

Protein Expression and Purification

Preparation and characterization of the RNase H domain of Moloney murine leukemia virus reverse transcriptase

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Abbreviations used: HIV-1, human immunodeficiency virus type 1; MMLV, Moloney murine leukemia virus; RNase H, ribonuclease H; RT, reverse transcriptase; PAGE, polyacrylamide gel electrophoresis

Moloney murine leukemia virus reverse transcriptase (MMLV RT) contains fingers, palm, thumb, and connection subdomains as well as an RNase H domain. The DNA polymerase active site resides in the palm subdomain, and the RNase H active site is located in the RNase H domain. The RNase H domain contains a positively charged α -helix called the C helix (H⁵⁹⁴GEIYRRR⁶⁰¹), that is thought to be involved in substrate recognition. In this study, we expressed three versions of the RNase H domain in *Escherichia coli*, the wild-type domain (WT) (residues Ile498–Leu671) and two variants that lack the regions containing the C helix (Ile593–Leu603 and Gly595–Thr605, which we called Δ C1 and Δ C2, respectively) with a strep-tag at the N-terminus and a deca-histidine tag at the C-terminus. These peptides were purified from the cells by anion-exchange, Ni²⁺ affinity, and Strep-Tactin affinity column chromatography, and then the tags were removed by proteolysis. In an RNase H assay using a 25-bp RNA-DNA heteroduplex, WT, Δ C1, and Δ C2 produced RNA fragments ranging from 7 to 16 nucleotides (nt) whereas the full-length MMLV RT (Thr24–Leu671) produced 14–20-nt RNA fragments, suggesting that elimination of the fingers, palm, thumb, and connection subdomains affects the binding of the RNase H domain to the RNA-DNA heteroduplex. The activity levels of WT, Δ C1, and Δ C2 were estimated to be 1%, 0.01%, and 0.01% of full-length MMLV RT activity, indicating that the C helix is important, but not critical, for the activity of the isolated RNase H domain.

Keywords: Moloney murine leukemia virus; reverse transcriptase; RNase H activity; template-primer; thermostabilization

1 **Introduction**

2
3 Retroviral reverse transcriptase (RT) possesses RNA- and DNA-dependent DNA
4 polymerase as well as RNase H activities. Moloney murine leukemia virus (MMLV) RT
5 is a 75-kDa monomer, comprised of the fingers (Thr24–Asp124 and Phe156–Ser195),
6 palm (Ile125–Phe155 and Pro196–Glu275), thumb (Gly276–Leu338), and connection
7 (Pro339–Asp468) subdomains and an RNase H domain (Arg469–Leu671). The active
8 site of the DNA polymerase activity is located in the palm subdomain while the RNase
9 H activity is in the RNase H domain [1, 2].

10 Due to its high catalytic activity and fidelity, MMLV RT is extensively used in cDNA
11 synthesis [3]. Thermostability of DNA polymerase activity of MMLV RT increases by
12 inactivating the RNase H activity by the mutation of the catalytically important residue,
13 Asp524 [4-6]. The same strategy was successful to improve the thermostabilities of RTs
14 from human immunodeficiency virus type-1 (HIV-1) [7] and avian myeloblastosis virus
15 (AMV) [5, 8].

16 The whole structure of MMLV RT has not been determined yet. From the crystal of
17 full-length MMLV RT (Thr24–Leu671), the structures of the fingers, palm, thumb, and
18 connection subdomains (Thr24–Asn479) have been determined, but that of the RNase H
19 domain has not [1, 9]. On the other hand, from the crystal of the isolated RNase H domain,
20 its whole structure has been determined [10]. In that study, the RNase H domain variant
21 lacking the polypeptide Ile593-Leu603 was successfully crystallized, while the wild-type
22 RNase H domain was not [10]. The deleted polypeptide region, Ile593-Leu603, contains
23 a positively charged α -helix called the C helix ($H^{594}GEIYRRR^{601}$). In xenotropic murine
24 leukemia virus-related virus (XMRV) RT, which has 95% amino-acid sequence homology

1 with MMLV RT, like MMLV RT, the structure of the isolated RNase H domain has been
2 determined whereas the whole structure has not [11-13]. Under these backgrounds, Das
3 *et al.* [1] and Cote *et al.* [2] have proposed the structural model of MMLV RT that the
4 RNase H domain is positioned far from the fingers/palm/thumb domain. However,
5 Pandey *et al.* have proposed another model that the polypeptide region Pro480-Arg506
6 of the RNase H domain, the structure of which has not been determined from the crystal
7 of the full-length molecule or the RNase H domain, interacts with the fingers/palm/thumb
8 subdomain, supporting the floor of the DNA polymerase active-site cleft. In MMLV RT,
9 it is difficult to clarify the interaction between the DNA polymerase and RNase H active
10 sites due to the lack of the crystal structure of the whole MMLV RT molecule.

11 The expression and purification of the isolated RNase H domains of MMLV RT in
12 *Escherichia coli* were previously reported [1, 14-16]. They were expressed without tags
13 [14, 16] or S-transferase fusion protein [1, 15]. In this study, we describe expression,
14 purification, and characterization of three versions of the RNase H domain of MMLV RT,
15 the wild-type domain and the two C helix-deficient variants. They were expressed with
16 N-terminal strep-tag and C-terminal deca-histidine tag in *E. coli*, purified from the cells,
17 and then the tags were removed by proteolysis. The enzyme preparations thus obtained
18 were characterized and compared with full-length MMLV RT.

19 20 **Materials and methods**

21 22 *Materials*

23
24 [γ -³²P]dATP (111 TBq/mmol) was purchased from PerkinElmer (Waltham, MI).

Oligonucleotides were from Fasmak (Atsugi, Japan). The RT concentration was determined by the method of Bradford [17] using Protein Assay CBB Solution (Nacalai Tesque, Kyoto, Japan) with bovine serum albumin (Nacalai Tesque) as standard.

Plasmids

We previously constructed the expression plasmid for C-terminally (His)₆-tagged MMLV RT (pET-MRT) by inserting the C-terminally (His)₆-tagged MMLV RT gene into the *Nde*I and *Eco*RI sites of pET-22b(+) plasmid (Merck Bioscience, Tokyo, Japan). The expression plasmids for the wild-type RNase H domain (Ile498–Leu671) (WT), its variant lacking Ile593–Leu603 (Δ C1), and its variant lacking Gly595–Thr605 (Δ C2), each with C-terminal (His)₆ tag (pET22b-RNaseH-WT, pET22b-RNaseH- Δ C1, and pET22b-RNaseH- Δ C2, respectively) were constructed as follows. Site-directed mutagenesis was carried out using the following nucleotide primers 5'-CAACACAAGTGGCATATGATCCTGGCCGAA-3' (497Nde) and 5'-TTCGGCCAGGATCATATGGCAGTTGTGTTG-3' (497Nde_cp) to change the nucleotide sequence CTTGAT encoding ⁴⁹⁶Leu⁴⁹⁷Asp into a *Nde*I recognition sequence CATATG. The resulting plasmid, pET-MRT(Nde), was digested with *Nde*I and self-ligated to produce the expression plasmid for WT, pET22b-RNaseH-WT. The 391-bp fragment (F1) was amplified from pET22b-RNaseH-WT using the following nucleotide primers 5'-TAATACGACTCACTATAGGG-3' (Pro) and 5'-GCCTTCTGATGTGAGATGGGCAGTAGCAAA-3' (DelI593-L603rev-4), and the 247-bp fragment (F2) was amplified using the following nucleotide primers 5'-TTTGCTACTGCCCATCTCACATCAGAAGGC-3' (DelI593-L603for-4) and 5'-

CTGAATTCTAGTGGTGGTGGTGGTGGAGGAGGGTAGAGGTGT-3' (MRT-HISB). The 608-bp fragment was amplified using the nucleotide primers Pro and MRT-HISB from the mixture of F1 and F2, digested with the restriction enzymes *Xba*I and *Eco*RI, and inserted in pET-22b(+) digested with *Xba*I and *Eco*RI to produce the expression plasmid for Δ C1, pET22b-RNaseH- Δ C1. The expression plasmid for Δ C2, pET22b-RNaseH- Δ C1 was constructed by the same method but using the nucleotide primers 5'-CTCTTTGCCTTCTGATGTGAGATGGGCAGT-3' (DelG595-T605rev-4) and 5'-ACTGCCCATATCCATTCAGAAGGCAAAGAG-3' (DelG595-T605for-4) instead of DelI593-L603rev-4 and DelI593-L603for-4, respectively.

The expression plasmids for WT, Δ C1, and Δ C2, each with an N-terminal strep tag and a C-terminal (His)₁₀ tag (pET52b-RNaseH-WT, pET52b-RNaseH- Δ C1, and pET52b-RNaseH- Δ C2, respectively) were constructed as follows. The 550-bp fragment was amplified with the following nucleotide primers 5'-TTTTGGTACCTTGATGTCCTGGCCGAAGCC-3' (497Kpn) and 5'-TTTTGAGCTCGAGGAGGGTAGAGGTGTC-3' (671Sac) from pET22b-RNaseH-WT, and the 517-bp fragments were amplified from 497Kpn and 671Sac from pET22b-RNaseH- Δ C1 or pET22b-RNaseH- Δ C2. These three fragments were digested with the restriction enzymes *Kpn*I and *Sac*I, and inserted in pET-52b(+) (Merck Bioscience) to produce pET52b-RNaseH-WT, pET52b-RNaseH- Δ C1, and pET52b-RNaseH- Δ C2, respectively.

Expression and purification of recombinant RNase H domain

E. coli strain BL21(DE3) [*F*⁻, *ompT*, *hsdS_B*(*r_B*-*m_B*-) *gal dcm* (DE3)] was transformed

with either pET52b-RNaseH-WT, pET52b-RNaseH- Δ C1, or pET52b-RNaseH- Δ C2. The overnight culture of the transformants (20 ml) was added to 2,000 ml of LB broth and in a 2-liter flask and incubated at 37°C under vigorous aeration by air-pump. When OD_{660} reached 0.6–0.8, 0.5 mL of 0.5 M IPTG was added and growth was continued at 30°C for 4 h. After centrifugation at $10,000 \times g$ for 10 min, the cells were harvested, suspended with 20 ml of 0.02 M potassium phosphate (pH 7.2), 2.0 mM dithiothreitol (DTT), 10% glycerol (buffer A) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and disrupted by sonication. After centrifugation at $20,000 \times g$ for 40 min, the supernatant was collected and applied to a column [25 mm (inner diameter) \times 120 mm] packed with Toyopearl DEAE-650M gel (Tosoh, Tokyo, Japan), previously equilibrated with buffer A. The bound RNase H domain was eluted with buffer A containing 100 mM NaCl and applied to a HisTrap column, previously equilibrated with 50 mM Tris-HCl (pH 8.3), 200 mM KCl, 2.0 mM DTT, 10% glycerol (buffer B). The bound RNase H domain was eluted with buffer B containing 250 mM imidazole and applied to a Strep-Tactin Superflow column (IBA, Göttingen, Germany), previously equilibrated with 100 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA (buffer C). The bound RNase H domain was eluted with buffer C containing 2.5 mM desthiobiotin, 50% glycerol. Two mg of the obtained RNase H domain was subjected to the digestion by 10 units of HRV 3C protease (Accelagen, San Diego, CA) to cleave the strept tag and 50 units of thrombin (GE Healthcare) to cleave the (His)₁₀ tag in 12.5 ml of 40 mM Tris-HCl (pH 7.5), 120 mM NaCl, 10% glycerol at 4°C for 36 h (buffer D). Then, the solution was three-fold diluted by adding 25 ml of water and further incubated at 4°C for 36 h. The digest was applied to a HisTrap column, previously equilibrated with buffer B. The RNase H domain without tags had weak binding ability to a HisTrap column, and it was collected from a HisTrap

column by eluting with buffer B containing 50 mM imidazole. In this buffer condition, the RNase H domain with deca-histidine tag was not eluted. The obtained RNase H domain without tags was applied to a PD-10 column packed with a Sephadex G-25 (GE Healthcare), previously equilibrated with 20 mM Tris-HCl (pH 7.0), containing 200 mM NaCl, 2.0 mM DTT, and 10% glycerol. The column was washed and eluted with the same buffer. Purified RNase H domain was stored at -80°C before use.

SDS-PAGE

SDS-PAGE of the RNase H domain was carried out in a 12.5% polyacrylamide gel under reducing conditions. Proteins (1.2 µg) were reduced by treatment with 2.5% (v/v) of 2-mercaptoethanol at 100°C for 10 min, and then applied onto the gel. A constant current of 40 mA was applied for 40 min. After electrophoresis, proteins were stained with Coomassie Brilliant Blue R-250. The molecular mass marker kit consisting of rabbit muscle phosphorylase B (97.2 kDa), bovine serum albumin (66.4 kDa), hen egg white ovalbumin (44.3 kDa), and bovine carbonic anhydrase (29.0 kDa) was purchased from Takara Bio Inc (Otsu, Japan).

CD spectroscopy

The CD spectra of the RNase H domain was measured with a 2-mm cell using a J-820 spectropolarimeter (Jasco, Tokyo, Japan) under the following condition: spectral range 200-250 nm; 25°C; 100 nm min⁻¹ scan rate; and 10 accumulations.

1 *Fluorescence-based RNase H assay*

2
3 Fluorescence-based RNase H activity assay was carried out according to the method
4 by Parniak *et al* [18]. Briefly, an RNA/DNA hybrid (named R18/D18) was prepared by
5 mixing the 3'-fluorescein modified 18-nucleotide (nt) RNA 5'-
6 GAUCUGAGCCUGGGAGCU-3' (R18) and 5'-dabcyl-modified complementary 18-nt
7 DNA (D18) with a molar ratio of 1.0:1.2 in 50 mM Tris-HCl buffer (pH 8.0) containing
8 60 mM KCl followed by the incubation at room temperature for 30 min. The RNase H
9 reaction was carried out in 50 mM Tris-HCl buffer (pH 8.0) containing 60 mM KCl, 5.0
10 mM MgCl₂, 0.25 µM R18/D18 (the concentration is expressed as that of R18), and
11 indicated concentrations of the RNase H domain or the full-length MMLV RT at 37°C.
12 An aliquot (800 µl) was taken from the reaction mixture at a predetermined time and
13 added to 0.5 M EDTA (160 µl). The fluorescence spectra of the reaction products were
14 measured with a Shimadzu RF-5300PC fluorescence spectrophotometer (Shimadzu,
15 Kyoto, Japan) under the following conditions: excitation wavelength, 490 nm; spectral
16 range 500-600 nm; 25°C; 120 nm min⁻¹ scan rate; and 3 accumulations. In each
17 measurement, the control baseline was obtained with the corresponding buffer in the
18 absence of protein.

20 *Radioisotope-based RNase H assay*

21
22 Radioisotope-based RNase H activity assay was carried out according to the method
23 by Álvarez *et al* [19]. Briefly, an RNA/DNA hybrid (named R25/D25) was prepared by
24 mixing 5'-[³²P]-labelled 25-nt RNA 5'-AUGUAUAGCCCUACCAGCAUUCTGG-3'

(R25) and unlabelled complementary 25-nt DNA (D25) by the method above described. The RNase H reaction (40 μ l) was carried out in 50 mM Tris-HCl (pH 8.0) buffer, containing 50 mM KCl, 0.1 mM MgCl₂, 20 nM R25/D25, and indicated concentrations of the RNase H domain or the full-length MMLV RT at 37°C. An aliquot (4 μ l) was taken from the reaction mixture at a predetermined time and added to 4 μ l of sample-loading buffer (10 mM EDTA, 90% (v/v) formamide, 3 mg/ml xylene cyanol FF, 3 mg/ml bromophenol blue, and 50 μ M 31D/21D. (31D/21D was prepared by mixing unlabelled 31-nt DNA 5'-TTTTTTTTTAGGATACATATGGTTAAAGTAT-3' (D31) and unlabelled complementary complementary 21-nt DNA 5'-ATACTTTAACCATATGTATCC-3' (D21) by the method above described and used as a carrier DNA.) The RNA fragments of the reaction products were analyzed by denaturing 20% polyacrylamide gel electrophoresis followed by image scanning with a BAS-2500 scanner (Fujifilm, Tokyo, Japan) using the program Multi Gauge version 2.2 (Fujifilm).

Results

Expression and purification of the RNase H domain

We initially attempted to express the wild-type RNase H domain (WT) (Ile498-Leu671) and the two C helix-deficient variants lacking Ile593-Leu603 (Δ C1) and Gly595-Thr605 (Δ C2), with C-terminal (His)₆ tags at the C-terminus. We constructed three expression plasmids, pET22b-RNaseH-WT, pET22b-RNaseH- Δ C1, and pET22b-RNaseH- Δ C2 that contain the T7 promoter, an ATG initiation codon, a 540- or 507-bp sequence encoding 180 or 169 amino acids comprising the intact or C helix-deficient

1 RNase H domain (Ile498–Leu671), and a (His)₆ tag, and a stop codon. SDS-PAGE
2 analysis of the soluble fractions of the *E. coli* transformants showed a 20-kDa protein
3 band corresponding to WT or a 19-kDa protein band corresponding to ΔC1 and ΔC2 (data
4 not shown). However, the RNase H domain could not be purified to homogeneity because
5 several contaminating proteins, presumably C-terminal (His)₆-tagged RNase H domain
6 degradation products, could not be completely removed (data not shown).

7 Next we attempted to express WT, ΔC1, and ΔC2 with both an N-terminal strep tag
8 and a C-terminal (His)₁₀ tag. We constructed three expression plasmids, pET52b-
9 RNaseH-WT, pET52b-RNaseH-ΔC1, and pET52b-RNaseH-ΔC2 (Fig. 1), that contain
10 the T7 promoter, an ATG initiation codon, a 711- or 678-bp sequences encoding 237 or
11 206 amino acids comprising a strep-tag, an HRV 3C protease recognition site, the intact
12 or C helix-deficient RNase H domain, a thrombin recognition site, and a (His)₁₀ tag, and
13 a stop codon. SDS-PAGE analysis of the soluble fractions showed a 25-kDa protein band
14 corresponding to WT in the transformants with pET52b-RNaseH-WT (lane 2 in Fig. 2A)
15 and a 24-kDa protein band corresponding to ΔC1 or ΔC2 in the transformants with
16 pET52b-RNaseH-ΔC1 or pET52b-RNaseH-ΔC2, respectively (data not shown).

17 To purify WT, ΔC1, and ΔC2, we used anion-exchange chromatography as the first
18 step, the Ni²⁺-sepharose chromatography as the second step, and the Strep-Tactin affinity
19 chromatography as the third step. At each step, each fraction was tested for the presence
20 of the 25-kDa or 24-kDa protein band by SDS-PAGE, and the active fractions were
21 pooled based on coomassie staining. The SDS-PAGE analysis showed that the active
22 fractions from the WT purification exhibited 25-kDa protein bands (Fig. 2A), and the
23 purified preparations of WT, ΔC1, and ΔC2 exhibited 25-kDa or 24-kDa protein bands
24 (Fig. 2B). In Fig. 2B, the 50 or 55 kDa faint bands were also observed; however their

orgins were unknown. Then, the N-terminal strep tag and C-terminal (His)₁₀ tag were removed by successive proteolysis with HRV 3C protease and thrombin, respectively. After proteolysis, the WT preparation exhibited a single 22-kDa protein band (Fig. 2C), the ΔC1 and ΔC2 preparations exhibited single 21-kDa protein bands (data not shown), and the protein bands corresponding to the 50 or 55 kDa faint bands observed in Fig. 2B were not observed (Fig. 2C for WT and data not shown for ΔC1 and ΔC2). From the 2 L cultures, 0.5–1.0 mg of purified WT, ΔC1, and ΔC2 were obtained. In 20 mM Tris-HCl (pH 7.0), 200 mM NaCl, 2.0 mM DTT, and 10% glycerol, WT, ΔC1, and ΔC2 with tags could be concentrated to 1–2 mg/ml, whereas without tags, these proteins could be concentrated to 10 mg/ml (data not shown), indicating that the removing of the tags increased the solubility.

To examine if the deletion of Ile593-Leu603 or Gly595-Thr605 affects structural change of the isolated RNase H domain, we made spectroscopic analyses. UV spectroscopy showed that purified WT, ΔC1, and ΔC2 exhibited spectra with a deep trough at approximately 250 nm and a peak at 275 nm (Fig. 3B for WT and data not shown for ΔC1 and ΔC2). CD spectroscopy showed that purified WT, ΔC1, and ΔC2 exhibited negative ellipticities at 202–250 nm with peaks at approximately 208 nm and 222 nm (Fig. 3B for WT and data not shown for ΔC1 and ΔC2). No appreciable changes were observed in UV and CD spectra, suggesting that ΔC1, and ΔC2 did not suffer from any global or drastic structural changes by the deletion.

Characterization of the RNase H domain

To analyze the RNase H activity of the isolated RNase H domains, we performed

1 fluorescence-based RNase H assay. An RNA/DNA hybrid (called R18/D18) consisting
2 of a 3'-fluorescein modified 18-nt RNA (R18) and a 5'-dabcyl-modified 18-nt DNA
3 (D18) was used as the substrate (Fig. 4A). R18/D18 is designed to emit strong
4 fluorescence when R18 is cleaved at a site close to the 3' end, and the fluorescein-labeled
5 RNA fragment dissociates from the complementary DNA strand. Figure 4B shows the
6 difference in the fluorescence spectra of R18/D18 before and after the reaction. Based on
7 the previous report, the MgCl₂ concentration was set at 5.0 mM [19]. When R18/D18 was
8 incubated without the full-length RT or the isolated RNase H domain for 30 min, there
9 was no change in the fluorescence spectra. When R18/D18 was incubated with 50 nM
10 HIV-1 RT or 1.8 μM WT for 30 min, the fluorescence increased with the peak at 515 nm.
11 When R18/D18 was incubated with ΔC1 or ΔC2 for 30 min, no fluorescence increase was
12 observed. These results suggest that WT has RNase H activity and ΔC1 and ΔC2 do not.

13 To further analyze the RNase H activity of the isolated RNase H domains, we used
14 a radioisotope-based RNase H assay. An RNA/DNA hybrid (R25/D25) consisting of a 5'-
15 [³²P]-labelled 25-nt RNA (R25) and an unlabeled complementary DNA (D25) was used
16 as the substrate (Fig. 5A). After the RNase H reaction, the RNA reaction products were
17 analyzed by denaturing PAGE. First we examined the effects of MgCl₂ concentration on
18 the RNase H activity of WT and found that reactions containing 0.1–0.2 mM yielded
19 relatively higher amounts of products than those containing other concentrations (0–15
20 mM) (data not shown). Thus, 0.1 mM was used in subsequent experiments.

21 Figure 5B shows the denatured PAGE analysis of the products obtained from the
22 reaction under various conditions. Unreacted R25/D25 showed only a single 25-nt RNA
23 band. When R25/D25 was incubated with 30 nM full-length MMLV RT, 16–20-nt bands
24 were detected at 15 s, and 14–17-nt bands were detected at 10 min. When R25/D25 was

1 incubated with 30 nM WT, only a 25-nt band was detected at both 15 s and 10 min. When
2 R25/D25 was incubated with 1.0 or 10 μ M WT, only a 25-nt band was detected at 15 s,
3 whereas various bands (mainly 7–16-nt bands) were detected at 10 min. This indicated
4 that the cleavage pattern of WT was different from that of the full-length MMLV RT and
5 that the activity of WT was considerably weaker than that of the full-length MMLV RT.
6 When R25/D25 was incubated with Δ C1 or Δ C2, various bands (mainly 7–16-nt bands)
7 were only detected at a 10 μ M and 10 min. Under all other reaction conditions tested,
8 only the uncleaved 25-nt band was detected. This indicated that although the activities of
9 Δ C1 and Δ C2 were weaker than that of WT, the cleavage patterns were the same as that
10 of WT.

11 Figure 5C compares the time courses of the cleavage patterns obtained in the
12 reactions with 30 nM full-length MMLV RT, 1 μ M WT, and 10 μ M Δ C1 and Δ C2. The
13 results of a densitometry trace of each band were shown in Fig. S1. In full-length MMLV
14 RT, 10–20-nt bands were detected, in which the amount of the 17-nt band was the highest
15 at 5–30 min and that of the 14-nt band was the highest at 60 and 120 min. In WT, Δ C1,
16 and Δ C2, 7–20-nt bands were detected, in which the amount of the 7- or 10-nt band was
17 higher than those of other bands. The rates of the decrease in the 25-nt bands in the
18 reaction with WT, Δ C1, and Δ C2 were 30%, 3%, and 3% of full-length MMLV RT,
19 respectively. Based on these enzyme concentrations (30 nM for full-length MMLV RT, 1
20 μ M for WT, and 10 μ M for Δ C1, and Δ C2), the activities of WT, Δ C1, and Δ C2 were
21 roughly estimated to be 1%, 0.01%, and 0.01% of full-length MMLV RT activity,
22 respectively.

Discussion

We expressed the isolated RNase H domain of MMLV RT with N-terminal strep-tag and C-terminal deca-histidine tag. The use of two tags has an advantage as it allows the removal of degraded products using one of the tags. In our initial attempt to express the RNase H domain with only a C-terminal (His)₆-tag, the degraded products could not be completely removed, as described in the Results section. In addition, the removing of the tags was important because it increased the solubility. Based on the SDS-PAGE (Fig. 2) and spectroscopic analysis (Fig. 3) results, we considered the purity of the RNase H preparations to be sufficient for use in structural and functional analysis.

The DNA in the 25-bp RNA/DNA heteroduplex used in the radioisotope-based RNase H assay lacks the primer 3'-terminus (Fig. 5A). The reaction with full-length MMLV RT produced 17–20-nt RNA fragments (Fig. 5B), indicating that the position at which the RNA strand of the RNA/DNA hybrid is cleaved is 17–20 bp upstream of the 3'-terminus of the primer. This is the same position as previously reported in assays of full-length MMLV RT using RNA/DNA hybrids with primer 3'-termini [20, 21]. This suggests that the DNA polymerase active site binds to the RNA/DNA hybrid in a similar manner whether it has the primer 3'-terminus or not.

Unlike the full-length MMLV RT, WT, $\Delta C1$, and $\Delta C2$ produced RNA fragments mainly ranging from 7 to 16 nt in length (Fig. 5B). We speculate a possible mechanism for the generation of fragments of these lengths as follows: For the full-length MMLV RT, the position at which the RNase H active site binds to the RNA strand of an RNA/DNA hybrid is predetermined, whereas for the isolated RNase H domains, the active site binding position is not; thus the cleavage site by the isolated RNase H domain

1 is different from that by the full-length MMLV RT. This suggests that elimination of the
2 fingers, palm, thumb, and connection subdomains affects the binding of the RNase H
3 domain to an RNA/DNA heteroduplex.

4 In this study, we expressed WT as well as $\Delta C1$ and $\Delta C2$ because the crystal structure
5 of MMLV RT $\Delta C1$ has been determined [1] and the structures of XMRV RT WT and $\Delta C2$
6 have been determined [22, 23]. In the RNase H assay using a fluorescent substrate, WT
7 exhibited activity, whereas $\Delta C1$ and $\Delta C2$ did not (Fig. 4). However, in the RNase H assay
8 using a radioactive substrate, all three exhibited the activity (Fig. 5). Therefore, we
9 concluded that the C helix is not critical for the activity of the isolated MMLV RT RNase
10 H domain. However, the RNase H activities of $\Delta C1$ and $\Delta C2$ were only 10% of WT
11 activity (Fig. 5C), suggesting that the C helix is important for activity. Similar results have
12 been reported for full-length MMLV RT, and the C helix has been shown to be important
13 for both DNA polymerase and RNase H activities [22, 23].

14 Unlike the MMLV RT RNase H domain, the RNase H domain of HIV-1 RT does not
15 contain the C helix, and the isolated HIV-1 RT RNase H domain lacks activity [24-27].
16 Interestingly, insertion of a basic loop near at the active site of the isolated RNase H
17 domain generated weak activity in the presence of Mn^{2+} [28]. Recently, Permanasari *et*
18 *al.* reported that addition of bacterial RNase HI or HII substrate binding domain to the
19 isolated RNase H domain also resulted in weak activity in the presence of Mn^{2+} [29].
20 These results supports the notion that the C helix is important for RNase H activity.

21 *E. coli* RNase HI contains the C helix [30] whereas the RNase H domains of MMLV
22 RT and HIV-1 RT are C helix-deficient. Comparison of the structure of $\Delta C1$ (PDB
23 accession codes SHB5) with that of *E. coli* RNase HI (3AA2) suggests that the C helix of
24 the RNase H domain of MMLV RT is positioned similarly to that of *E. coli* RNase HI.

1 Comparison of the structure of $\Delta C1$ with a complex containing the isolated HIV-1 RT
2 RNase H domain and an RNA/DNA heteroduplex (1HYS) suggests that the C helix in the
3 RNase H domain of MMLV RT interacts with the RNA strand of the RNA/DNA
4 heteroduplex. Therefore, the C helix is thought to contribute to substrate recognition, as
5 reported previously by Nowotny *et al.* [31].

6 In conclusion, we expressed the RNase H domain of MMLV RT in *E. coli* and
7 purified it to homogeneity. Characterization of the recombinant proteins suggested that
8 the C helix is involved in the substrate recognition and that the fingers, palm, thumb, and
9 connection subdomains are involved in positioning of the cleavage sites in the RNA strand
10 of an RNA/DNA heteroduplex. These recombinant RNase H domain proteins might be
11 suitable for use in structural and functional analyses to elucidate the mechanisms of
12 thermostabilization of MMLV RT through site-directed mutagenesis. An extensive study
13 is currently underway to prepare crystals of WT, $\Delta C1$, and $\Delta C2$, with and without a
14 mutation of the catalytic Asp (Asp524).

16 **Acknowledgments**

18 This study was supported in part (K.Y.) by Grants-in-Aid for Scientific Research
19 (no. 21580110) from the Japan Society for the Promotion of Science and the Salt Science
20 Research Foundation.

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Figure legends

Fig. 1. Expression plasmids for the RNase H domain. The structures of pET-RNaseH-WT, pET-RNaseH- Δ C1, and pET-RNaseH- Δ C2 are shown. The “498”, “671”, and asterisk indicate Ile498, Leu671, and the termination codon. The amino acid sequences of strep tag, HRV 3C protease recognition site, thrombin recognition site, and (His)₁₀ are underlined.

Fig. 2. SDS-PAGE analysis. CBB-stained 12.5% SDS-polyacryl amide gel is shown. (A) Active fractions of each purification stage for the wild-type RNase H (WT). Lanes: marker proteins (lane 1), soluble fractions of the total extracts (lane 2), active fractions of ion-exchange chromatography (lane 3), Ni²⁺ affinity chromatography (lane 4), and Strep-Tactin affinity chromatography (lane 5). (B) Purified enzyme preparations with N-terminal strep tag and C-terminal (His)₁₀ tag. Lanes: marker proteins (lane 1), WT (lane 2), RNaseH- Δ C1 (lane 3), and RNaseH- Δ C2 (lane 4). (C) Purified WT preparations. Lanes: marker proteins (lane 1), before removal of fused tags (lane 2), and after removal of fused tags (lane 3).

Fig. 3. UV and CD spectroscopic analysis of purified WT. (A) UV spectra. (B) CD spectra.

Fig. 4. RNase H activity of the RNase H domain using a fluorescent substrate. (A) Sequences of the RNA-DNA hybrid, R18/D18. (B) Fluorescence spectra of R18/D18 with excitation at 490 nm. The reaction was carried out with either of 50 nM HIV-1 RT, 1.8 μ M WT, 1.8 μ M Δ C1, and 1.8 μ M Δ C2 for 30 min at pH 8.0 at 37°C.

Fig. 5. RNase H activity of the RNase H domain using a radioactive substrate. (A) Sequences of the RNA-DNA hybrid, R25/D25. (B, C) Patterns of denatured PAGE. The enzyme concentrations were 30 nM for full-length MMLV RT, 1 μ M for WT, and 10 μ M for Δ C1 and Δ C2. The reaction times were 15 s and 10 min (B) and 15 s, 30 s, 1 min, 5 min, 15 min, 30 min, 1 h, and 2 h (C). The reaction was carried out with 1.8 μ M WT, Δ C1, or Δ C2 for 30 min at pH 8.0 at 37°C.

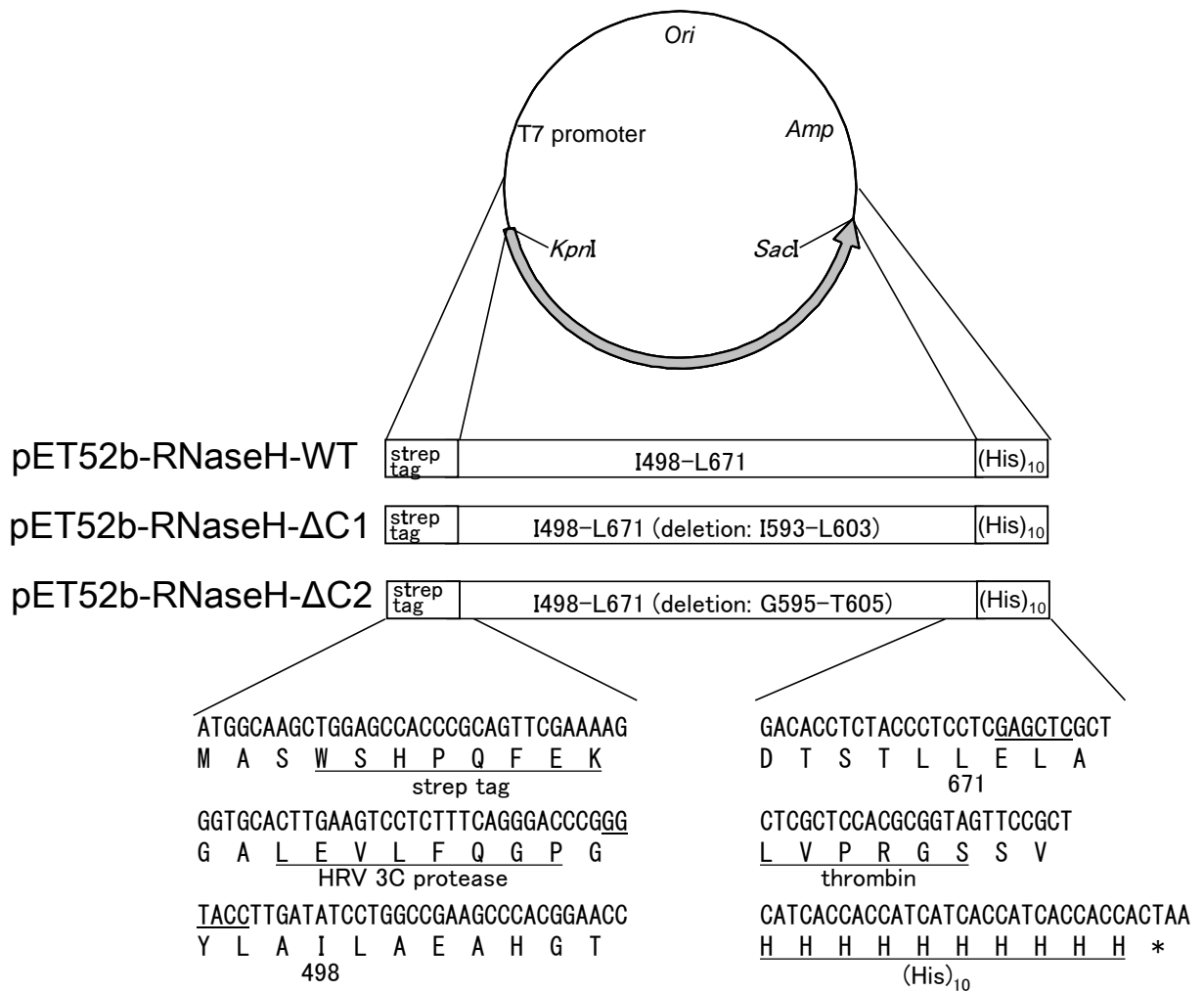


Fig. 1

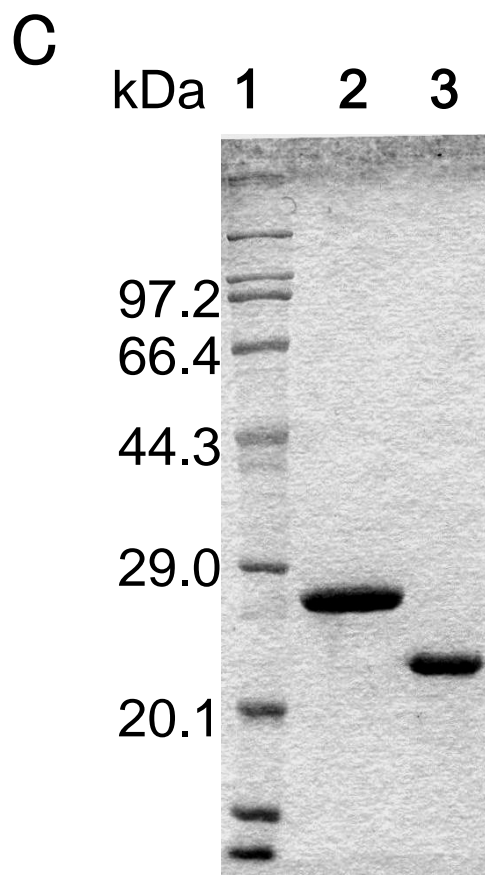
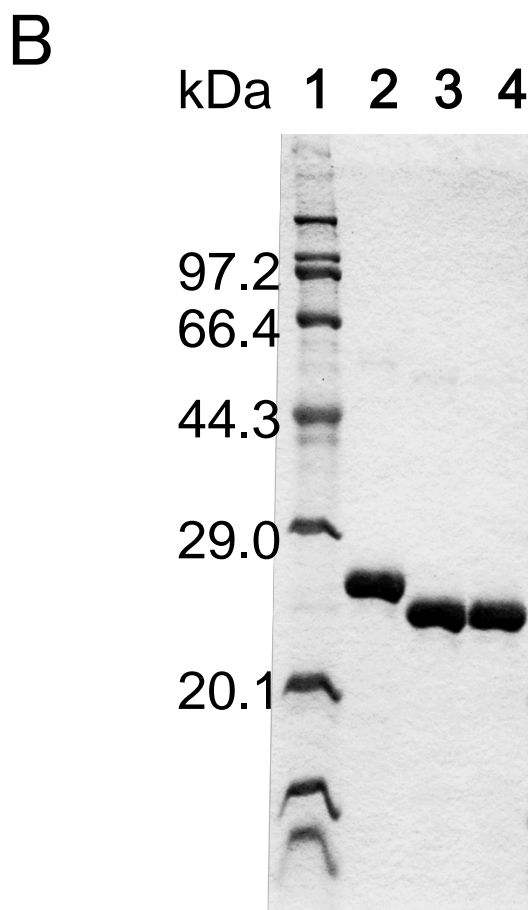
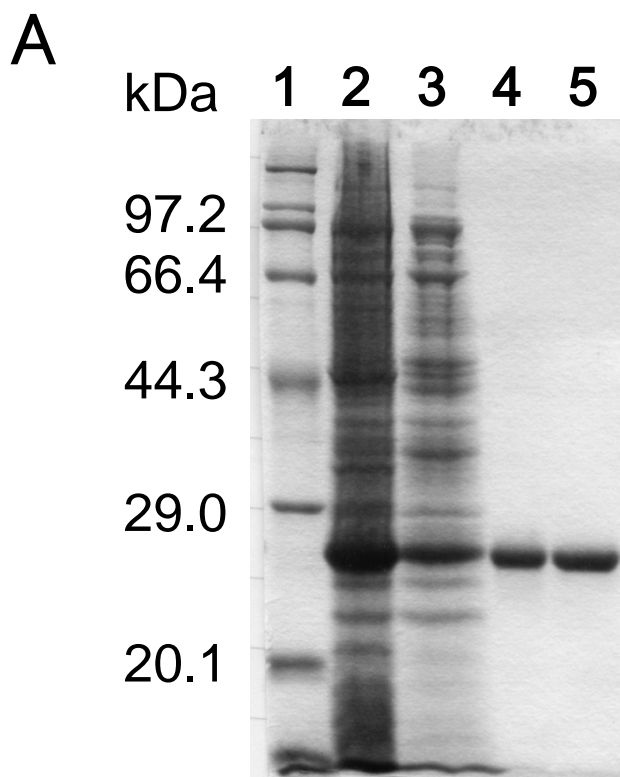


Fig. 2

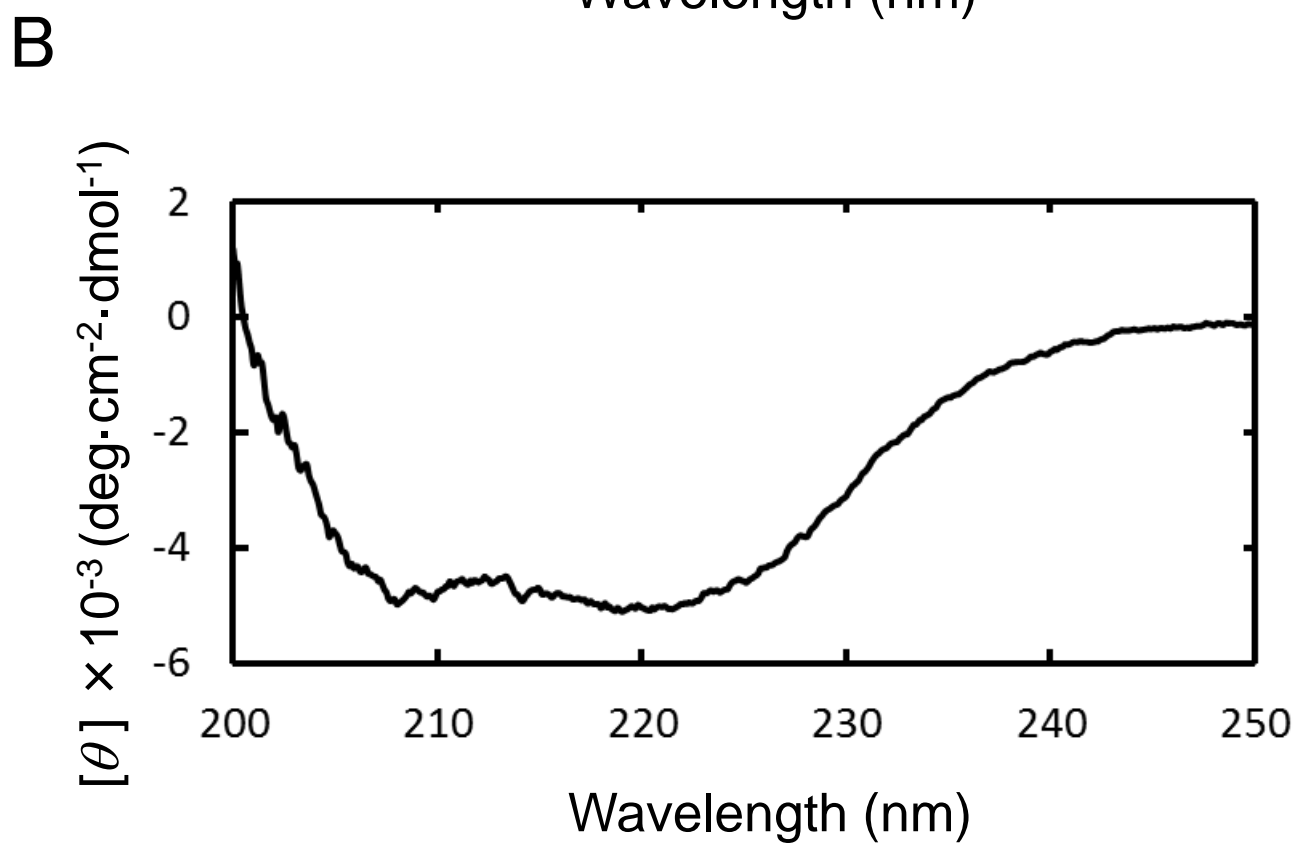
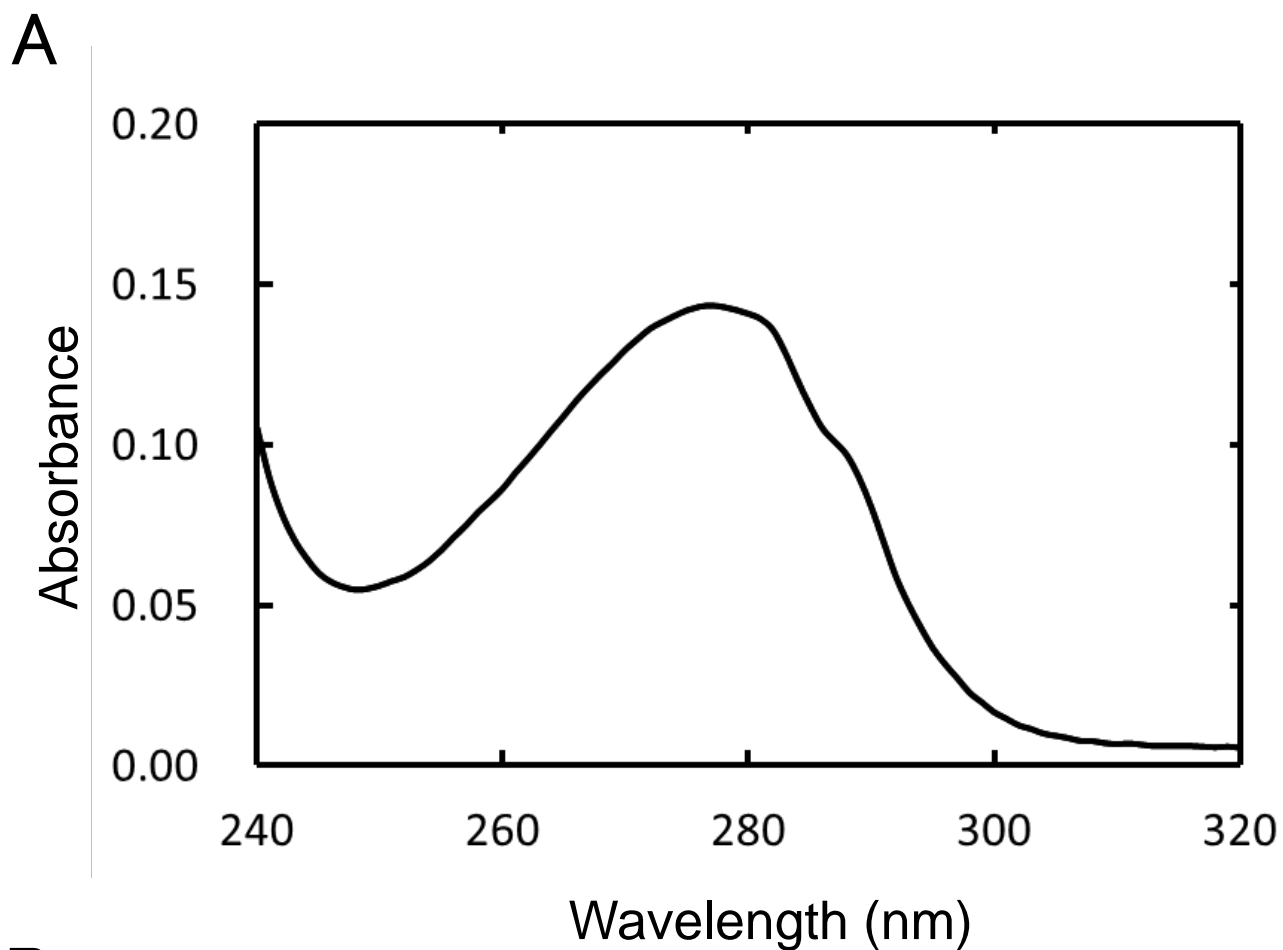


Fig. 3

A

R18: 5'-GAUCUGAGCCUGGGAGCU-fluorescein-3'
D18: 3'-CTAGACTCGGACCCTCGA-DABCYL-5'

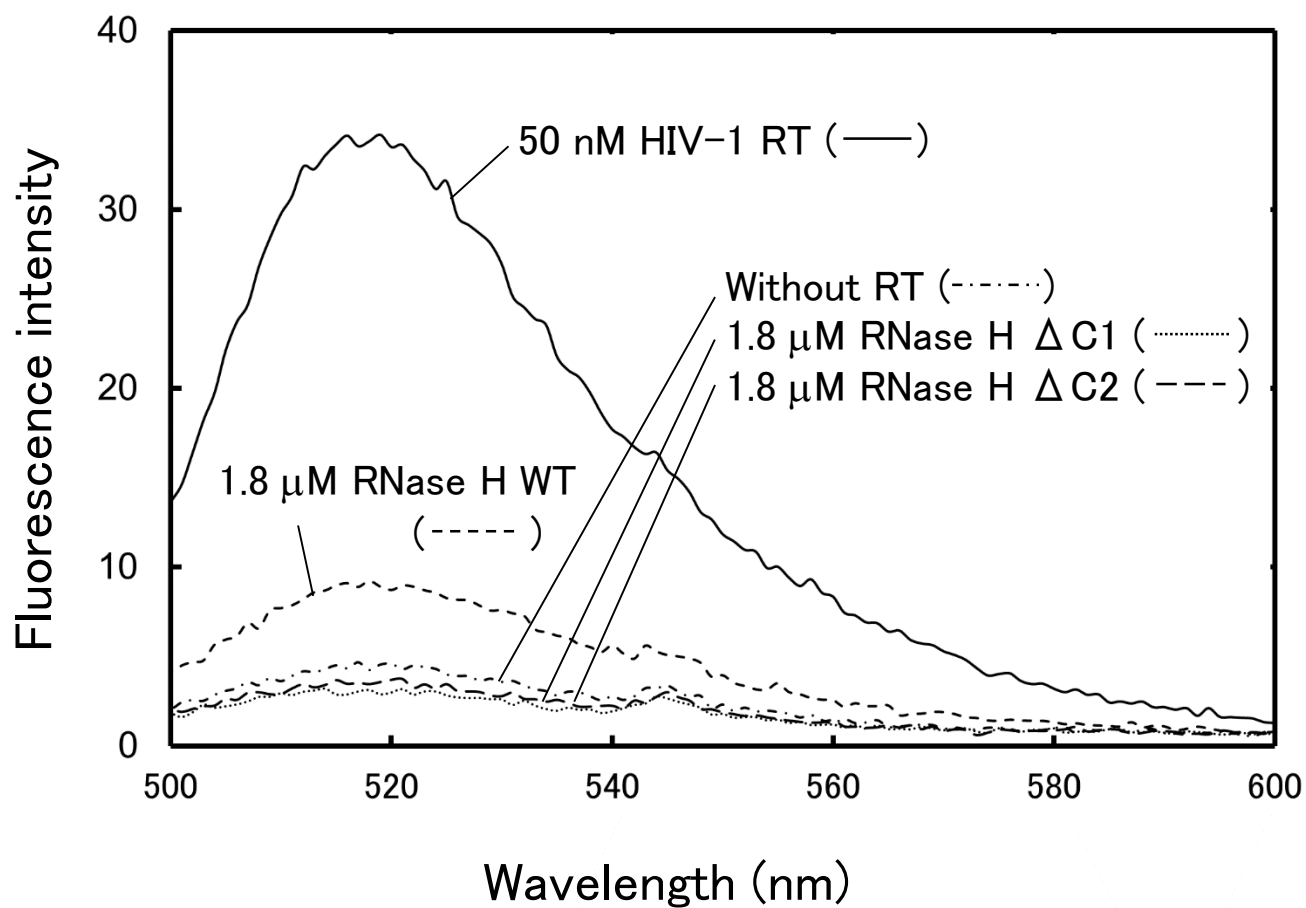
B

Fig. 4

R25: 5' - [32P] AUGUAUAGCCCUACCAGCAUUCTGG - 3'
D25: 3' - TACATATCGGGATGGTCGTAAGACC - 5'

Figure 1: Gel electrophoresis image showing RT-PCR products for fMMLV, WT, $\Delta C1$, and $\Delta C2$ constructs. The gel has 12 lanes. Lanes 1-4 are for RT, WT, $\Delta C1$, and $\Delta C2$ at 0.03 μM . Lanes 5-8 are for WT, $\Delta C1$, and $\Delta C2$ at 1 μM . Lanes 9-12 are for WT, $\Delta C1$, and $\Delta C2$ at 10 μM . The gel shows bands at various sizes, with a prominent band at 25 nt. The $\Delta C1$ and $\Delta C2$ constructs show a shift in the 25 nt band compared to the WT construct.

Fig. 5

C

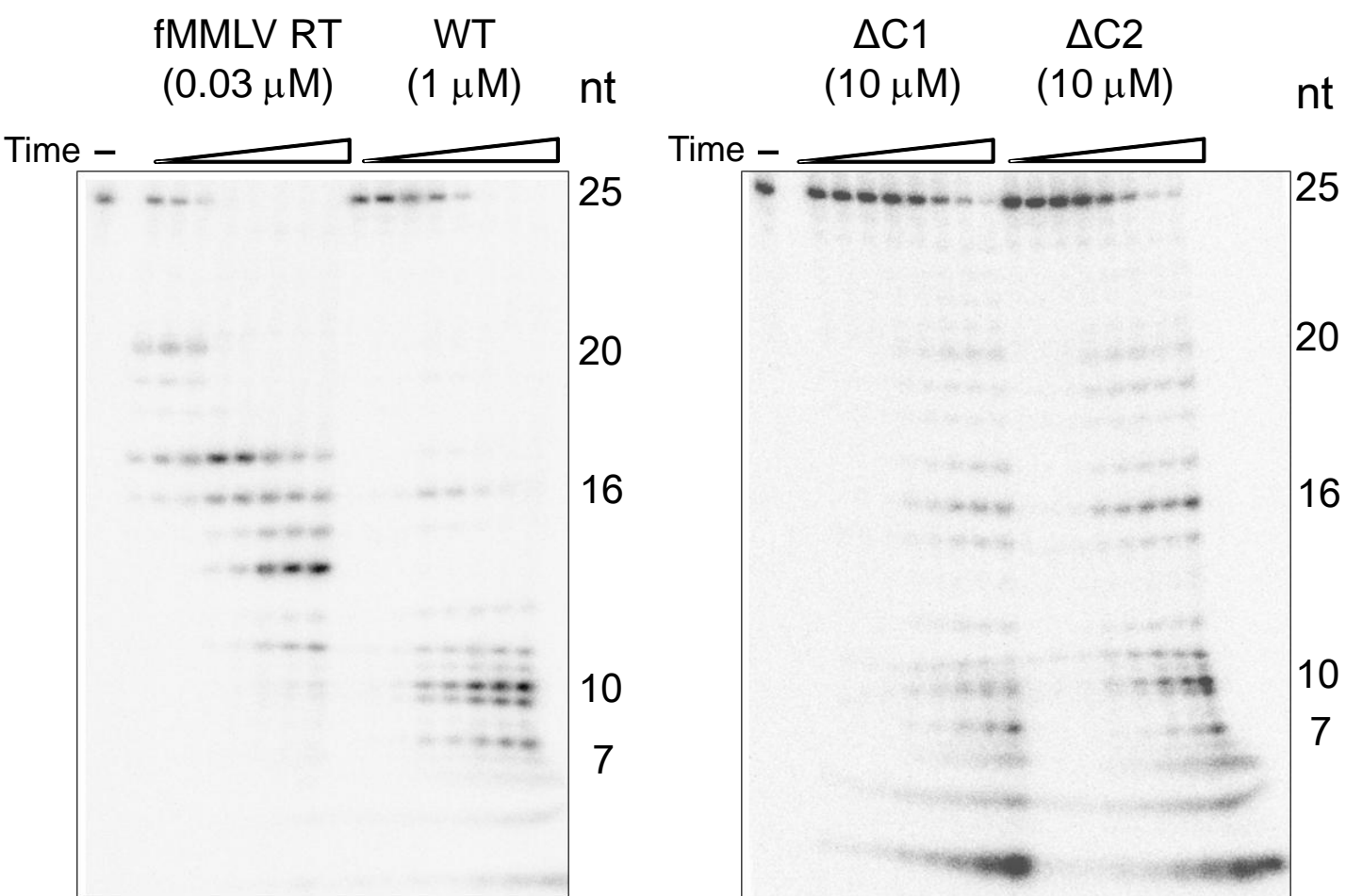


Fig. 5

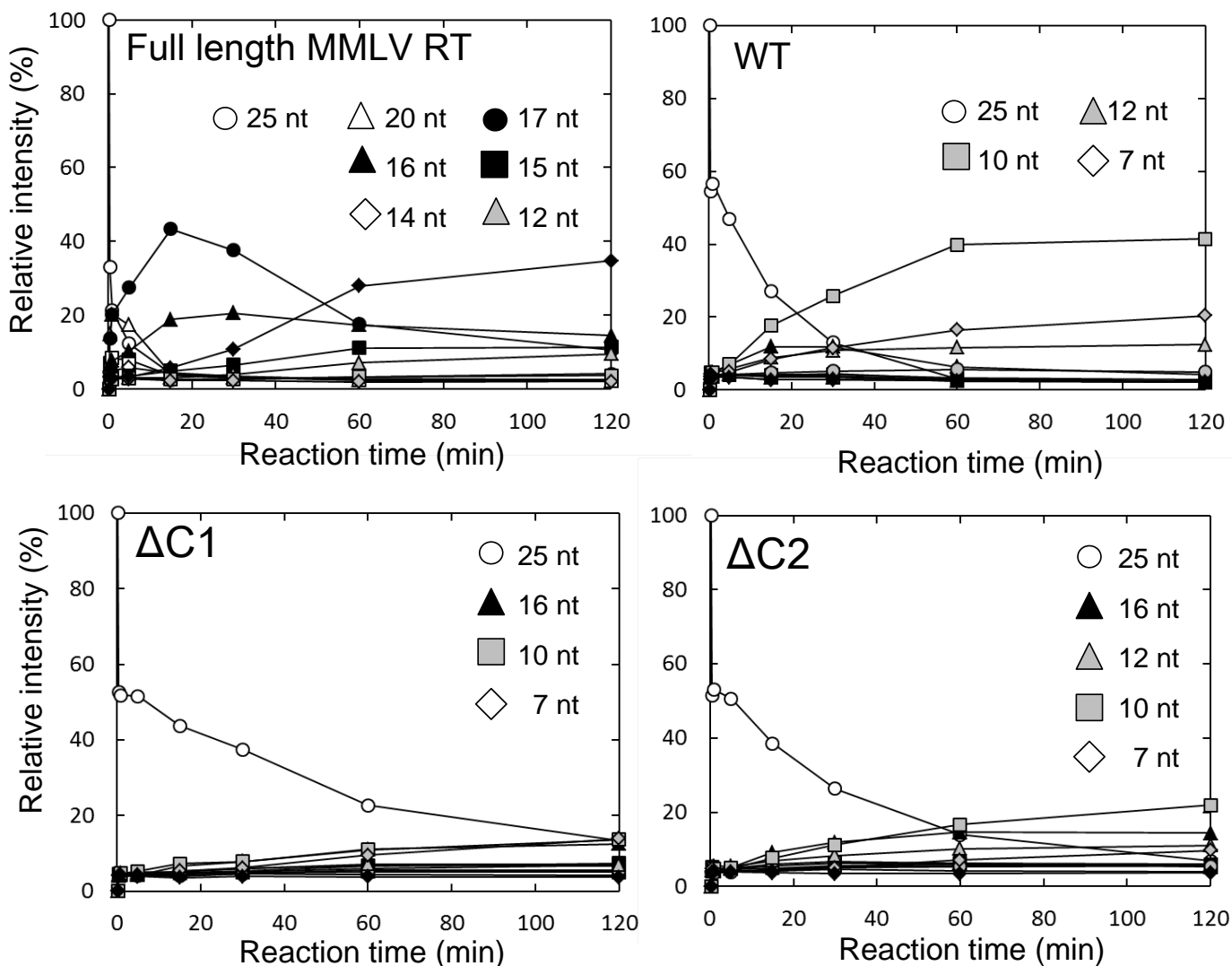


Fig. S1. Time course of the amounts of RNA fragments in the RNase H reaction. The result of the densitometry traces of the bands in Fig. 5C is shown. The relative intensity is defined as the ratio of the amount of each band to those of all 7–25-nt bands. Symbols for RNA fragments (nt): 25 (open circle), 20 (open triangle), 19 (open square), 18 (open diamond), 17 (solid circle), 16 (solid triangle), 15 (solid square), 14 (solid diamond), 13 (gray circle), 12 (gray triangle), 10 (gray square), and 7 (gray diamond).