RNase P-dependent Cleavage of Polycistronic mRNAs within Their Downstream Coding Regions in *Escherichia coli*

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M1 RNA, the catalytic subunit of *Escherichia coli* RNase P, is an essential ribozyme that processes the 5' leader sequence of tRNA precursors (ptRNAs). Using KS2003, an *E. coli* strain generating only low levels of M1 RNA, which showed growth defects, we examined whether M1 RNA is involved in polycistronic mRNA processing or degradation. Microarray analysis of total RNA from KS2003 revealed six polycistronic operon mRNAs (acpP-fabF, cysDNC, flgAMN, lepAB, phoPQ, and puuCBE) showing large differences in expression between the adjacent genes in the same mRNA transcript compared with the KS2001 wild type strain. Model substrates spanning an adjacent pair of genes for each polycistronic mRNA were tested for RNase P cleavage *in vitro*. Five model RNAs (cysNC, flgMN, lepAB, phoPQ, and puuBE) were cleaved by RNase P holoenzyme but not by M1 RNA alone. However, the cleavages occurred at non-ptRNA-like cleavage sites, with much less efficiency than the cleavage of ptRNA. Since cleavage products generated by RNase P from a polycistronic mRNA can have different *in vivo* stabilities, our results suggest that RNase P cleavage may lead to differential expression of each cistron.

Key Words : C5 protein, M1 RNA, Microarray, Polycistronic mRNA, RNase P

Introduction

RNase P was characterized initially as an RNA-processing enzyme that generates the mature 5' ends of tRNAs in all organisms.[1] *Escherichia coli* RNase P contains two components, a single RNA subunit (M1 RNA) and a small basic protein subunit (C5 protein). M1 RNA can cleave precursor tRNAs (ptRNAs) without C5 protein *in vitro*.[2] In addition to ptRNA, RNase P is known to process various RNAs, such as 4.5S RNA, tmRNA, and phages P1 and P7 antisense C4 RNA precursors.[3-6] As several polycistronic mRNAs have also been found to be substrates for RNase P, it has been suggested that this enzyme plays multiple roles in regulation of gene expression.[7,8] Li and Altman[8] showed that *tna*, *secG*, *rbs*, and *his* operons contain RNase P cleavage sites. These authors searched for new mRNA substrates of RNase P by comparing mRNA levels between *E. coli rnpA49* cells grown at 30 °C and 43 °C. The *rnpA49* strain generates mutant C5 protein that is inefficiently assembled with M1 RNA at 43 °C, causing a deficiency of RNase P holoenzyme in the cell.[9] This previous study might not have identified all mRNA substrates of RNase P due to two possible limitations. First, since mRNAs were prepared at 43 °C, mRNA substrates poorly expressed at this high temperature might not be present at adequate levels. Second, it is possible that mRNAs might be cleaved by M1 RNA alone regardless of the presence of C5 protein. Recently, we reported that KS2003, an M1 RNA processing-defective mutant, generates much less M1 RNA compared to the wild-type strain.[10] Using this M1 RNA-deficient strain, we performed high-density microarray analysis in the present study. From the microarray data we selected six polycistronic mRNAs that showed large differences in mRNA expression between adjacent genes. We analyzed whether these polycistronic mRNAs might be substrates of M1 RNA or RNase P holoenzyme *in vitro*. Analysis of the model substrates suggested that five polycistronic mRNAs were substrates of RNase P holoenzyme, but none of them were cleaved by M1 RNA alone. Our results suggest that RNase P-mediated post-transcriptional regulation is achieved by regulating the stability of each cistron through cleavage of a polycistronic mRNA by RNase P.

Experimental Section

Bacterial strains. *E. coli* KS2001 (wild type Δ*zha-295::kan* control), and KS2003 (with a mutation GAUUU to CCCCA at the *rne*-dependent site, Δ*zha-295::kan*)[10] are derivatives of the same original strain, MG1655.

Cell culture and total RNA preparation. Overnight cultures grown in rich medium (LB) with 30 μg/mL kanamycin at 37 °C were diluted 100-fold into fresh medium and incubated at 37 °C with agitation for 17 h 30 min (A_{600} ~3.6). Total RNAs were extracted from cells using a Qiagen RNeasy Mini kit. For quality control of RNA samples for microarray analysis, total RNA samples were examined for integrity and purity. Each sample showed a 23S/16S ratio of > 1.8 and the 260 nm/280 nm absorption ratio was > 2.0.

Microarray analysis. Total RNA samples were sent to Affymetrix for microarray analysis using the Affymetrix standard protocol to measure mRNA levels on GeneChip® *E. coli* Genome 2.0 arrays. Each RNA sample was assayed

independently. From the microarray data, genes whose expression differed by more than twofold in KS2003 compared to KS2001 were identified. Amongst these genes, polycistronic operon mRNAs showing large differences in expression between the adjacent genes in the same mRNA transcript compared with KS2001 were chosen for further analysis.

Preparation of RNA substrates. Model substrates spanning an adjacent pair of genes for each polycistronic mRNA were prepared from the corresponding DNA templates by *in vitro* transcription.[11] The DNA templates for acpPfabF, cysNC, flgMN, lepAB, phoPQ, and puuBE were obtained by PCR from chromosomal DNA of MG1655 with suitable pairs of primers: primer T7 acpPfabF-up, 5'-AGGTAATACGACTCACTATAGGGCCACCAGGCGTAAG-3' and primer acpPfabF-dn, 5'-AGGCATCCATCTTGCGCTGTTCTTTGCGCGAG-3' for acpPfabF; primer T7 cysNC-up, 5'-AGGTAATACGACTCACTATAGGGTGCCGGTATGTG-3' and primer cysNC-dn, 5'-TCCAGCGCCCCGGCGACCGTTG-3' for cysNC; primer T7 flgMN-up, 5'-AGGTAATACGACTCACTATAGGGCAAAATTGCCGATG-3' and primer flgMN-dn, 5'-TTGCTGTATTGGGTTCTTTCCTGCGTAAC-3' for flgMN; primer T7 lepAB-up, 5'-AGGTAATACGACTCACTATAGGGCGATATCAGCCGTAAG-3' and primer lepAB-dn, 5'-CATAAAATGCCCGTCACCAGTGTGGCAATC-3' for lepAB; primer T7 phoPQ-up, 5'-AGGTAATACGACTCACTATAGGGACGTCTGCGCA-3' and primer phoPQ-dn, 5'-CAACTTATTGTTTTCCCACTTCGCAAGGGTATAG-3' for phoPQ; primer T7 puuBE-up, 5'-AGGTAATACGACTCACTATAGGGCGCACGCTGCGTG-3' and primer puuBE-dn, 5'-ATCAGGGTGGCGATGTCCGGTATTCAGCAC-3' for puuBE. Each PCR product was used to generate RNA substrates synthesized by T7 RNA polymerase. Precursor $tRNA^{Phe}$ ($ptRNA^{Phe}$) was prepared as a run-off transcript by transcription with T7 polymerase using *Bst*N1-cleaved pBO201 (Park, B. H. and Lee, Y., unpublished results). The RNAs prepared by *in vitro* transcription were purified in 5% (w/v) polyacrylamide gels containing 7 M urea prior to use. RNA was internally labeled by *in vitro* transcription with [α-^{32}P]CTP or posttranscriptionally labeled by incorporation of a labeled group at the 5' terminus of the transcript. To prepare 5' end-labeled RNA, RNA was incubated with calf intestinal alkaline phosphatase[12] to generate free 5'-OH groups, and then labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP.

RNase P cleavage assay and measurement of kinetic parameters. M1 RNA and C5 protein were prepared as described.[13] M1 RNA and the RNA substrates were separately renatured by heating for 3 minutes at 80 °C in nuclease-free water, after which the solution cooled at room temperature. The RNase P holoenzyme was assembled by mixing M1 RNA and C5 protein in a ratio of 1:10 in buffer L (50 mM Tris-HCl; pH 8.0, 100 mM NH_4Cl, 10 mM $MgCl_2$) at 37 °C. The RNA substrate was incubated either with the holoenzyme in buffer L or with M1 RNA in buffer H (50 mM Tris-HCl; pH 8.0, 100 mM NH_4Cl, 100 mM $MgCl_2$) at 37 °C. The cleavage reaction was stopped with an equal volume of Gel Loading Buffer II (Ambion). The cleavage products were electrophoresed on 8% (w/v) denaturing gels containing 7 M urea and quantitated by using a Molecular Dynamics PhosphoImager. Experiments measuring single turnovers were performed under conditions where the enzyme was in excess over the substrate ([E]/[S] ≥ 2.5).

Results

Using total RNA prepared from KS2003 cells, which express much less M1 RNA than do wild-type cells, we performed microarray assays to examine differential mRNA expression under the M1 RNA-deficient condition. We identified genes whose expression was changed more than twofold by M1 RNA deficiency. The microarray analysis of total RNA prepared from cells in the stationary phase revealed that 60 genes were expressed at much higher levels, and 74 genes at much lower levels, in KS2003 than in the KS2001 control wild-type strain. We chose to examine six polycistronic mRNAs (acpP-fabF, cysDNC, flgMN, lepAB, phoPQ, and puuBE), which showed large differences in expression between adjacent genes in the same polycistronic mRNAs (Fig. 1).

The model RNAs including the intergenic regions for these polycistronic mRNAs were synthesized as molecules 200 to 300 nt in size *in vitro* to determine whether they might be substrates for RNase P. The start and stop sites for each model RNA on the *E. coli* chromosome are listed in Table 1. The six model RNAs were analyzed for RNase P cleavage by M1 RNA alone or by RNase P holoenzyme. The $ptRNA^{phe}$ was used as a positive control substrate for RNase P. The main RNase P cleavage products are indicated by boxes in Figure 2. Five RNAs (cysNC, flgMN, lepAB, phoPQ, and puuBE), but not acpPfabF RNA, were cleaved by RNase P holoenzyme. These results suggest that five

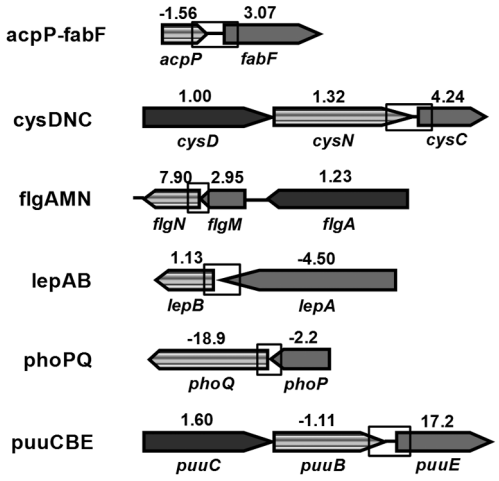

Figure 1. Gene structures of polycistronic mRNAs used as substrate candidates for RNase P. The number above the name of the gene stands for the fold-change in the corresponding mRNA level in KS2003 compared with the KS2001 wild type strain, as given by microarray data. Minus (–) means a decrease in mRNA level. The regions corresponding to model substrate RNAs for each polycistronic mRNA are boxed.

Table 1. RNA targets for the RNase P cleavage assay

Polycistronic mRNA	Model RNA	Size (nt)	Region on *E. coli* chromosome[a]
acpP-fabF	acpPfabF	323	1151060 → 1151381
cysDNC	cysNC	287	2872155 → 2871869
flgAMN	flgMN	264	1129114 → 1128852
lepAB	lepAB	210	2703481 → 2703273
phoPQ	phoPQ	300	1189089 → 1188790
puuCBE	puuBE	292	1363462 → 1363753

[a]Coordinates based on those of Blattner *et al.*[15]

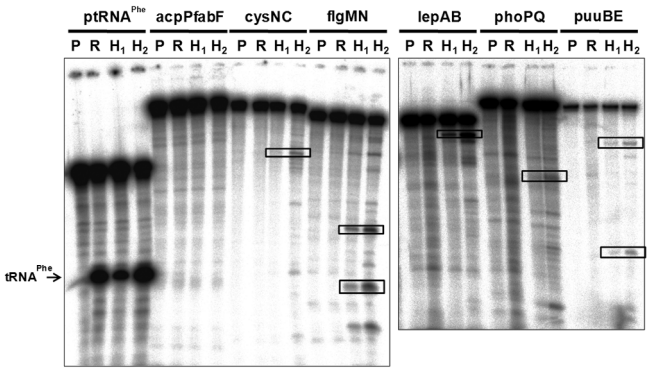

Figure 2. RNase P cleavage reaction. Model substrate RNAs for each polycistronic mRNA were prepared by *in vitro* transcription. The ptRNA^{phe} (100 nM) and model substrate RNAs (50 nM) labeled at the 5' ends were incubated with RNase P for 40 min at 37 ^{o}C. The acpPfabF model RNA (323 nt), cysNC model RNA (287 nt), flgMN model RNA (264 nt), lepAB model RNA (210 nt), phoPQ model RNA (300 nt), and puuBE model RNA (292 nt), were used. Lane P, C5 protein 200 nM; Lane R, M1 RNA 55 nM; Lane H_1, RNase P holoenzyme 2 nM; Lane H_2, RNase P holoenzyme 20 nM. Reaction products were separated on an 8% (w/v) polyacrylamide gel containing 7 M urea. The cleavage products of model substrate RNAs by RNase P holoenzyme are indicated in boxes.

mRNAs might be substrates of RNase P in the cell. However, none of the RNA substrates were cleaved by M1 RNA alone, suggesting that C5 protein is essential for RNase P cleavage of mRNA substrates.

To determinate the cleavage sites, we generated RNA ladders from the same RNA substrates by alkaline hydrolysis and RNase T1 digestion. The main cleavage sites of each RNA substrate were estimated by matching the bands of cleaved products to the ladders and assigned to each polycistronic mRNA sequence (Fig. 3). The secondary structures of five model RNA substrates were predicted by RNase V1 digestion and calculations with the Zuker program (www.bioinfo.rpi.edu/applications/mfold),[14] and the structures of phoPQ and puuBE are shown as representatives in Figure 4. The RNase V1-cleavage sites are mostly distributed within the predicted double-stranded region. No cleavage site of RNase P in these model RNAs corresponds to the classical model RNase P cleavage site which is located at the 5' base of a helix in the structure drawn. The RNase P cleavage sites of the model RNAs are located at the middle of a stem or in a loop.

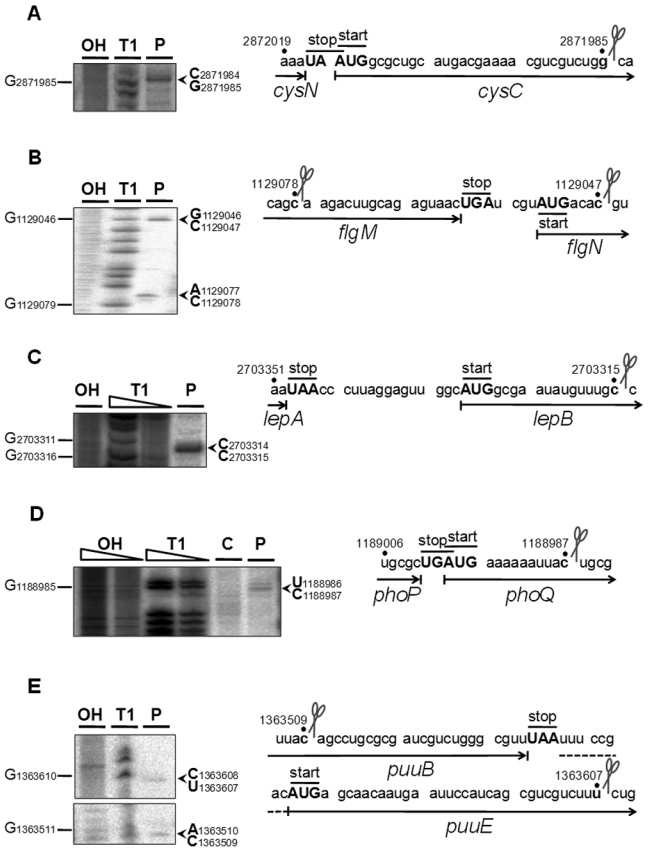

Figure 3. Determination of cleavage sites of RNase P in model substrate RNAs. RNase P reactions were carried out with RNA substrates labeled with ^{32}P at the 5' ends. Reaction products were separated on an 8% (w/v) polyacrylamide gel containing 7 M urea. Lane OH, partial alkaline hydrolysis; Lane T1, partial RNase T1 digestion of each RNA substrate; Lane P, product RNA cleaved by RNase P (50 nM of RNA substrates, 20 nM of RNase P, 37 ^{o}C, 40 min). The predicted cleavage sites are indicated by arrows and the numbers of these positions on the *E. coli* MG1655 chromosome are shown on the right sides of the gels. The positions of cleavage sites are also shown in the corresponding mRNA sequences. A, cysNC model RNA; B, flgMN model RNA; C, lepAB model RNA; D, phoPQ model RNA; E, puuBE model RNA.

To compare the cleavage efficiencies of ptRNA^{Phe} and polycistronic mRNA, the single turnover RNase P activity was measured by RNase P cleavage assay. The k_{cat} and K_M values of the cleavage reactions of ptRNA^{Phe} and lepAB RNA are shown in Table 2. The lepAB model RNA was chosen as representative of the five model RNA substrates because RNase P cleavage efficiency of lepAB RNA was one of the highest seen (Fig. 2). The single turnover k_{cat}/K_M value for lepAB RNA was about 200-fold lower than that for ptRNA^{Phe}. These data suggest that RNase P cleaves polycistronic mRNAs with low efficiency although RNase P processes precursor tRNA with high efficiency.

Table 2. Single turnover kinetic parameters in RNase P reactions

Substrates	K_M, μM	k_{cat}, min^{-1}	k_{cat}/K_M, min$^{-1}\cdot\mu$M^{-1}
ptRNA^{Phe}	0.0065	0.16	24
lepAB	0.015	0.0019	0.13

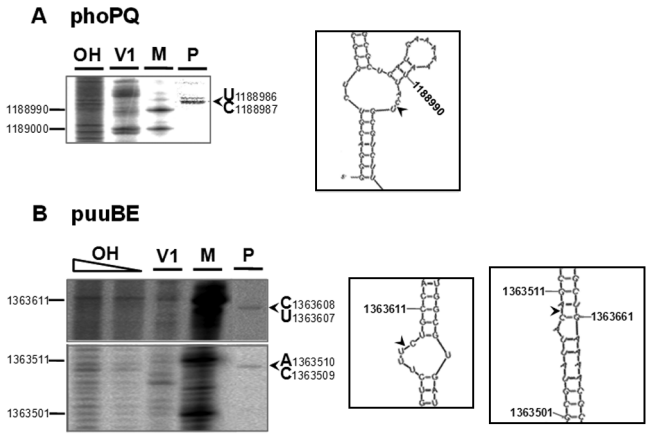

Figure 4. RNase V1-probing of model substrate RNAs. RNAs transcribed *in vitro* were [^{32}P]-labeled at the 5' ends and 0.2 pmol of each labeled RNA was treated with 0.005 units of RNase V1 at room temperature for 15 min. The reaction products were separated on an 8% (w/v) polyacrylamide sequencing gel containing 7 M urea. Two representative model substrates, phoPQ (A) and puuBE (B), are shown in the figure. Lane OH, partial alkaline hydrolysis ladders; lane M, RNA Decade^{TM} markers (Ambion). Lane P, products cleaved by RNase P (50 nM of RNA substrates, 20 nM of RNase P, 37 ^{o}C, 40 min). The numbers to the right of the gels indicate the positions of RNase P cleavage sites on the bacterial chromosome. The predicted secondary structures near cleavage sites are shown on the right sides of the figure. The RNase P cleavage sites are indicated by arrows.

Discussion

M1 RNA can itself cleave ptRNA in the absence of C5 protein *in vitro*. All known mRNA substrates for RNase P are not cleaved by M1 RNA alone, but by RNase P holoenzyme. We wondered if there might be any mRNA substrates cleaved by M1 RNA alone. We initially sought to discover new mRNA substrates that could be cleaved not only by RNase P holoenzyme but also by M1 RNA alone by analyzing mRNAs from M1 RNA-deficient cells. Through microarray analysis we selected six polycistronic mRNAs with large differences in expression levels of adjacent genes in the same operons. Since model substrates for five of them were cleaved by RNase P, the expression differences of adjacent genes given by the microarray data could result from RNase P cleavage of the corresponding mRNAs. However, M1 RNA alone did not cleave any of the five model RNAs, suggesting that cleavage of mRNA by RNase P strictly requires C5 protein.

When the RNase P cleavage sites of the model RNAs were mapped in secondary structures predicted by mfold and RNase V1 probing, we found that the cleavage sites seemed to differ from those in pre-tRNA substrates. The single turnover kinetic parameter k_{cat}/K_M was determined by RNase P cleavage assay using several different substrate concentrations. The k_{cat}/K_M value of lepAB model RNA, chosen as the representative of five model RNAs, is about 200-fold lower than that of ptRNA^{phe}. The low cleavage rate of mRNA by RNase P is mainly because of much lower turnover rates (k_{cat}). The lower cleavage efficiency of mRNAs suggests that RNase P may be utilized for controling the intracellular concentration of mRNAs carefully rather than affording complete processing as in the case with precursor tRNAs; RNase P may play a role in the fine-tuning of mRNA degradation. Alternatively, certain factors may be needed for activation of RNase P cleavage activity; these factors may play an important role in regulating the intracellular level of specific mRNAs.

We have shown that some polycistronic mRNAs are cleaved by RNase P and that this cleavage could lead to differential expression of cistrons in each polycistronic mRNA. However, it remains to be demonstrated how such differential expression is controlled in the cell and what the biological consequences of this regulation may be. Since we have identified several new mRNA substrates for RNase P, in addition to previously known mRNA substrates, it seems likely that mRNA substrates for RNase P are more abundant than expected and there may be a RNase P-mediated global regulating system controlling mRNA levels in *E. coli*.

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