FEBS Letters 583 (2009) 419–424

FEBS Letters

journal homepage: www.FEBSLetters.org

Novel function of C5 protein as a metabolic stabilizer of M1 RNA

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ARTICLE INFO

Article history:
Received 30 October 2008
Revised 4 December 2008
Accepted 17 December 2008
Available online 27 December 2008

Edited by Ulrike Kutay

Keywords:
M1 RNA
C5 protein
RNase P
RNA stability

ABSTRACT

***Escherichia coli* RNase P is a ribonucleoprotein composed of a large RNA subunit (M1 RNA) and a small protein subunit (C5 protein). We examined if C5 protein plays a role in maintaining metabolic stability of M1 RNA. The sequestration of C5 protein available for M1 RNA binding reduced M1 RNA stability in vivo, and its reduced stability was recovered via overexpression of C5 protein. In addition, M1 RNA was rapidly degraded in a temperature-sensitive C5 protein mutant strain at non-permissive temperatures. Collectively, our results demonstrate that the C5 protein metabolically stabilizes M1 RNA in the cell.**

1. Introduction

Escherichia coli RNase P was initially characterized as a tRNA processing enzyme that removes extraneous 5′ sequences from precursor tRNAs to generate mature 5′ termini [1]. In addition to assisting with tRNA processing, RNase P also contributes to the processing of other non-tRNA substrates (e.g. 4.5S RNA and tmRNA) and assists with decay of several mRNAs [2–6]. The RNase P holoenzyme is composed of two subunits, including a large RNA subunit (M1 RNA, 377 nucleotides) and a small basic protein (C5 protein, 119 amino acids). While M1 RNA can perform catalytic reactions in the absence of C5 protein in vitro [7], both components are essential in vivo [8]. C5 protein stabilizes the catalytically active conformation of M1 RNA [9,10] and modulates the substrate specificity of the RNase P reaction [11–16].

M1 RNA is a metabolically stable RNA [17,18]. However, it has not yet been known whether M1 RNA is intrinsically stable or whether this RNA requires additional factors to maintain its stability. Since the depletion of one or more proteins that are part of ribonucleoprotein complexes can decrease the endogenous level of their RNA components [19], C5 protein could be a factor required for metabolic stability of M1 RNA. Studies with temperature-sensitive C5 protein mutant *E. coli* strains demonstrated that cell growth at the non-permissive temperature results in substantially reduced M1 RNA levels compared to wild-type cells [20,21]. In addition, it has recently been shown that overexpression of RNase P protein leads to increased steady-state levels of RNase P RNA in *Bacillus subtilis* [22]. However, these studies provide no direct evidence of the involvement of C5 protein in the maintenance of the metabolic stability of M1 RNA, because the cellular RNA levels could be affected by an alteration of transcription.

In this study, we sought to determine if C5 protein is required for the metabolic stability of M1 RNA using a new approach where C5 protein is sequestered by overexpression of enzymatically defective but C5 protein-binding-competent variants of M1 RNA. Our findings revealed that C5 protein metabolically stabilizes M1 RNA in vivo. This phenomenon of C5 protein-dependent M1 RNA stability, although observed before, has never been investigated in such detail.

2. Materials and methods

2.1. Bacterial strains and plasmids

The *E. coli* K-12 strain JM109 was used for plasmid propagation and RNA analysis. The *E. coli* temperature-sensitive C5 protein mutant isogenic strains NHY312 (*rnpA*$^{+}$) and NHY322 (*rnpA49*) [21] were also used for RNA analysis. Plasmid pLM1, a derivative of the pGEM3 vector, which contains the *rnpB* sequence with flanking sequences spanning positions –270 to +1286 [23], was used to construct additional plasmids capable of overexpressing truncated M1 RNAs. The deletions were introduced by gene splicing via overlap extension (SOEing) PCR mutagenesis, as previously described [24], to generate plasmids, pLMdP3, pLMdP8/9, and pLMdP12. The C5 protein-expressing plasmid, pACTC5, was a derivative of

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0014-5793/$34.00
doi:10.1016/j.febslet.2008.12.040

the pACYC184 vector, which can co-exist with pGEM3 [25]. The *rnpB*-containing fragments in the pLM plasmid series were also cloned into the *Bam*HI-*Hin*dIII sites of pACYC184 to generate pACYC derivatives that overexpressed truncated M1 RNAs. The primers used in this study are described in Table 1. Plasmid pMC5 [26] was used to express C5 protein as a fusion with maltose-binding protein (MBP).

2.2. In vitro transcription of RNA

DNA templates used for the in vitro transcription of M1 RNA and truncated M1 RNA derivatives were obtained by PCR from pLM1 or corresponding deletion plasmids (Table 1). In vitro transcription was performed using T7 RNA polymerase and RNA was purified by gel elution. The in vitro transcripts contained extra GG sequences at their 5′ ends.

2.3. Gel mobility shift assays

MBP-C5 protein was purified from cell containing pMC5 and gel mobility shift assays between MBP-C5 protein and the truncated M1 RNA derivatives were performed, as previously described [27].

2.4. Preparation of RNA bound to MBP-C5 protein

JM109 cells containing both pMC5 and a pACYC184 derivative that overexpressed truncated M1 RNA were grown to an A_{600} of 0.5. Expression and purification of MBP-C5 protein were carried out, as described previously [28], except that cell extracts were prepared by grinding the cell pellet with alumina in the presence of 10 mM ribonucleoside vanadyl complex (NEB) and protease inhibitor cocktail (Pharmacia).

2.5. Total cellular RNA preparation

JM109 cells containing plasmids were grown overnight (37 °C) in LB containing ampicillin (50 mg/ml) or tetracycline (25 μg/ml), diluted 1:100 in the same medium, and grown to an A_{600} of approximately 0.5 at 37 °C. When necessary, expression of C5 protein was induced via the addition of 0.1 mM IPTG, followed by 10 min incubation. For RNA stability assays, rifampicin (150 μg/ml) was added to the cultures to stop transcription. Cultures were then analyzed at different time intervals. In case of *rnpA49* isogenic strains, cells were grown overnight at 30 °C, diluted (1:100) into the fresh media, and grown to an A_{600} of 0.25. Cultures were divided and incubated at 43 °C for 10 min before the rifampicin treatment. Total cellular RNA was prepared by hot phenol extraction, as described previously [23].

2.6. Northern blot analysis

The RNA samples were subjected to Northern blot analysis, as described previously [18]. The probes used for the M1 RNA detection were an anti-M1 RNA riboprobe [23], or stem-specific oligonucleotide probes. The stem-specific probes were used to detect intact M1 RNA, but not truncated derivatives. tRNA, 6S RNA and 5S RNA were analyzed with antisense oligonucleotide probes (Table 1). The internally ^{32}P-labeled anti-M1 RNA riboprobe was prepared by T7 RNA polymerase using the *Hin*dIII-treated pLMd23 as a template [23]. Oligonucleotide probes were ^{32}P-labeled at their 5′ ends.

2.7. Immunoblot analysis

Total cell lysates or the purified MBP-C5 protein fractions were subjected to immunoblot analysis using C5 protein-specific antibody. Antibody to C5 protein, generated by coupling residues 57–71 to bovine serum albumin, was provided by Ab Frontier.

2.8. Cell growth analysis

A single colony from each strain was inoculated into 1.5 ml LB containing ampicillin (50 μg/ml). The overnight culture was then diluted 1:100 in 30 ml of fresh medium containing ampicillin (50 μg/ml) in a 250-ml Erlenmeyer flask and continued to be grown at 37 °C. Cell growth was monitored by recording A_{600} values at various times.

3. Results

3.1. Overexpression of truncated M1 RNA decreases the metabolic stability of M1 RNA

To determine if C5 protein affects metabolic stability of M1 RNA, we sequestered C5 protein available for M1 RNA binding in vivo. We hypothesized that overexpression of a competitor RNA for C5 protein binding would reduce the amount of C5 protein available for the formation of the RNase P holoenzyme. To test this hypothesis, first, we constructed plasmids overexpressing P3-, P8/9- and P12-deleted M1 RNA derivatives (Fig. 1). The P3 domain of

Table 1
Sequences of the oligonucleotides used in this study.

Names	Sequences (5′–3′)	Description
1_pLM1	GGG GTACCGATTGGTGTCGCAAAC G	Generation of deleted *rnpB*
4_pLM1	CGAATTCTCCGGACAATGGCTTTGAGG	Generation of deleted *rnpB*
2_P3	GCCCTATGGAGCCCGGACTTTCCTCGCGACTGTCTGGTCAGC	Generation of P3-deleted *rnpB*
3_P3	GAGGAAAGTCCGGGCTCCATAGGGC	Generation of P3-deleted *rnpB*
2_P8/9	CGGTTTGCTCTCTGTTGCACTGGTCCACCCTGCCCTATGG AGCC	Generation of P8/9-deleted *rnpB*
3_P8/9	GACCAGTGCAACAGAGAGCAAACCG	Generation of P8/9-deleted *rnpB*
2_P12	TGCGCTCTTACCGCACCCTTTCACCGTTTGCTC TCTGTTGCACTG	Generation of P12-deleted *rnpB*
3_P12	GGTGAAAGGGTGCGGTAAGAGCGCA	Generation of P12-deleted *rnpB*
5_pACYC	GAGGATCCCCGGGTACCGATTGGTGTC	Cloning of deleted *rnpB* into pACYC184
6_pACYC	CCCAAGCTTCCGGACAATGGCTTTGAGG	Cloning of deleted *rnpB* into pACYC184
T7-M1(+1)	AGGTAATACGACTCACTATAGGGAAGCTGACCAGACAGTC	DNA template for in vitro transcription
M1 (+377)	AGGTGAAACT GACCGATAAG CCGGGTTC	DNA template for in vitro transcription
Anti-rnpB-P3	GTCTCCCCCGAAGAGGACGACGACG	Stem-specific probe
Anti-rnpB-P8	GGTTTCCCCCCCAGGCGTTACCTGG	Stem-specific probe
Anti-rnpB-P12	GATCCCGCTTGCGCGGGCCATC	M1 RNA probe
Anti-tRNA^{Tyr}	ATTTACAGTCTGCTCCCTTTGGCCGCT	tRNA^{Tyr} probe
Anti-tRNA^{Phe}	GGACTCGGAATCGAACCAAG	tRNA^{Phe} probe
Anti-6S	CCGACCGGTATTTCTCTGAGATGTTCGCA	6S RNA probe
Anti-5S	CGGCATGGGGTCAGGTGG	5S RNA probe

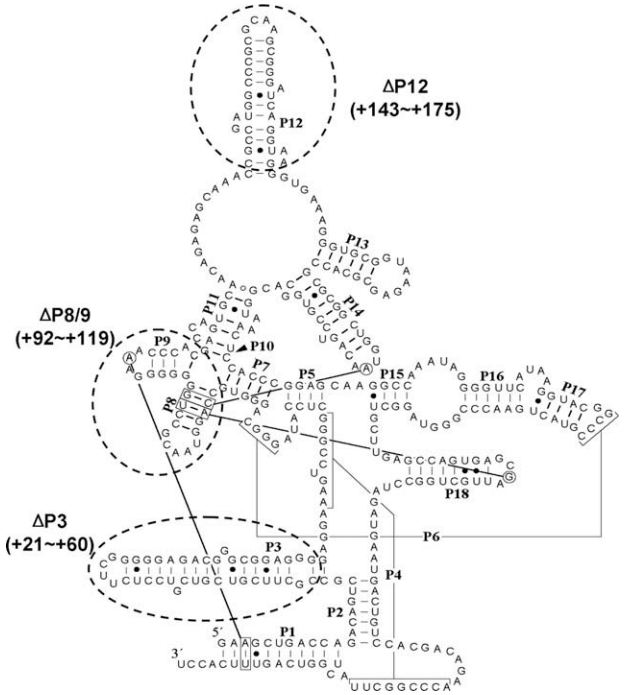

Fig. 1. Deleted regions in the truncated M1 RNA derivatives. Deletion regions (dotted circle) within the truncated M1 RNA derivatives are indicated in the secondary structure of M1 RNA [29].

M1 RNA interacts with C5 protein in the RNase P holoenzyme, whereas the P8/9 and P12 regions are not essential for the interaction [30,31]. We assessed binding of these truncated M1 RNA derivatives to C5 protein. Electrophoretic gel mobility assays with MBP-C5 protein revealed that P8/9-deleted M1 RNA had a binding affinity similar to that of intact M1 RNA. However, binding affinity was very poor in P3-deleted M1 RNA (Fig. 2A). Unfortunately, we

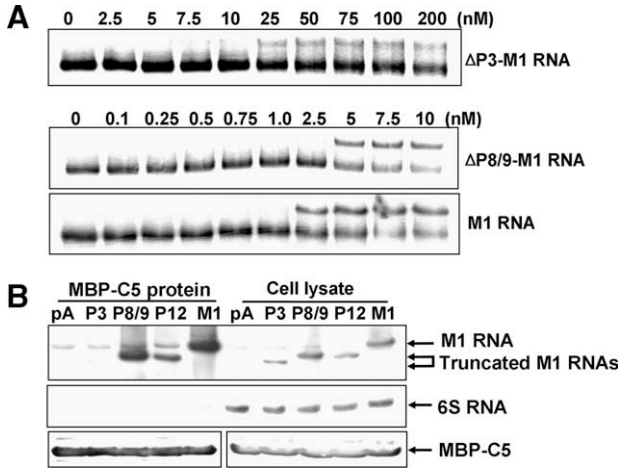

Fig. 2. Binding of truncated M1 RNA derivatives to C5 protein. (A) ^{32}P-labeled M1 RNA derivatives (0.5 nM) were incubated with increasing amounts of MBP-C5 protein, as indicated. Electrophoretic gel mobility shift assays were performed to assess interactions between RNA and protein. (B) MBP-C5 protein was purified from lysates of cells overexpressing both truncated M1 RNA and MBP-C5 protein. RNA extracted from the purified protein, or RNA extracted from the cell lysates as total cellular RNA, was subjected to Northern blot analysis using an anti-M1 probe. The following RNAs were overexpressed: P3, P3-deleted M1 RNA; P8/9, P8/9-deleted M1 RNA; P12, P12-deleted M1 RNA; M1, intact M1 RNA; pA, vector (pACYC184) control. The contents of MBP-C5 protein in the purified fractions or lysates were also assessed via immunoblot analysis using C5 protein-specific antibody.

were not able to generate a reproducible binding affinity for P12-deleted M1 RNA, as this RNA was partially degraded during incubation with the MBP-C5 fusion protein (data not shown). Instead, we assessed the binding ability of P12-deleted M1 RNA to C5 protein in the cell (Fig. 2B). The MBP-C5 protein was purified from *E. coli* cells overexpressing both MBP-C5 protein and truncated M1 RNAs. Truncated M1 RNAs bound to the purified MBP-C5 protein were examined via northern blot analysis. High concentrations of P12-deleted M1 RNA and P8/9-deleted RNA were observed to bind to the MBP-C5 protein, whereas the signal of P3-deleted M1 RNA remained very low. These data as well as in vitro binding analysis assured that overexpression of the P8/9- and P12-deleted M1 RNA derivatives could sequester C5 protein available for M1 RNA binding within cells.

We then investigated the stability of M1 RNA in cells overexpressing truncated M1 RNA derivatives (Fig. 3). In cells overexpressing P3-deleted M1 RNA and control cells, the $t_{1/2}$ values of endogenous M1 RNA were 39 and 59 min, respectively. However, the $t_{1/2}$ values in cells overexpressing P8/9- or P12-deleted M1 RNA decreased to 10 and 8.6 min, respectively. These data indicate that the sequestration of C5 protein via overexpression of C5 protein-interacting RNA reduces the metabolic stability of M1 RNA.

3.2. Overexpression of C5 protein restores the metabolic stability of M1 RNA

We analyzed the metabolic stability of M1 RNA in cells co-overexpressing truncated M1 RNA derivatives and C5 protein (Fig. 4). C5 protein was overexpressed via IPTG induction of pACTC5. Co-overexpression of C5 protein restored the metabolic stability of M1 RNA in cells overexpressing P8/9- or P12-deleted M1 RNA. Excess C5 protein also slightly increased the metabolic stability of M1 RNA in cells overexpressing P3-deleted variants or control cells. These data clearly support the role of C5 protein in the metabolic stabilization of M1 RNA in vivo.

3.3. The mutation rnpA49 decreases the metabolic stability of M1 RNA

To further confirm that C5 protein is required for M1 RNA stability, we used an *rnpA49* strain carrying the thermosensitive mutant phenotype in C5 protein function [32]. At 43 °C (non-permissive temperature), the half-life of M1 RNA in *rnpA49* cells significantly decreased with the value of 7.3 min, compared to 40 min in *rnpA*$^+$ cells (Fig. 5). This result shows that functional C5 protein is required for M1 RNA stability in vivo, in line with previous findings [20]. It has been proposed that the *rnpA49* mutation leads to a defect in the assembly of the mutant C5 protein into RNase P holoenzyme at the non-permissive temperature [33], because the temperature-sensitive phenotype of *rnpA49* cells is complemented by expression of excess M1 RNA [20], and the addition of excess M1 RNA to in vitro RNase P reconstitution reactions with the mutant C5 protein increases its enzymatic activity [33]. Thus, our data suggest that assembly of RNase P holoenzyme is essential for maintaining the metabolic stability of M1 RNA in the cell.

3.4. C5 protein depletion retards tRNA processing and cell growth

We examined how depletion of C5 protein might affect the catalytic activity of RNase P in vivo (Fig. 6A). Northern blot analysis of total RNA isolated from cells overexpressing P8/9- or P12-deleted M1 RNA derivatives revealed some defects in tRNA^{Tyr} and tRNA^{Phe} processing with accumulation of their precursors, whereas these defects were not observed in cells overexpressing P3-deleted M1 RNA. We further examined the growth of cells overexpressing P8/9- or P12-deleted M1 RNA (Fig. 6B). Growth was slower in these cells, compared with cells overexpressing the P3-deleted M1 RNA

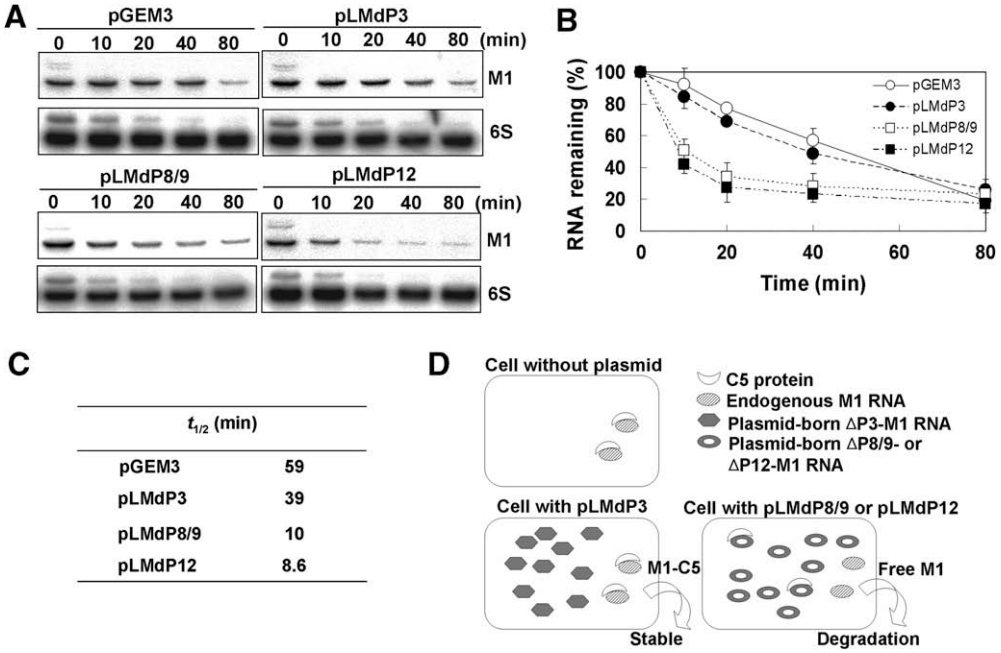

Fig. 3. Analysis of M1 RNA stability in cells overexpressing truncated M1 RNA derivatives. (A) Effects of overexpressing the truncated M1 RNA derivatives on the metabolic stability of endogenous M1 RNAs. Total cellular RNA was isolated from cells containing plasmids at the indicated times after the rifampicin treatment. RNAs were subjected to Northern blot analysis using stem-specific probes covering the deletion region of the corresponding plasmid-encoded M1 RNA variant to quantitate endogenous M1 RNA exclusively. Each truncated RNA does not hybridize with the corresponding stem-specific probe due to the stem deletion. Each RNA sample was analyzed along with RNA from cells containing the empty vector pGEM3 in the same Northern filter. (B) The band intensity of endogenous M1 RNA was normalized to that of 6S RNA. The remaining RNA (% of initial level) was calculated as a ratio of the amount of RNA at each indicated time to the amount of RNA at the rifampicin addition point. (C) The $t_{1/2}$ values were determined by linear regression of data obtained from four independent experiments. (D) Possible mechanism of M1 RNA instability via the overexpression of P8/9- or P12-deleted M1 RNA. When overexpressed RNA sequesters C5 protein, free M1 RNA is rapidly degraded.

or control cells. Therefore, it is likely that this slow growth arose from less RNase P activity.

4. Discussion

In this study, we examined the role of C5 protein in the metabolic stability of *E. coli* M1 RNA. We developed a novel method for sequestration of C5 protein in the cell. Reducing the amount of C5 protein available for RNase P holoenzyme assembly decreased the metabolic stability of M1 RNA and reduced the half-life of this RNA from 60 min to 10 min. Therefore, C5 protein appears to be required for the metabolic stability of M1 RNA in vivo. In addition, M1 RNA is rapidly degraded in *rnpA49* cells at the non-permissive temperature when C5 protein is not functional [20] (Fig. 5). This finding further supports the role of C5 protein in the maintenance of M1 RNA stability. It has been well established that C5 protein play roles in stabilizing the catalytically active conformation of the ribozyme M1 RNA [9,10] and modulating the substrate specificity [11–15]. Therefore, our results suggest that C5 protein functions not only as a cofactor in the ribozyme reactions, but also as a metabolic stabilizer of M1 RNA in vivo.

In light of our findings, we propose a working model for the stabilization of M1 RNA (Fig. 7). First, M1 RNA is transcribed from *rnpB*. The primary transcript, pM1 RNA, is then processed at the 3′ end to generate M1 RNA [23,34,35]. The pM1 RNA is metabolically unstable, with a half-life of 5 min, because of polyadenylation-dependent degradation at the 3′ end [18]. However, this polyadenylation does not occur when the 3′ end undergoes maturation by processing, resulting in a metabolically stable RNA with a half-life of 60 min. The stability of M1 RNA can be sustained only in the presence of C5 protein. Without C5 protein, M1 RNA rapidly degrades with a half-life of 10 min.

C5 protein sequestration by overexpression of truncated M1 RNA gave rise to some production of mature tRNA, although precursor tRNAs accumulated. These data suggest that cells with depleted levels of free C5 protein retained residual RNase P activity, perhaps by some intact M1 RNAs winning in competition with truncated derivatives for C5 protein binding. Alternatively, ribonucleoprotein complexes (composed of truncated M1 RNAs and C5 protein) may have retained some catalytic activities [36,37].

It has been hypothesized that life originated from an RNA-dependent environment, where RNA operated as the functional molecule [38]. As life evolved, many of the tasks performed by RNA were transferred to proteins. In addition, it has been speculated that some RNP complexes (e.g. RNase P, ribosomes, and spliceosomes) represent vestiges of the transition from a RNA-driven environment to the contemporary world of proteins [7,39–42]. During this transition, proteins were thought to assist with the tasks performed by RNA molecules, as the protein components of contemporary RNP complexes function as cofactors, whereas catalytic activities are retained by some RNAs. Given that RNA molecules are chemically and biologically unstable, it also remains possible that proteins emerged in the RNA world during the early evolution of life to prevent the degradation of RNA molecules. Our findings support this possibility by demonstrating that C5 protein acting as a protein cofactor is also essential for the stability of M1 RNA.

As C5 protein is only one-tenth the size of M1 RNA, it is very interesting that this small protein is capable of protecting such a large RNA molecule from degradation. The site for the initial cleavage as a committed step of M1 RNA turnover may exist in the M1 RNA region that interacts with C5 protein. Alternatively, the cleavage site may be located at another region, as the M1 RNA structure changes with C5 protein binding and may prevent the ribonuclease from accessing that site. It is also possible that C5 binding might affect the metabolic stability of M1 RNA by altering

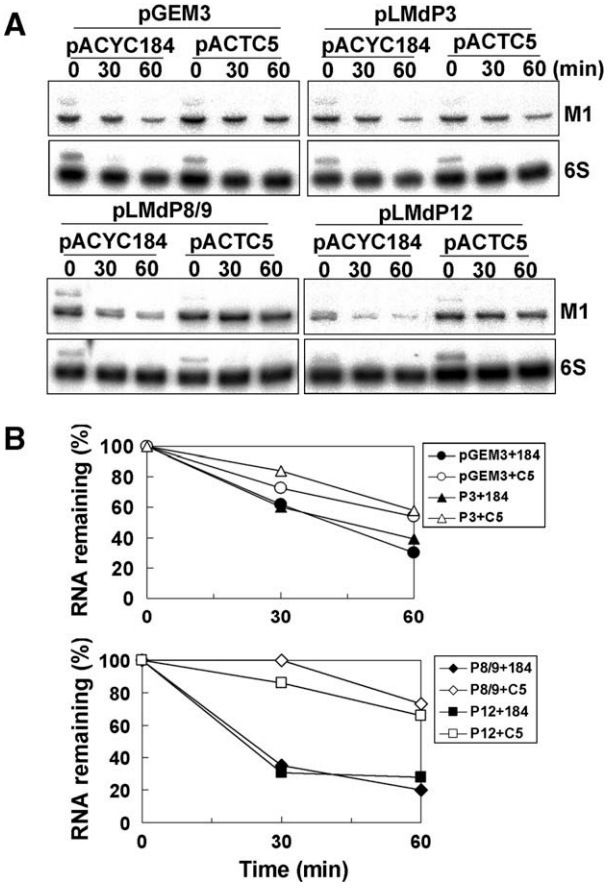

Fig. 4. Effects of C5 protein overexpression on the stability of M1 RNA. (A) The stability of M1 RNA in cells overexpressing truncated M1 RNA, in conjunction with overexpression of C5 protein from pACTC5, was examined by Northern blot analysis using stem-specific probes. (B) The remaining RNA (% of initial level) was calculated, as in Fig. 3B. 184, pACYC184; C5, pACTC5; P3, pLMdP3; P8/9, pLMdP8/9; P12, pLMdP12.

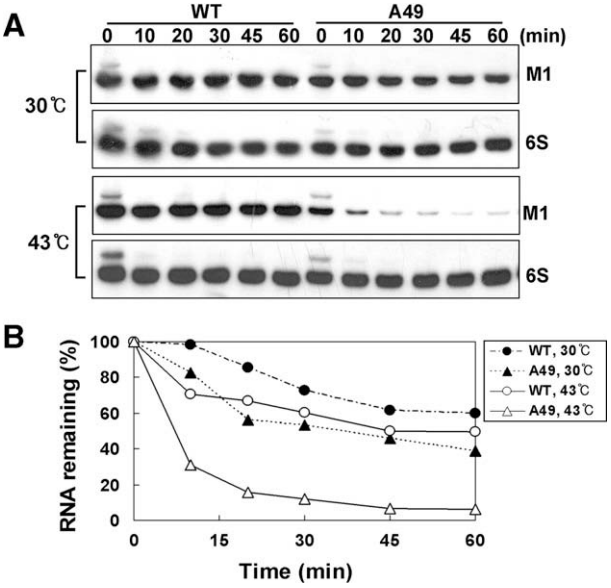

Fig. 5. Stability of M1 RNA in *rnpA49* cells. (A) Rifampicin was added to *rnpA*$^{+}$ (WT) and *rnpA49* (A49) cells grown at 30 °C or 43 °C. Total cellular RNA was isolated from each strain at the indicated times and subjected to Northern blot analysis using the antisense M1 RNA riboprobe. (B) The remaining RNA (% of initial level) was calculated, as in Fig. 3B.

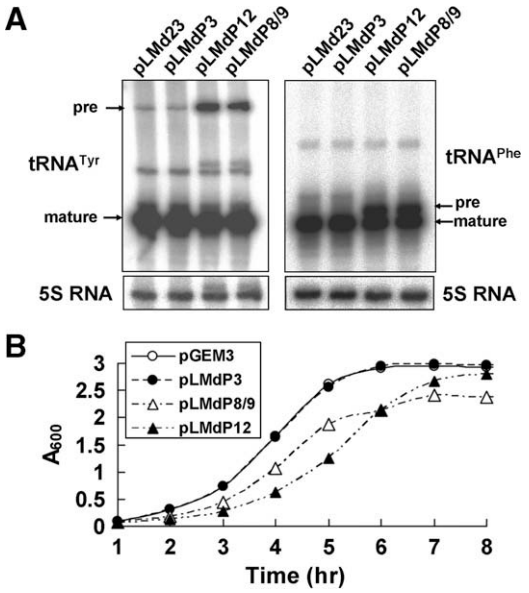

Fig. 6. Effects of C5 protein depletion on tRNA processing and cell growth. (A) Northern blot analysis was performed on total RNAs isolated from cells containing pLMdP3, pLMdP8/9, or pLMdP12. Membranes were probed with anti-tRNA^{Tyr} (left) and anti-tRNA^{Phe} (right) oligonucleotides. (B) Growth of cells overexpressing truncated M1 RNA derivatives.

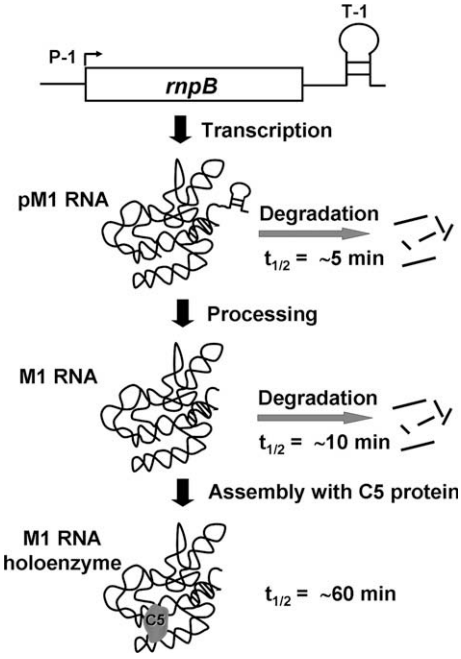

Fig. 7. A model for the maintenance of stable M1 RNA in *E. coli*. A primary *rnpB* transcript (pM1 RNA) is metabolically unstable, with a half-life of 5 min. Processing at the 3′ end increases the stability of M1 RNA, resulting in a half-life of 10 min. The processed product (M1 RNA) binds to C5 protein, forms RNase P holoenzyme and becomes metabolically stable.

other rate-determining steps in RNA degradation, such as polyadenylation of the 3′ end [43] or removal of pyrophosphate at the 5′ end [44]. Although the initial cleavage site and the enzymes responsible for M1 RNA decay remain unknown, the identification of C5 protein as a metabolic stabilizer of M1 RNA provides a tool that can be used to examine the mechanism by which a protein protects RNA from degradation in the cell.

Acknowledgements

We thank Prof. S. Altman for providing *rnpA49* isogenic strains. This work was supported by the 21C Frontier Microbial Genomics and Application Center Program (No. MG08-0201-2-0) and grants (Nos. R01-2005-000-10293-0 and R01-2008-000-11660-0) from the Korea Science and Engineering Foundation.

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