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NOTE

RNase G Participates in Processing of the 5′-end of 23S Ribosomal RNA

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In *Escherichia coli*, primary rRNA transcripts must be processed by a complex process in which several ribonucleases are involved in order to generate mature 16S, 23S, and 5S rRNA molecules. While it is known that RNase G, a single-stranded RNA-specific endoribonuclease encoded by the *rng* gene, plays an active role in the maturation of the 5′-end of 16S rRNA, its involvement in the maturation of the 5′-end of 23S rRNA remains unclear. Here we show that *E. coli* cells deleted for the *rng* gene accumulate the 23S rRNA precursor containing an extra 77 nucleotides at its mature 5′-end. *In vitro* cleavage assays show that RNase G cleaves synthetic RNA containing a sequence encompassing the 5′-end to 77 nucleotides upstream of mature 23S rRNA at two sites present in single-stranded regions. Our results suggest the involvement of RNase G in the processing of the 5′-region of 23S rRNA precursors.

***Keywords*:** rRNA processing, Rng, RNase G, 23S rRNA

In *Escherichia coli*, primary rRNA transcript, which is termed 30S precursor, is initially cleaved by RNase III, a double-stranded RNA specific endoribonuclease, to generate precursors to the mature 16S, 23S, and 5S rRNA (Robertson *et al.*, 1968; Young and Steitz, 1978; Bram *et al.*, 1980). However, rRNA can be also processed by alternative pathways that do not require RNase III (Gegenheimer *et al.*, 1977). *E. coli* cells with the *rnc* gene encoding RNase III deleted accumulated precursors of 16S, 23S, and 5S rRNA, which eventually were further processed to become rRNA molecules with both termini that are similar to mature rRNAs and assembled into ribosomes (King *et al.*, 1984; Srivastava and Schlessinger, 1988).

RNase G, a single-stranded RNA specific endoribonuclease encoded by the *rng* gene, is actively involved in the maturation process of the 5′-end of 16S rRNA (Li *et al.*, 1999a; Wachi *et al.*, 1999). A recent study showed that ribosomes containing unprocessed 16S rRNA in *E. coli* cells with the *rng* gene deleted exhibited defects in proofreading function in protein synthesis, indicating the importance of 16S rRNA maturation induced by RNase G (Roy-Chaudhuri *et al.*, 2010).

E. coli cells that have a deleted *rnc* gene accumulate large amounts of several processing intermediates containing -4 to 96 nucleotides at the 5′-end of 23S rRNA (King *et al.*, 1984). Based on this observation, it has been suggested that the processing of the 5′-end of 23S rRNA involves unknown endoribonucleases since a 5′ to 3′ exoribonuclease has not been discovered in *E. coli* (Zuo and Deutscher, 2001). However, it remains unclear whether RNase G plays a role in the maturation of the 5′-end of 23S rRNA. Based on these previous

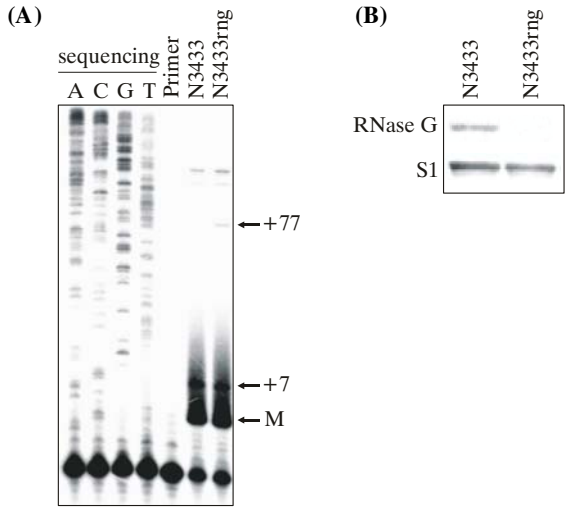

Fig. 1. Effects of deletion of the *rng* gene on the 5′-end processing of 23S rRNA. (A) Primer extension analysis of the 5′-end of 23S rRNA. Total RNA was prepared from N3433 and N3433*rng* strains and hybridized with 5′-end-labeled primer (23S rRNA-5′). Synthesized cDNA products were analyzed on a 10% polyacrylamide gel containing 8 M urea. Sequencing ladders were produced using the same primer used in cDNA synthesis and PCR DNA encompassing the 23S rRNA gene as template. The cDNA band corresponding to the 5′-end of mature 23S rRNA is indicated by M. A processing product of RNase III, which contains seven extra nucleotides, and a 23S rRNA precursor accumulated in N3433*rng* are indicated by +7 and +77, respectively. (B) Western blot analysis of RNase G. Total proteins from N3433 and N3433*rng* were separated by SDS-PAGE and analyzed by immunoblotting with polyclonal antibodies to RNase G and ribosomal protein S1.

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findings, we investigated whether RNase G participates in the processing of the 5′-end of 23S rRNA.

In order to determine the effects of *rng* deletion on the 5′-end maturation of 23S rRNA in *E. coli*, we performed primer extension analysis on 23S rRNA from wild-type *E. coli* strain N3433 (*Hfr*, *lacZ43*, *λ*-, *relA1*, *spoT1*, *thi-1*) (Goldblum and Apririon, 1981) and an isogenic strain with the *rng* gene deleted (N3433*rng*) (Lee *et al.*, 2002). Cultures were grown to an optical

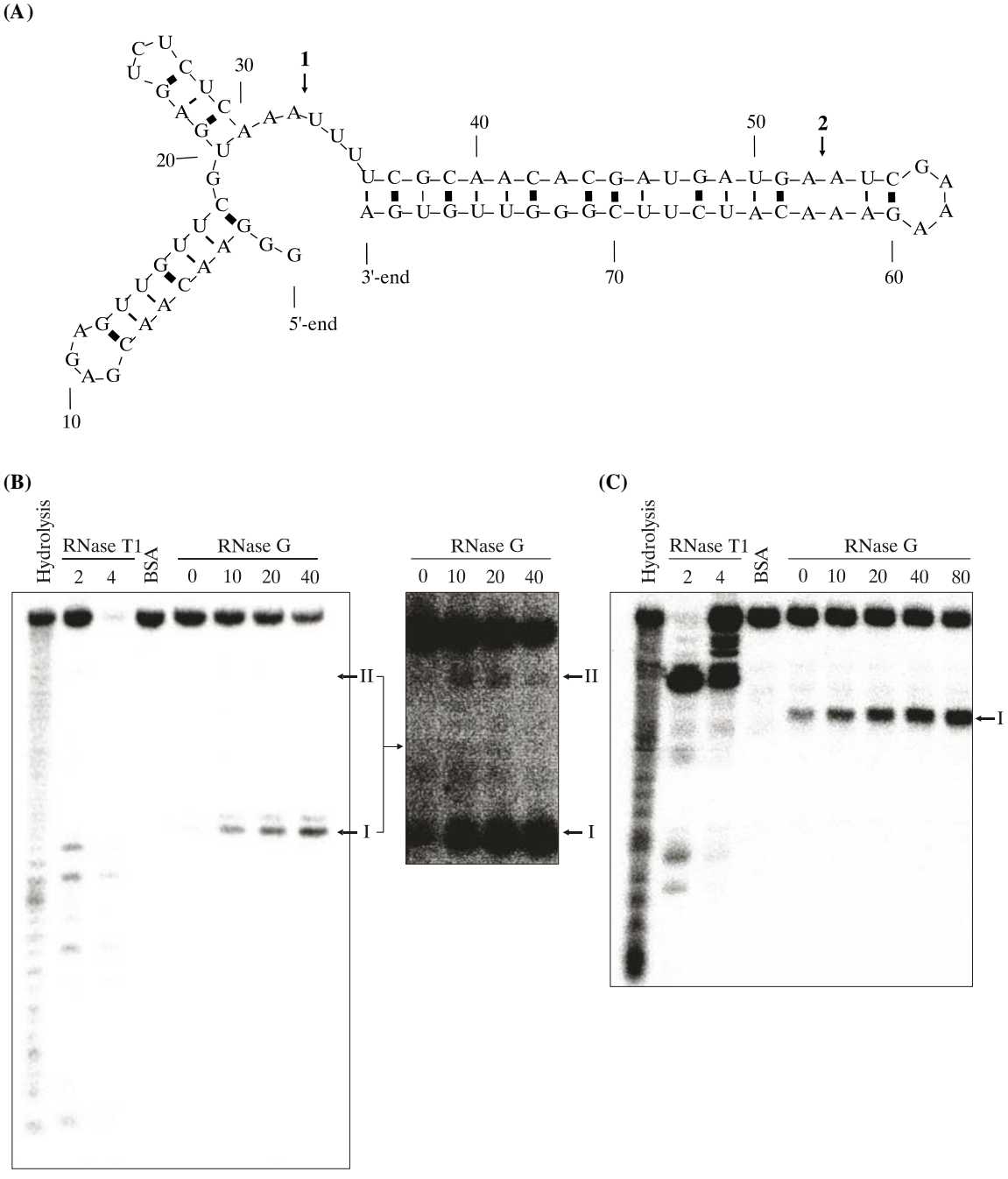

Fig. 2. Identification of RNase G cleavage sites in the 5′ region of 23S rRNA. (A) Predicted secondary structure of 23S-79-RNA. The secondary structure was deduced using the M-fold program and RNase T1 digestion of 23S-79-RNA shown in (B) and (C). RNase G cleavage sites identified in (B) and (C) are denoted by 1 and 2. B. *In vitro* cleavage of 5′ [^{32}P]-end-labeled synthetic 23S-79-RNA. (C) *In vitro* cleavage of 3′ [^{32}P]-end-labeled synthetic 23S-79-RNA. End-labeled 23S-79-RNA (0.5 pmole) was incubated with 0.5 μg of purified RNase G in the presence of 0.25 μg/ml of yeast tRNA and 20 U of RNaseOUT (Invitrogen, USA) in cleavage buffer (30 mM Tris-HCl; pH 7.9, 160 mM NaCl, 10 mM $MgCl_2$, 0.1 mM DTT, 0.1 mM EDTA; pH 8.0). Cleavage reactions were incubated at 37°C. Samples were withdrawn at indicated time intervals (min) and separated on a 10% polyacrylamide gel containing 8 M urea. Size markers were generated by alkaline hydrolysis and RNase T1 digestion of labeled RNA.

density at 600 nm (OD_{600})=0.6, and total RNA samples were prepared using an RNeasy Miniprep kit (QIAGEN, Germany). The precursors of 23S rRNA were determined via a primer extension method. 23S rRNA were hybridized with the [γ-^{32}P] 5′-end labeled primer, 23S rRNA-5′ (5′-ATCCACCGTGTAC GCTTAGT), containing a sequence complimentary to the 5′-terminus of 23S rRNA, and the annealed primer was extended through the 5′-end of the 23S rRNA precursors using AMV reverse transcriptase. The extension reaction contained a mixture of four kinds of deoxynucleotides. The extended cDNA fragments were separated on a 10% polyacrylamide gel. Sequencing ladders were produced using the same primer used in cDNA synthesis and pRNA122 (Lee *et al.*, 2001) was used as template. The results showed that a 23S rRNA precursor that contains an extra 77 nucleotides at its 5′-end accumulated in N3433*rng* (Fig. 1A). Western blot analysis of total protein from N3433*rng* confirmed that this strain does not express RNase G protein (Fig. 1B). Western blot analysis was carried out as described previously (Sim *et al.*, 2010). These results suggest that RNase G participates in the 5′-end processing of 23S rRNA and may play a role in an alternative processing pathway for the 5′-end maturation of 23S rRNA that does not require RNase III. This RNase III-independent pathway may produce 23S rRNA processing intermediates containing either three or seven extra nucleotides at its 5′-end (Bram *et al.*, 1980; Sirdeshmukh and Schlessinger, 1985).

The results from the primer extension experiment showed that RNase G is involved in the processing of the 5′-end of 23S rRNA. However, the processing intermediate of 23S rRNA, which contained an extra 77 nucleotides at its 5′-end and accumulated in N3433*rng,* was not detected in wild-type *E. coli* (N3433), probably because it is a fast processing intermediate. For this reason, an *in vitro* cleavage assay was carried out to identify RNase G cleavage sites in the upstream portion of the 5′-end of 23S rRNA (Fig. 2A). Affinity-purified RNase G (Lee *et al.*, 2002) was incubated with synthetic RNA that contains a sequence encompassing the 5′-end to 79 nucleotides upstream of the mature 23S rRNA sequence (23S-79-RNA). 23S-79-RNA used in the cleavage assay was synthesized using the MEGAscript™ T7 kit (Ambion, USA) and PCR DNA as a template, according to the manufacturer's instructions. *In vitro*-synthesized 23S-79-RNA was treated with calf intestinal phosphatase (CIP) and 5′-end labeled using T4 polynucleotide kinase and [γ-^{32}P] ATP. [^{32}P]-labeled 23S-79-RNA was purified from a 6% acrylamide gel containing 8 M urea. PCR-generated DNA was prepared using pRNA122 (Lee *et al.*, 2001) as a template and the following primers: 23S rRNA-79 (5′-TAATACGACTCACTATAGGGAACAAC GAGAGTTGTTCGTGAGT) and 23S rRNA-0R (5′-TCACA ACCCGAAGATGTTT). RNase G cleaved 5′-[^{32}P]-end-labeled 23S-79-RNA and generated one major (32 ntd-long, band I in Fig. 2B) and one minor cleavage product (52 ntd long, band II in Fig. 2B) corresponding to sites at 47 (site 1) and 27 (site 2) nucleotides, respectively, upstream of the 5′-end of the mature 23S rRNA, which were deduced from the ladder generated by alkaline hydrolysis of 5′-[^{32}P]-end-labeled 23S-79-RNA (lane 1 in Fig. 2B). Analysis of predicted secondary structure and RNase T1 digestion of 23S-79-RNA indicated that these RNase G cleavage sites were located in single-stranded regions of the RNA (Figs. 2A and B). The identified RNase G cleavage sites were further characterized using 3′-[^{32}P]-end-labeled 23S-79-RNA (Fig. 2C). This RNA was synthesized using T4 RNA ligase and [γ-^{32}P] pCp and used to examine whether or not the minor cleavage product (band II in Fig. 2B) generated by RNase III cleavage at site 2 was fully detected because of further cleavage by RNase G at site 1, which removes an RNA fragment containing 5′-[^{32}P]-end-labeled sequence. However, the cleavage of 3′-[^{32}P]-end-labeled 23S-79-RNA by RNase G did not yield detectable amounts of the cleavage product generated by RNase G cleavage at site 2, indicating that the cleavage site 1 is a major RNase G cleavage site. Cleavage activity of RNase G on 3′- [^{32}P]-end-labeled 23S-79-RNA was ~90% lower compared to that on 5′-[^{32}P]-end-labeled 23S-79-RNA, which reflects a well-known enzymatic property of RNase G; it cleaves RNA substrates with a 5′-monophosphate group much more efficiently than it does those with a 5′-hydroxyl group (Tock *et al.*, 2000).

In summary, our experimental results show that RNase G participates in the processing of the 5′-end of 23S rRNA, a finding that has not been previously detected by northern blot analysis of 23S rRNA precursors (Li *et al.*, 1999b). Considering that ribosomes containing unprocessed 16S rRNA in *rng*-deleted *E. coli* showed defects in proofreading function during protein synthesis and increased sensitivity to aminoglycoside antibiotics, neomtcin, and paromomycin (Roy-Chaudhuri *et al.,* 2010), it is possible that RNase G-mediated processing of the 5′ region of 23S rRNA becomes important under certain physiological conditions, which remains to be further investigated.

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