Identification of the first archaeal Type 1 RNase H gene from *Halobacterium* sp. NRC-1: archaeal RNase HI can cleave an RNA–DNA junction

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All the archaeal genomes sequenced to date contain a single Type 2 RNase H gene. We found that the genome of a halophilic archaeon, *Halobacterium* sp. NRC-1, contains an open reading frame with similarity to Type 1 RNase H. The protein encoded by the Vng0255c gene, possessed amino acid sequence identities of 33 % with *Escherichia coli* RNase HI and 34 % with a *Bacillus subtilis* RNase HI homologue. The *B. subtilis* RNase HI homologue, however, lacks amino acid sequences corresponding to a basic protrusion region of the *E. coli* RNase HI, and the Vng0255c has the similar deletion. As this deletion apparently conferred a complete loss of RNase H activity on the *B. subtilis* RNase HI homologue protein, the Vng0255c product was expected to exhibit no RNase H activity. However, the purified recombinant Vng0255c protein specifically cleaved an RNA

strand of the RNA/DNA hybrid *in vitro*, and when the Vng0255c gene was expressed in an *E. coli* strain MIC2067 it could suppress the temperature-sensitive growth defect associated with the loss of RNase H enzymes of this strain. These results *in vitro* and *in vivo* strongly indicate that the *Halobacterium* Vng0255c is the first archaeal Type 1 RNase H. This enzyme, unlike other Type 1 RNases H, was able to cleave an Okazaki fragment-like substrate at the junction between the 3'-side of ribonucleotide and 5'-side of deoxyribonucleotide. It is likely that the archaeal Type 1 RNase H plays a role in the removal of the last ribonucleotide of the RNA primer from the Okazaki fragment during DNA replication.

Key words: archaea, catalytic mechanism, *Halobacterium*, Okazaki fragment, RNA–DNA junction, RNase H.

INTRODUCTION

RNase H endonucleolytically cleaves only the RNA strand of an RNA/DNA hybrid [1]. RNase H activity has been suggested to be involved in important cellular functions, such as DNA replication, repair and transcription [2–6]. The genes encoding RNase H enzymes have been found in all organisms whose genome sequences have been determined [7,8]. Some organisms have multiple RNase H genes in their genomes. For example, *Escherichia coli* has two RNase H genes, *rnhA* encoding RNase HI [9] and *rnhB* encoding RNase HII [10], which display little amino acid sequence similarity. The RNase HI and HII show differences in specific activities, divalent metal ion preferences and specificities for cleavage sites [7,10,11]. Because these two are not paralogous, RNases H are classified into two major families, Type 1 and Type 2 RNases H based on the amino acid sequence similarities with the two *E. coli* enzymes [8]. The Type 1 family (*E. coli* RNase HI orthologues) includes bacterial RNases HI, eukaryotic RNases H1 and retroviral RNase H domains of reverse transcriptases, and the Type 2 family (*E. coli* RNase HII orthologues) includes bacterial RNases HII and HIII, archaeal RNases HII and eukaryotic RNases H2. The Type 2 RNase H has been found in all of the three domains, whereas the Type 1 RNase H has not been found in archaeal genomes [7,8]. Therefore, the RNase H enzymes of the Type 2 family are more universal than those of the Type 1 family. Database searches carried out by Ohtani et al. [8] showed a single *rnh* gene encoding Type 2 RNase H per archaeal genome, suggesting that the RNase HII is the only RNase H in archaeal cells [8]. However, presence of multiple *rnh* genes in some bacterial and all eukaryotic genomes promoted us to investigate Type 1 RNase H genes in archaeal genomes.

As a result of searches on database, we found three hypothetical proteins what could be orthologues of Type 1 RNase H, Vng0255c of *Halobacterium* sp. NRC-1, PAE1792 of *Pyrobaculum aerophilum* and ST0753 of *Sulfolobus tokodaii*. However, amino acid sequences of these putative Type 1 RNase H homologues exhibit the highest similarity to that of *Bacillus subtilis* RNase HI homologue (YpdQ). Because RNase H activity of the YpdQ protein has never been detected, the activity of these three archaeal proteins remained to be determined. The Vng0255c gene from *Halobacterium* sp. NRC-1 was chosen for biochemical and enzymic analyses, because only this has a histidine residue in a position that would allow it to function in a manner similar to His¹²⁴ of *E. coli* RNase HI. We report here that Vng0255c from *Halobacterium* exhibits RNase H activity *in vivo* and *in vitro*, and is the first Type 1 RNase H gene cloned from archaea.

EXPERIMENTAL

Cells, plasmids and materials

The genome of a halophilic archaeon, *Halobacterium* sp. NRC-1, was kindly donated by Professor A. Yamagishi (Department of Molecular Biology, Tokyo University of Pharmacy and Life Science, Hachioji, Japan). The *E. coli* mutant strains MIC2067 [12] and MIC2067(DE3) [11] have been described elsewhere. The plasmid pET-11a was purchased from Novagen (Madison, WI, U.S.A.). The plasmid pHASH117, which is a derivative from pBR322 [13], and *B. subtilis* 168 genomic DNA solution were kindly donated by Dr Y. Ohashi and Dr H. Ohshima (Institute for Advanced Biosciences, Keio University, Tsuruoka, Japan). Restriction enzymes and modifying enzymes were from TaKaRa

Abbreviations used: Halo-RNase HI, *Halobacterium* RNase HI, RT, reverse transcriptase; ts, temperature-sensitive.

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Bio (Kyoto, Japan). *Crotalus atrox* phosphodiesterase I was purchased from Sigma (St. Louis, MO, U.S.A.). Recombinant *E. coli* RNase HI was prepared according to the method described by Kanaya et al. [14].

DNA manipulations

PCR was performed for 30 cycles using a GeneAmp PCR system 2700 (Applied Biosystems, Foster City, CA, U.S.A.). Reagents for PCR, Ex-Taq HotStart version (TaKaRa Bio) or KOD-Plus (Toyobo, Osaka, Japan) were used according to the manufacturer's instructions. DNA sequences were determined by using a Prism 3100 DNA sequencer (Applied Biosystems).

In vivo complementation assay for RNase H activity

Plasmid pHASH-Halo1 was constructed for complementation assays by ligating the DNA fragment containing the *Halobacterium* Vng0255c gene (*Halo-rnhA*) to the *EcoRV* site of pHASH117. The DNA fragment was amplified by PCR using *Halobacterium* sp. NRC-1 genome as a template. The primer sequences were 5'-TATGCCAGTCGTCGAGTCCGACATCCAGAC-3' for the 5'-primer and 5'-TTCAGGCATCGTCGAGGGCCTCGTTGGCGA-3' for the 3'-primer. The *E. coli* mutant strain MIC2067 [12] can form colonies at 30 °C, but not at 42 °C. The temperature-sensitive (ts) growth phenotype can be relieved by introduction of a functional RNase H gene. This strain was transformed with the plasmid pHASH-Halo1, spread on Luria-Bertani medium plates containing 50 µg/ml ampicillin and 30 µg/ml chloramphenicol, and incubated at 30 °C and 42 °C. The plasmid pBR860, which carries the *E. coli rnhA* gene [15], was used as a positive control, and the pHASH117 was used as a negative control for the complementation experiment.

Plasmid construction, over-expression and purification of Halo-RNase HI (*Halobacterium* RNase HI)

Plasmid pET-Halo1 for over-expression was constructed by ligating the DNA fragment containing the *Halo-rnhA* gene to the *NdeI*–*Bam*HI site of pET-11a. The DNA fragment was amplified by PCR using *Halobacterium* sp. NRC-1 genomic DNA as a template. The primer sequences were 5'-CGGGGTGACCTGACCTCATATGCCAGTCGTCGAGTGC-3' for the 5'-primer and 5'-GCCGCGTCGGATCCCTTATCAGGCATCGTCGAGGGCCTCGTTGGC-3' for the 3'-primer, where underlined bases show the positions of the *NdeI* (5'-primer) and *Bam*HI (3'-primer) sites. For overproduction, *E. coli* MIC2067(DE3) was transformed with pET-Halo1 and grown in Luria-Bertani medium containing 0.1 % glucose, 50 µg/ml ampicillin and 30 µg/ml chloramphenicol, at 30 °C. When the D_{600} of the culture reached around 0.5, isopropyl β-D-thiogalactoside was added to the culture medium (final concentration, 0.3 mM) and induction was continued for an additional 4 h. Cells were harvested by centrifugation at 6000 g for 5 min. The following protein purification was carried out at 4 °C. Cells were suspended in 20 mM Tris/HCl (pH 8.0) containing 1 mM EDTA (TE buffer), disrupted by sonication with an ultrasonic disruptor UD-201 from Tomy Corp. (Tokyo, Japan), and centrifuged at 30 000 g for 30 min. The supernatant was applied to a column (4 ml) of DE52 (Whatman, Fairfield, NJ, U.S.A.) equilibrated with TE buffer. The protein was eluted from the column by a linear gradient of NaCl from 0 to 0.5 M in TE buffer. The Vng0255c protein-containing fractions at an NaCl concentration of 0.2 M were pooled and dialysed against 5 mM sodium phosphate (pH 7.0). The dialysed solution was

Table 1 Oligomeric RNA/DNA substrates

Deoxyribonucleotides and ribonucleotides are shown by uppercase and lowercase letters respectively. The asterisk indicates the fluorescently-labelled site.

Substrate	Sequence
12-bp RNA/DNA	5'-*cggagaugacgg-3' 3'-GCCTCTACTGCC-5'
RNA9-DNA/DNA	5'-uugcaugccTGCAGGTCG*-3' 3'-AACGTACGGACGTCCAGC-5'
RNA1-DNA/DNA	5'-cTGCAGGTCG*-3' 3'-AACGTACGGACGTCCAGC-5'

applied to a column (4 ml) of hydroxyapatite Bio-Gel HT gel (Bio-Rad, Hercules, CA, U.S.A.) equilibrated with 5 mM sodium phosphate (pH 7.0), followed by elution from the column by a linear gradient of sodium phosphate from 5 to 200 mM. Fractions containing the protein were pooled, dialysed against 20 mM sodium acetate (pH 5.5) and applied to a column (4 ml) of Toyopearl SuperQ-650M (Tosoh Corp., Yamaguchi, Japan) equilibrated with 20 mM sodium acetate (pH 5.5). Fractions at around 0.2 M NaCl, eluted by a 0–0.5 M linear NaCl gradient, contained the pure protein. They were combined, concentrated and used for further analyses.

Plasmid construction, over-expression and purification of a *B. subtilis* RNase HI homologue

The plasmid pET-Bsu1 used for the over-expression studies was constructed by ligating the DNA fragment containing the *ypdQ* gene to the *NdeI*–*Bam*HI site of pET-11a. The DNA fragment was amplified by PCR using *B. subtilis* 168 genomic DNA as a template. The primer sequences were 5'-AAGGAGTTCCATATGCTTACAGAAATATAT-3' for the 5'-primer and 5'-GCGCGCGGATCCCTTATTAATTCTTTTCATTTCAG-3' for the 3'-primer, where underlined bases show the positions of the *NdeI* (5'-primer) and *Bam*HI (3'-primer) sites. Overproduction of the *B. subtilis* RNase HI homologue (YpdQ) in *E. coli* MIC2067(DE3) and sonication lysis were carried out by a procedure similar to that described above for Halo-RNase HI. The supernatant obtained after sonication lysis was applied to a column (4 ml) of DE52 (Whatman) equilibrated with TE buffer (pH 8.0). The flow-through fraction containing the protein was dialysed against 5 mM sodium phosphate (pH 7.0) and applied to a column (4 ml) of hydroxyapatite Bio-Gel HT gel (Bio-Rad) equilibrated with 5 mM sodium phosphate (pH 7.0). The protein was eluted from the column by a linear gradient of sodium phosphate from 5 to 200 mM. Fractions around 70 mM sodium phosphate containing the pure YpdQ protein were combined, concentrated and used for further analyses.

Cleavage of oligomeric substrates

The end-labelled RNA strands and the complementary DNAs were chemically synthesized by Prologo (Paris, France). A fluorescent tag (6-carboxy-fluorescein) was used for the end-labelling of the RNA strands. The RNA/DNA duplexes (0.5 µM) were prepared by hybridizing the end-labelled RNA strands with a 2.0 molar equivalent of complementary DNA oligomers. The sequences of substrates are shown in Table 1. Hydrolysis of the substrate was carried out at 37 °C for 15 min in 10 mM Tris/HCl (pH 8.5) containing 10 mM MnCl₂, 10 mM NaCl, 1 mM 2-mercaptoethanol and 50 µg/ml BSA for Halo-RNase HI and

B. subtilis YpdQ, and in 10 mM Tris/HCl (pH 8.0) containing 10 mM MgCl₂, 50 mM NaCl, 1 mM 2-mercaptoethanol and 50 µg/ml BSA for *E. coli* RNase HI. The effect of divalent metal ions on the cleavage activity was analysed in the presence of 10 mM MgCl₂, CoCl₂, NiCl₂, CuCl₂, CaCl₂ and ZnCl₂ in place of MnCl₂. The activity at different pH values was measured in a reaction buffer solution containing 10 mM sodium acetate (pH 4.1–5.2), 10 mM Pipes (pH 6.1–7.3), 10 mM Tris/HCl (pH 7.1–8.8) or 10 mM glycine/NaOH (pH 8.3–10.0). Products were analysed on a 20% polyacrylamide gel containing 7 M urea and quantified using the Molecular Imager FX (Bio-Rad). When the hybrid duplex in which RNA is 5'-end labelled was used as a substrate, products were identified by comparing their patterns of migration on the gel with those of the oligonucleotides generated by partial digestion of RNA with snake venom phosphodiesterase [16]. One unit is defined as the amount of enzyme required to hydrolyse 1 µmol substrate per min at 37 °C. The specific activity was defined as the enzymic activity per mg of protein.

Substrate specificity

The 12-bp double-stranded RNA and DNA, and the RNA/DNA duplex with 5'-end-labelled DNA for DNase H activity which cleaves a DNA strand of the RNA/DNA hybrid, were prepared as described for the construction of the RNA/DNA hybrid. The sequences of the 5'-end-labelled strands of all substrates are the same as that of the RNA of the 12-bp RNA/DNA hybrid. The reactions and product analyses were carried out as described above.

Mutagenesis

The genes encoding the mutant proteins were constructed by site-directed mutagenesis, as described previously [17]. They were designed to alter the codon for histidine (CAC) to that for tryptophan (TGG) or alanine (GCA) for Halo-RNase HI mutant proteins, and the codon for Trp¹⁰⁹ (TGG) to that for histidine (CAT) for *B. subtilis* YpdQ-W109H. Plasmids for the over-expression of mutant proteins were constructed by ligating the DNA fragments to the *Nde*I–*Bam*HI site of pET-11a. The Halo-RNase HI and *B. subtilis* YpdQ mutant proteins were overproduced and purified as described for the wild-type proteins.

Protein purity and concentration

The purity of each protein was determined by SDS/PAGE on 15% polyacrylamide gel followed by staining with Coomassie Brilliant Blue. The protein concentrations were determined from the extent of UV absorption with A₂₈₀^{0.1%} values of 1.22 for Halo-RNase HI wild-type, 1.47 for Halo-H179W (Halo-RNase HI where His¹²⁴ → Trp), 0.57 for *B. subtilis* YpdQ, 0.22 for YpdQ-W109H and 2.02 for *E. coli* RNase HI. The value for *E. coli* RNase HI was experimentally determined [18]. Other values were calculated by using ε values of 1576 M⁻¹·cm⁻¹ for tyrosine and 5225 M⁻¹·cm⁻¹ for tryptophan at 280 nm [19].

RESULTS

RNase HI homologue from *Halobacterium* sp. NRC-1

When RNase H homologue genes were searched by BLASTP from the genomic database of *Halobacterium* sp. NRC-1 [20], two genes with high scores were found. One was the *rnh* gene encoding an archaeal RNase HIII (Type 2 RNase H) orthologue,

Figure 1 Multiple alignments of Type 1 RNase H sequences

Halo, B.sub., HIV-1, and *E. coli* represent *Halobacterium* Vng0255c (Halo-RNase HI), *B. subtilis* YpdQ, an RNase H domain of HIV-1 RT and *E. coli* RNase HI respectively. Numbers represent the positions of amino acid residues that start from the initiator methionine for each protein. The asterisks indicate the conserved amino acid residues, which are involved in catalytic function of *E. coli* RNase HI. Lines below the sequences indicate the secondary structure of *E. coli* RNase HI [42]. The boxed region forms a basic protrusion in the *E. coli* RNase HI structure.

which is typically found in other archaeal genomes. The other was the Vng0255c gene that has been annotated as a hypothetical protein. The protein encoded by the Vng0255c gene, comprising 199 amino acid residues with a calculated molecular mass of 20980 Da and an isoelectric point (pI) of 4.20, shows significant identity in amino acid sequence with those of Type 1 RNases H, for example, 33% with *E. coli* RNase HI (Figure 1). Detailed comparison with that of *E. coli* RNase HI revealed two unusual sequence characteristics, an N-terminal extension and lack of the region corresponding to the basic protrusion [21]. Although Vng0255c has an N-terminal extension as found in eukaryotic RNases H1 [22], the amino acid sequence similarity between the extended regions is negligible. The basic protrusion is important for substrate binding in *E. coli* RNase HI [23]. In addition, absence of the basic protrusion renders the proteins inactive, as observed in the RNase H domains of retroviral reverse transcriptases (RTs) [24–26] and in an inactive RNase HI homologue (YpdQ) from *B. subtilis* [7]. The Vng0255c from *Halobacterium* showed 34% amino acid sequence identities with *B. subtilis* YpdQ. The amino acid residues involved in divalent metal-ion binding and catalytic function, corresponding to Asp¹⁰, Glu⁴⁸, Asp⁷⁰, His¹²⁴ and Asp¹³⁴ of *E. coli* RNase HI [27], were all conserved in the Vng0255c (Figure 1).

Complementation assay

E. coli MIC2067 shows an RNase H-dependent ts growth defect [12]. It grows normally at 30 °C, but is unable to form colonies at 42 °C. To examine whether the *Halobacterium* Vng0255c gene complements the ts phenotype of this strain, MIC2067 was transformed with pHASH-HaloI, in which the expression level of the Vng0255c was controlled by the promoter P_{spac} and the SD sequence (AAAGGAGG) of the parental plasmid [13]. As shown

Figure 2 Effect of *Halo-rnhA* on the *ts* growth of *E. coli* mutant MIC2067

MIC2067 cells transformed with each plasmid were incubated on a Luria–Bertani plate containing 50 µg/ml ampicillin and 30 µg/ml chloramphenicol, at either 30 °C or 42 °C. The plasmids pHASH-Halo1 and pBR860 contain *Halo-rnhA* and *E. coli rnhA* genes respectively. The plasmid pHASH117 was examined as a negative control.

Figure 3 SDS/PAGE of the purified proteins

All recombinant proteins were purified as described in the Experimental section. Samples were subjected to SDS/PAGE (15 % gel) and stained with Coomassie Brilliant Blue: M, low-molecular-mass standards kit (Amersham); H, *Halobacterium* Vng0255c (Halo-RNase HI); B, *B. subtilis* YpdQ; E, *E. coli* RNase HI. Molecular masses are indicated on the left-hand side of the gel.

in Figure 2, the resultant MIC2067 transformants formed colonies at 42 °C, suggesting that the Halo-RNase HI exhibits enzymic activity *in vivo*. Because the Vng0255c protein also exhibited an RNase H activity *in vitro*, as described below, we will refer to this protein as RNase HI (Halo-RNase HI), while the Vng0255c gene is designated *rnhA*.

Over-expression and purification

We used *E. coli* MIC2067(DE3), in which λ(DE3) is integrated into the chromosome, to over-express a gene in the pET vector [11], as a host for overproduction of Halo-RNase HI or *B. subtilis* YpdQ. The λ(DE3) is a recombinant phage carrying the cloned T7 RNA polymerase gene for T7 promoter of the pET vector. The protein purified from this strain must be free from *E. coli* RNase HI and HII, because both the *rnhA* and *rnhB* genes of MIC2067(DE3) were disrupted. The production levels of the recombinant Halo-RNase HI and *B. subtilis* YpdQ were estimated to be roughly 25 and 30 mg/l of culture respectively, and these levels were sufficient for monitoring the purification steps. Most of each protein was accumulated intracellularly in a soluble form. The proteins were purified to apparent homogeneity (Figure 3), as described in the Experimental section. The amount of the protein purified from 1 litre of culture was approx. 10 and 14 mg for Halo-RNase HI and *B. subtilis* YpdQ respectively.

Figure 4 Effect of the divalent metal ion concentrations on the Halo-RNase HI activity

The activities were determined at 37 °C for 15 min with Halo-RNase HI in 10 mM Tris/HCl (pH 8.5) containing 10 mM NaCl, 1 mM 2-mercaptoethanol, 50 µg/ml BSA, and various concentrations of MnCl₂ (●), MgCl₂ (◇), NiCl₂ (▲), or CoCl₂ (×), by using a 12-bp RNA/DNA hybrid as a substrate. The errors, which represent the 67 % confidence limits, are within 30 % of the values reported.

In vitro assay for RNase H activity

The assay for RNase H activity was carried out as described in the Experimental section using the 12-bp RNA/DNA hybrid molecule as a substrate. Halo-RNase HI exhibited activity in the presence of Mn²⁺, Mg²⁺, Co²⁺ and Ni²⁺, but not in the presence of Cu²⁺, Ca²⁺ or Zn²⁺ or in the absence of the divalent metal ions. As shown in Figure 4, Halo-RNase HI showed maximal activity at 20, 100, 1 and 100 mM in the presence of MnCl₂, MgCl₂, CoCl₂ and NiCl₂ respectively, and preferred MnCl₂ and MgCl₂ to CoCl₂ and NiCl₂. The specific activities determined in the presence of 20 mM MnCl₂ or 100 mM MgCl₂ were approx. 20-fold higher than those in the presence of 1 mM CoCl₂ or 100 mM NiCl₂ (Figure 4). Cleavage patterns in the presence of 20 mM MnCl₂, 100 mM MgCl₂, 1 mM CoCl₂ or 100 mM NiCl₂ are shown in Figure 5. Halo-RNase HI preferred to cleave the substrate at all cleavable sites from g⁵ to c¹⁰ in each of the metal ions tested. However, in the presence of MnCl₂, cleavages were observed at all phosphodiester bonds, except for c¹–g². In the presence of MgCl₂, CoCl₂ or NiCl₂, no cleavages were observed at c¹–g², g²–g³, g³–a⁴ and c¹⁰–g¹¹. The same substrate was cleaved by *E. coli* RNase HI at a⁶–u⁷, u⁷–g⁸ and a⁹–c¹⁰, as described previously [7,28].

The cleavage activities of Halo-RNase HI in the presence of 10 mM MnCl₂ or 100 mM MgCl₂ increased exponentially as the pH increased from 4 to 10. The activity seemed to be almost proportional to the concentration of hydroxyl ions. The activity of Halo-RNase HI remained between 70 and 100 % at concentrations of NaCl or KCl ranging from 0 to 2.5 M. Because the solubility of divalent metal ions decreased and RNA/DNA substrates might be destabilized at high pH conditions, a pH of 8.5 in the presence of 10 mM MnCl₂ was adopted as the standard cleavage reaction.

The specific activities of Halo-RNase HI in the presence of Mg²⁺ and Mn²⁺ are compared with those of *E. coli* RNase HI in Table 2. In the presence of Mg²⁺, the specific activity of the Halo-RNase HI was 40-fold lower than that of *E. coli* RNase HI.

The 3'-end-labelled RNA–DNA/DNA substrates containing one or nine ribonucleotide(s) were also examined. These substrates were used as a model substrate of the RNA primer of the Okazaki-fragment during lagging strand synthesis in DNA replication. When the substrate containing one ribonucleotide was used, neither Halo-RNase HI nor *E. coli* RNase HI cleaved its RNA–DNA junction (data not shown). When the substrate containing

Figure 5 Cleavage of oligomeric RNA/DNA substrates in the presence of various divalent metal ions by Halo-RNase HI

(A) A 12-bp RNA/DNA hybrid was incubated at 37 °C for 15 min with Halo-RNase HI in the presence of 20 mM MnCl₂, 100 mM MgCl₂, 100 mM NiCl₂ or 1 mM CoCl₂. The concentration of the substrate is 0.5 μM. Products were separated on a 20 % polyacrylamide gel containing 7 M urea, as described in the Experimental section. M represents products resulting from partial digestion of the 12-bp RNA with snake venom phosphodiesterase. (B) Cleavage sites of the substrates are denoted by arrows. Differences in the size of the arrows reflect the relative cleavage intensities at the indicated position. Black and grey arrows represent the first cleavage sites and the following cleavage sites respectively. Deoxyribonucleotides and ribonucleotides are denoted with uppercase and lowercase letters respectively.

Table 2 Comparison of the specific activities of the enzymes in the presence of either Mg²⁺ or Mn²⁺ ions

The hydrolysis of the 12-bp RNA/DNA hybrids with enzyme was carried out at 37 °C for 15 min under the conditions described in the Experimental section. The concentrations of the metal ions were optimal values for RNase H activities of enzymes. *B. subtilis* YpdQ exhibited no RNase H activity under any examined conditions. Errors, which represent the 67 % confidence limits, are within 30 % of the values reported.

Enzyme	Metal	Specific activity (units/mg)
Halo-RNase HI	MgCl ₂ (100 mM)	0.44
	MnCl ₂ (20 mM)	0.46
<i>E. coli</i> RNase HI	MgCl ₂ (10 mM)	17.4
	MnCl ₂ (1 μM)	0.68
<i>B. subtilis</i> YpdQ	MgCl ₂ (10 mM)	< 0.0001
	MnCl ₂ (10 mM)	< 0.0001

9-mer ribonucleotides was used, Halo-RNase HI cleaved the RNA–DNA junction in contrast to no cleavage by *E. coli* RNase HI (Figure 6).

Substrate specificities

A series of 12-mer single-stranded RNA and DNA, double-stranded RNA and DNA, and RNA/DNA hybrid labelled at the 5'-end of DNA, were incubated with the enzyme in the presence of 10 mM MnCl₂ or 100 mM MgCl₂. No cleavage for these substrates by the Halo-RNase HI (results not shown) indicated that Halo-RNase HI specifically cleaves the RNA strand of the RNA/DNA hybrid.

Comparison with *B. subtilis* RNase HI homologue

Although *B. subtilis* YpdQ shows 34 % amino acid sequence identity with Halo-RNase HI, it exhibited neither RNase H nor other nuclease activity [7]. Re-examination of the highly purified recombinant *B. subtilis* YpdQ used in the present study confirmed

the previous results [7] under all reaction conditions (Table 2 and Figure 6C). The all-or-none RNase H activity between Halo-RNase HI and *B. subtilis* YpdQ could be attributed to the replacement of the histidine residue at the position equivalent to His¹²⁴ for *E. coli* RNase HI. His¹²⁴ is part of the active site and is conserved in other active Type 1 RNases H, including the Halo-RNase HI, but replaced by tryptophan in *B. subtilis* YpdQ (Figure 1). Mutant proteins Halo-RNase HI H179W and *B. subtilis* YpdQ W109H (Trp¹⁰⁹ → His in *B. subtilis* YpdQ) were constructed and purified to examine the effect of the His → Trp replacement on RNase H activity. *B. subtilis* YpdQ-W109H showed no gain of RNase H activity. The far- and near-UV CD spectra of the YpdQ-W109H and those of the wild-type protein showed little difference, suggesting that both proteins are similarly folded (results not shown). In contrast, conversion of histidine into tryptophan for Halo-H179W resulted in a level of RNase H activity similar to that of the wild-type Halo-RNase HI. Because the difference of RNase H activity between the wild-type *E. coli* RNase HI and H124A mutant increased dramatically as the pH decreased [29], the influence of pH on the RNase H activities of the wild-type Halo-RNase HI and H179W mutant was also analysed. However, their activity-pH profiles were almost the same (results not shown). This suggested that the histidine residue at this position is not important for the catalytic mechanism of Halo-RNase HI. Halo-RNase HI contains four more His residues, His²⁹, His³³, His⁴⁰ and His⁷¹, at its N-terminal extension (Figure 1). Although we examined a possible contribution of other histidine residues to RNase H activity *in vitro* using five constructed His → Ala mutant proteins containing H179A, the results suggested that none of these histidine residues were likely to be involved in the catalytic function for Halo-RNase HI (results not shown).

DISCUSSION

Halo-RNase HI

Vng0255c, a Type 1 RNase H homologue, was identified in the *Halobacterium* sp. NRC-1 genome, and its gene product exhibited

Figure 6 Cleavage of Okazaki fragment-like substrate

Hydrolysis of the 3'-end-labelled RNA–DNA containing 9-mer RNA hybridized to the cDNA by Halo-RNase HI (**A**), *E. coli* RNase HI (**B**) or *B. subtilis* YpdQ (**C**). The RNA–DNA/DNA hybrids were incubated at 37 °C for 15 min with Halo-RNase HI or *B. subtilis* YpdQ in 10 mM Tris/HCl (pH 8.5) containing 10 mM MnCl₂, 10 mM NaCl, 1 mM 2-mercaptoethanol and 50 μg/ml BSA, or with *E. coli* RNase HI in 10 mM Tris/HCl (pH 8.0) containing 10 mM MgCl₂, 50 mM NaCl, 1 mM 2-mercaptoethanol and 50 μg/ml BSA. Product separation was carried out as described in the legend for Figure 5. M represents the 3'-end-labelled RNA–DNA containing one ribonucleotide. Then, one base shorter product (black arrowhead) than M shows that the RNA–DNA junction of the RNA9–DNA/DNA substrate has been cleaved. Cleavage sites are shown as described in Figure 5(B).

RNase H activity both *in vivo* and *in vitro*. The Vng0255c product is an RNase H, and the gene designated as *rnhA* is the first archaeal Type 1 RNase H gene to produce an active enzyme. This result suggests that, in addition to bacterial and eukaryotic genomes, some archaeal genomes also contain the Type 1 RNase H gene. Halo-RNase HI cleaved the 12-bp RNA/DNA substrate at multiple sites, and appeared to be an exonuclease, as shown in Figure 5. However, it was able to cleave the RNA region of a DNA–RNA–DNA/DNA substrate (results not shown), suggesting that it cleaves the RNA in an endonucleolytic manner. Halo-RNase HI can cleave the RNA–DNA junction of an RNA–DNA/DNA substrate containing a 9-mer ribonucleotides, but cannot cleave the junction of the substrate containing a single ribonucleotide (Figure 6A). Probably, it requires an upstream double-stranded foothold of moderate length to access the RNA–DNA junction. The presence of only a single ribonucleotide at the 5'-side of the RNA–DNA junction will not be sufficient for Halo-RNase HI to access the RNA–DNA junction. However, *E. coli* RNase HI never cleaves the RNA–DNA junction (Figure 6B). This junction cleavage activity is unique for Halo-RNase HI.

Physiological functions

The physiological function of Halo-RNase HI remains to be determined. One of the proposed functions of RNase H is the removal of RNA primers from the Okazaki fragment during lagging-strand DNA synthesis. It has been reported that the known cellular RNases H could not cleave an RNA–DNA junction, and left one or several ribonucleotide(s) at the 5'-end of the DNA strand in Okazaki fragment-like substrates [3,30,31]. Many studies have indicated that the RNA primers can be completely removed by DNA polymerase I in bacteria [32] or by flap endonuclease in archaea [33] and eukarya [3]. However, the substrate specificity of Halo-RNase HI for cleavage on the RNA–DNA junction was clearly defined as shown in Figure 6(A). This suggests that the enzyme possesses a potential activity to remove the RNA primer completely from the Okazaki fragment.

Why is *B. subtilis* YpdQ inactive?

Our results showed that the *B. subtilis* RNase HI homologue (YpdQ) was inactive. Despite its similarity to YpdQ, Halo-RNase HI was active. The major differences between these two proteins are an amino acid substitution at the position corresponding to an active-site His¹²⁴ for *E. coli* RNase HI and an N-terminal extension in Halo-RNase HI. However, *B. subtilis* YpdQ W109H did not exhibit RNase H activity *in vitro*, but its CD spectra suggested that the wild-type YpdQ and W109H mutant proteins were folded into similar structures. In addition, the *B. subtilis* *ypdQ* W109H-mutated gene cannot complement an RNase H-dependent ts growth phenotype of *E. coli* MIC3001 (N. Ohtani and S. Kanaya, unpublished work). The results of site-directed mutagenesis suggested that a His¹⁷⁹ residue in Halo-RNase HI is not involved in catalytic function, unlike that in *E. coli* RNase HI. Consequently, we conclude that the replacement of histidine by tryptophan was not responsible for the difference in activity between the two proteins.

We constructed a Halo-RNase HI mutant, the first 60 amino acid residues of which are deleted, to examine whether the N-terminal extension is important for its activity. However, the mutant protein was not overproduced in *E. coli* cells and did not complement the ts growth of *E. coli* MIC2067 (results not shown). Although the N-terminal extension may be important for the stability of Halo-RNase HI, it remained unclear whether it is important for its activity.

Catalytic mechanism

The amino acid residues (Asp¹⁰, Glu⁴⁸, Asp⁷⁰, His¹²⁴ and Asp¹³⁴) involved in the divalent metal-ion binding and catalytic function of *E. coli* RNase HI were also conserved in Halo-RNase HI (Figure 1). The results of site-directed mutagenesis on Halo-RNase HI suggested that none of the histidine residues, containing His¹⁷⁹ corresponding to the His¹²⁴ for *E. coli* RNase HI, influenced RNase H activity. Interestingly, the histidine residue corresponding to the His¹²⁴ for *E. coli* RNase HI is not conserved in the RNase H

Table 3 Halo-RNase HI homologues

Organism	Gene name
Bacteria	
<i>B. subtilis</i>	<i>ypdQ</i> *
<i>Enterococcus faecalis</i>	<i>ebsB</i>
<i>Leptospira interrogans</i>	<i>rnhA</i>
<i>Mycobacterium tuberculosis</i>	Rv2228c†
<i>Mycobacterium leprae</i>	ML1637†
<i>Streptomyces coelicolor</i>	SC02299†
<i>Streptomyces avermitilis</i>	SAV5877†
<i>Corynebacterium glutamicum</i>	<i>rnhA</i> (Cgl2236)†‡
<i>Corynebacterium efficiens</i>	CE2133†
<i>Thermobifida fusca</i>	Tfus2822†
Archaea	
<i>Halobacterium</i> sp. NRC-1	<i>Halo-rnhA</i> (Vng0255c)
<i>Sulfolobus tokodaii</i>	ST0753
<i>Pyrobaculum aerophilum</i>	PAE1792
Eukarya	
<i>Arabidopsis thaliana</i>	At3g01410 (putative RNase H)
<i>Oryza sativa</i>	OSJNB0011A08.1 (putative RNase)

* See reference [7].

† The product of the *rnhA*-like gene from bacteria classified to actinomycetales has an additional C-terminal domain homologous to a CobC protein.

‡ See reference [36].

domain of RT of the Gypsy family of retro-elements [34,35] and the recently cloned *Corynebacterium glutamicum* RNase HI [36]. However, both the RTs of the Gypsy family and *C. glutamicum* RNase HI exhibit RNase H activity. In *E. coli* RNase HI, the catalytic mechanisms in the one Mg^{2+} -form [37], one Mn^{2+} -form and two Mn^{2+} -form [38] have been proposed. In the two Mn^{2+} -form (attenuation form), the second metal ion, instead of His¹²⁴, is proposed to facilitate the formation of an attacking hydroxide ion [38], because the His¹²⁴ → Ala mutation does not seriously affect the activity of *E. coli* RNase HI at high Mn^{2+} concentrations allowing the attenuation form [39]. In the case of Halo-RNase HI, the effect of 10 mM $MnCl_2$ in the standard reaction condition was not attenuation, but activation, as shown in Figure 4. Therefore the catalytic mechanism for Halo-RNase HI may be slightly different from that proposed for *E. coli* RNase HI.

Substrate binding

The RNase H domain of HIV-1 RT is inactive [25,26]. The most notable difference between the inactive domain from HIV-1 and the active *E. coli* protein is a basic protrusion region, which is present in *E. coli*, but absent from the HIV-1 homologue. The basic protrusion region has been reported to be important for substrate binding for *E. coli* RNase HI [23]. However, the basic protrusion-deletion mutant exhibits no Mg^{2+} -dependent RNase H activity, while it maintains an Mn^{2+} -dependent RNase H activity, suggesting that the basic protrusion region is not essential for RNase H activity [40]. Halo-RNase HI also lacked a region corresponding to the basic protrusion and exhibited an RNase H activity both in the presence of $MnCl_2$ and in the presence of $MgCl_2$. Hence, the basic protrusion region is not crucial for the RNase H activity of Type 1 enzymes. In Halo-RNase HI, a region corresponding to an α IV helix of *E. coli* RNase HI forms a positive-charge cluster consisting of five arginines and one lysine residues (Figure 1). This region may be important for substrate binding for Halo-RNase HI.

Halo-RNase HI homologues and their evolutionary relationship

Two archaeal genomes also contain Type 1 RNase HI homologue genes. These are the ST0753 gene from *S. tokodaii* and PAE1792 gene from *P. aerophilum*. Furthermore, the Halo-RNase HI homologue genes could be also found in the bacterial and eukaryotic genomes (Table 3). A common feature among them is the lack of a basic protrusion region as in the RNase H domain of retroviral RT. This led us to speculate that these Type 1 RNase H homologues might be derived by horizontal gene transfer through a retrovirus. The working hypothesis may explain the clear contrast that cellular Type 1 RNases H cannot cleave the Okazaki fragment-like substrate at the RNA–DNA junction, but Halo-RNase HI possesses similar activity to that of HIV-1 RT [41]. Further analyses of other Type 1 RNase H homologues containing archaeal RNase HI (as listed in Table 3) will help to clarify their origins and their relationship with retrovirus RT.

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REFERENCES

- 1 Crouch, R. J. and Dirksen, M.-L. (1982) Ribonucleases H in Nuclease (Linn, S. M. and Crouch, R. J., eds.), pp. 211–241, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- 2 Kogoma, T. and Foster, P. L. (1998) Physiological function of *E. coli* RNase HI. In Ribonucleases H (Crouch, R. J. and Toulme, J. J., eds.), pp. 39–66, INSERM, Paris.
- 3 Qiu, J., Qian, Y., Frank, P., Wintersberger, U. and Shen, B. (1999) *Saccharomyces cerevisiae* RNase H(35) functions in RNA primer removal during lagging-strand DNA synthesis, most efficiently in cooperation with Rad27 nuclease. *Mol. Cell. Biol.* **19**, 8361–8371.
- 4 Rumbaugh, J. A., Murante, R. S., Shi, S. and Bambara, R. A. (1997) Creation and removal of embedded ribonucleotides in chromosomal DNA during mammalian Okazaki fragment processing. *J. Biol. Chem.* **272**, 22591–22599.
- 5 Drolet, M., Phoenix, P., Menzel, R., Masse, E., Liu, L. F. and Crouch, R. J. (1995) Overexpression of RNase H partially complements the growth defect of an *Escherichia coli* Δ *topA* mutant: R-loop formation is a major problem in the absence of DNA topoisomerase I. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 3526–3530.
- 6 Arudchandran, A., Cerritelli, S., Narimatsu, S., Itaya, M., Shin, D. Y., Shimada, Y. and Crouch, R. J. (2000) The absence of ribonuclease H1 or H2 alters the sensitivity of *Saccharomyces cerevisiae* to hydroxyurea, caffeine and ethyl methanesulphonate: implications for roles of RNases H in DNA replication and repair. *Genes Cells* **5**, 789–802.
- 7 Ohtani, N., Haruki, M., Morikawa, M., Crouch, R. J., Itaya, M. and Kanaya, S. (1999) Identification of the genes encoding Mn^{2+} -dependent RNase HII and Mg^{2+} -dependent RNase HIII from *Bacillus subtilis*: classification of RNases H into three families. *Biochemistry* **38**, 605–618.
- 8 Ohtani, N., Haruki, M., Morikawa, M. and Kanaya, S. (1999) Molecular diversities of RNases H. *J. Biosci. Bioeng.* **88**, 12–19.
- 9 Kanaya, S. and Crouch, R. J. (1983) DNA sequence of the gene coding for *Escherichia coli* ribonuclease H. *J. Biol. Chem.* **258**, 1276–1281.
- 10 Itaya, M. (1990) Isolation and characterization of a second RNase H (RNase HII) of *Escherichia coli* K-12 encoded by the *rnhB* gene. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 8587–8591.
- 11 Ohtani, N., Haruki, M., Muroya, A., Morikawa, M. and Kanaya, S. (2000) Characterization of ribonuclease HIII from *Escherichia coli* overproduced in a soluble form. *J. Biochem. (Tokyo)* **127**, 895–899.
- 12 Itaya, M., Omori, A., Kanaya, S., Crouch, R. J., Tanaka, T. and Kondo, K. (1999) Isolation of RNase H genes that are essential for growth of *Bacillus subtilis* 168. *J. Bacteriol.* **181**, 2118–2123.
- 13 Ohashi, Y., Ohshima, H., Tsuge, K. and Itaya, M. (2003) Far different levels of gene expression provided by an oriented cloning system in *Bacillus subtilis* and *Escherichia coli*. *FEMS Microbiol. Lett.* **221**, 125–130.

- 14 Kanaya, S., Kohara, A., Miyagawa, M., Matsuzaki, T., Morikawa, K. and Ikehara, M. (1989) Overproduction and preliminary crystallographic study of ribonuclease H from *Escherichia coli*. *J. Biol. Chem.* **264**, 11546–11549
- 15 Haruki, M., Noguchi, E., Akasaka, A., Oobatake, M., Itaya, M. and Kanaya, S. (1994) A novel strategy for stabilization of *Escherichia coli* ribonuclease HI involving a screen for an intragenic suppressor of carboxyl-terminal deletions. *J. Biol. Chem.* **269**, 26904–26911
- 16 Jay, E., Bambara, R., Padmanabham, P. and Wu, R. (1974) DNA sequence analysis: a general, simple and rapid method for sequencing large oligodeoxyribonucleotide fragments by mapping. *Nucleic Acids Res.* **1**, 331–353
- 17 Kanaya, S., Oobatake, M., Nakamura, H. and Ikehara, M. (1993) pH-dependent thermostabilization of *Escherichia coli* ribonuclease HI by histidine to alanine substitutions. *J. Biotechnol.* **28**, 117–136
- 18 Kanaya, S., Kimura, S., Katsuda, C. and Ikehara, M. (1990) Role of cysteine residues in ribonuclease H from *Escherichia coli*. Site-directed mutagenesis and chemical modification. *Biochem. J.* **271**, 59–66
- 19 Goodwin, T. W. and Morton, R. A. (1946) The spectrophotometric determination of tyrosine and tryptophan in proteins. *Biochem. J.* **40**, 628–632
- 20 Ng, W. V., Kennedy, S. P., Mahairas, G. G., Berquist, B., Pan, M., Shukla, H. D., Lasky, S. R., Baliga, N. S., Thorsson, V., Sbrogna, J. et al. (2000) Genome sequence of *Halobacterium* species NRC-1. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 12176–12181
- 21 Katayanagi, K., Miyagawa, M., Matsushima, M., Ishikawa, M., Kanaya, S., Nakamura, H., Ikehara, M., Matsuzaki, T. and Morikawa, K. (1992) Structural details of ribonuclease H from *Escherichia coli* as refined to an atomic resolution. *J. Mol. Biol.* **223**, 1029–1052
- 22 Cerritelli, S. M., Fedoroff, O. Y., Reid, B. R. and Crouch, R. J. (1998) A common 40 amino acid motif in eukaryotic RNases H1 and caulimovirus ORF VI proteins binds to duplex RNAs. *Nucleic Acids Res.* **26**, 1834–1840
- 23 Kanaya, S., Katsuda-Nakai, C. and Ikehara, M. (1991) Importance of the positive charge cluster in *Escherichia coli* ribonuclease HI for the effective binding of the substrate. *J. Biol. Chem.* **266**, 11621–11627
- 24 Davies, 2nd, J. F., Hostomska, Z., Hostomsky, Z., Jordan, S. R. and Matthews, D. A. (1991) Crystal structure of the ribonuclease H domain of HIV-1 reverse transcriptase. *Science* **252**, 88–95
- 25 Becerra, S. P., Clore, G. M., Gronenborn, A. M., Karlstrom, A. R., Stahl, S. J., Wilson, S. H. and Wingfield, P. T. (1990) Purification and characterization of the RNase H domain of HIV-1 reverse transcriptase expressed in recombinant *Escherichia coli*. *FEBS Lett.* **270**, 76–80
- 26 Hostomsky, Z., Hostomska, Z., Hudson, G. O., Moomaw, E. W. and Nides, B. R. (1991) Reconstitution *in vitro* of RNase H activity by using purified N-terminal and C-terminal domains of human immunodeficiency virus type 1 reverse transcriptase. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 1148–1152
- 27 Kanaya, S. (1998) Enzymatic activity and protein stability of *E. coli* ribonuclease HI. In *Ribonucleases H* (Crouch, R. J. and Toulme, J. J., eds.), pp. 1–38, INSERM, Paris
- 28 Kanaya, E. and Kanaya, S. (1995) Kinetic analysis of *Escherichia coli* ribonuclease HI using oligomeric DNA/RNA substrates suggests an alternative mechanism for the interaction between the enzyme and the substrate. *Eur. J. Biochem.* **231**, 557–562
- 29 Kashiwagi, T., Jeanteur, D., Haruki, M., Katayanagi, K., Kanaya, S. and Morikawa, K. (1996) Proposal for new catalytic roles for two invariant residues in *Escherichia coli* ribonuclease HI. *Protein Eng.* **9**, 857–867
- 30 Eder, P. S., Walder, R. Y. and Walder, J. A. (1993) Substrate specificity of human RNase H1 and its role in excision repair of ribose residues misincorporated in DNA. *Biochimie* **75**, 123–126
- 31 Chai, Q., Qiu, J., Chapados, B. R. and Shen, B. (2001) *Archaeoglobus fulgidus* RNase HI in DNA replication: enzymological functions and activity regulation via metal cofactors. *Biochem. Biophys. Res. Commun.* **286**, 1073–1081
- 32 Ogawa, T. and Okazaki, T. (1984) Function of RNase H in DNA replication revealed by RNase H defective mutants of *Escherichia coli*. *Mol. Gen. Genet.* **193**, 231–237
- 33 Sato, A., Kanai, A., Itaya, M. and Tomita, M. (2003) Cooperative regulation for Okazaki fragment processing by RNase HI and Fen-1 purified from a hyperthermophilic archaeon, *P. furiosus*. *Biochem. Biophys. Res. Commun.* **309**, 247–252
- 34 Lener, D., Budihas, S. R. and Le-Grice, S. F. (2002) Mutating conserved residues in the ribonuclease H domain of Ty3 reverse transcriptase affects specialized cleavage events. *J. Biol. Chem.* **277**, 26486–26495
- 35 Levin, H. L. (1996) An unusual mechanism of self-primed reverse transcription requires the RNase H domain of reverse transcriptase to cleave an RNA duplex. *Mol. Cell. Biol.* **16**, 5645–5654
- 36 Hirasawa, T., Kumagai, Y., Nagai, K. and Wachi, M. (2003) A *Corynebacterium glutamicum* *rrhA recG* double mutant showing lysozyme-sensitivity, temperature-sensitive growth, and UV-Sensitivity. *Biosci. Biotechnol. Biochem.* **67**, 2416–2424
- 37 Kanaya, S., Oobatake, M. and Liu, Y. (1996) Thermal stability of *Escherichia coli* ribonuclease HI and its active site mutants in the presence and absence of the Mg^{2+} ion. Proposal of a novel catalytic role for Glu⁴⁸. *J. Biol. Chem.* **271**, 32729–32736
- 38 Tsunaka, Y., Haruki, M., Morikawa, M., Oobatake, M. and Kanaya, S. (2003) Dispensability of Glu⁴⁸ and Asp¹³⁴ for Mn^{2+} -dependent activity of *E. coli* ribonuclease HI. *Biochemistry* **42**, 3366–3374
- 39 Keck, J. L., Goedken, E. R. and Marqusee, S. (1998) Activation/attenuation model for RNase H. A one-metal mechanism with second-metal inhibition. *J. Biol. Chem.* **273**, 34128–34133
- 40 Keck, J. L. and Marqusee, S. (1996) The putative substrate recognition loop of *Escherichia coli* ribonuclease H is not essential for activity. *J. Biol. Chem.* **271**, 19883–19887
- 41 Gotte, M., Maier, G., Onori, A. M., Cellai, L., Wainberg, M. A. and Heumann, H. (1999) Temporal coordination between initiation of HIV⁺-strand DNA synthesis and primer removal. *J. Biol. Chem.* **274**, 11159–11169
- 42 Katayanagi, K., Miyagawa, M., Matsushima, M., Ishikawa, M., Kanaya, S., Ikehara, M., Matsuzaki, T. and Morikawa, K. (1990) Three-dimensional structure of ribonuclease H from *E. coli*. *Nature (London)* **347**, 306–309

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