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Cooperative regulation for Okazaki fragment processing by RNase HII and FEN-1 purified from a hyperthermophilic archaeon, *Pyrococcus furiosus*

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Abstract

A reconstitution system that recapitulates the processing of Okazaki-primer RNA was established by the heat-stable recombinant enzymes RNase HII and FEN-1 (termed *Pf*-RNase HII and *Pf*-FEN-1, respectively) prepared from a hyperthermophilic archaeon, *Pyrococcus furiosus*. A 35-mer RNA–DNA/DNA hybrid substrate mimicking an Okazaki fragment was used to investigate the properties of the processing reaction in vitro at 50 °C. *Pf*-RNase HII endonucleolytically cleaves the RNA primer region, but does not cut the junction between RNA and DNA. Removal of the RNA of the RNA–DNA junction was brought about by *Pf*-FEN-1 after *Pf*-RNase HII digestion. In the presence of 0.25–5 mM $MnCl_2$, *Pf*-FEN-1 alone weakly cleaved the junction. The addition of *Pf*-RNase HII to the reaction mixture increased removal efficiency and optimal *Pf*-FEN-1 activity was achieved at an equal amount of the two enzymes. These results indicate that there are at least two steps in the degradation of primer RNA requiring a step-specific enzyme. It is likely that *Pf*-RNase HII and *Pf*-FEN-1 cooperatively process Okazaki fragment during lagging-strand DNA replication.

Keywords: RNase H; FEN-1; Okazaki fragment; Archaea; *P. furiosus*

Discontinuous DNA polymerization is initiated by primer RNA synthesis in the lagging-strand DNA replication that forms Okazaki fragments [1]. The RNA primers of Okazaki fragments should be removed prior to DNA-joining steps by DNA ligase. Thus, primer RNA processing is critical for lagging-strand DNA synthesis. So far, two principle models for the removal of primer RNA have been proposed [2] based on the different enzymes used: (a) RNase H cleaves the RNA region attached to the 5′-end of the Okazaki fragment, leaving a single ribonucleotide adjacent to the RNA–DNA junction. Flap endonuclease 1 (FEN-1) then removes the remaining ribonucleotide; and (b) a helicase such as Dna2 displaces the RNA segment. FEN-1 then endonucleolytically cleaves the branch point, releasing the displaced RNA. An in vitro reconstitution system will be helpful for distinguishing between these two models.

We have been working on RNA metabolism in the hyperthermophilic archaeon *Pyrococcus furiosus* [3] and this organism has several advantages for biochemical analysis. For instance, in *P. furiosus*, the encoded proteins are mostly heat-stable and easy to handle without inactivation, and most of the genes involved in nucleic acid metabolism in archaea are similar to those found in eukarya. The regulation mechanisms are simpler in the former than in the latter. In this paper, we describe an archaeal in vitro reconstitution system that recapitulates the processing of RNA in Okazaki substrate, an RNA–DNA/DNA hybrid substrate mimicking Okazaki fragment. Because previous in vitro reconstitution systems could not rule out the possibility that the purified

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fraction contains a small amount of undesirable nuclease activities, the use of heat-stable purified recombinant proteins provide a system which is better able to minimize trace amounts of impurities in the reaction conditions. We cloned the genes that encode *Pf*-RNase HII and *Pf*-FEN-1 and prepared highly purified recombinant products. We present evidence that these two enzymes play an important role in model (a) above. It should be noted that equal amounts of each enzyme are required for effective primer RNA processing.

Materials and methods

Cloning and construction of expression vectors. The genomic DNA of *P. furiosus* DSM3638 prepared using a GNOME kit (BIO101, La Jolla, CA, USA) was partially digested with *Sau*3AI. The resulting DNA fragments were fractionated by electrophoresis on a 0.7% agarose gel. Fragments of approximately 15 kb in size were prepared from the gel and used for the templates for PCR cloning. Both *Pf*-RNase HII and *Pf*-FEN-1 genes were cloned into the pET-23b expression vector (Novagen, Madison, WI, USA) via polymerase chain reaction (PCR) using the following site- specific primers: HA (5′-TAACTCGAGAGGTTTCT TAAAGAATTTATCAAGC-3′) and HS (5′-AATCATATGAAAAT AGGGGGAATTGACGAAGC-3′) for *Pf*-RNase HII [National Center for Biotechnology Information (NCBI) protein database Accession No. NP_579510], and Flap-A (5′-TAACTCGAGTCTCTTGAACC AACTTTCAAGGGTT-3′) and Flap-S (5′-TTACATATGGGTGTC CCAATTGGTGAGATTA-3′) for *Pf*-FEN-1 [NCBI protein database Accession No. NP_579143]. These oligonucleotides were designed to provide *Nde*I and *Xho*I sites at the 5′ and 3′ termini, respectively. The resulting vectors, pET-rnh2 and pET-fen1, encode full-length *Pf*-RNase HII and *Pf*-FEN-1 proteins with six-histidine tags at their C-terminal ends. The nucleotide sequences of the insert DNA were determined and confirmed to be identical to those in the database.

Complementation of Escherichia coli RNases H mutant MIC2067. An *E. coli* mutant MIC2067 can form colonies at 30 °C but not at 42 °C due to a deficiency of two *rnh* genes (*rnhA339::cat* and*rnhB716::kam*) [4]. The temperature-sensitive growth phenotype is suppressed by delivering functional RNase H genes from various organisms. The plasmid pET-rnh2 expressing *Pf*-RNase HII was used to transform the MIC2067 and the transformants were selected on LB plates containing 100 μg/ml ampicillin at 30 and 42 °C. Plasmid pSK760, which carries the transcription of the *E. coli rnhA* gene, was used as a positive control [5], while pBR322 was used as a non-complementing control.

RNA isolation and reverse transcription-polymerase chain reaction. Total RNA was prepared by using an RNeasy Protect Bacteria Mini kit (Qiagen, Germany) according to the instructions of the manufacturer. Reverse transcription-polymerase chain reaction (RT-PCR) for *Pf*-RNase HII mRNA was carried out using ReverTra Dash Kit (Toyobo Biochemicals, Japan) as follows: 200 ng total RNA was reverse transcribed with *Pf*-RNase HII-specific primer RNH-A (5′-AGTTTCCCAGGTTCGTCTGACTATT-3′). The resulting cDNA was amplified with two primers, RNH-A and RNH-S (5′- ATGAAA ATAGGGGGAATTGACGAAG-3′). PCR products were separated on 3% NuSieve agarose gel electrophoresis and stained with ErBr.

Expression and purification of his-tagged recombinant proteins. *E. coli* strain BL21(DE3) was transformed with expression plasmids. The transformants growing logarithmically in LB medium containing 50 μg/ml ampicillin at 37 °C were supplemented with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After 14–16 h of further growth at 30 °C, the cells were harvested by centrifugation (5000*g* for 5 min at 4 °C) and the recombinant proteins were extracted by sonication (0.5 min) in a buffer (Buffer A) containing 20 mM Tris–HCl (pH 8.0), 5 mM imidazole, 500 mM NaCl, and 0.1% NP40. These extracts were heat-treated at 85 °C for 15 min to kill the endogenous ribonuclease-related proteins from *E. coli* and centrifuged at 12,000*g* for 10 min at 4 °C to remove debris. The recombinant *Pf*-RNase HII and *Pf*-FEN-1 proteins were purified using an Ni^{2+}–Sepharose column following the manufacturer's instructions (Amersham, NJ, USA) and the eluted protein peaks were dialyzed against Buffer B, which contained 50 mM Tris–HCl (pH 7.5), 1 mM ethylenediaminetetraacetic acid (EDTA), 0.2% Tween 20, 7 mM 2-mercaptoethanol, and 10% glycerol.

In vitro assay system for Okazaki substrate processing. The end-labeled (either at 5′ FAM or 3′ FITC) 35-base RNA–DNA oligonucleotides, 5′-GCGAAUUUAGGGCGAgagcaaacttctctacctct-3′ or 3′ FITC end-labeled 21-base RNA–DNA oligonucleotide 5′-Agagcaaacttctcta cctct-3′ in which ribonucleotides are denoted with uppercase letters and deoxyribonucleotides with lowercase letters, were chemically synthesized by Hokkaido System Science (Hokkaido, Japan). These oligonucleotides were annealed to their 35-base complementary DNA 5′-agagguagagaaguuugcucucgcccuaaauucgc-3′ and the resulting duplexes (or partially duplexes) mimicking Okazaki fragment were used for substrates. Standard processing assays were carried out in a final volume of 20 μl containing 20 mM Tris–HCl (pH 8.0), 1 mM DTT, 50 mM KCl, 4 mM $MnCl_2$, 400 pmol of the substrate, and 20–120 ng of the purified recombinant *Pf*-RNase HII and/or *Pf*-FEN-1 proteins. The reaction mixture was incubated at 50 °C for 15 min and quenched with an equal volume of the stop solution [8 M urea, 1 M Tris–HCl (pH 7.2), and a small amount of blue dextran (Sigma Chemical, St. Louis, MO, USA)]. In some experiments, $MgCl_2$ was used instead of $MnCl_2$. Samples were denatured at 70 °C for 5 min and the cleavage products were separated on 20% polyacrylamide gel containing 8 M urea. Gel images were visualized and analyzed by Molecular Imager FX Pro (Bio-Rad Laboratories, Hercules, CA, USA).

Results and discussion

Cloning of genes encoding for RNase HII and FEN-1 from P. furiosus and preparation of the recombinant proteins

Bacteria and eukaryotic cells contained two or more RNase H genes in their genome, while the hyperthermophilic archaeon *P. furiosus* possessed only one gene classified as RNase HII in its genome [6]. We cloned the gene by PCR and confirmed the identity of the clones by determining their nucleotide sequence. The gene encoded a protein that was 224 amino acids long and had a calculated molecular weight of 25.3 kDa. This protein showed 31% similarity to RNase HII [NCBI protein database Accession No. NC_000913] from *E. coli* [7] and 58% similarity to RNase HII [NCBI protein database Accession No. BAA32803] from *Thermococcus kodakaraensis* KOD1 [8]. We constructed an expression plasmid for the RNase HII-related protein in *P. furiosus* and found that the resulting plasmid, which we named pET-rnh2, successfully complemented the temperature-sensitive growth defect of the *E. coli* RNase H mutant strain MIC2067 (Fig. 1A). Since the T7 promoter in pET vector is leaky, the *P. furiosus* RNase HII-related gene expression was observed without induction of the

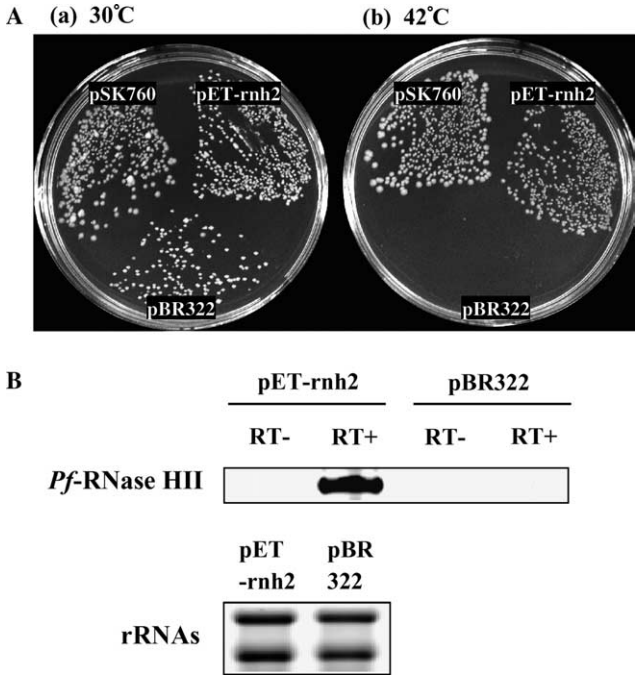

Fig. 1. (A) Effect of *Pf*-RNase HII on the temperature-sensitive growth of *E. coli* mutant MIC2067. Cells were grown on an LB plate containing 100 μg/ml of ampicillin either at 30 °C for 24 h (a) or at 42 °C for 16 h (b). The plasmids used were pSK760, pET-rnh2, and pBR322 (see Materials and methods). (B) RT-PCR analysis of *Pf*-RNase HII gene expression in *E. coli* mutant MIC2067. Mutant MIC2067 cells that transformed with either plasmid pET-rnh2 or pBR322 were grown in LB medium containing 50 μg/ml of ampicillin at 30 °C for 24 h. Total RNAs were prepared and RT-PCR analysis for *Pf*-RNase HII transcript was carried out using either reverse transcribed (RT+) or mock transcribed (RT−) RNAs. PCR products were separated on 3% NuSieve gel electrophoresis and stained with EtBr. For normalization of rRNAs, total RNA was separated on 1.2% denaturing agarose gel electrophoresis and stained with EtBr. Gel images were visualized and analyzed by Molecular Imager FX Pro (Bio-Rad Laboratories, Hercules, CA, USA).

gene. We confirmed the presence of the transcript from *P. furiosus* RNase HII-related gene by RT-PCR in a reverse-transcription-dependent manner (Fig. 1B). These observations strongly indicate that the *P. furiosus* RNase HII-related genes are expressed in *E. coli* and that the product functions as RNase H. We refer to the protein as *Pf*-RNase HII hereafter. On the other hand, the FEN-1 protein from *P. furiosus*, referred to in the present report as *Pf*-FEN-1, has previously been identified [9,10]. We also isolated the gene by PCR-mediated cloning and confirmed the presence of the *Pf*-FEN-1 consisting of 340-amino-acid residues with a calculated molecular weight of 38.7 kDa. The expression vector of *Pf*-FEN- 1 (pET-fen1) did not complement the RNase H mutant MIC2067 (data not shown).

In order to obtain large amounts of proteins *Pf*-RNase HII and *Pf*-FEN-1 for biochemical study, the recombinant C-terminal his-tagged proteins were overexpressed in *E. coli* strain BL21(DE3) and purified to

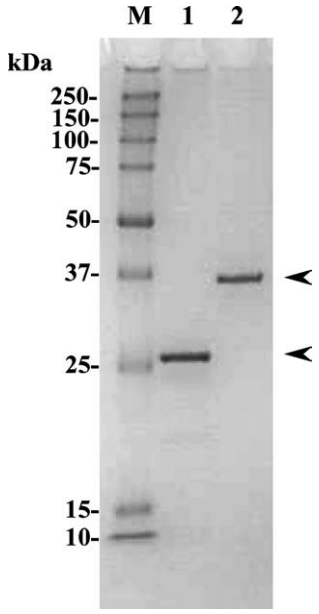

Fig. 2. Purified recombinant *Pf*-RNase HII and *Pf*-FEN-1. Peak samples from Ni^{2+}–Sepharose column chromatography described in Materials and methods above were dialyzed and analyzed by SDS–polyacrylamide gel electrophoresis on a 10–20% gel. The gel was stained with Coomassie brilliant blue. Arrowheads indicate the positions of the purified enzymes: (1) *Pf*-RNase HII and (2) *Pf*-FEN-1. M, size markers (Bio- Rad).

near homogeneity by heat treatment at 85 °C for 15 min followed by Ni^{2+}–Sepharose column chromatography. The purified proteins revealed a molecular weight of about 25 and 37 kDa for *Pf*-RNase HII and *Pf*-FEN-1, respectively, as determined by SDS–polyacrylamide gel electrophoresis (Fig. 2). These findings are consistent with the values determined from the deduced amino acid sequences for the genes. Our purified *Pf*-FEN-1 exhibited structure-specific nuclease activity when unannealed 5′-flap substrate was used (data not shown), as previously reported [11].

Both Pf-RNase HII and Pf-FEN-1 are required for the effective processing of an Okazaki substrate

We used a 35-mer RNA–DNA/DNA hybrid substrate because it mimics an Okazaki fragment. The substrate was FAM-labeled at the 5′ end of the RNA strand to enable monitoring RNA degradation. As shown in Fig. 3, the recombinant *Pf*-RNase HII cleaved at several sites and the pattern of cleavage was similar to that found for other archaeal RNases H such as *T. kodakaraensis* KOD1 [8] or *A. fulgidus* [12]. Specifically, the enzyme cleaved the 5′ phosphodiester bond of the last ribonucleotide at the RNA–DNA junction, but did not cleave at the bond between RNA and DNA. These results again indicate that the RNase HII-related gene found in *P. furiosus* encodes a protein with ribonuclease H activity. As the activity depends completely on

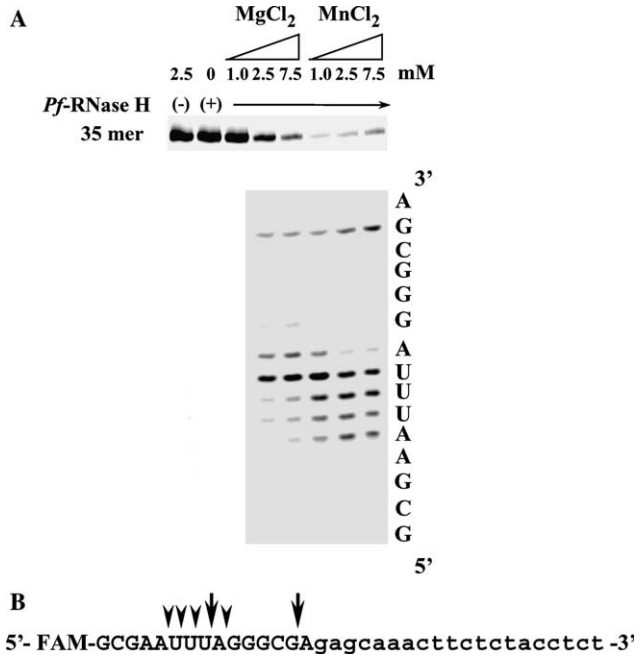

Fig. 3. Cleavage specificity of the purified recombinant *Pf*-RNase HII on Okazaki substrate. (A) Denaturing polyacrylamide gel analysis of cleavage reactions. 5′ FAM-labeled Okazaki substrate was used for the processing assay. The reaction was carried out using 80 ng of purified *Pf*-RNase HII with various concentrations of $MgCl_2$ or $MnCl_2$. (B) Graphical representation of cleavage sites by *Pf*-RNase HII. Ribonucleotides are denoted with uppercase letters and deoxyribonucleotides with lowercase letters. Arrows and arrowheads represent major and minor cleavage sites, respectively.

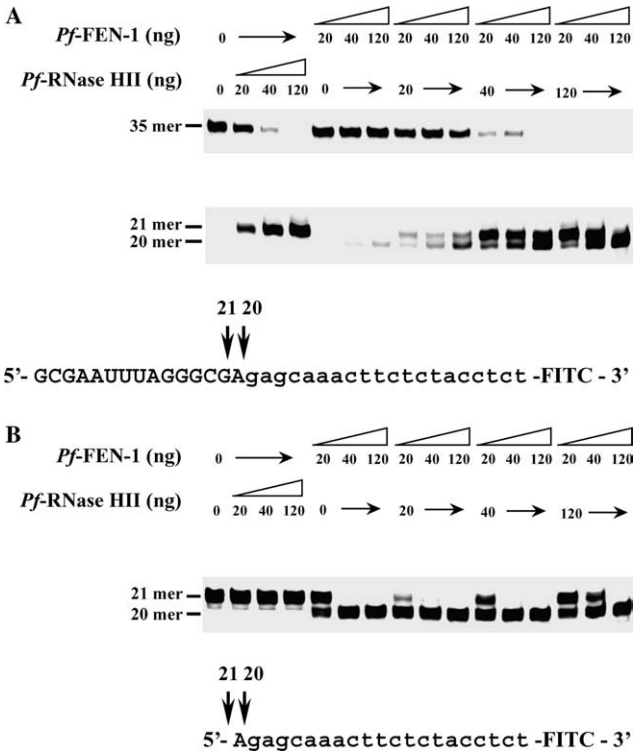

Fig. 4. Both *Pf*-RNase HII and *Pf*-FEN-1 are required for the effective processing of Okazaki substrate. The 3′ FITC-labeled Okazaki substrates, either a 35-base RNA–DNA/35-base DNA (A) or a 21-base RNA–DNA/35-base DNA (B), were used for the processing assay. The reaction was carried out using 4 mM $MnCl_2$ with varying amounts of the recombinant proteins. Graphical representations of cleavage sites by *Pf*-RNase HII and *Pf*-FEN-1 are shown at the bottom of each column. Ribonucleotides are denoted with uppercase letters and deoxyribonucleotides with lowercase letters. Arrowheads represent the cleavage sites that correspond to the 20- or 21-mer bands in (A) and (B).

divalent metal ions, we examined the effect of two divalent ions such as Mg^{2+} and Mn^{2+} on enzymatic activity. Similar to RNases HII from *E. coli* [7,13] and *M. jannaschii* [14], *Pf*-RNase HII was found to prefer the Mn^{2+} ion. In contrast, RNases HII from *T. kodakaraensis* KOD1 and *A. fulgidus* preferred the Mg^{2+} ion rather than the Mn^{2+} ion. Preferential cleavage of the 5′ phosphodiester bond of the last ribonucleotide at the RNA–DNA junction was observed in the presence of 1–7.5 mM $MnCl_2$ as shown in Fig. 3. In the presence of the same concentration of $MgCl_2$, on the other hand, the enzyme preferentially cleaved the phosphodiester bond in the middle of the RNA sequence (between U^8 and A^9), suggesting that cleavage specificity of *Pf*-RNase HII is partially regulated by divalent metal ions. Further research is necessary to substantiate ion-dependent regulation of enzymes. We also confirmed that the 35-mer DNA strand remains intact after processing reaction (data not shown).

To analyze the processing step of the single remaining ribonuclotide left by *Pf*-RNase HII digestion, the same Okazaki substrate was prepared with the exception that it was 3′-end labeled with FITC. The effects of coincubation of both enzymes *Pf*-RNase HII and *Pf*-FEN-1 on this substrate were investigated. *Pf*-RNase HII again cleaved the 5′ phosphodiester bond of the last ribonucleotide at the RNA–DNA junction in this substrate and a 21-mer product was accumulated (Fig. 4A). When the *Pf*-FEN-1 was added to this reaction product, a 20-mer band was detected in a dose-dependent manner indicating that one base was processed. These results indicate that *Pf*-RNase HII provides a suitable substrate for *Pf*-FEN-1. The optimal conditions for RNA primer degradation were obtained using nearly equal amounts of the two enzymes. In a similar processing experiment using enzymes purified from calf thymus, Huang et al. [15] report that the best molar ratio of RNase H:FEN-1 (the authors refer to the nuclease as RTH-1 in their manuscript) is approximately 1:1000 for processing of primer RNA in vitro. In the present study, we achieved high reliability by establishing an in vitro reconstitution system using highly purified recombinant proteins heat-treated at 85 °C for 15 min to rule out possible contaminated nuclease activities from *E. coli*.

In order to confirm that *Pf*-FEN-1 removes the remaining one ribonucleotide, we tested a 21-mer RNA–DNA/35-mer DNA substrate that is only one ribonu-

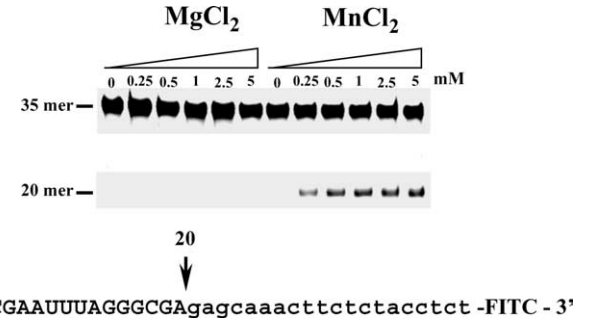

Fig. 5. *Pf*-FEN-1 alone weakly cleaved the RNA–DNA junction in the presence of 0.25–5 mM $MnCl_2$. The 3′ FITC-labeled 35-base RNA–DNA/35-base DNA was used for the processing assay. The reaction was carried out using 120 ng each of the purified enzymes at 0–5 mM of $MgCl_2$ or $MnCl_2$. See Fig. 4 legend in detail.

cleotide and 20 of deoxyribonucleotides in the end-labeling strand. As shown in Fig. 4B, the 21-mer substrate is no longer digested by *Pf*-RNase HII but *Pf*-FEN-1 effectively removes the ribonucleotide in a dose-dependent manner. In higher concentration of *Pf*-RNase HII, the processing activity at the RNA–DNA junction by *Pf*-FEN-1 is inhibited, confirming that *Pf*-RNase HII is unable to remove the last one ribonucleotide and that the enzyme is required for providing a suitable substrate for *Pf*-FEN-1. Finally, the effects of Mg^{2+} and Mn^{2+} ions on the *Pf*-FEN-1 activity are shown in Fig. 5. Our results indicate that, in the presence of 0.25–5 mM Mn^{2+} ion, a weak but distinct 20-mer band was detected on *Pf*-FEN-1 enzyme digestion. The result indicated that *Pf*-FEN-1 alone directly digested the RNA–DNA junction without involvement of *Pf*-RNase HII in vitro.

In conclusion, there are at least two steps for primer RNA processing of Okazaki fragment and each step requires the specific enzymes for the in vitro reaction at 50 °C: (1) *Pf*-RNase HII-dependent step and (2) *Pf*-FEN-1-dependent step. The first step is required for providing the suitable template structure for the second step. Given that *Pf*-FEN-1 alone is able to cleave the junction between DNA and RNA of Okazaki fragment in the presence of $MnCl_2$, *Pf*-FEN-1 may account for the entire processing reaction. Nevertheless, we propose that the nuclease activity possessed by some DNA polymerases plays a role in primer RNA removal [16]. The recombinant *Pfu* DNA polymerase [NCBI protein database Accession No. P80061] isolated in a separate experiment did not produce any significant effect on the processing reaction in our system (unpublished observations). Moreover, it has been demonstrated that proliferating cell nuclear antigen (PCNA) interacts with FEN-1 and stimulates its activity [17,18]. It is intriguing as to how other factors, including PCNA and DNA ligase [19,20], were involved in the RNA processing reaction examined in this study. Several models for primer RNA processing of Okazaki fragment have been proposed. In the quest to determine which model is an accurate representation of the biological phenomenon, our reconstitution system using purified heat-stable enzymes may provide a basic environment for further analysis of the molecular mechanisms of primer RNA processing.

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