# Dual Function of RNase E for Control of M1 RNA Biosynthesis in *Escherichia coli*†

Jae-hyeong Ko,‡ Kook Han,‡ Yool Kim,‡ Soyeong Sim,‡ Kwang-sun Kim,‡ Sang-Joon Lee,‡ Bongrae Cho,§ Kangryul Lee,|| and Younghoon Lee*,‡

*Department of Chemistry and Center for Molecular Design and Synthesis, Korea Advanced Institute of Science and Technology, Daejeon 305-701, Korea, Department of Applied Chemistry, Division of Applied Science, Cheongju University, Cheongju 360-764, Korea, and Department of Chemistry, Catholic University of Korea, Bucheon 420-743, Korea*

*Received August 1, 2007; Revised Manuscript Received October 5, 2007*

ABSTRACT: M1 RNA, the gene product of *rnpB*, is the catalytic subunit of RNase P in *Escherichia coli*. M1 RNA is transcribed from a proximal promoter as pM1 RNA, a precursor M1 RNA, and then is processed at its 3′ end by RNase E. In addition to pM1 RNA, large *rnpB*-containing transcripts are produced from unknown upstream promoters. However, it is not known yet how these large transcripts contribute to M1 RNA biosynthesis. To examine their biological relevance to M1 RNA biosynthesis, we constructed a model upstream transcript, upRNA, and analyzed its cellular metabolism. We found that upRNA was primarily degraded rather than processed to M1 RNA in the cell and that this degradation occurred in RNase E-dependent manner. The in vitro cleavage assay with the N-terminal catalytic fraction of RNase E showed that the M1 RNA structural sequence in upRNA was much more vulnerable to the enzyme than the sequence in pM1 RNA. Considering that RNase E is a processing enzyme involved in 3′ end formation of M1 RNA, our results imply that this enzyme plays a dual role in processing and degradation to achieve tight control of M1 RNA biosynthesis.

M1 RNA is the catalytic RNA subunit of the ribonucleoprotein enzyme RNase P, which is responsible for the removal of 5′ precursor sequences during the biosynthesis of tRNA in *Escherichia coli* (*1*). It is transcribed as a precursor of M1 RNA (pM1 RNA) from the P-1 promoter of the *rnpB* gene, and processing of pM1 RNA is initiated by cleavage at the 3′ end by RNase E (*2*−*6*). A study by Lundberg and Altman on large *rnpB*-containing upstream transcripts yielded interesting findings (*4*). Under conditions where RNase E was inactive, three transcripts were observed, and the sizes of these transcripts were significantly larger than those of the expected RNAs from known promoters (*7*, *8*). To investigate the metabolic pathway of the transcripts, they constructed a model substrate that was a precursor of M1 RNA with extra 270 nucleotides at the 5′ end, and showed that several cleavages by RNase P and unknown enzymes occurred within the upstream region in vitro. From those findings, it was proposed that large transcripts might become functional M1 RNAs (*4*). The fact that these transcripts accumulated substantially in the *rne* temperature-sensitive (*rne*$^{ts}$) mutant strain at nonpermissive temperatures implies that RNase E, the product of the *rne* gene, might be involved in the metabolic pathway of *rnpB*-containing upstream transcripts (*4*). However, it has not been clarified yet how RNase E is involved in metabolism of these large transcripts and how they would contribute to M1 RNA biosynthesis.

RNase E is an essential endoribonuclease required for the maturation of a number of stable RNAs, such as 5S rRNA (*9*, *10*), M1 RNA (*4*, *5*, *11*), 10Sa RNA (*12*), 16S rRNA (*13*), and tRNA (*14*, *15*). On the other side, this ribonuclease acts as an important regulatory enzyme that governs the principal pathway for the degradation of RNA I (*16*, *17*) and several mRNAs, such as those for S15 (*18*, *19*), S20 (*20*), and bacteriophage T4 (*21*). RNase E is a core enzyme of the degradosome complex that is thought to have a general role in RNA degradation (*22*, *23*).

This study set out to answer how *rnpB*-containing upstream transcripts are metabolized and how RNase E is involved in this process. We constructed upRNA, a model transcript containing extra upstream sequences at the 5′ end of M1 RNA, and analyzed its metabolic pathway in vivo and in vitro. We investigated the role of RNase E in this process with in vivo and in vitro analyses. From the data from upRNA, we conclude that *rnpB*-containing upstream transcripts are primarily degraded and not processed to M1 RNA. Since RNase E is involved in 3′ processing of M1 RNA, these results imply that this enzyme plays a dual role as the processing and degradation enzyme to achieve tight control of M1 RNA biosynthesis.

## EXPERIMENTAL PROCEDURES

*Bacterial Strains and Plasmids.* The *E. coli* strains used were JM109 (*24*), MCE$^{+}$, MCE$^{-}$, KS2001, and KS2003.

† This work was supported by Grant MG05-0202-3-0 from the 21C Frontier Microbial Genomics and Application Center Program, Grant R01-2005-000-10293-0 from MOST (KOSEF), the Center for Molecular Design and Synthesis at KAIST, and the Brain Korea 21 project.

* To whom correspondence should be addressed. Phone: +82-42-869-2832. Fax: +82-42-869-2810. E-mail: Younghoon.Lee@kaist.ac.kr.

‡ Korea Advanced Institute of Science and Technology.

§ Cheongju University.

|| Catholic University of Korea.

10.1021/bi701528j CCC: $40.75 
Published on Web 12/20/2007

MCE$^+$ (*rne*$^+$ K12r$^-$m$^+$) and MCE$^-$ (*rne-3071* K12r$^-$m$^+$), kindly provided by A. J. Carpousis, were RNase E$^+$ and RNase E$^{ts}$ isogenic strains derived from MC1061 (*25*). KS2003 (MG1655 Δ*zha-295::kan* CCCCA) carried the CCCCA mutation at the *rne*-dependent site of *rnpB*, whereas KS2001 (MG1655 Δ*zha-295::kan*) was the isogenic strain carrying the wild-type sequence at the *rne*-dependent site (*26*). Plasmid vectors, pSS6 and pLM1, were used to construct template plasmids for the generation of model RNA (upRNA). pSS6 contains a *tac* promoter (*27*), and pLM1 is a derivative of pGEM3 (Promega) containing *rnpB* from −270 to +1286 with respect to its transcription (*28*). The pSSLM1 plasmid was constructed by subcloning the *Hinc*II-*Stu*I 1135 bp fragment of pLM1 into the *Sma*I site of pSS6. To deactivate the P-1 promoter in pSSLM1, the −10 region of *rnpB* was mutated from 5′-TATACTG-3′ to 5′-CTCGAGC-3′ using QuikChange site-directed mutagenesis kit (Stratagene) with primers of For-10 (5′-CGCGCAAACCCTCCTCGAGCCGCGCCGAAGCTGAC-3′) and Rev-10 (5′-GTCAGCTTCGGCGCGGCTCGAGGAGGGTTTGCGCG-3′). The resultant plasmid was designated pSSM10. For the in vitro preparation of upRNA, a template plasmid, pLM1+415, was constructed by introducing a *Dra*I site at position +415 of *rnpB* in plasmid pLM1 with primers of PM1Dra-5 (5′-GCTTCGGCGGGTTTTTGCTTTAAAAGGGGCAGAAAGATGAAT GAC-3′) and PM1Dra-3 (5′-GTCATTCATCTTTCTGCCCCTTTTAAAGCAAAAACCCGCCGAAGC-3′). For the preparation of precursor M1 RNA in vitro, pSPM1 was constructed. PCR was performed with *E. coli* genomic DNA, rnpsp6 (5′-GGAAGCTTATTTAGGTGACACTATAGAAGCTGACCAGACAGT-3′) and rnpdra (5′-GGGAATTCTTTAAAGCAAAAACCCGCCGAAGC-3′). PCR products were cut with *Hin*dIII and *Eco*RI and subcloned into pUC19 vector. *Dra*I-cleaved pSPM1 was used as template for in vitro transcription of precursor M1 RNA. For the preparation of M1 RNA, pGER3 was used as described previously (*27*).

pRNEN is an expression plasmid for the His-tagged N-terminal catalytic half of RNase E containing the 1584 bp fragment (encoding 528 residues) of the *rne* gene (*11*).

*Preparation of Total Cellular RNA. E. coli* cells containing expression plasmids of upRNA were grown overnight in LB media containing 50 μg/mL of ampicillin. The overnight culture was diluted (1:100) into fresh media and grown at 30 °C to an $OD_{600}$[1] of 0.4. Then IPTG solution was added to the cell culture at appropriate concentrations, and the culture was further incubated for another 10 min. Following induction, total cellular RNAs were prepared from cultures, as described previously (*29*). In the case of MCE$^+$ and MCE$^-$ cells, the cultures grown at 30 °C were divided and incubated at 44 °C for 10 min before IPTG induction. In case of KS2001 and KS2003, the overnight cultures were diluted 1:100 and grown to the stationary phase for 16 h at 37 °C.

For RNA stability assays, cells cultured overnight were diluted (1:100) into fresh media and grown at 30 °C to an $OD_{600}$ of 0.4. Where required, the temperature was elevated to 44 °C and cells were incubated for another 10 min. IPTG was added into the culture, followed by further incubation for 10 min. Rifampicin was added to the culture at a final concentration of 100 μg/mL to stop further transcription. Cultures were dispensed at different time intervals, and total RNA was prepared.

*Reverse Transcription Polymerase Chain Reaction.* Total RNA (5 μg) from both the MCE$^+$ and MCE$^-$ strains grown at 30 or 44 °C was employed for RT-PCR. As a control, RNA was pretreated before RT-PCR with RNase (Promega) or with DNase (Ambion) at 37 °C for 20 min, according to the manufacturer's instructions. The primers used for RT-PCR were 5′-GTTACCTGGCACCCTGCCCT-3′ (+100R; reverse, complementary to sequences between +81 and +100 of *rnpB*) and 5′-GGCGCGCAAACCCTCTATAC-3′ (−28F; forward, corresponding to sequences between −28 and −9 of *rnpB*), or 5′-GCTTCGTCGTCGTCCTCTTC-3′ (+22F; forward, corresponding to sequences between +22 and +41 of *rnpB*). AccuPower RT/PCR Premix (Bioneer) was used for RT-PCR using the manufacturer's protocol. In brief, RNA was mixed with the reverse primer, incubated at 70 °C for 5 min, and kept on ice. The forward primer was added to the mixture, and cDNA synthesis was performed at 42 °C for 60 min. The mixture was incubated at 94 °C for 5 min to inactivate reverse transcriptase. The following PCR conditions were employed: 16 cycles at 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min.

*Northern Analysis.* For northern analysis, RNA samples (20 μg) were electrophoresed on a 5% polyacrylamide gel containing 7 M urea and electrotransferred to Hybond N+ (Amersham Biosciences) with a Hoefer Semiphor Semi-Dry transfer unit at 180 mA for 45 min. To increase transferring efficiency of large RNAs, the polyacrylamide gel was immersed in an alkaline solution (50 mM NaOH, 1 mM EDTA) for 15 min, followed by a neutralization solution of 45 mM Tris−borate, pH 8.0, 1 mM EDTA, for 15 min prior to electroblotting. Following transfer, the membrane was hybridized with oligo-1 (5′-GCTCTCTGTTGCACTGGTCG-3′), which was complementary to the region from +119 to +138 of *rnpB*, or anti-M1 riboprobe (*5*), as described previously (*5*, *29*). The anti-M1 was internally labeled with [α-$^{32}$P]CTP during in vitro transcription of *Hin*dIII-cleaved pMTd23 (*5*) as a template. The oligo-1 was labeled at the 5′ end with [γ-$^{32}$P]ATP and T4 polynucleotide kinase. For detecting 6S RNA, the anti-6S riboprobe was prepared using *Hin*dIII-digested pKS200, as described previously (*30*). The membrane was exposed to imaging plate or X-ray film and then analyzed with BAS-1500 (Fuji).

*In Vitro Reaction by the Purified N-Terminal Half of RNase E.* The N-terminal half of RNase E (NTH-RNase E) was expressed and purified according to a previous report (*11*). The upRNA and pM1 RNA substrates were prepared by in vitro transcription using pLM1+415 and pSPM1 as templates. The plasmid DNAs were digested with *Dra*I, followed by runoff transcription with SP6 RNA polymerase (Promega). The upRNA transcript encompassed positions −270 to +415 of *rnpB*, including an extra 45 bases corresponding to the vector sequence at the 5′ end. The pM1 RNA transcript encompassed positions +1 to +415 of *rnpB*. The RNAs were labeled at the 5′ end with [γ-$^{32}$P]ATP and T4 polynucleotide kinase.

The labeled RNA molecules were renatured by heating for 3 min at 80 °C in 25 mM Tris−Cl, pH 8.0 and 100 mM

[1] Abbreviations: DTT, dithiothreitol; IPTG, isopropylthio-β-D-galactoside; NTH-RNase E, N-terminal half of RNase E; OD, optical density.

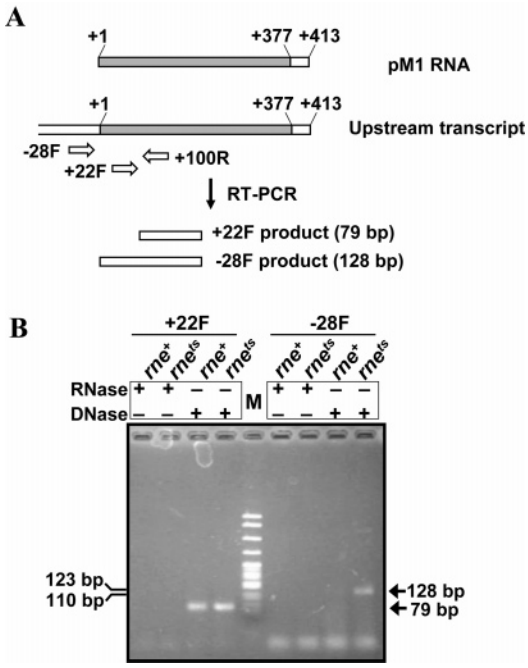


FIGURE 1: Levels of endogenous upstream transcripts of *rnpB*. (A) Schematic representation of RT-PCR experiments. The mature M1 RNA is marked in gray. Total cellular RNAs were extracted from $MCE^+$ and $MCE^-$ cells at 44 °C. When required, the RNA samples were treated with RNase (Promega) or DNase (Ambion). RNAs were reverse-transcribed into the corresponding cDNA sequences by RT-PCR. Reverse transcription was performed with the +100R primer complementary to nucleotides +81 to +100 of *rnpB*. PCR was performed with a pair of the −28F primer (corresponding to −28 to −9 of *rnpB*) and +100R primer or the +22F primer (corresponding to +22 to +41 of *rnpB*) and +100R primer. (B) Analysis of RT-PCR products. RT-PCR products are electrophoresed on a 3% agarose gel and stained with ethidium bromide. The PCR product derived from upstream transcripts was indicated by the arrow. In lane M, $\lambda$ DNA fragments produced by *Bst*EII treatment are employed as size markers.

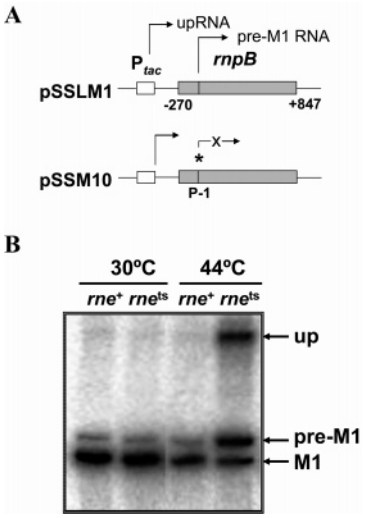


FIGURE 2: Expression of upRNA in vivo. (A) Schematic representation of the expression plasmids of upRNA. An upRNA expression plasmid, pSSLM1, contains both the *tac* promoter and the region encompassing −270 to +847 of *rnpB*. pSSM10, a derivative of pSSLM1, has a mutation (indicated by an asterisk) at the −10 region of the P-1 promoter of *rnpB* for the promoter inactivation. (B) upRNA expression in $MCE^+$ (*rne*$^+$) and $MCE^-$ (*rne*$^{ts}$) cells. Total cellular RNAs were prepared from $MCE^+$ and $MCE^-$ cells containing pSSLM1 grown in the presence of 0.06 mM IPTG at the indicated temperatures. The RNAs were separated on a 5% polyacrylamide gel containing 7 M urea and analyzed by northern blotting. The 5′ end labeled oligo-1 was used as the probe. Up, pre-M1, and M1 stand for upRNA, precursor M1 RNA, and M1 RNA, respectively.

NaCl, and cooling down at room temperature. RNA substrates of 0.2 pmol were incubated with 15 or 30 ng of NTH-RNase E in 50 $\mu$L of reaction buffer (25 mM Tris−Cl, pH 8.0, 10 mM $MgCl_2$, 100 mM NaCl, 0.1% Triton X-100, 1 mM DTT) including 20 $\mu$g/mL of yeast total RNA at 37 °C for 10 min. We assumed that the RNase P reaction condition would be the optimum condition to maintain the M1 RNA structure. Since the typical RNase P reaction is carried out at 10 mM $Mg^{2+}$ and 100 mM monovalent ion, those concentrations were chosen for the RNase E reaction. Yeast total RNA was added to prevent substrate RNA or cleavage products from degradation by, if any, contaminated RNases. It has been known that the addition of yeast RNA at 20 $\mu$g/mL does not affect the RNase E cleavage but inhibits RNA degradation by other RNases (*5*, *11*). Reaction products were analyzed on a 3% or 5% polyacrylamide sequencing gel containing 8 M urea. We also employed the RNase E cleavage reactions in the absence of yeast total RNA and obtained basically the same cleavage patterns.

*Enzymatic Probing of RNA.* upRNA and pM1 RNA molecules prepared by in vitro transcription were labeled at the 3′ end with [$^{32}$P]pCp and RNA ligase. RNA substrates were renatured, as described above. The refolded RNA of 0.2 pmol was incubated with RNase A, RNase T1, or RNase V1 (Ambion) in 20 $\mu$L of the RNase E reaction buffer including yeast tRNA 1 $\mu$g, and with S1 nuclease (Ambion) in the same buffer but containing an additional 10 mM $ZnCl_2$ at 37 °C for 30 min. The cleavage products were recovered by ethanol precipitation and electrophoresed on a 4% polyacrylamide gel containing 8 M urea.

## RESULTS

*Cellular Levels of Endogenous rnpB Upstream Transcripts.* Previous work showed that large *rnpB* upstream transcripts of 0.6, 2.1, and 5 kb exist in a temperature-sensitive mutant strain of RNase E at the nonpermissive temperature (*4*). However, it is not clear how much they are synthesized in the cell. We attempted to compare the cellular level of the large transcripts with that of mature M1 RNA. For this purpose, total RNAs from the wild-type $MCE^+$ and the *rne*$^{ts}$ mutant strain $MCE^-$ grown at 44 °C were subjected to RT-PCR using primers −28F or +22F as the forward primer and +100R as the reverse primer. Forward primer −28F was designed to correspond to the region upstream of the 5′ end of M1 RNA to quantitate transcripts containing sequences of the upstream region. On the other hand, forward primer +22F was specific for a sequence within mature M1 RNA to quantitate transcripts containing the sequence for M1 RNA (Figure 1A). The +22F PCR product of 79 bp was detected in both $MCE^+$ and $MCE^-$ cells, whereas the −28F PCR product of 128 bp was in the $MCE^-$ cells only (Figure 1B). The PCR products disappeared by the RNase treatment, but not the DNase treatment, indicating that they were driven from *rnpB* transcripts. When the amount of the endogenous upstream transcripts relative to M1 RNA calculated as the ratio of [−28F product] to ([+22F product] − [−28F product]), its value was about 50%.

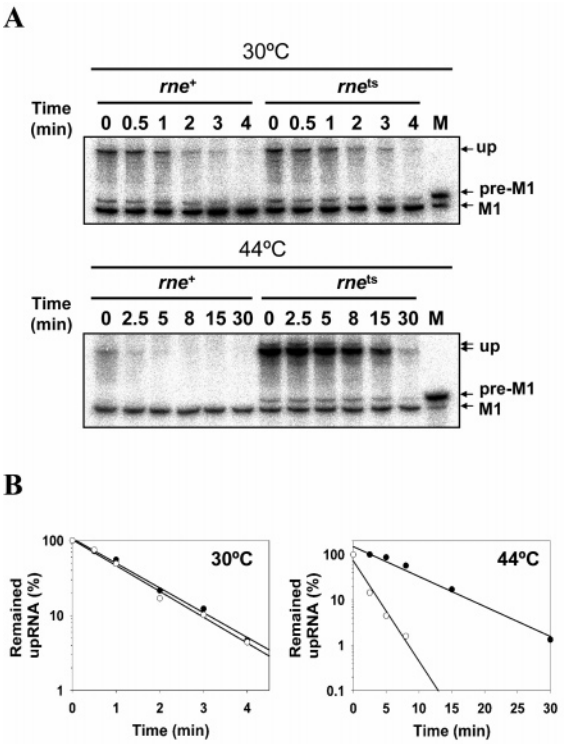


FIGURE 3: *rne* dependency of upRNA stability. (A) Rifampicin was added to MCE$^+$ (*rne*$^+$) and MCE$^-$ (*rne*$^{ts}$) cells containing pSSLM1 grown in the presence of 0.06 mM IPTG at 30 or 44 °C. Total cellular RNAs were isolated at the indicated times and subjected to northern analysis using the 5′ end labeled oligo-1 as the probe. In lane M, precursor M1 RNA (413 nt) and M1 RNA (377 nt) prepared by in vitro transcription were used as size markers. Up, pre-M1, and M1 stand for upRNA, precursor M1 RNA, and M1 RNA, respectively. The data show one representative experiment of three performed. (B) The remaining RNA % was calculated as a ratio of the amount of RNA at the indicated times with respect to that of the zero time point after the rifampicin addition. Open and filled circles represent *rne*$^+$ and *rne*$^{ts}$ cells, respectively.

*Construction of a Model Substrate and Its Expression.* Since multiple *rnp*B-containing upstream transcripts are present in the cell, we designed a model substrate to systematically investigate their metabolic pathway. Initially, we constructed the template plasmid for a model RNA by subcloning the *rnpB* fragment into pSS6 (Figure 2A). The resulting plasmid, pSSLM1, contained a fragment encompassing nucleotides −270 to +847 of the *rnpB* gene linked to the *tac* promoter so that the amount of a primary transcript of 742 nt, named upRNA, could be regulated by the IPTG concentration in vivo. pSSLM1 also produces pM1 RNA from the native P-1 promoter of *rnpB* because the P-1 promoter exists in pSSLM1. Therefore, excess M1 RNA produced from the P-1 promoter on the high-copy plasmid could highly contribute to the steady-state concentration of mature M1 RNA in the plasmid-containing cells. This high concentration of M1 RNA may cause problems in evaluating the contribution of upRNA to M1 RNA biogenesis. Therefore, we also constructed another template plasmid for upRNA, pSSM10, in which P-1 promoter activity was disrupted via the −10 mutation of TATACTG to CTCGAGC (Figure 2A). However, it should also be noted that upRNA produced by pSSM10 may undergo a different metabolic pathway due to the mutated sequence at the −10 region. Thus, in this study, we used both pSSLM1 and pSSM10 to rule out any possible effects of the −10 mutation and effects masked by transcription from the strong P-1 promoter, respectively.

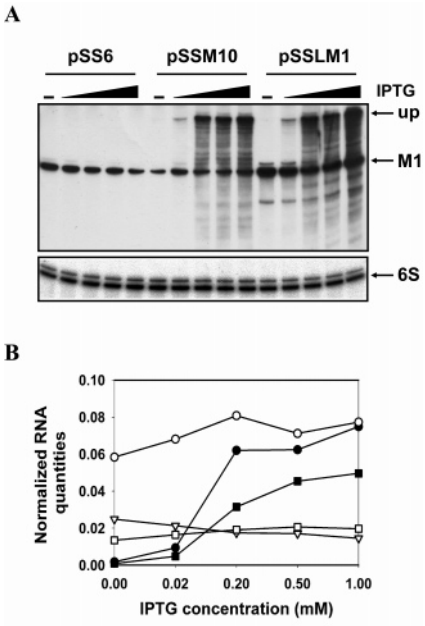


FIGURE 4: Steady-state levels of M1 RNA and upRNA in cells treated with different concentrations of IPTG. (A) Total cellular RNAs were prepared from JM109 cells containing pSS6, pSSLM1, and pSSM10 and subjected to northern analysis. For differential expression of upRNA, cells were treated with IPTG for 10 min at final concentrations of 0, 0.02, 0.2, 0.5, and 1 mM. The probe for northern analysis was an internally labeled anti-M1. (B) The band intensities of upRNA and M1 RNA were normalized to that of 6S RNA and plotted as a function of IPTG concentration. Open and filled symbols represent M1 RNA and upRNA, respectively. ○ and ●, cells containing pSSLM1; □ and ■, cells containing pSSM10; ▽, cells containing pSS6.

*RNase E Dependency of the Steady-State Concentration of upRNA.* To examine whether upRNA accumulates in the absence of RNase E, we performed northern analysis after inducing the expression of upRNA in RNase E-deficient cells (*rne*$^{ts}$). When induced with 0.06 mM IPTG, upRNA accumulated at the nonpermissive temperature in *rne*$^{ts}$ cells, whereas it was barely detected in *rne*$^+$ cells. (Figure 2B). We then examined more quantitatively the effect of RNase E on the turnover of upRNA by comparing the half-lives of upRNA between *rne*$^+$ and *rne*$^{ts}$ cells. For these experiments, we modified the method for northern analysis by partially alkaline-hydrolyzing RNAs in the polyacrylamide gel prior to the membrane transfer of RNA. This modified protocol highly increased the northern signal by improving transferring efficiency of large RNAs in the polyacrylamide gel, to the extent that upRNA in *rne*$^+$ cells was readily detected. At 30 °C (permissive temperature), the half-life of upRNA in *rne*$^+$ cells was 1.4 min, similar to 1.5 min in *rne*$^{ts}$ cells (Figure 3). However, at 44 °C (nonpermissive temperature), the half-life of upRNA in *rne*$^{ts}$ cells significantly increased with the value of 13 min, compared to 2.1 min in *rne*$^+$ cells (Figure 3). These data indicate that RNase E is involved in the turnover of upRNA in vivo.

*Fate of upRNA.* To determine whether upRNA undergoes processing to M1 RNA or degradation, we analyzed the change of M1 RNA levels as the expression of upRNA increased. When the IPTG concentration increased from 0.02

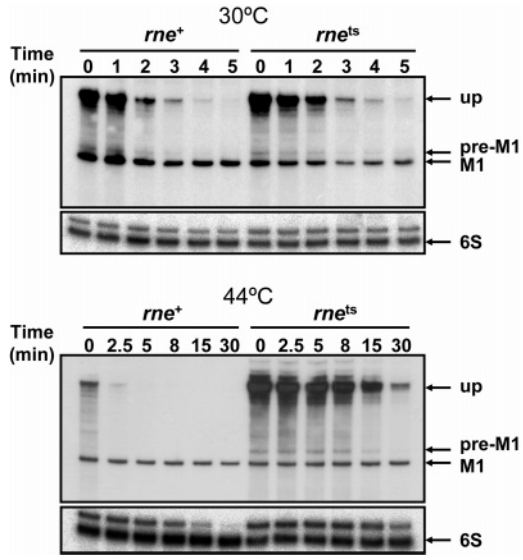


FIGURE 5: Fate of upRNA in cells containing pSSM10. Rifampicin was added to MCE$^+$ (*rne*$^+$) and MCE$^-$ (*rne*$^{ts}$) cells containing pSSM10 grown in the presence of 0.1 mM IPTG at 30 or 44 °C. Total cellular RNAs were isolated at the indicated times and subjected to northern analysis using the 5′ end labeled oligo-1 as the probe.

to 1 mM in cells containing pSSLM1 or pSSM10, the steady-state concentrations of upRNA increased about 10-fold in both cells. However, M1 RNA levels were nearly identical in spite of the increase in the upRNA levels (Figure 4). Instead, the amount of degradation fragments extensively increased with the increase of IPTG concentration. These data show that upRNA scarcely contributes to M1 RNA biosynthesis. We analyzed in detail the fate of upRNA produced from pSSM10 that contained the inactivated P-1 promoter. P-1 transcripts in cells containing this plasmid were expected to be produced only from chromosomal *rnpB* gene. Using cells containing pSSM10, RNA stability assays were performed (Figure 5). About 95% of upRNA disappeared in *rne*$^+$ cells in 5 min after the treatment of rifampicin at 30 °C. However, the change in M1 RNA levels was not observed. In *rne*$^{ts}$ cells, at the nonpermissive temperature (44 °C), upRNA was highly accumulated as in cells containing pSSLM1. We also found that the rifampicin treatment under this condition did not increase M1 RNA levels. This result suggests that upRNA does not undergo processing to generate M1 RNA even in the absence of degradation by RNase E.

It is possible that a small increase of mature M1 RNA could not be detected over the background signals of the pre-existing M1 RNA. To exclude this possibility, we used M1 RNA-deficient *E. coli* strain KS2003, where M1 RNA levels are very low due to the mutation of the *rne*-dependent site of *rnpB* (*26*). KS2003 cells mimic *rnpB*-knockout cells, particularly in the stationary phase (*26*). In stationary phase cells of KS2003 containing pSSM10, therefore, any mature M1 RNA generated from upRNA could be more clearly observed if present. However, we did not detect M1 RNA in IPTG-induced KS2003 cells containing pSSM10 (Figure 6). The data of KS2003 cells also suggest that upRNA is not processed to M1 RNA.

It might be argued that excess M1 RNA may not be stable in vivo in the absence of C5 protein, the protein component

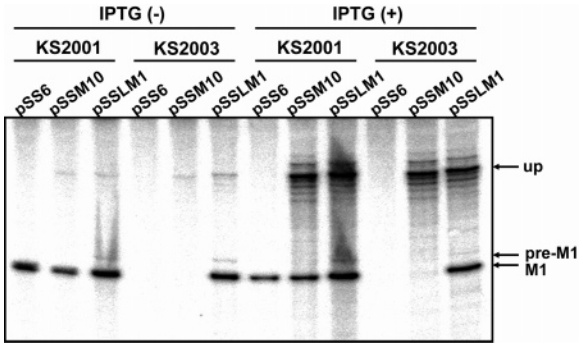


FIGURE 6: Expression of upRNA in M1 RNA-deficient cells. Total cellular RNAs were prepared from KS2001 and KS2003 containing pSS6, pSSLM1, and pSSM10 and subjected to northern analysis, as in Figure 3. For the induction of upRNA, cells were treated with 0.1 mM IPTG.

of RNase P, and thereby additional M1 RNA derived from upRNA could not be detected. This is not the case because excess M1 RNA generated from the P-1 promoter on pSSLM1 in the absence of IPTG was observed (Figure 4). Furthermore, M1 RNA derived from upRNA was not observed in M1 RNA-deficient KS2003 cells even though M1 RNA produced from the P-1 promoter on pSSLM1 was observed in these cells (Figure 6). Taken together, these results suggest that little amount of M1 RNA derived from upRNA is not due to its rapid degradation in the absence of C5 protein.

*Cleavage of upRNA by RNase E in Vitro*. To test whether RNase E is directly involved in metabolism of upRNA, we performed an in vitro cleavage assay of upRNA using the purified NTH-RNase E as the catalytic domain, which alone can carry out the RNase E reaction in vitro (*11*, *31*). upRNA was synthesized by in vitro transcription and labeled at the 5′ end with $^{32}$P. The in vitro transcribed upRNA of 730 nt carried the extra sequences of the 45 nt vector and 270 nt upstream sequences at the 5′ end of pM1 RNA. We compared the cleavage reaction of upRNA with that of pM1 RNA that leads to cleavage of pM1 RNA at positions +378/+379 (*11*). Cleavage sites were estimated using G-specific cleavage products by RNase T1. Cleavage sites in the M1 RNA region of upRNA were further deduced by comparing G-specific cleavage patterns of upRNA and pM1 RNA because they share the M1 RNA sequence. upRNA was cleaved at positions around +378/+379 by NTH-RNase E (Figures 7 and 9), as expected from the fact that upRNA also had the 3′ processing site. In addition, we found that NTH-RNase E cleaved upRNA in the upstream sequence (positions −172, −157, −147, −116, and −102) and within the M1 RNA structural sequence (positions +4, +8, +97, +134/+136, +166, +255, and +329) (Figures 7 and 9). The cleavages of the upRNA within the M1 RNA structural sequence were surprising because pM1 RNA did not generate those cleavages under the same condition. This finding supports the hypothesis that RNase E is directly involved in the degradation of upRNA by cleaving the M1 RNA structural region.

To examine whether the susceptibility of upRNA to RNase E cleavage would have resulted from structural differences in the M1 RNA sequence between upRNA and pM1 RNA, we probed their secondary structures using S1 nuclease and RNase A (specific to the single-stranded region) and RNase

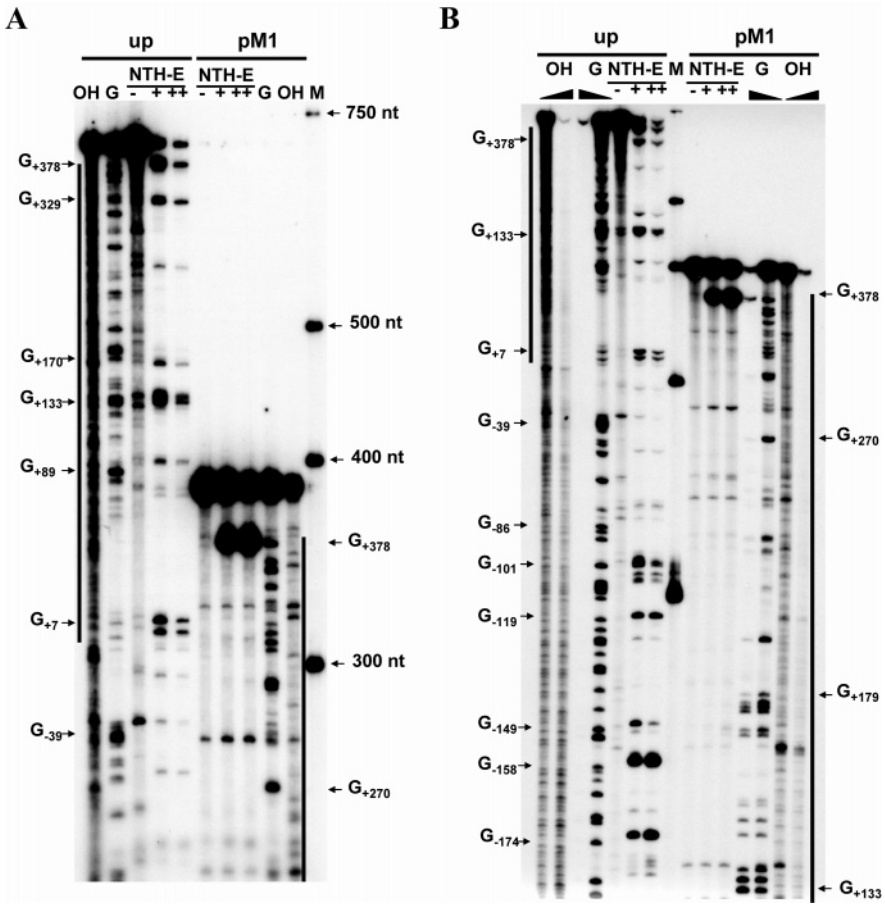


FIGURE 7: In vitro processing of upRNA by the N-terminal half of RNase E. (A) upRNA and pM1 RNA transcribed in vitro were $^{32}$P-labeled at the 5′ end, and 0.2 pmol of the labeled RNAs were treated with 15 (in lane "+") or 30 ng (in lane "++") of the N-terminal half of RNase E (NTH-RNase E) at 37 °C for 10 min. Reaction products were separated on a 3% polyacrylamide sequencing gel containing 8 M urea. M, RNA Century-Plus markers (Ambion); OH, partial alkaline hydrolysis ladders; G, G-specific (RNase T1) cleavage products; −, no enzyme. The region of upRNA corresponding to M1 RNA was marked with a solid bar. (B) The RNA substrates were assayed independently, as in (A), and the reaction products were separated on a 5% polyacrylamide sequencing gel containing 8 M urea.

V1 (specific to the double-stranded region). In this probing experiment, the RNA molecules labeled with [$^{32}$P]pCp at the 3′ end were used. Although in vitro transcripts were heterogeneous with the 3′ ends of mainly positions +415 and +413 (*32*), we are able to clearly compare the cleavage patterns (Figures 8 and 9). We also assigned cleavage products by analyzing combined cleavage patterns expected from the heterogeneous +415 and +413 ends along with not only information on secondary structures deduced from cleavage patterns of S1 nuclease, RNase A, and RNase V1 cleavages but also the proposed secondary structure of M1 RNA (*33*). The cleavage patterns by all the enzymes in the M1 RNA region between the upRNA and pM1 RNA were almost the same, except for a minor difference in RNase A and RNase V1 cleavages at the region of +373 to +377, which might result from base-pairing with 5′ upstream sequences in upRNA. The global similarity of the enzymatic probing data for the M1 RNA sequence between upRNA and pM1 RNA suggests that the vulnerability of upRNA to degradation by RNase E is not due to the alteration in the folding of its M1 RNA region.

## DISCUSSION

M1 RNA transcripts can be generated from both the proximal P-1 promoter and unknown distal upstream promoters in *E. coli* (*4*, *8*). Biogenesis of M1 RNA by the P-1 transcription and subsequent 3′ processing has been extensively studied (*2*−*6*), whereas that from upstream transcripts has not been clear. In this study, we analyzed upRNA, a model *rnpB*-containing upstream transcript in *E. coli*, and showed that upRNA was primarily degraded and not processed to M1 RNA. Therefore, upRNA makes little contribution to M1 RNA biosynthesis under normal cellular conditions. This degradation of upRNA supports a model that the biosynthesis of M1 RNA is tightly regulated: only transcripts from the proximal P-1 promoter give rise to M1 RNA through 3′ processing, whereas those from upstream promoters are degraded.

Lundberg and Altman (*4*) showed that the multicopy plasmid carrying the upstream 6.6 kb region of *rnpB* with inactivated P-1 promoter complements the growth of a temperature-sensitive M1 RNA-deficient strain at restrictive temperatures. This complementation phenomenon could be explained on the basis of our results. First, the inactivated P-1 promoter may have enough residual activity to permit cell growth. Another possible explanation is that minimal amounts of mature M1 RNA for cell growth may be produced from upstream transcripts although most of them are degraded. Alternatively, upstream transcripts may directly function without processing until they are degraded, because

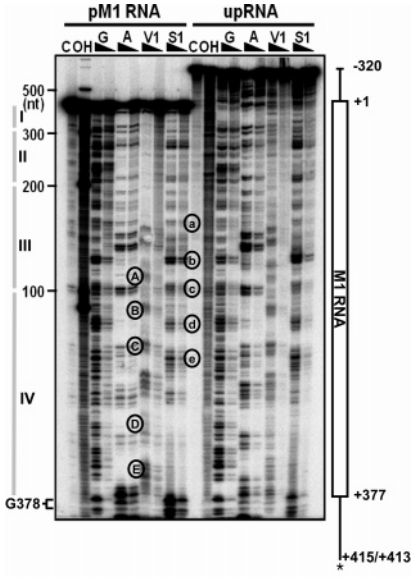


FIGURE 8: Structure probing of pM1 RNA and upRNA. RNAs transcribed in vitro were $^{32}$P-labeled at the 3′ end, and 0.2 pmol of the labeled RNAs were treated with 0.1 and 0.01 pg of RNase A, 0.05 and 0.005 units of RNase V1, or 0.3 and 0.03 units of S1 nucleases at 37 °C for 15 min. The reaction products were separated on a 4% polyacrylamide sequencing gel containing 8 M urea. C, untreated RNA as a control; G, G-specific (RNase T1) cleavage products, OH, partial alkaline hydrolysis ladders mixed with RNA Century-Plus markers (Ambion). $G_{378}$ cleavage products are indicated on the left. The region of M1 RNA is divided into four parts on the left: I, +1 to +115; II, +116 to +215; III, +216 to +315; IV, +316 to +377. Some cleavage sites by RNase V1 and S1 nuclease are indicated by capital and small letters within the circle in the figure, respectively. The schematic structure of upRNA with an asterisk at the position of label is shown in the right.

it has been known that M1 RNA with extra nucleotides at the 3′ end or 5′ end remains catalytically active in vitro (*34*−*36*).

An interesting finding of this study is the dual mode of action of RNase E on *rnpB* expression. RNase E is an essential enzyme required for processing of M1 RNA by removing extra nucleotides at the 3′ end of pM1 RNA (*4*, *5*, *11*). We showed here that the enzyme can also initiate the degradation of upRNA. This mode of action may be similar to that of initiation of mRNA degradation by RNase E (*21*, *37*−*39*). NTH-RNase E did not cleave within the M1 RNA sequence in pM1 RNA, but cleaved in upRNA, which may be a major mechanism of the RNase E-dependent degradation of upRNA. A plausible explanation for the cleavage difference between pM1 RNA and upRNA is the substrate specificity of RNase E. RNase E has been known to cleave RNAs with unpaired nucleotides at the 5′ end more efficiently than those with 5′-terminal base-pairing (*20*, *40*). pM1 RNA has a rigid stem structure at the 5′ end (*5*), whereas upRNA has extended sequences from the 5′ end of pM1 RNA. Therefore, potential cleavage sites within the M1 RNA structural sequence in upRNA may be more susceptible to RNase E than those in pM1 RNA even though the M1 RNA region in upRNA folds like that in pM1 RNA. Even when cleavages occur within the upstream sequence in the first place, the cleaved RNAs bearing monophosphates at the 5′ end could be preferred substrates for RNase E (*41*−*44*).

We do not exclude the possibility that the folding of upRNA is different from that of natural large upstream transcripts, thereby leading to different metabolic pathways. However, it is noteworthy that the endogenous transcripts exist as multiple forms having different 5′ ends but sharing the M1 RNA sequence at the 3′ region. In this regard, upRNA is similar to the endogenous transcripts. Furthermore, our results suggest that the sequence extension from the 5′ end of M1 RNA is responsible for rapid degradation of upRNA. Therefore, it is likely that the endogenous transcripts follow the same metabolic pathway of upRNA. Here, we propose a model that entails two modes for RNase E involvement in the regulation of biosynthesis of M1 RNA (Figure 10). In one mode, transcripts from the P-1 promoter

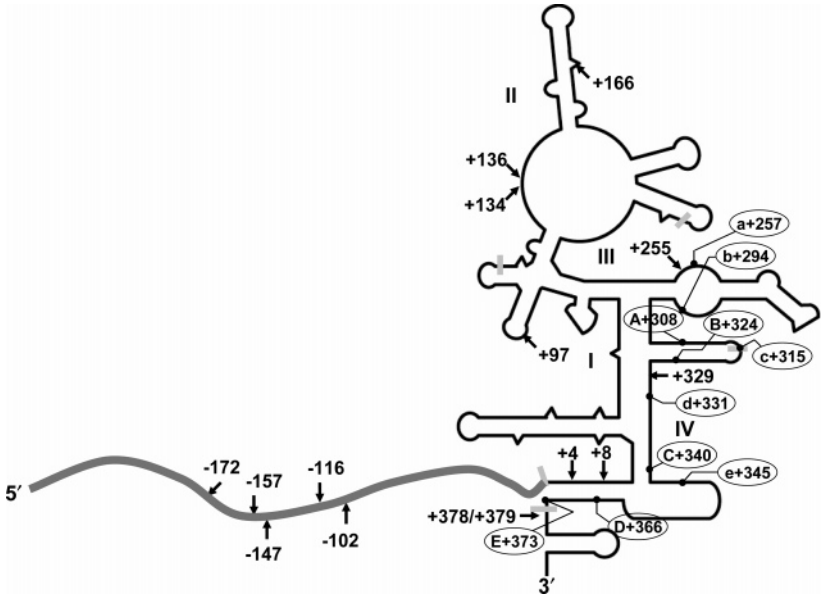


FIGURE 9: Schematic representation of the cleavage sites by RNase E, RNase V1, and S1 nuclease. A secondary structure of M1 RNA proposed by Hass et al. (*33*). is shown without its sequence. The tertiary base-pairing interactions are not shown here. The four parts (I, II, III, and IV) of the M1 RNA region indicated in Figure 8 are also presented. The 270 nt upstream sequence of upRNA is indicated by the thick line. The arrows indicate cleavage sites of upRNA by RNase E. Capital and small letters within the circle indicate cleavage sites by RNase V1 and S1 nuclease, respectively.

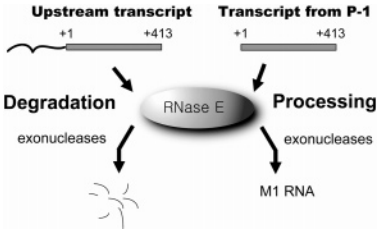


FIGURE 10: Proposed model for the dual mode of RNase E on the biosynthesis of M1 RNA. In degradation mode, transcripts from upstream promoters were cleaved by RNase E and subsequently degraded by other cellular factors (left). In processing mode, RNase E cleaves a primary transcript from the P-1 promoter at the +378/+379 position as a processing enzyme (*11*), and exoribonucleases trim the intermediate +378/+379 product at the 3′ end to mature M1 RNA (*6*) (right).

are driven into the processing pathway, processed at the 3′ end, and transformed to mature M1 RNA. In the other mode, larger *rnpB*-containing upstream transcripts are degraded by RNase E and other possible cellular factors. M1 RNA is not produced in this pathway, and degradation products are recycled.

The biological meaning of the degradation mode of RNase E action is currently unclear for upstream transcripts. Four plausible ORFs (*garP*, *garL*, *garR*, and *garK*) are located upstream of the *rnpB* gene, and the stop codon of the last ORF *garK* is located within the P-1 promoter of *rnpB*. If these genes are expressed, their transcripts could be extended to the *rnpB* terminator (*4*). In that case, the regulation of possible production of mature M1 RNA from the extended transcripts could not be coordinated with regulation of expression from the proximal P-1 promoter. Therefore, the cell may need an additional machinery to remove upstream transcripts for the tight regulation of M1 RNA biosynthesis.

## REFERENCES


1. Guerrier-Takada, C., Gardiner, K., Marsh, T., Pace, N., and Altman, S. (1983) The RNA moiety of ribonuclease P is the catalytic subunit of the enzyme, *Cell 35*, 849−857.
2. Gurevitz, M., Jain, S. K., and Apirion, D. (1983) Identification of a precursor molecular for the RNA moiety of the processing enzyme RNase P, *Proc. Natl. Acad. Sci. U.S.A. 80*, 4450−4454.
3. Sakamoto, H., Kimura, N., and Shimura, Y. (1983) Processing of transcription products of the gene encoding the RNA component of RNase P, *Proc. Natl. Acad. Sci. U.S.A. 80*, 6187−6191.
4. Lundberg, U., and Altman, S. (1995) Processing of the precursor to the catalytic RNA subunit of RNase P from *Escherichia coli*, *RNA 1*, 327−334.
5. Kim, S., Kim, H., Park, I., and Lee, Y. (1996) Mutational analysis of RNA structures and sequences postulated to affect 3′ processing of M1 RNA, the RNA component of *Escherichia coli* RNase P, *J. Biol. Chem. 271*, 19330−19337.
6. Li, Z., Pandit, S., and Deutscher, M. P. (1998) 3′ exoribonucleolytic trimming is a common feature of the maturation of small, stable RNAs in *Escherichia coli*, *Proc. Natl. Acad. Sci. U.S.A. 95*, 2856−2861.
7. Motamedi, H., Lee, Y., and Schmidt, F. J. (1984) Tandem promoters preceding the gene for the M1 RNA component of *Escherichia coli* ribonuclease P, *Proc. Natl. Acad. Sci. U.S.A. 81*, 3959−3963.
8. Lee, Y., Ramamoorthy, R., Park, C.-U., and Schmidt, F. J. (1989) Sites of initiation and pausing in the *Escherichia coli rnpB* (M1 RNA) transcript, *J. Biol. Chem. 264*, 5098−5163.
9. Gegenheimer, P., Watson, N., and Apirion, D. (1977) Multiple pathways for primary processing of ribosomal RNA in *Escherichia coli*, *J. Biol. Chem. 252*, 3064−3073.
10. Ghora, B. K., and Apirion, D. (1978) Structural analysis and *in vitro* processing to p5 rRNA of a 9S RNA molecule isolated from an *rne* mutant of *E. coli*, *Cell 15*, 1055−1066.
11. Sim, S., Kim, K. S., and Lee, Y. (2002) 3′-End processing of precursor M1 RNA by the N-terminal half of RNase E, *FEBS Lett. 529*, 225−231.
12. Lin-Chao, S., Wei, C. L., and Lin, Y. T. (1999) RNase E is required for the maturation of *ssrA* RNA and normal *ssrA* RNA peptide-tagging activity, *Proc. Natl. Acad. Sci. U.S.A. 96*, 12406−12411.
13. Li, Z., Pandit, S., and Deutscher, M. P. (1999) RNase G (CafA protein) and RNase E are both required for the 5′ maturation of 16S ribosomal RNA, *EMBO J. 18*, 2878−2885.
14. Ow, M. C., and Kushner, S. R. (2002) Initiation of tRNA maturation by RNase E is essential for cell viability in *E. coli*, *Genes Dev. 16*, 1102−1115.
15. Li, Z., and Deutscher, M. P. (2002) RNase E plays an essential role in the maturation of *Escherichia coli* tRNA precursors, *RNA 8*, 97−109.
16. Lin-Chao, S., and Cohen, S. N. (1991) The rate of processing and degradation of antisense RNAI regulates the replication of ColE1-type plasmids *in vivo*, *Cell 65*, 1233−1242.
17. Xu, F., Lin-Chao, S., and Cohen, S. N. (1993) The *Escherichia coli pcnB* gene promotes adenylation of antisense RNA I of ColE1-type plasmids *in vivo* and degradation of RNAI decay intermediates, *Proc. Natl. Acad. Sci. U.S.A. 90*, 6756−6760.
18. Regnier, P., and Hajnsdorf, E. (1991) Decay of mRNA encoding ribosomal protein S15 of *Escherichia coli* is initiated by an RNase E-dependent endonucleolytic cleavage that removes the 3′ stabilizing stem and loop structure. *J. Mol. Biol. 217*, 283−292.
19. Hajnsdorf, E., Braun, F., Haugel-Nielsen, J., Le Derout, J., and Regnier, P. (1996) Multiple degradation pathways of the *rpsO* mRNA of *Escherichia coli*. RNase E interacts with the 5′ and 3′ extremities of the primary transcript, *Biochimie 78*, 416−424.
20. Mackie, G. A., and Genereaux, J. L. (1993) The role of RNA structure in determining RNase E-dependent cleavage sites in the mRNA for ribosomal protein S20 *in vitro*, *J. Mol. Biol. 234*, 998−1012.
21. Mudd, E. A., Carpousis, A. J., and Krisch, H. M. (1990) *Escherichia coli* RNase E has a role in the decay of bacteriophage T4 mRNA, *Genes Dev. 4*, 873−881.
22. Py, B., Causton, H., Mudd, E. A., and Higgins, C. F. (1994) A protein complex mediating mRNA degradation in *Escherichia coli*, *Mol. Microbiol. 14*, 717−729.
23. Miczak, A., Kaberdin, V. R., Wei, C.-L., and Lin-Chao, S. (1996) Proteins associated with RNase E in a multicomponent ribonucleolytic complex, *Proc. Natl. Acad. Sci. U.S.A. 93*, 3865−3869.
24. Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors, *Gene 33*, 103−119.
25. Carpousis, A. J., Van Houwe, G., Ehretsmann, C., and Krisch, H. M. (1994) Copurification of *E. coli* RNAase E and PNPase: evidence for a specific association between two enzymes important in RNA processing and degradation, *Cell 76*, 889−900.
26. Kim, K., Sim, S., Ko, J., and Lee, Y. (2005) Processing of M1 RNA at the 3′ end protects its primary transcript from degradation, *J. Biol. Chem. 280*, 34667−34674.
27. Park, B. H., Choi, Y. N., Park, J. W., Sim, S., Gil, M. C., Kim, S., Kim, M., and Lee, Y. (1998) Expression of C5 protein, the protein component of *Escherichia coli* RNase P, from the *tac* promoter, *Mol. Cells 8*, 96−100.
28. Lee, Y. M., Lee, Y., and Park, C.-U. (1989) Transcription termination in the M1 RNA gene of *Escherichia coli*, *Korean Biochem. J. 22*, 276−281.
29. Ko, J., and Lee, Y. (2006) RNA-conjugated template-switching RT-PCR method for generating an *Escherichia coli* cDNA library for small RNAs, *J. Microbiol. Methods 64*, 297−304.
30. Kim, K., and Lee, Y. (2004) Regulation of 6S RNA biogenesis by switching utilization of both sigma factors and endoribonucleases, *Nucleic Acids Res. 32*, 6057−6068.
31. Redko, Y., Tock, M. R., Adams, C. J., Kaberdin, V. R., Grasby, J. A., and McDowall, K. J. (2003) Determination of the catalytic parameters of the N-terminal half of *Escherichia coli* ribonuclease E and the identification of critical functional groups in RNA substrates, *J. Biol. Chem. 278*, 44001−44008.
32. Kim, S., Sim, S., and Lee, Y. (1999) *In vitro* analysis of processing at the 3′-end of precursors of M1 RNA, the catalytic subunit of

*Escherichia coli* RNase P: multiple pathways and steps for the processing, *Nucleic Acids Res. 27*, 895−901.
33. Hass, E. S., Brown, J. W., Pitulle, C., and Pace, N. R. (1994) Further perspective on the catalytic core and secondary structure of ribonuclease P RNA, *Proc. Natl. Acad. Sci. U.S.A. 91*, 2527−2531.
34. Guerrier-Takada, C., and Altman, S. (1984) Catalytic activity of an RNA molecule prepared by transcription *in vitro*, *Science 223*, 285−286.
35. Lawrence, N. P., and Altman, S. (1986) Site-directed mutagenesis of M1 RNA, the RNA subunit of *Escherichia coli* ribonuclease P: The effects of an addition and small deletions on catalytic function, *J. Mol. Biol. 191*, 163−175.
36. Guerrier-Takada, C., and Altman, S. (1986) M1 RNA with large terminal deletions retains its catalytic activity, *Cell 45*, 177−183.
37. Coburn, G. A., and Mackie, G. A. (1999) Degradation of mRNA in *Escherichia coli*: an old problem with some new twists, *Prog. Nucleic Acid Res. Mol. Biol. 62*, 55−108.
38. Nierlich, D. P., and Murakawa, G. J. (1996) The decay of bacterial messenger RNA, *Prog. Nucleic Acid Res. Mol. Biol. 52*, 153−216.
39. Carpousis, A. J., Vanzo, N. F., and Raynal, L. C. (1999) mRNA degradation: A tale of poly(A) and multiprotein machines, *Trends Genet. 15*, 24−28.
40. Bouvet, P., and Belasco, J. G. (1992) Control of RNase E-mediated RNA degradation by 5′-terminal base pairing in *E. coli*, *Nature 360*, 488−491.
41. Tock, M. R., Walsh, A. P., Carroll, G., and McDowall, K. J. (2000) The CafA protein required for the 5′-maturation of 16S rRNA is a 5′-end-dependent ribonuclease that has context-dependent broad sequence specificity, *J. Biol. Chem. 275*, 8726−8732.
42. Jiang, X., and Belasco, J. G. (2004) Catalytic activation of multimeric RNase E and RNase G by 5′-monophosphorylated RNA, *Proc. Natl. Acad. Sci. U.S.A. 101*, 9211−9216.
43. Mackie, G. A. (1998) Ribonuclease E is a 5′-end-dependent endonuclease, *Nature 395*, 720−723.
44. Jiang, X., Diwa, A., and Belasco, J. G. (2000) Regions of RNase E important for 5′-end-dependent RNA cleavage and autoregulated synthesis, *J. Bacteriol. 182*, 2468−2475.

BI701528J