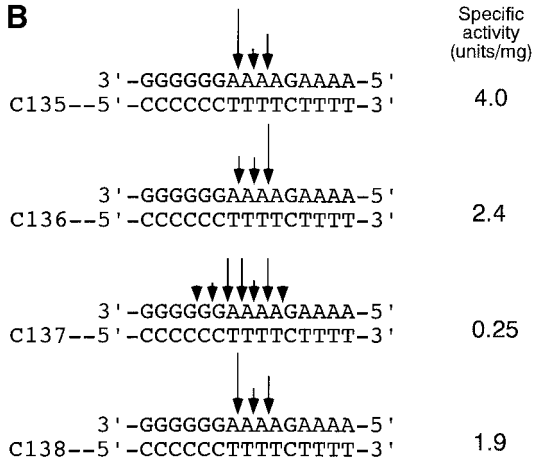


Fig. 2. Cleavage of the PPT-RNA with DNA-linked TRNHs. (**A**) Autoradiograph of cleavage reactions. The hydrolyses of the 5′ end-labeled PPT-RNA (10 pmol) with 0.16 ng (0.008 pmol) of the d15-C135/TRNH (lane 3), 0.16 ng (0.008 pmol) of the d15-C136/TRNH (lane 4), 1.6 ng (0.08 pmol) of the d15-C137/TRNH (lane 5) and 0.16 ng (0.008 pmol) of the d15-C138/TRNH (lane 6) were carried out at 65°C for 15 min and the hydrolyzates were separated on a 20% polyacrylamide gel containing 7 M urea as described under Materials and methods. The concentration of the substrate is 1.0 μM. Lane 1, partial digest of the PPT-RNA with snake venom phosphodiesterase; lane 2, untreated PPT-RNA. (**B**) The sites and extents of cleavages by the DNA-linked TRNHs. Cleavage sites of the PPT-RNA with various DNA-linked TRNHs are shown by arrows. The difference in the size of the arrows reflects the relative cleavage intensity at the indicated position. The upper and lower sequences represent RNA and DNA, respectively. C135–C138 represent the TRNH variants linked at the 5′-terminus of the 15-mer DNA. The specific activities of these DNA-linked TRNHs are also shown.

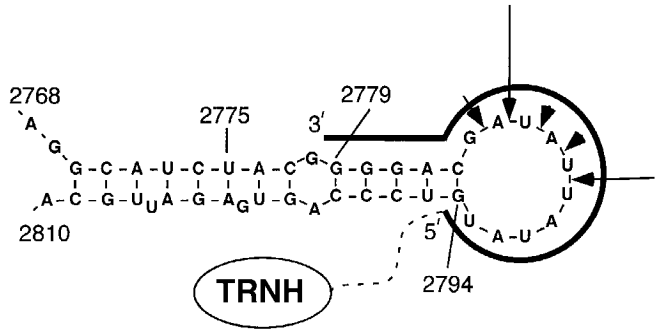


Fig. 3. Proposed secondary structure of MS2 RNA at 2768–2810. The secondary structure of MS2 RNA at 2768–2810 proposed by Fiers *et al.* (1976) is shown. The numbers represent the positions of the nucleotides in the MS2 RNA sequence. The interaction between the d16-C135/TRNH and target RNA sequence is also schematically shown. For the d16-C135/TRNH, the protein, linker and DNA portions are indicated by TRNH, broken line and thick line, respectively. Cleavage sites of MS2 RNA with the d16-C135/TRNH are shown by arrows. The difference in the size of the arrows reflects the relative cleavage intensity at the indicated position.

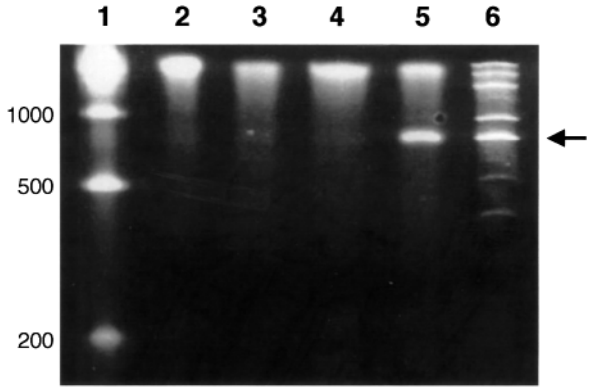


Fig. 4. Cleavage of MS2 RNA with DNA-linked TRNHs. Hydrolyzates of MS2 RNA (7 pmol) with 1 pmol of the d8-C135/TRNH (lane 3), d12-C135/TRNH (lane 4), d16-C135/TRNH (lane 5) and d20-C135/TRNH (lane 6) at 65°C for 15 min were separated on a 3.5% polyacrylamide gel containing 7 M urea and visualized with ethidium bromide staining. Lane 1, 0.2–10 kb RNA size markers (Perfect RNA Markers, Novagen); lane 2, untreated MS2 RNA. The numbers along the gel represent the size of each RNA marker in the bases. The 800-base RNA fragment produced upon cleavage at the target site is shown by an arrow.

site-specifically among those examined. When the d16-C135/TRNH was incubated with MS2 RNA at different temperatures ranging from 30 to 80°C, it cleaved MS2 RNA most efficiently at 65°C and very poorly at 30 and 80°C (data not shown). This result is consistent with the previous one, in which the PPT-RNA was used as a substrate (Haruki *et al.*, 2000).

Determination of the cleavage site(s)

To determine the cleavage site(s) of MS2 RNA, the ~800-base RNA fragment produced upon hydrolysis of MS2 RNA with the d16-C135/TRNH at 65°C for 15 min was extracted from the gel and used as a template for the primer extension reaction. The 16-mer primer labeled at the 5′-end is complementary to MS2 RNA at 2795–2810. The reaction products were separated on urea-gel and detected by autoradiography (Figure 5). The band detected at A16 is the 16-mer primer which is not extended. The bands detected at A22–T26 represent the extended products. These results are summarized in Figure 3. The d16-C135/TRNH cleaved MS2 RNA most efficiently at 2785–2786 (A–U). It also efficiently cleaved MS2 RNA at

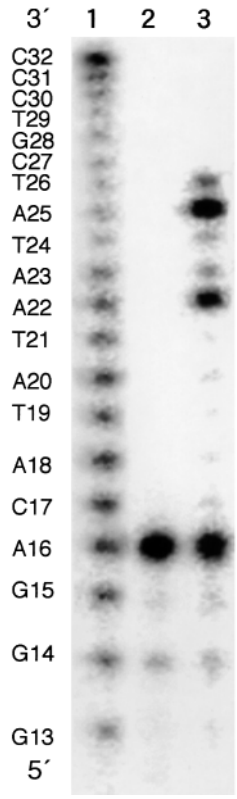


Fig. 5. Identification of the primer extension products. The primer extension products were separated on a 20% polyacrylamide gel containing 7 M urea and were analyzed with Instant Imager. The primer extension reaction was carried out as described under Materials and methods. Lane 1, partial digest of the ^{32}P-labeled 32-mer DNA, which is complementary to the MS2 RNA sequence at 2779–2810, with snake venom phosphodiesterase; lane 2, the ^{32}P-labeled 16-mer DNA used as a primer, which is complementary to the MS2 RNA sequence at 2795–2810; lane 3, the primer extension products.

2788–2789 (U–U), but cleaved 2784–2785 (G–A), 2786–2787 (U–A) and 2787–2788 (A–U) with much lower efficiency.

Discussion

DNA-linked RNase H site-specifically cleaves RNA at the region where its DNA adduct forms a DNA–RNA hybrid. However, this DNA–RNA hybrid interacts with RNase H in a constrained manner, because it is cross-linked to RNase H. Therefore, the cross-linking site is one of the factors that determine the site specificity and hydrolytic efficiency of DNA-linked RNase H. In this study, we constructed four types of the DNA-linked TRNHs, in which the 15-mer DNA complementary to the PPT-RNA was cross-linked at positions 135, 136, 137 and 138. Comparison of the specific activities of these DNA-linked TRNHs for the cleavage of the PPT-RNA indicated that position 135 is the most appropriate cross-linking site. However, the specific activities of the d15-C136/TRNH and d15-C138/TRNH were lower than that of the d15-C135/TRNH by only ~50%, suggesting that the enzymatic activity of DNA-linked TRNH is fairly tolerant to the substitution of the cross-linking site. For the construction of these DNA-linked TRNHs, a 27 Å linker, which is the longest linker among those commercially available, was used. The flexibility of this linker may cancel the constraint caused by a shift of the cross-linking site. Therefore, it seems unlikely that alteration of the cross-linking site to other potential sites dramatically improves the hydrolytic efficiency of DNA-linked RNase H.

The d15-C137/TRNH cleaved the PPT-RNA not only with greatly reduced efficiency but also in a less strict site-specific manner, indicating that position 137 is not suitable for cross-linking. Because the side chain of Cys137, whose position is equivalent to that of Ala137 (Figure 1), faces the catalytic site of the protein and is located very close to it, the interaction between the cross-linked substrate and enzyme may be severely limited. It is noted that the Arg138→Cys mutation greatly reduced the enzymatic activity (Table I). Nevertheless, the specific activity of the d15-C138/TRNH was comparable to that of the d15-C136/TRNH. Hence the Arg138→Cys mutation seriously affects the enzymatic activity only when the enzyme interacts with the unlinked substrate. These results strongly suggest that Arg138 is involved in substrate binding. Arg138 is conserved in *E.coli* RNase HI and the mutation of this residue to Cys also greatly reduced the enzymatic activity by 80% (S.Kanaya, unpublished work).

The d15-C135/TRNH and d15-C138/TRNH cleaved the PPT-RNA most preferentially at A8–A9, whereas the d15-C136/TRNH cleaved it most preferentially at A6–A7. According to the three-dimensional models of C135/TRNH, C136/TRNH and C138/TRNH, the side chains of Cys135, Cys136 and Cys138, which are substituted for Arg135, Glu136 and Arg138, respectively, face away from the active site of the protein. These models are not shown, but the positions of the β-carbons of these cysteine residues are nearly identical with those of Arg135, Glu136 and Arg138 shown in Figure 1. Therefore, the linker between the DNA adduct and the protein must form a loop in order to bring the DNA–RNA substrate in contact with the active site of the protein. The distance between Cys135 and the active site of the protein is comparable to that between Cys138 and the active site of the protein, whereas the distance between Cys136 and the active site of the protein is considerably larger than the others. This may be the reason why the most preferable cleavage site of the PPT-RNA was shifted by two by changing the cross-linking site from position 135 or 138 to position 136. The 27 Å linker may be too short for the d15-C136/TRNH to permit the interaction between the cross-linked DNA–RNA substrate and the protein so that the PPT-RNA is cleaved at A8–A9.

Of the four DNA-linked TRNHs which differ in size of the DNA adduct, only the d16-C135/TRNH cleaved MS2 RNA at the target site alone at 65°C (Figure 4). The d8-C135/TRNH and d12-C135/TRNH could not cleave MS2 RNA at this temperature, probably because the DNA adducts in these DNA-linked TRNHs are too short to form a stable DNA–RNA hybrid at 65°C. These DNA-linked TRNHs could not efficiently cleave MS2 RNA even at <40°C, probably because the tertiary structure of MS2 RNA is too stable to permit the formation of a DNA–RNA hybrid between the DNA adduct and the target RNA at these temperatures. In addition, the catalytic efficiency of DNA-linked TRNH has been reported to be greatly reduced at <40°C (Haruki *et al.*, 2000). In contrast, the d20-C135/TRNH efficiently cleaved MS2 RNA, but in a poor site-specific manner. It cleaved MS2 RNA not only at the target site but also at several alternative sites. These alternative sites remained to be determined. However, MS2 RNA has two potential sequences UGGCGAGACGAUACGAUGGG at 2642–2661 and CACAGUGACGACUUUACAGC at 1872–1891 that can be recognized by the d20-C135/TRNH. Twelve out of the 20 bases in these sequences

(underlined bases) are conserved in the target sequence at 2775–2794. If MS2 RNA were cleaved at the former site, then 2.65 and 0.92 kb fragments would be produced. Likewise, if MS2 RNA were cleaved at the latter site, then 1.88 and 1.69 kb fragments would be produced. According to the PAGE results shown in Figure 4, several RNA fragments, in addition to the 0.8 kb fragment, were produced upon cleavage of MS2 RNA with the d20-C135/TRNH. Of these, the largest fragment may represent uncleaved MS2 RNA. The other three fragments with sizes larger than 0.8 kb may represent the RNA fragments produced upon cleavage at these alternative sites.

The d16-C135/TRNH cleaved MS2 RNA not only site-specifically but also in a catalytic manner, because ~50% of MS2 RNA was cleaved at a substrate:enzyme ratio of 7:1 (Figure 4). These results indicate that the size of the DNA adduct is important for the DNA-linked RNase H to cleave RNA both efficiently and site-specifically. If the DNA adduct were too short, DNA-linked RNase H would not cleave RNA efficiently, because its DNA adduct cannot form a stable DNA–RNA hybrid with the target sequence. Alternatively, if the DNA adduct were too long, DNA-linked RNase H would also cleave RNA at the additional sites, because its DNA adduct can form a stable DNA–RNA hybrid with the sequences which are not fully complementary.

The d16-C135/TRNH cleaved MS2 RNA at the multiple positions within the target sequence (Figure 3). It has been shown previously that the DNA-linked enzyme with the 9-mer DNA adduct cleaves RNA at the unique position within the target sequence (Kanaya *et al.*, 1992, 1994; Uchiyama *et al.*, 1994). Therefore, only the DNA-linked RNases H with relatively short DNA adducts may be able to cleave RNA at the unique position. However, MS2 RNA can be efficiently cleaved by the DNA-linked TRNHs with relatively long DNA adducts only at $>50°C$. In addition, it was not efficiently cleaved by the DNA-linked ERNHs at $<40°C$ (H.Chon, unpublished work), probably because the tertiary structure of MS2 RNA is too stable to permit the formation of a DNA–RNA hybrid at these temperatures. These results suggest that highly structured RNA, such as MS2 RNA, can be site-specifically cleaved by a thermostable DNA-linked RNase H at high temperatures, but at the multiple positions within the target sequence. It is noted that the major cleavage site nearest the 5′-end of the hybrid region of RNA was the phosphodiester bond between the seventh and eighth residues. This result is consistent with the proposal that the DNA residues complementary to the RNA residues, which are located six or seven residues upstream from the cleavage site, interact with the basic protrusion of the enzyme (Kanaya and Kanaya, 1995; Iwai *et al.*, 1996).

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