A role of RnlA in the RNase LS activity from *Escherichia coli*

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Escherichia coli ribonuclease LS is a potential antagonist of bacteriophage T4. When the T4 *dmd* gene is defective, RNase LS cleaves T4 mRNAs and antagonizes T4 reproduction. Our previous work demonstrated that *E. coli* *rnlA* is essential for RNase LS activity. Here we show that His-tagged RnlA cleaves T4 *soc* RNA at one of the sites also cleaved by RNase LS in a cell extract. The cleavage activities of His-tagged RnlA and the RNase LS activity in a cell extract were inhibited by Dmd encoded by T4 phage. Fractionation of the RNase LS activity in a cell extract showed that it sedimented through a sucrose density gradient as a 1000-kDa complex that included RnlA. Pull-down experiments revealed more than 10 proteins associated with His-tagged RnlA. Among these, triose phosphate isomerase exhibited a remarkable affinity to RnlA. These results suggest that RnlA plays a central role in RNase LS activity and that its activity is regulated by multiple components.

Key words: RnlA, RNase LS, *Escherichia coli*, Dmd, T4 phage

INTRODUCTION

In *Escherichia coli*, endonucleases play a critical role at the initial and rate-limiting steps of mRNA degradation (Kushner, 2002). RNase E is considered to trigger the degradation of many mRNAs (Coburn and Mackie, 1999; Bernstein et al., 2002). This RNase interacts through its C-terminal scaffold region with PNPase, RhlB RNA helicase, enolase and other proteins to form a complex and functions as an mRNA-decay machine (Morita et al., 2005; Caruthers et al., 2006). *E. coli* has additional endonucleases such as RNases I, III, G, P and Z that can cleave mRNAs, although their spectrum of action is limited (Bardwell et al., 1989; Cannistraro and Kennell, 1991; Alifano et al., 1994; Kaga et al., 2002; Perwez and Kushner 2006). Two additional endonucleases have been identified, which target various species of mRNA under certain physiological conditions. RelE cleaves mRNA positioned at the ribosomal A site when bacterial cells are starved for amino acids (Pedersen et al., 2003). MazF cleaves mRNAs when cells undergo programmed cell death under various stressful conditions (Aizenman et al., 1996; Zhang et al., 2003; Hazan et al., 2004). Thus,

E. coli has developed many mRNA endonucleases to adapt to different conditions. We found yet another endonuclease, RNase LS, which targets numerous mRNAs encoded by phage T4. This RNase also plays a role in *E. coli* mRNA turnover, although its effect is modest in comparison to that of RNase E (Otsuka and Yonesaki, 2005). In addition, RNase LS is involved in the metabolism of small RNA, because a 307-nucleotide fragment possessing an internal sequence of 23S rRNA accumulates to a high level in cells deficient in RNase LS (Otsuka and Yonesaki, 2005). When a T4 *dmd* mutant infects *E. coli*, this RNase is activated after early and middle genes of T4 phage are expressed (Kai et al., 1998; Ueno and Yonesaki, 2001; Otsuka et al., 2003) and causes rapid degradation of most mRNAs at late stages, leading to a defect in T4 phage growth (Kai et al., 1996). Therefore, RNase LS is a potential antagonist of T4 replication and *dmd* is required for overcoming the activity of RNase LS.

RNase LS has rather broad sequence specificity, preferentially cleaving RNA 3' to pyrimidines (Kai et al., 1996; Kai and Yonesaki, 2002; Kanesaki et al., 2005). Some of cleavages by RNase LS can be linked tightly to polypeptide chain elongation and termination (Kanesaki et al., 2005; Yamanishi and Yonesaki, 2005). Thus, together with activation after T4 infection, RNase LS has unique and complex functional properties. Nevertheless, information about its structure and biochemical proper-

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ties is entirely lacking. Recently, we found that *rnIA* is essential for RNase LS activity both *in vivo* and *in vitro* (Otsuka and Yonesaki, 2005). In this study, we attempted an initial approach to understand the molecular basis of RNase LS activity, concentrating on the relation between RnIA and RNase LS. Our results indicate that RnIA plays a central role in the RNase LS activity and that the activity of RnIA is regulated by multiple components.

MATERIALS AND METHODS

Phages and bacterial strains Our wild-type strain of bacteriophage is T4D. T4 *amSF16* or dK contains an amber mutation or a deletion in *dmd*, respectively (Kai et al., 1996; Ueno and Yonesaki, 2001). The *E. coli* K-12 strains MH1 (*sup⁰ hsdR ΔlacX74 rpsL*), TY9114 (MH1 *rna::kan*), TY0482 (MH1 *rnIA2*) and TY0324 (MH1 *rnIA::kan*) were described previously (Otsuka and Yonesaki, 2005).

Plasmids In order to construct a plasmid expressing Dmd, a 210-bp DNA fragment containing T4 *dmd* was amplified by PCR with T4 DNA as a template and the primers 5'-ccgaattcggttaaagaggagattg and 5'-ccgaattcttatcctcggaatccactt. The amplified DNA fragment was digested with *EcoRI* and cloned downstream of a λ phage promoter, P_L, in the plasmid pNT45 (Fujisawa et al., 1985) to construct pGE615.

pETN expressing RnIA was constructed as follows. A DNA fragment containing *rnIA* was amplified by PCR with the DNA from *E. coli* strain MG1655 as a template and the primers 5'-ggaattccatatgacaatcaggagttac and 5'-aactgcagaactcaaacaatatataag. The DNA fragment was digested with *NdeI* and *PstI*, and ligated to pET21a(+) (Novagen) previously digested with the same enzymes to construct pETN.

pQErnIA was constructed for purification of His-tagged RnIA. A DNA fragment containing the *rnIA* was amplified by PCR with the DNA from *E. coli* strain MG1655 as a template and the primers 5'-cggtatccacaatcaggagttac and 5'-aactgcagaactcaaacaatatataag. The DNA fragment was digested with *BamHI* and *PstI*, and cloned into a vector pQE-80L (Qiagen) to construct pQErnIA.

Assay for RNA cleavage *in vitro* T4 *soc* RNA was synthesized *in vitro* by T7 RNA polymerase (Toyobo Inc.) with a DNA fragment containing the *soc* coding region downstream of the T7 promoter (Ueno and Yonesaki, 2002). Thusly synthesized *soc* RNA has the same 5'-end as does *soc* mRNA *in vivo*, while they have different lengths in the 3' untranslated region. RNA cleavage assays were performed in a 20- μ l reaction mixture containing 55 mM Tris-HCl (pH 8.0), 76 mM potassium acetate, 11 mM magnesium acetate, 1.5 mM dithiothreitol

(DTT), 1.6% (w/v) polyethylene glycol #6000, 0.1 pmol of *soc* RNA, 1 mM each of 4 NTPs and cell extract or His-tagged RnIA. The reactions were incubated for 60 min at 30°C and were terminated by adding 10 μ l of 0.3% sodium dodecyl sulfate (SDS) and 0.6 M sodium acetate (pH 5.2). RNAs were extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform and then precipitated with ethanol. Recovered RNAs were used for primer extension analysis. Primer extension analysis was performed by using *soc* primer 2 (5'-gttattaaccagttacttc) as described previously (Kai and Yonesaki, 2002).

Preparation of cell extract for RNA cleavage assay

Bacterial cell extracts were prepared according to a method described previously (Nirenberg, 1963; Stanley and Wahba, 1967). Briefly, cells were grown to 3×10^8 cells/ml in 300 ml of LB medium, harvested by centrifugation and washed twice with ice-cold TMCK buffer (10 mM Tris-HCl (pH 7.5), 10 mM magnesium acetate, 30 mM KCl, 0.5 mM DTT). The cells were stored at -80°C until used in the following procedures, which were performed at 4°C. The frozen cells were thawed and ground with 0.75 g of aluminum oxide. Subsequently, 150 μ l of TMCK buffer containing 1.5 units of RNase-free DNase (Nippon Gene Inc.) were added to the cell paste. The suspension was kept on ice for 10 min, and the aluminum oxide was removed by centrifugation at $15,000 \times g$ for 20 min to obtain a clear lysate. This lysate was centrifuged at $30,000 \times g$ for 30 min to prepare S30, and then S30 was centrifuged at $100,000 \times g$ for 2 hr to obtain a supernatant (S100) and a precipitate. The precipitate was suspended with 150 μ l of TMCK buffer (P100). For further fractionation, we added ammonium chloride to P100 to a final concentration of 1 M and kept the solution on ice overnight. Then, the sample was centrifuged at $100,000 \times g$ for 2 hr. The supernatant was dialyzed against TMCK buffer (HSS). The pellet was suspended in TMCK buffer to the same volume as the HSS (HSP). All the fractions prepared in this manner were stored at -80°C. Approximate protein concentrations in each fraction were: S30, 40 mg/ml; S100, 3 mg/ml; P100, 35 mg/ml; HSP, 28 mg/ml; HSS, 6 mg/ml.

Purification of His-tagged RnIA TY9114 cells harboring pQErnIA were grown at 30°C to a density of 3×10^8 cells/ml in 1 l of LB medium. After adding IPTG to a final concentration of 0.2 mM, the culture was incubated for 30 min. Cells were spun down, washed and suspended with 12 ml of lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole and 1 mM phenylmethylsulfonyl fluoride, pH 8.0). The cells were disrupted by an ultrasonic generator, UD-201 (Tomy Seiko, Co., Ltd.), at a duty of 30% for 5 min. The lysate was centrifuged at $10,000 \times g$ for 20 min and the His-tagged RnIA was

purified from the supernatant by a Ni-beads column (Ni-NTA Superflow, Qiagen) according to the manufacturer's instructions. The fraction containing His-tagged RnlA was eluted with 250 mM imidazole. This fraction was dialyzed against TMCK buffer containing 20% (v/v) glycerol and then loaded onto a DEAE cellulose (Whatman) column. The flow-through fractions containing His-tagged RnlA were combined and loaded onto a Heparin sepharose CL-6B (Amersham Biosciences) column. His-tagged RnlA was eluted at 0.25–0.30 M potassium acetate, dialyzed against TMCK buffer containing 50% (v/v) glycerol and stored at -20°C . SDS-containing polyacrylamide gel electrophoresis (SDS-PAGE) revealed that the His-tagged RnlA was more than 95% pure.

Purification of Dmd MH1 cells were co-transformed with pGE615 and pNT204 (Fujisawa et al., 1985), the latter of which encodes a temperature-sensitive λ CI repressor. Transformed cells were incubated at 30°C to a density of 2×10^8 cells/ml in 500 ml LB medium, and the culture was shifted to 42°C for 2 hr to induce Dmd protein. The cells were harvested and washed with buffer A (20 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 5 mM β -mercaptoethanol). The cells were suspended in 4.3 ml of buffer A containing 2.2 mg of lysozyme and incubated on ice for 15 min. Then the cell suspension was frozen at -80°C and then thawed at room temperature for 2 cycles to lyse the cells. After 5 $\mu\text{g}/\text{ml}$ pancreatic DNase I and 10 mM MgCl_2 had been added and incubated for 1 hr at 4°C , the lysate was centrifuged at $11,000 \times g$ for 10 min. An equal volume of $(\text{NH}_4)_2\text{SO}_4$ -saturated water was added to the supernatant and the resulting precipitate was collected by centrifugation at $11,000 \times g$ for 10 min. The precipitate was suspended in buffer A, dialyzed against the same buffer and loaded onto a DEAE-cellulose column. Dmd was eluted at 0.15–0.20 M NaCl and the fractions containing Dmd were combined. Pooled fractions were dialyzed against buffer A and loaded onto an Affi-Gel Blue column. The flow-through fractions containing Dmd were combined. Proteins in the pooled fractions were precipitated with 50%-saturated $(\text{NH}_4)_2\text{SO}_4$, collected by centrifugation and suspended in buffer A. Finally, the proteins were loaded on a Sephadex G-50 molecular-sieve column and were eluted with buffer A containing 0.1 M NaCl. Fractions containing Dmd were dialyzed overnight against buffer A containing 70%-saturated $(\text{NH}_4)_2\text{SO}_4$. The precipitate was collected by centrifugation for 10 min at $11,000 \times g$, suspended in buffer B (20 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 5 mM β -mercaptoethanol, 50% (v/v) glycerol, 0.1 M NaCl) and then dialyzed overnight against the same buffer. Analysis of the final fraction by SDS-PAGE revealed that the Dmd was more than 95% pure.

Antibodies and Western blotting analysis BL21(DE3)

(Novagen) harboring pETN was grown to a density of 3×10^8 cells/ml in 1 l of LB at 30°C and RnlA was induced by adding IPTG to 0.2 mM and incubating for 2.5 hr. Cells were harvested, washed and suspended in a buffer consisting of 50 mM potassium phosphate (pH 7.0) and 1 mM EDTA. After the cells were disrupted by sonication, the precipitate was collected by centrifugation at $11,000 \times g$ for 10 min. The precipitate was suspended by sonication in a buffer consisting of 10 mM potassium phosphate (pH 7.0), 1 mM EDTA and 4% Triton-X100, and incubated at 30°C for 30 min with rotation. After the precipitate was recovered by centrifugation at $11,000 \times g$ for 10 min, the extraction with Triton X-100 as above was repeated until the supernatant became clear. To remove Triton X-100, the precipitate was suspended in water and centrifuged at $20,000 \times g$ for 20 min twice. After the sample was subjected to SDS-PAGE, proteins were stained by Coomassie brilliant blue. The band containing RnlA was cut off the gel and RnlA was eluted electrophoretically. The purified RnlA was used to raise antibody against RnlA in a rabbit (Nikka Techno Service, Hitachi, Japan). For Western blotting, proteins were separated by SDS-PAGE and transferred onto a PVDF membrane. Membranes were blocked for 60 min at room temperature with 5% skim milk in TBS containing 0.05% Tween-20 and then incubated for 60 min with a rabbit polyclonal anti-RnlA antibody (1: 10,000) or a mouse monoclonal anti-His antibody (Amersham Bioscience, 1: 3000). Next, membranes were incubated for 60 min with HRP-coupled donkey anti-rabbit antibody (Amersham Bioscience, 1: 5000) or HRP-coupled sheep anti-mouse antibody (Amersham Bioscience, 1: 5000). Protein bands were detected with Chemi-Lumi One kit (Nacalai tesque, Kyoto, Japan) and LAS image analyzer (Fujifilm). The band intensities were quantified using the NIH image program.

RESULTS

RNA cleavage by His-tagged RnlA *E. coli rnlA* is essential for RNase LS activity in a cell extract as well as *in vivo* (Otsuka et al., 2003; Otsuka and Yonesaki, 2005). In order to investigate whether or not RnlA itself has endonucleolytic activity, we attempted to purify the protein. The *rnlA* gene encodes a protein of 357 amino acids with a predicted molecular mass of 40 kDa. When RnlA was over-expressed from a plasmid, the protein was recovered in insoluble fractions when a cell extract was subjected to low-speed centrifugation. In spite of our efforts, attempts to solubilize the protein under non-denaturing conditions were not successful. Subsequently, we constructed a plasmid expressing His-tagged RnlA. His-tagged RnlA was functional *in vivo*, because introduction of the recombinant gene into *rnlA* mutant cells was sufficient to suppress the growth of T4 *dmd* mutant (data not shown). When His-tagged RnlA was

over-expressed, it remained soluble after low-speed centrifugation and we were able to purify it as described in MATERIALS AND METHODS. After various amounts of the His-tagged RnIA were mixed with T4 *soc* RNA and incubated at 30°C, RNAs were recovered and examined for cleavage. Because the cleavage activity in a cell extract as well as *in vivo* had been analyzed by primer extension, we adopted this technique to characterize the activity associated with purified His-tagged RnIA, which allowed us to compare the activity with that characterized previously. Within the range of amounts of His-tagged RnIA used in the assay, *soc* RNA was found to be cleaved at nucleotide position 207 (Fig. 1) relative to the 5'-end of *soc* RNA, the cleavage at which is the most prominent among those cleaved by RNase LS *in vivo* (Kai and Yonesaki, 2002; Otsuka and Yonesaki, 2005). This result indicates that His-tagged RnIA exhibits an endonuclease activity. Absence of prominent accumulation of the product of cleavage at nucleotide position 207 probably came from downstream cleavages that were difficult to detect by primer extension. Other cleavages found with cell extracts (Figs. 2 and 3; Otsuka and Yonesaki, 2005) as well as *in vivo* (Kai and Yonesaki, 2002; Otsuka et al., 2003; Otsuka and Yonesaki, 2005) were not detected using the purified protein. Inspection through a time-course of a cleavage reaction using His-tagged RnIA also revealed no cleavage upstream of position 207 (data not shown).

In order to explore whether the cleavage activity by His-tagged RnIA relates to RNase LS activity, we investigated the effects of T4 Dmd protein on the cleavage reaction. Because the mRNA cleavage by RNase LS occurs when *dmd* is defective (Kai et al., 1996; Kai and Yonesaki, 2002), this gene should have an inhibitory effect on RNase LS activity. Dmd (7 kDa) consists of 60 amino acids and is expressed immediately after infection (Kai et al., 1998). Dmd was purified as described in MATERIALS AND METHODS and included in the RNA cleavage reaction by His-tagged RnIA (Fig. 1). Addition of 1 ng of Dmd did not show any significant effect on cleavage at position 207. Addition of 10 ng modestly reduced the cleavage activity and 100 ng suppressed cleavage at position 207 to the background level. Thus, Dmd clearly inhibited the cleavage activity of His-tagged RnIA. This result strongly suggests that RnIA is the endonuclease responsible for RNase LS activity.

The site of cleavage at position 207 is 3' to G. RNase LS cleaves *soc* RNA *in vivo* at multiple sites including this site (Kai and Yonesaki, 2002; Otsuka and Yonesaki, 2005). The other sites are 3' to pyrimidines as found for the preference of RNase LS (Kai et al., 1996; Kai and Yonesaki, 2002; Kanesaki et al., 2005). Thus, His-tagged RnIA selected only a site with an exceptional nucleotide. This observation raised the possibility that selection of the exceptional nucleotide for cleavage may

Fig. 1. Cleavage of *soc* RNA by His-tagged RnIA. After various amounts of His-tagged RnIA were incubated at 30°C for 1 hr with 12 ng of *soc* RNA synthesized *in vitro*, *soc* RNAs were recovered and analyzed for the sites of cleavage by primer extension (lanes 1–6) as described in MATERIALS AND METHODS. The band indicated by F corresponds to the full-length *soc* RNA. The band of cleavage at nucleotide 207 (relative to the 5'-end of *soc* RNA) is shown by an arrow. A set of sequence ladders for wild-type *soc* obtained by the dideoxy method are in lanes 7–10. In order to examine the effect of T4 Dmd on the cleavage activity of His-tagged RnIA, 100 ng of His-tagged RnIA was tested for *in vitro* cleavage of *soc* RNA in the presence of various amounts of Dmd (lanes 11–14).

simply reflect its accessibility by RnIA and the cleavage activity of RnIA might be modulated when it acts as RNase LS with multiple cleavage sites, or that RNase LS might consist of multiple components including RnIA. Therefore, we attempted to characterize the RNase LS

activity in a cell extract.

Cleavages of *soc* RNA by cell extracts After *soc* RNA

had been incubated with an extract from TY9114 cells, *soc* RNA was recovered and analyzed by primer extension (Fig. 2A). Using S30 of the cell extract (see MATERIALS

Fig. 2. Cleavages of *soc* RNA by cell extracts. (A) TY9114 cell extracts were fractionated as described in MATERIALS AND METHODS. Each fraction was incubated at 30°C for 1 hr with *soc* RNA (12 ng) and cleavage sites of *soc* RNA were detected by primer extension as in Fig. 1. The band indicated by F corresponds to the full-length *soc* RNA. The band of cleavage at position 59 corresponds to a product cleaved by RNase E. The bands of cleavages at positions 135, 153 and 207 correspond to cleavage products by RNase LS *in vivo*. Lane 1, 1 μ l of S30; lane 2, 1 μ l of S100; lane 3, 1 μ l of P100; lane 4, 1 μ l of HSP; lane 5, 1 μ l of HSS; lane 6, 1 μ l of HSP plus 1 μ l of HSS. (B) The cleavage activity derived from TY9114 cells was assayed in the presence of 2 μ l of P100 and Dmd. The amount of Dmd was as follows: lane 1, 0 ng; lane 2, 1 ng; lane 3, 10 ng; lane 4, 100 ng; lane 5, 1000 ng. (C) HSS, HSP or HSS+HSP were examined for *in vitro* cleavages of *soc* RNA. Lane 1, 1 μ l of P100; lanes 2–5, 1 μ l, 2 μ l, 4 μ l or 8 μ l of HSS, respectively; lanes 6–9, 1 μ l, 2 μ l, 4 μ l or 8 μ l of HSP, respectively; lanes 10–13, 2 μ l of HSP plus 0, 1 μ l, 2 μ l or 4 μ l of HSS, respectively.

AND METHODS), we detected several cleavages (lane 1). In previous work, we suggested that the cleavage at position 59 was attributable to RNase E (Otsuka et al., 2003). Indeed, with S30 derived from cells carrying a temperature-sensitive allele of RNase E, this cleavage was significantly reduced when the cleavage reaction was performed at 42°C in contrast to a reaction at 30°C. In contrast, activity was similar at both temperatures with an extract derived from cells carrying a wild-type allele of RNase E (data not shown). Cleavages at positions 135, 153 and 207 were identical to those detected *in vivo* for RNase LS (Kai and Yonesaki, 2002). These cleavages were not detected when S30 of the *rnla2* or *rnla::kan* cell extract was used instead (Otsuka and Yonesaki, 2005; data not shown). Next, the RNase LS activity in S30 was fractionated by ultracentrifugation and divided into supernatant (S100) and precipitate (P100). The cleavage activity for production of bands at positions 135, 153 and 207 was found in P100 but not in S100 (Fig. 2A, lanes 2 and 3). Because this result suggested that Rnla included in P100, we attempted to clarify the distribution of Rnla in each fraction from TY9114 cells by western blotting with anti-Rnla. However, endogenous Rnla remained below the detectable level. Instead, therefore, we followed the distribution of His-tagged Rnla expressed from a plasmid, pQErnla. As we expected, His-tagged Rnla was present in the S30 and P100, but not in S100 (data not shown).

The effects of Dmd on cleavages of *soc* RNA at positions 135, 153 and 207 with P100 are shown in Fig. 2B. These cleavages were inhibited with increasing amount of Dmd, as with His-tagged Rnla. In contrast to these cleavages, the cleavage at position 59 by RNase E was not affected by Dmd at any concentration.

Next, P100 was further treated with 1 M NH_4Cl and again ultracentrifuged to obtain the supernatant (HSS) and the precipitate (HSP). The cleavages at positions 135, 153 and 207 were not detected with HSS (Fig. 2A, lane 5) and the cleavage at position 207 was detected with HSP, whereas the specific activity was less than one-tenth compared to that of P100 (lane 4). The weak activity associated with HSP was reproducible. Interestingly, 4-fold stronger activity was recovered when both fractions were included together in the cleavage reaction (lane 6). The requirement of both HSP and HSS for efficient cleavage was further confirmed as shown in Fig. 2C. An increase in the amount of HSP alone increased cleavage at positions 135, 153 and 207 (lanes 6–9), whereas an increase of HSS did not lead to any significant activity (lanes 2–5). When a constant amount of HSP was added together with increasing amounts of HSS, the cleavage activity was increased 3-fold to 10-fold, depending on the cleavage site (lanes 10–13). These results suggest that RNase LS consists of multiple components and some components required for efficient cleavage were dissociated

from others during fractionation.

Sedimentation of cleavage activity through a sucrose density gradient In order to estimate the size of RNase LS, P100 from wild-type cells was separated on a 5–20% sucrose density gradient and sedimentation of RNase LS activity was investigated. First, we tested each fraction for its ability to cleave *soc* RNA at positions 135, 153 and 207. However, no activity was detected in any fraction (data not shown). This result could be expected, because, first, components included in P100 were diluted during sedimentation through a sucrose gradient and second, some components essential for RNase LS activity might have become separated. In order to

Fig. 3. Sedimentation of the cleavage activity through a sucrose gradient. (A) P100 (0.2 ml containing 3.0 mg protein) prepared from TY9114 cells was layered onto a 5-ml 5–20% (v/v) sucrose linear density gradient prepared in TMCK buffer and was centrifuged for 3 hr at $50,000 \times g$ in a RPS 55T-2 rotor (Hitachi Koki). Twenty-nine fractions of equal volume were collected from the top. Each fraction was measured for absorbance at 260 nm and we confirmed that the peak of absorbance corresponds to a peak of ribosomes by electrophoresis of RNA from each fraction. Symbols: A_{260} , open circle; cleavage activity in the presence of HSS, closed circle; cleavage activity in the presence of HSP, closed triangle. (B) The cleavage of *soc* RNA at nucleotide 135 was examined as in Fig. 2 for each fraction in the presence of either 18 μg of HSS (upper panel) or 28 μg of HSP (lower panel) from TY9114 cells. The band intensities for cleavage at position 135 were quantified by the NIH image program and expressed in arbitrary units.

examine the second possibility, we performed reconstitution experiments. When each fraction was assayed in the presence of either HSS or HSP, cleavage at position 135 could be detected. Inability to detect other cleavages relating to RNase LS activity was probably caused by dilution. In the assays including HSS, the cleavage activity appeared just behind a peak of ribosome (Fig. 3). From the sedimentation velocity, we estimated that the cleavage activity in the presence of HSS roughly corresponded to a mass of 1000 kDa, based on the mass (2500 kDa) of the 70S ribosome. With including HSP, the activity appeared at the top of sucrose density gradient (Fig. 3). These results suggest that components required for RNase LS can be separable under the present conditions, such as high-salt wash, and that this RNase is present as a large complex. In this connection, it is notable that HSS had no stimulatory effect on the cleavage activity of His-RnlA alone (data not shown).

Proteins associated with RnlA In experiments similar to Fig. 2C, we examined fractions from the *rnlA2* or *rnlA::kan* cell extract in combinations with those from wild-type cell extract and found that HSP and HSS from mutant cell extract were defective and normal in relation to RNase LS activity, respectively (data not shown). This result strongly suggested that RnlA was present in the HSP or 1000-kDa complex. To clarify this possibility, P100 prepared from cells expressing His-RnlA was centrifuged through a 5–20% linear sucrose gradient and the fractions were analyzed by western blots with antibody against the His tag. His-tagged RnlA was broadly distributed from the middle of the gradient to the bottom with a peak in the vicinity of a peak of ribosomes (Fig. 4A and B). The cleavage activity in each fraction was examined in the presence of HSS (Fig. 4A and C). The peak of cleavage activity appeared just behind a peak of ribosomes and the distribution of the activity was nearly consistent with the distributions of His-tagged RnlA. As a control, purified His-tagged RnlA remained in the top fraction of the gradient when centrifuged alone (data not shown).

In order to identify proteins associated with RnlA, the sucrose-gradient fractions containing His-RnlA were pooled and mixed with Ni-agarose to absorb His-RnlA. The agarose was spun down and extensively rinsed with wash buffer and the bound proteins were eluted by high concentration of imidazole. After the bound fractions were separated by SDS-PAGE, proteins were stained with Coomassie blue (Fig. 5, lane 1). In addition to His-RnlA, more than 10 proteins were detected, the proportions of which were comparable to that of His-RnlA. To identify these proteins, we determined their N-terminal sequences. The results for identified proteins and their structural genes were listed in the figure. In contrast, when corresponding sucrose-gradient fractions obtained from

Fig. 4. Sedimentation of His-tagged RnlA through a sucrose density gradient. (A) P100 prepared from TY9114 cells transformed with pQErnlA was centrifuged through a sucrose density gradient as in Fig. 3. Each fraction was measured for absorbance at 260 nm, for *in vitro* cleavage of *soc* RNA and for the distribution of His-tagged RnlA. A_{260} , open circle; cleavage at nucleotide 135, closed triangles; quantity of His-tagged RnlA, closed circles. (B) His-tagged RnlA in gradient fractions was detected by western blotting with anti-His tag. (C) The cleavage activity was measured as in Fig. 3, by using gradient fractions in the presence of 15 μ g of HSS from TY9114 cells.

cells not expressing His-RnlA were subjected to pull-down by Ni-agarose, no protein was detected after elution from the agarose (data not shown).

As seen in the figure, the ratio of proteins pulled down together with His-RnlA are not strictly stoichiometric, suggesting loose association of some proteins. In order to remove loosely associating proteins, we washed Ni-agarose with 1 M NaCl and then eluted with imidazole. Only two proteins, His-RnlA and triose phosphate isomerase (TpiA; Mr = 27 kDa), were eluted from the agarose

Fig. 5. Pull-down and elution of His-tagged RnlA. The fractions from No. 10 to 16 in Fig. 4 were pooled (1.2 ml) and mixed with 20 μ l of Ni-NTA agarose beads by end-over-end rotation for 1 hr at 4°C. After the agarose beads were span down, they were washed five times with TMCK containing 20 mM imidazole and bound proteins were eluted with TMCK buffer containing 250 mM imidazole. The bound fractions were separated on a 15%-polyacrylamide gel containing SDS and transferred to a PDVF membrane. Proteins were visualized by staining with Coomassie Blue. His-tagged RnlA, indicated by an arrowhead, was confirmed by western blot with anti-His tag. A piece of PDVF membrane containing each protein band numbered from 1 to 12 was cut out for analysis of N-terminal sequences.

(Fig. 5, lane 2). From three independent experiments, the relative amount of TpiA to His-RnlA was calculated to be 0.6–0.7, corresponding to a molar ratio of 0.9–1.1. So far, we have been able to separate these two proteins with SDS plus heat, but not by SDS alone or with other non-denaturing agents (data not shown).

DISCUSSION

An *E. coli* endonuclease, RNase LS, cleaves mRNAs of bacteriophage T4 and *E. coli*. Previous work revealed that *rnlA* is essential for RNase LS activity *in vitro* as well as *in vivo* (Otsuka et al., 2003; Otsuka and Yonesaki, 2005). In this study, His-tagged RnlA cleaved *soc* RNA at the same position as one of the cleavage sites of RNase LS *in vivo* and both this activity and RNase LS activity in a cell extract were inhibited by T4 Dmd, which did not inhibit the cleavage by RNase E. T4 *dmd* is required for blocking RNase LS *in vivo*. Considering that Dmd is expressed immediately after T4 infection and suppresses RNase LS activity (Kai et al., 1998), our present results strongly suggest that RnlA plays a central role in the RNase LS activity.

Two lines of evidence suggest that RnlA is a component of the 1000-kDa complex. First, His-tagged RnlA showed a sedimentation rate similar to that of the 1000-kDa complex through a sucrose density gradient of P100.

Second, pull-down experiments revealed more than 10 proteins associated with His-tagged RnlA.

As exemplified by analysis on the mRNAs of T4 phage late genes *soc* and *23* and the middle gene *uvsY*, RNase LS preferentially cleaves 3' to pyrimidines (Kai et al., 1996; Kai and Yonesaki, 2002; Kanesaki et al., 2005), showing a rather broad sequence specificity. In addition, *soc* RNA and *uvsY* RNA are remarkably stabilized when translation initiation is impaired by disruption of the Shine-Dalgarno sequence or the initiation codon, suggesting a dependency of cleavage on translation. Indeed, some of cleavages by RNase LS can be linked tightly to polypeptide chain elongation and termination (Kai and Yonesaki, 2002; Kanesaki et al., 2005; Yamanishi and Yonesaki, 2005). However, the other cleavages are independent of translation, which is consistent with the observation that the residual activity of *soc* RNA degradation is still significant even in the absence of translation. Therefore, the RNA cleavages are likely to be promoted by two different mechanisms, one dependent on translation and the other independent of translation. Because *rnlA* is essential for RNase LS activity and a mutation of this gene can eliminate both mechanisms (Otsuka and Yonesaki, 2005), RNase LS activity could account for these mechanisms. Thus, RNase LS would exhibit its activity in a complicated manner. Cleavage by His-tagged RnlA is not completely identical to those found

with the RNase LS *in vivo*. The only site at nucleotide position 207 cleaved by His-tagged RnlA is 3' to G; as noted above, the preference of RNase LS is 3' to pyrimidines. The selection of the only site with an exceptional nucleotide suggests that the cleavage activity of RnlA could be modulated in a form of complex. We identified 12 proteins pulled down by Ni beads from the fraction containing His-RnlA. Among these, triose phosphate isomerase (TpiA) is especially notable in two aspects. First, it stoichiometrically binds to RnlA with a high affinity. Second, TpiA is known to exist in a large complex containing proteins involved in carbohydrate metabolism (Mowbray and Moses, 1976). These facts may imply that some of the other 11 proteins associate with RnlA via TpiA. In this context, we preliminarily observed that a mutant lacking *tpiA* partially permitted the growth of T4 *dmd* mutant, suggesting that TpiA is required for sufficient activity of RNase LS. Our present study also suggests that another component required for sufficient activity is separated from RnlA by high-salt wash or centrifugation through a sucrose density gradient of P100. These observations may support the idea that the cleavage activity of RnlA is modulated by interaction with other proteins.

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REFERENCES

- Aizenman, E., Engelberg-Kulka, H., and Glaser, G. (1996) An *Escherichia coli* chromosomal "addiction module" regulated by guanosine 3',5'-bispyrophosphate: a model for programmed bacterial cell death. *Proc. Natl. Acad. Sci. USA* **93**, 6059–6063.
- Alifano, P., Rivellini, F., Piscitelli, C., Arraiano, C. M., Bruni, C. B., and Carlomagno, M. S. (1994) Ribonuclease E provides substrates for ribonuclease P-dependent processing of a polycistronic mRNA. *Genes Dev.* **8**, 3021–3031.
- Bardwell, J. C. A., Regnier, P., Chen, S.-M., Nakamura, Y., Grunberg-Manago, M., and Court, D. L. (1989) Autoregulation of RNase III operon by mRNA processing. *EMBO J.* **8**, 3401–3407.
- Bernstein, J. A., Khodursky, A. B., Lin, P.-H., Lin-Chao, S., and Cohen, S. N. (2002) Global analysis of mRNA decay and abundance in *Escherichia coli* at single-gene resolution using two-color fluorescent DNA microarrays. *Proc. Natl. Acad. Sci. USA* **99**, 9697–9702.
- Cannistraro, V. J., and Kennell, D. (1991) RNase I*, a form of RNase I, and mRNA degradation in *Escherichia coli*. *J. Bacteriol.* **173**, 4653–4659.
- Caruthers, J. M., Feng, Y., McKay, D. B., and Cohen, S. N. (2006) Retention of core catalytic functions by a conserved minimal ribonuclease E peptide that lack the domain required for tetramer formation. *J. Biol. Chem.* **281**, 27046–27051.
- Coburn, G. A., and Mackie, G. A. (1999) Degradation of mRNA in *Escherichia coli*: an old problem with some new twists. *Mol. Biol.* **62**, 55–108.
- Fujisawa, H., Yonesaki, T., and Minagawa, T. (1985) Sequence of the T4 recombination gene, *UvsX*, and its comparison with that of the *recA* gene of *Escherichia coli*. *Nucleic Acids Res.* **13**, 7473–7481.
- Hazan, R., Sat, B., and Engelberg-Kulka, H. (2004) *Escherichia coli mazEF*-mediated cell death is triggered by various stressful conditions. *J. Bacteriol.* **168**, 3663–3669.
- Kaga, N., Umitsuki, G., Nagai, K., and Wachi, M. (2002) RNase G-dependent degradation of the *eno* mRNA encoding a glycolysis enzyme enolase in *Escherichia coli*. *Biosci. Biotechnol. Biochem.* **66**, 2216–2220.
- Kai, T., Selick, E. H., and Yonesaki, T. (1996) Destabilization of bacteriophage T4 mRNAs by a mutation of gene 61.5. *Genetics* **144**, 7–14.
- Kai, T., Ueno, H., and Yonesaki, T. (1998) Involvement of other bacteriophage T4 genes in the blockade of protein synthesis and mRNA destabilization by a mutation of gene 61.5. *Virology* **248**, 148–155.
- Kai, T., and Yonesaki, T. (2002) Multiple mechanisms for degradation of bacteriophage T4 *soc* mRNA. *Genetics* **160**, 5–12.
- Kanesaki, T., Hamada, T., and Yonesaki, T. (2005) Opposite roles of the *dmd* gene in the control of RNase E and RNase LS activities. *Genes Genet. Syst.* **80**, 241–249.
- Kushner, S. R. (2002) mRNA decay in *Escherichia coli* comes of age. *J. Bacteriol.* **184**, 4658–4665.
- Morita, T., Maki, K., and Aiba, H. (2005) RNase E-based ribonucleoprotein complexes: mechanical basis of mRNA destabilization mediated by bacterial noncoding RNAs. *Genes Dev.* **19**, 2176–2186.
- Mowbray, J., and Moses, V. (1976) The tentative identification in *Escherichia coli* of a multienzyme complex with glycolytic activity. *Eur. J. Biochem.* **66**, 25–36.
- Nirenberg, M. W. (1963) Cell-free synthesis directed by messenger RNA. *Methods in Enzymology* **6**, 17–23.
- Otsuka, Y., Ueno, H., and Yonesaki, T. (2003) *Escherichia coli* endoribonucleases involved in the cleavage of bacteriophage T4 mRNAs. *J. Bacteriol.* **185**, 983–990.
- Otsuka, Y., and Yonesaki, T. (2005) A novel endoribonuclease, RNase LS, in *Escherichia coli*. *Genetics* **169**, 13–20.
- Pedersen, K., Zavialov, A. V., Pavlov, M. Y., Elf, J., Gerdes, K., and Ehrenberg, M. (2003) The bacterial toxin RelE displays codon-specific cleavage of mRNAs in the ribosomal A site. *Cell* **112**, 131–140.
- Perwez, T., and Kushner, S. R. (2006) RNase Z in *Escherichia coli* plays a significant role in mRNA decay. *Mol. Microbiol.* **60**, 723–737.
- Stanley, W. M. Jr., and Wahba, A. J. (1967) Chromatographic purification of ribosomes. *Methods in Enzymology* **12**, 524–526.
- Ueno, H., and Yonesaki, T. (2001) Recognition and specific degradation of bacteriophage T4 mRNAs. *Genetics* **158**, 7–17.
- Ueno, H., and Yonesaki, T. (2002) Role of *Escherichia coli* Hfq in late-gene silencing of bacteriophage T4 *dmd* mutant. *Genes Genet. Syst.* **77**, 301–308.
- Yamanishi, H., and Yonesaki, T. (2005) RNA cleavage linked with ribosomal action. *Genetics* **171**, 419–425.
- Zhang, YI, Zhang, K. P., Hoeflich, K. P., Ikura, M., Qing, G., and Inouye, M. (2003) MazF cleaves cellular mRNAs specifically at ACA to block protein synthesis in *Escherichia coli*. *Mol. Cell* **12**, 913–923.