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Cleavage of a DNA–RNA–DNA/DNA chimeric substrate containing a single ribonucleotide at the DNA–RNA junction with prokaryotic RNases HII

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Abstract **We have analyzed the cleavage specificities of various prokaryotic Type 2 ribonucleases H (RNases H) on chimeric DNA–RNA–DNA/DNA substrates containing one to four ribonucleotides. RNases HII from *Bacillus subtilis* and *Thermococcus kodakaraensis* cleaved all of these substrates to produce a DNA segment with a 5′-monoribonucleotide. Consequently, these enzymes cleaved even the chimeric substrate containing a single ribonucleotide at the DNA–RNA junction (5′-side of the single ribonucleotide). In contrast, *Escherichia coli* RNase HI and *B. subtilis* RNase HIII did not cleave the chimeric substrate containing a single ribonucleotide. These results suggest that bacterial and archaeal RNases HII are involved in excision of a single ribonucleotide misincorporated into DNA.**

Key words: Type 2 ribonuclease H; DNA–RNA–DNA/DNA heteroduplex; DNA–RNA junction; *Bacillus subtilis*; *Thermococcus kodakaraensis*

1. Introduction

Ribonuclease H (RNase H) degrades only the RNA strand of an RNA/DNA heteroduplex [1]. The enzyme is ubiquitously present in various organisms. Single bacterial and eukaryotic cells often contain two different RNases H, which show little sequence similarity with each other [2,3]. Based on the difference in the amino acid sequences, RNases H are classified into two major families, Type 1 and Type 2 RNases H [2,3]. Type 1 enzymes include bacterial RNases HI, eukaryotic RNases H1, and the RNase H domains of reverse transcriptases. Type 2 enzymes include bacterial RNases HII and HIII, archaeal RNases HII, and eukaryotic RNases H2. According to this classification, mammalian RNases H are classified into RNases H1 and H2. However, they have been classified into Class I (RNase HI) and Class II (RNase HII) RNases H, based on the difference in the biochemical properties of the enzymes purified from the cells [4]. Class I RNase H from calf thymus (RNase HI) has recently been shown to consist of two subunits with molecular masses of 32 kDa and 21 kDa [5]. This 32 kDa subunit is equivalent to RNase H2 and is enzymatically active as judged by renaturation gel assay.

It has been suggested that mammalian RNases HI are involved in the removal of Okazaki fragments together with flap endonuclease-1 (FEN-1) [6,7]. These mammalian enzymes specifically recognize an RNA–DNA junction region and cleave RNA–DNA/DNA heteroduplex to produce a DNA segment with a 5′-monoribonucleotide, which is then digested by FEN-1. In addition to these mammalian enzymes, yeast RNase H(35) [8], which is a Type 2 RNase H [9], archaeal RNases HII [10–12], and bacterial RNase HII [2] exhibit similar cleavage specificity on RNA–DNA/DNA or DNA–RNA–DNA/DNA substrates. Coordination of Type 2 RNase H with FEN-1 in Okazaki fragment removal has also been proposed for yeast RNase H(35) [8] and archaeal RNase HII [10,12]. A yeast strain lacking both of the RNase H(35) and FEN-1 genes has been shown to exhibit lethal phenotype [8]. In bacteria, which lack FEN-1, Okazaki fragments are removed by RNase H and the 3′→5′ exonuclease activity of DNA polymerase I [13].

The major RNase H purified from K562 human erythroleukemia cells hydrolyzes a DNA–RNA–DNA/DNA substrate containing a single ribonucleotide at the DNA–RNA junction (5′-side of the single ribonucleotide), suggesting that it is involved in ribonucleotide excision from genomic DNA during DNA replication [14]. This RNase H has been designated as RNase H(1) (terminology is altered from RNase H1 to avoid confusion in this report). It remained to be determined whether human RNase H(1) is related to RNase H1 or H2. However, its enzymatic properties and subunit structure are similar to those of calf thymus RNase HI [5], suggesting that it is related to RNase H2. Therefore, an ability to cleave DNA–RNA junction may be one of the characteristics common to Type 2 RNases H. However, it remained to be determined whether prokaryotic Type 2 RNases H cleave a DNA–RNA junction as well.

Several prokaryotic Type 2 RNases H, such as *Escherichia coli* RNase HII [15,16], *Bacillus subtilis* RNases HII and HIII [2], and RNases HII from hyperthermophilic archaea *Thermococcus kodakaraensis* KOD1 [11] and *Archaeoglobus fulgidus* [10,12], have been overproduced in *E. coli*, purified, and biochemically characterized. In this report, we analyzed cleavage specificities of *B. subtilis* RNase HII, *B. subtilis* RNase HIII, and *T. kodakaraensis* RNase HII in comparison with that of *E. coli* RNase HI using chimeric DNA–RNA–DNA/DNA

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heteroduplexes containing one to four ribonucleotides as substrates.

2. Materials and methods

2.1. Materials

[γ-^{32}P]ATP (> 5000 Ci/mmol) was obtained from Amersham. *Crotalus durissus* phosphodiesterase was from Boehringer Mannheim. DNA–RNA–DNA chimeric oligonuclotides (5′-CGTCCC[rA]$_{1-4}$CCGTGC-3′) and their complementary DNA oligonucleotides were chemically synthesized by Fasmac Co.

2.2. Enzyme preparation

E. coli RNase HI [17], *T. kodakaraensis* RNase HII [11], and *B. subtilis* RNases HII and HIII [2] were overproduced in *E. coli* and purified as reported previously. The concentrations of these proteins were determined from the UV absorption on the basis that the absorbance at 280 nm of a 0.1% solution is 2.0 for *E. coli* RNase HI, 0.63 for *T. kodakaraensis* RNase HII, 0.93 for *B. subtilis* RNase HII, and 0.62 for *B. subtilis* RNase HIII. These values, except that of *E. coli* RNase HI which has been experimentally determined [18], were calculated by using ε of 1576 M^{-1} cm^{-1} for Tyr and 5225 M^{-1} cm^{-1} for Trp at 280 nm [19].

2.3. Cleavage of DNA–RNA–DNA/DNA heteroduplexes

The DNA–RNA–DNA strands were ^{32}P-labeled at the 5′-end. These ^{32}P-labeled DNA–RNA–DNA strands (1.0 μM) were hybridized with 1.5 molar equivalents of the complementary DNA strands to produce hybrid duplexes. These duplexes are designated as [rA]$_n$ substrates, in which n represents the number of adenosines. Hydrolysis of the substrate was carried out at 30°C for 15 min in 10 mM Tris–HCl (pH 8.0) containing 50 mM NaCl, 1 mM 2-mercaptoethanol, 0.1 mg/ml bovine serum albumin (BSA), and 10 mM $MgCl_2$ (for *E. coli* RNase HI and *T. kodakaraensis* RNase HII), 10 mM Tris–HCl (pH 8.0) containing 50 mM KCl, 1 mM 2-mercaptoethanol, 0.1 mg/ml BSA, and 10 mM $MnCl_2$ (for *B. subtilis* RNase HII), or 10 mM Tris–HCl (pH 8.5) containing 100 mM NaCl, 1 mM 2-mercaptoethanol, 0.1 mg/ml BSA, and 50 mM $MgCl_2$ (for *B. subtilis* RNase HIII). The hydrolysates were separated on a 20% polyacrylamide gel containing 7 M urea and were analyzed with Instant Imager (Packard). These hydrolysates were identified by comparing their migrations on the gel with those of the oligonucleotides generated by the partial digestion of the ^{32}P-labeled DNA–RNA–DNA with snake venom phosphodiesterase [20].

For the determination of the kinetic parameters, the concentrations of the substrate were varied from 0.1 to 1.0 μM. The amount of enzyme was controlled such that the ratio of the hydrolyzed substrate did not exceed 30% of the total. The hydrolysis of the substrate with the enzyme follows Michaelis–Menten kinetics, and the kinetic parameters, K_m and V_{max}, were determined from the Lineweaver–Burk plot.

3. Results

It has previously been shown that *E. coli* RNase HI, which represents Type 1 RNases H, cleaves the 20 bp chimeric DNA–RNA–DNA/DNA substrate containing four ribonucleotides (adenosines) (dT_7–rA_4–dT_9/$dA_7T_4A_9$) exclusively at the middle of these adenosines [21]. However, its cleavage efficiency dramatically decreased as the number of adenosines in the chimeric substrates decreased below three. As a result, *E. coli* RNase HI could not cleave the chimeric substrate containing one or two adenosines. In contrast, human RNase H(1), which may represent mammalian Type 2 RNases H, cleaved the dT_7–rA_4–dT_9/$dA_7T_4A_9$ exclusively at the phosphodiester bond between the third and fourth adenosines [14]. In addition, it cleaved even the chimeric substrate containing single adenosine at the 5′-side of this adenosine. To examine whether bacterial and archaeal Type 2 RNases H show similar substrate specificity to that of human RNase H(1), a series of chimeric DNA–RNA–DNAs (5′-CGTCCC[rA]$_{1-4}$CCGTGC-3′) were synthesized. These DNA sequences are arbitrary. The 6-mer DNA is flanked on either side of a stretch of adenosines in these chimeric DNAs, because 6–7 bp of DNA/RNA hybrid has been suggested to be sufficient to cover the substrate-binding site of *E. coli* RNase HI [22], and the substrate-binding site of *E. coli* RNase HI has been suggested to accommodate longer substrates than those of archaeal and bacterial RNases HII [12,23].

Cleavage of the [rA]$_{1-4}$ substrates with *T. kodakaraensis* RNase HII is shown in Fig. 1A and summarized in Fig. 1B. *T. kodakaraensis* RNase HII cleaved the [rA]$_4$ substrate preferentially at the phosphodiester bond between the third and fourth adenosines. This result agrees with that obtained using the 29 bp DNA–RNA–DNA/DNA substrate [11]. The enzyme cleaved the [rA]$_3$ substrate preferentially at the phosphodiester bond between the second and third adenosines, and the [rA]$_2$ substrate preferentially at the middle of the adenosines.

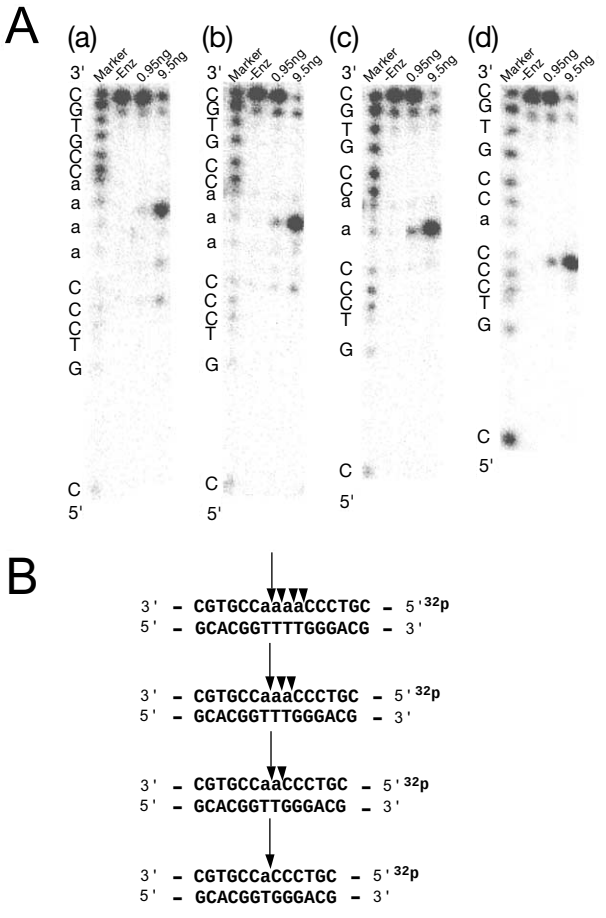

Fig. 1. Cleavage of DNA–RNA–DNA/DNA heteroduplexes by *T. kodakaraensis* RNase HII. A: The 5′-end-labeled DNA–RNA–DNA containing four (a), three (b), two (c), or one (d) adenosine(s) hybridized to the complementary DNA was hydrolyzed with *T. kodakaraensis* RNase HII at 30°C for 15 min and the hydrolysates were separated on a 20% polyacrylamide gel containing 7 M urea as described in Section 2. The concentration of the substrate is 1.0 μM. Partial digest of each DNA–RNA–DNA with snake venom phosphodiesterase was used as a marker. B: Cleavage sites of each DNA–RNA–DNA/DNA heteroduplex with *T. kodakaraensis* RNase HII are shown by arrows. The difference in the size of arrows reflects the relative cleavage intensities at the indicated position. Deoxyribonucleotides are shown by capital letters and ribonucleotides are shown by small letters.

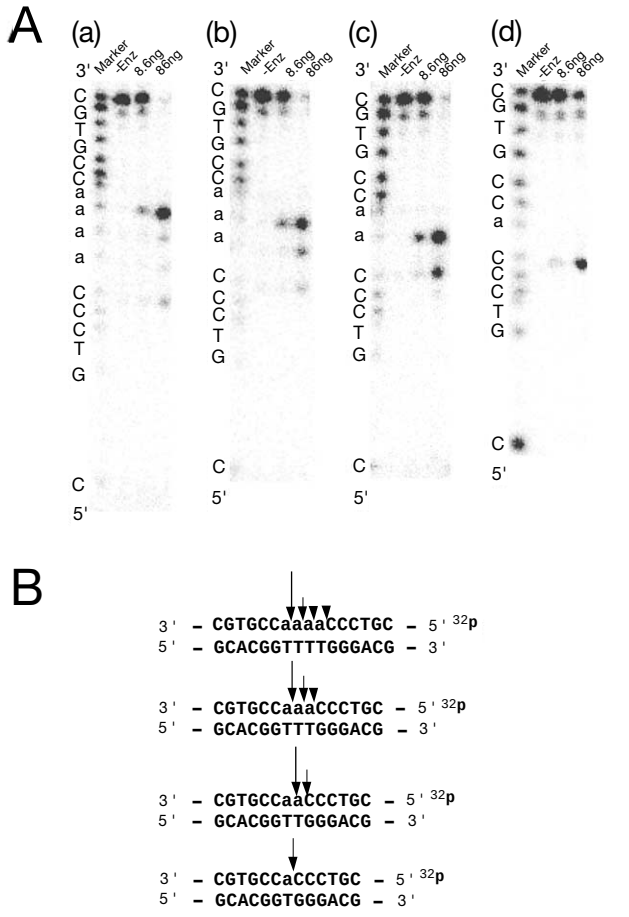

Fig. 2. Cleavage of DNA–RNA–DNA/DNA heteroduplexes by *B. subtilis* RNase HII. A: Hydrolysis of the 5′-end-labeled DNA–RNA–DNA containing four (a), three (b), two (c), or one (d) adenosine(s) hybridized to the complementary DNA with *B. subtilis* RNase HII and separation of the hydrolysates were carried out as described in the legend for Fig. 1. B: Cleavage sites of each DNA–RNA–DNA/DNA heteroduplex with *B. subtilis* RNase HII are shown as described in the legend for Fig. 1.

It also cleaved the $[rA]_1$ substrate exclusively at the DNA–RNA junction.

Cleavage of the $[rA]_{1-4}$ substrates with *B. subtilis* RNase HII is shown in Fig. 2A and summarized in Fig. 2B. *B. subtilis* RNase HII cleaved these substrates in a similar manner as did *T. kodakaraensis* RNase HII. However, *B. subtilis* RNase HII cleaved the $[rA]_1$ substrate less effectively than *T. kodakaraensis* RNase HII, because the amount of *B. subtilis* RNase HII required for complete cleavage of this substrate is at least 10 times larger than that of *T. kodakaraensis* RNase HII. In addition, *B. subtilis* RNase HII cleaved the $[rA]_{2-4}$ substrates less site-selectively than *T. kodakaraensis* RNase HII. Both enzymes cleaved these substrates at minor sites as well, which include the DNA–RNA junction and all possible RNA–RNA linkages. However, *B. subtilis* RNase HII cleaved at these minor sites more efficiently than *T. kodakaraensis* RNase HII.

Cleavage of the $[rA]_{1-4}$ substrates with *B. subtilis* RNase HIII is shown in Fig. 3A and summarized in Fig. 3B. *B. subtilis* RNase HIII cleaved the $[rA]_4$ substrate at the phosphodiester bonds between the first and second, the second and third, and the third and fourth adenosines to similar extents. It has previously been shown that *B. subtilis* RNase HIII cleaves the 29 bp DNA–RNA–DNA/DNA substrate preferentially at the phosphodiester bond between the second and third adenosines, and less preferentially at those between the first and second, and the third and fourth adenosines [2]. This disagreement might be due to the difference in the length and/or sequence of the DNAs flanking both sides of tetra-adenosine. *B. subtilis* RNase HIII cleaved the $[rA]_3$ substrate at the phosphodiester bonds between the first and second, and the second and third adenosines, but with much less efficiency. It cleaved the $[rA]_2$ substrate quite poorly at the phosphodiester bond between the first and second adenosines, and did not cleave the $[rA]_1$ substrate. Because the amount of *B. subtilis* RNase HIII required for complete cleavage of the $[rA]_4$ substrate is at least twice as much as that of *B. subtilis* RNase HII, *B. subtilis* RNase HIII less effectively cleaved even the $[rA]_4$ substrate than *B. subtilis* RNase HII.

Cleavage of the $[rA]_{1-4}$ substrates with *E. coli* RNase HI is shown in Fig. 4A and summarized in Fig. 4B. *E. coli* RNase HI cleaved the $[rA]_4$ substrate almost exclusively at the middle of the tetra-adenosine. It cleaved the $[rA]_3$ substrate at the phosphodiester bond between the first and second adenosines

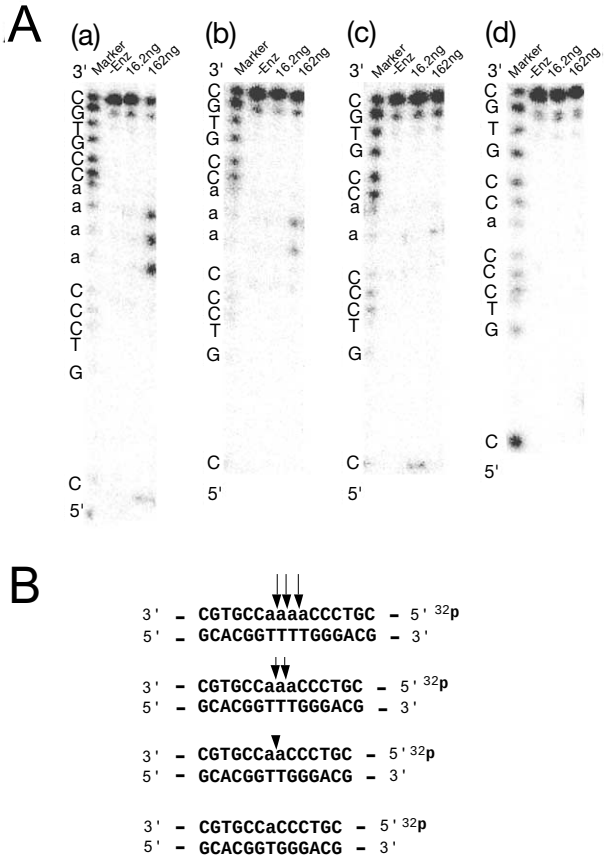

Fig. 3. Cleavage of DNA–RNA–DNA/DNA heteroduplexes by *B. subtilis* RNase HIII. A: Hydrolysis of the 5′-end-labeled DNA–RNA–DNA containing four (a), three (b), two (c), or one (d) adenosine(s) hybridized to the complementary DNA with *B. subtilis* RNase HIII and separation of the hydrolysates were carried out as described in the legend for Fig. 1. B: Cleavage sites of each DNA–RNA–DNA/DNA heteroduplex with *B. subtilis* RNase HIII are shown as described in the legend for Fig. 1.

with much less efficiency, and did not cleave the $[rA]_2$ and $[rA]_1$ substrates. These results agree with those obtained using the 20 bp DNA–RNA–DNA/DNA substrate [21].

The kinetic parameters of *T. kodakaraensis* RNase HII and *B. subtilis* RNase HII for hydrolysis of the $[rA]_1$ and $[rA]_4$ substrates are summarized in Table 1. The K_m and k_{cat} values of these enzymes for hydrolysis of the $[rA]_1$ substrate are comparable to those for hydrolysis of the $[rA]_4$ substrate. These results suggest that reduction in the number of ribonucleotides in the chimeric substrate from four to one seriously affects neither the binding affinity nor the hydrolysis rate. When the kinetic parameters of *T. kodakaraensis* RNase HII are compared with those of *B. subtilis* RNase HII, the K_m value of the former enzyme is comparable to that of the latter, whereas the k_{cat} value of the former enzyme is higher than that of the latter by roughly 20-fold, whatever the substrate is. Hence, these two enzymes differ mainly in the hydrolysis rate.

4. Discussion

In this study, we showed that *T. kodakaraensis* RNase HII and *B. subtilis* RNase HII cleaved even the $[rA]_1$ substrate at the DNA/RNA junction. In contrast, *E. coli* RNase HI and *B. subtilis* RNase HIII did not cleave this substrate. These results suggest that this cleavage specificity is a characteristic common to bacterial RNase HII, archaeal RNases HII, and eukaryotic RNases H2. In addition, the current results, as well as the previous ones [2,10–12], indicate that bacterial RNase HII, archaeal RNases HII, and eukaryotic RNases H2 cleave RNA–DNA/DNA and DNA–RNA–DNA/DNA substrates containing multiple ribonucleotides to produce a DNA segment with a single ribonucleotide at the 5′-terminus, whereas *E. coli* RNase HI and *B. subtilis* RNase HIII do not. Thus, Type 2 RNases H, except for bacterial RNases HIII, are clearly distinct from Type 1 RNases H in substrate specificities.

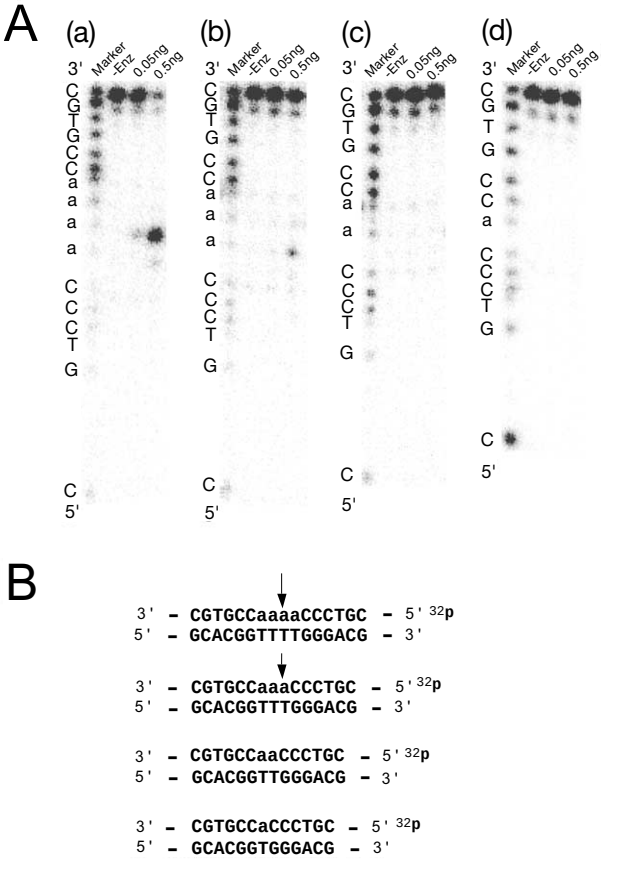

Fig. 4. Cleavage of DNA–RNA–DNA/DNA heteroduplexes by *E. coli* RNase HI. A: Hydrolysis of the 5′-end-labeled DNA–RNA–DNA containing four (a), three (b), two (c), or one (d) adenosine(s) hybridized to the complementary DNA with *E. coli* RNase HI and separation of the hydrolysates were carried out as described in the legend for Fig. 1. B: Cleavage sites of each DNA–RNA–DNA/DNA heteroduplex with *E. coli* RNase HI are shown as described in the legend for Fig. 1.

Table 1
Kinetic parameters of *T. kodakaraensis* RNase HII and *B. subtilis* RNase HII

Enzyme	Substrate	K_m (μM)	k_{cat} (min^{-1})
T. kodakaraensis RNase HII	$[rA]_4$	0.58	11.5
	$[rA]_1$	0.56	8.0
B. subtilis RNase HII	$[rA]_4$	0.43	0.52
	$[rA]_1$	0.21	0.34

Hydrolyses of the substrates were carried out at 30°C for 15 min as described in Section 2. Errors, which represent the 67% confidence limits, are all at or below ±20% of the values reported.

The kinetic studies indicate that *T. kodakaraensis* RNase HII, as well as *B. subtilis* RNase HII, interact with the $[rA]_1$ and $[rA]_4$ substrates with similar affinities and cleave these substrates with similar hydrolysis rates. In contrast, the kinetic studies of *E. coli* RNase HI using the dT_7–$(rA)_n$–dT_9/$dA_7(T)_nA_9$ substrates have previously shown that reduction in the number of ribonucleotides in the chimeric substrate from four to three does not seriously affect the binding affinity, but seriously affects the hydrolysis rate [21]. These results suggest that the $[rA]_1$ substrate binds to *T. kodakaraensis* RNase HII and *B. subtilis* RNase HII such that the RNA/DNA hybrid region can contact the active-sites of these enzymes, whereas it binds to *E. coli* RNase HI such that the RNA/DNA hybrid region cannot contact the active site of the enzyme. This difference may be ascribed to the structural difference in the substrate-binding sites of these enzymes.

Comparison of the crystal structures of *E. coli* RNase HI [24,25] and *T. kodakaraensis* RNase HII [23], which represent those of Type 1 and Type 2 RNases H, revealed that these two enzymes share a main chain fold consisting of a five-stranded β-sheet and two α-helices. In addition, steric configurations of the four acidic active-site residues are conserved in these two structures. These results suggest that Type 1 and Type 2 RNases H hydrolyze substrates by a similar mechanism. However, *E. coli* RNase HI and *T. kodakaraensis* RNase HII differ in the location of the substrate-binding domain. The former contains it as an internal domain termed basic protrusion, whereas the latter contains it as an extra C-terminal domain. According to the model for a complex between DNA/RNA hybrid and *E. coli* RNase HI [26] or *T. kodakaraensis* RNase HII [23], the length of the substrate covered by *T. kodakaraensis* RNase HII is shorter than that covered by *E. coli* RNase HI. Therefore, *T. kodakaraensis* RNase HII may interact with a substrate less strictly than does *E. coli* RNase HI and thereby accommodate a variety of substrates. This may be the reason why *T. kodakaraensis* RNase HII recognizes and cleaves even the chimeric substrate containing a single ribonucleotide.

B. subtilis RNase HII lacks either an internal or C-terminal substrate-binding domain. Instead, it has a long N-terminal extension, which has been suggested to form an alternative substrate-binding domain [27]. Because *B. subtilis* RNase HII cleaves the chimeric substrate containing a single ribonucleotide as well, the length of the substrate covered by this enzyme may be similar to that covered by *T. kodakaraensis* RNase HII.

B. subtilis RNase HIII is a member of Type 2 RNases H. Nevertheless, its behavior on the $[rA]_{1-4}$ substrates was different from those of other Type 2 enzymes, but similar to that of *E. coli* RNase HI. Consistent results have been reported previously [2]. The reason as to why this enzyme functionally resembles a Type 1 enzyme, instead of a Type 2 enzyme, remains to be determined.

The physiological roles of RNases H still remain unclear, although they are thought to be involved in DNA replication and repair [28]. The observation that *T. kodakaraensis* RNase HII and *B. subtilis* RNase HII cleaved the DNA–RNA–DNA/DNA substrate with a single ribonucleotide suggests that bacterial and archaeal RNases HII, as well as eukaryotic Type 2 RNases H, are involved in excision of a single ribonucleotide misincorporated into DNA. Most of the bacteria and eukaryotes, such as *E. coli*, yeast, and human, contain both Type 1 and Type 2 RNases H within a single cell [3]. Whether their in vivo functions are cooperative, complementary, or independent is of great interest.

Takara Bio Inc. has developed a novel DNA amplification method using DNA–RNA chimeric primers, RNase H, and strand-exchanging DNA polymerase (International Publication Numbers WO 00/56877, 2000 and WO 02/16639, 2002). In this method, named 'Isothermal and Chimeric primer-Initiated Amplification of Nucleic Acids' (ICAN™), RNase H introduces a nick in the extended product at the RNA derived from the chimeric primers. The strand-exchanging DNA polymerase synthesizes the complementary strand from the nick. The dispensability of the denaturation–hybridization step of primer in this method enables isothermal amplification of DNA. However, *E. coli* RNase HI, which cannot cleave the DNA–RNA junction, is used for this method, resulting in ribonucleotides left in the amplified DNA. Therefore, Type 2 enzymes with an ability to cleave the DNA–RNA junction, such as *T. kodakaraensis* RNase HII and *B. subtilis* RNase HII, may be more useful for this method than *E. coli* RNase HI.

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