

Implication of RNases PH in Turnover of M1 RNA

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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 tRNA processing, RNase P is involved in the processing of other non-tRNA substrates (e.g., 4.5S RNA and tmRNA) and the decay of several mRNAs.²⁻⁶ The RNase P holoenzyme is composed of two subunits, a large RNA subunit (M1 RNA, 377 nucleotides) and a small basic protein (C5 protein, 119 amino acids). M1 RNA is the catalytic subunit that can perform the reaction as a ribozyme in the absence of C5 protein *in vitro*,⁷ but both the protein and RNA components are essential *in vivo*.⁸ C5 protein stabilizes the catalytically active conformation of M1 RNA^{9,10} and modulates its substrate specificity.¹¹⁻¹⁶ Previously, we have shown that the turnover rate of free M1 RNA increases when C5 protein is sequestered by truncated M1 RNA transcripts, such as P12-deleted M1 RNA, in the cell, suggesting that the protein also functions as a metabolic stabilizer of M1 RNA.¹⁷

The size of C5 protein is only one-tenth of M1 RNA. Therefore, it is an interesting issue how this small protein can protect such a large RNA molecule from degradation. To address this issue, in this study, we tried to identify RNases responsible for the rapid turnover of free M1 RNA. For this purpose, we introduced into cells plasmid pLMd12, which overproduced P12-deleted M1 RNA derivative as a C5 protein-interacting RNA, so that this plasmid-borne, overexpressed M1 RNA derivative could sequester C5 protein available for M1 RNA binding and thereby generate a protein-free form of M1 RNA within cells.¹⁷ Generally RNA degradation is initiated by endoribonucleolytic cleavage and RNase E plays an important role in this endoribonucleolytic cleavage.^{18,19} Therefore, we investigated the turnover rate of free M1 RNA in strains GW20 and GW11, which showed a temperature-sensitive RNase E phenotype and a RNase G mutant phenotype, respectively.²⁰ Total RNAs prepared from GW20 and GW11 cells containing plasmid pLMdP12 were analyzed. The cellular levels of M1 RNA level after the treatment of rifampicin that inhibits transcription were monitored by northern blot analysis and then the metabolic stability of M1 RNA was assessed (Fig. 1). We observed no decrease of the M1 RNA stability in both cells. Surprisingly the M1 RNA stability was not decreased in the wild type control strain GW10 either. This contrasts our previous report that the sequestration of C5 protein *via* overexpression of C5 protein-interacting RNA, such as P12-deleted M1 RNA, reduces the metabolic stability

of M1 RNA.¹⁷ Since we had previously analyzed the M1 RNA stability in strain JM109, this discrepancy may be due to the difference of strains used. Therefore, we examined the M1 RNA stability in various strains with overexpressing P12-deleted M1 RNA (Fig. 2). MC1000 and DH5 α strains showed the decrease of M1 RNA stability like JM109 strain, while MG1655 strain showed little change of M1 RNA stability, as shown in GW10, suggesting that the overexpression of P12-deleted M1 RNA was not effective in MG1655 and GW10. One possibility for the lack of the M1 RNA stability-decreasing effect of P12-deleted M1 RNA in MG1655 and GW10 is that these strains would express P12-deleted M1 RNA at the lower level or C5 protein at the higher level than MC1000, JM109, and DH5 α , and thereby C5 protein would not be sequestered enough to generate protein-free M1 RNA. To test this possibility, expression levels of the truncated M1 RNA and C5 protein were compared among these strains. Northern blot analysis showed that the levels of P12-deleted M1 RNA were comparable or even higher in MG1655 and GW10 than in JM109 and MC1000 (Fig. 3A), and western analysis showed that the levels of C5 protein in MG1655 and GW10 cells were also similar to those in JM109 and MC1000 (Fig. 3B). These data suggest that the ineffectiveness of plasmid-borne P12-deleted M1 RNA on the M1 RNA stability in MG1655 or GW10 is not due to low expression of

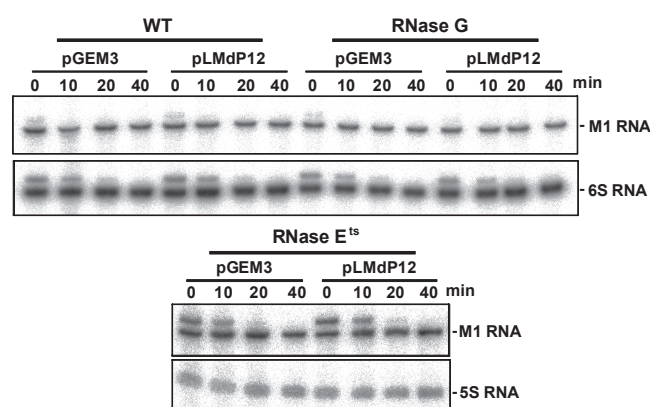


Figure 1. Effects on sequestration of C5 protein on M1 RNA stability in endoribonuclease-deficient cells. Total cellular RNA was isolated from strains GW10 (wild type), GW11 (*rng*⁻), and GW20 (*rne*^{ts}) containing plasmids at the indicated times after the rifampicin treatment. Each RNA sample (30 μ g) was fractionated on a 5% polyacrylamide gel containing 7 M urea and analyzed by northern blotting. A stem P12-specific probe was used as a probe for M1 RNA.

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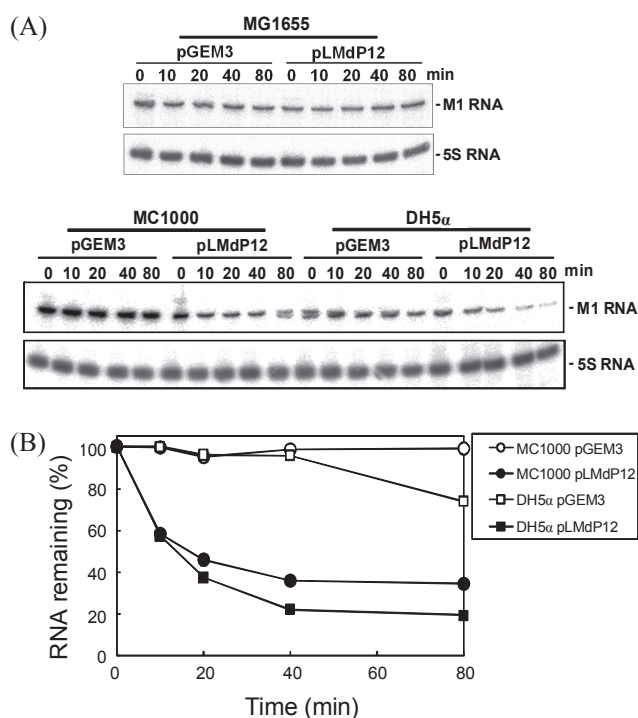


Figure 2. Comparison of effects of C5 protein sequestration on M1 RNA stability among various strains. (A) Total cellular RNA isolated from strains MG1655, MC1000, and DH5 α were analyzed as in Fig. 1. (B) The band intensity of endogenous M1 RNA was normalized to that of 5S RNA. The remaining RNA (% 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.

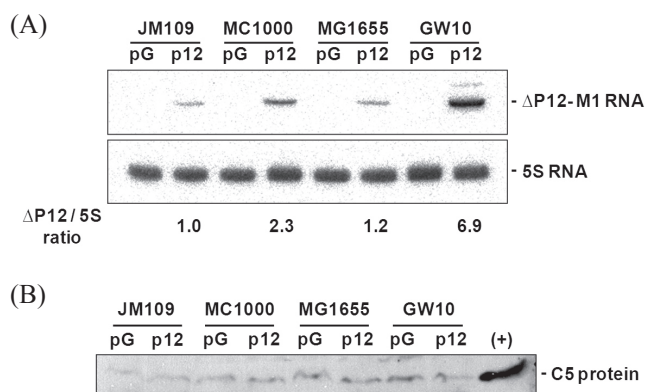


Figure 3. Comparison of expression levels of P12-deleted M1 RNA and C5 protein in different strains. (A) P12-deleted M1 RNA levels in different strains were analyzed by northern blotting with an anti-M1 RNA riboprobe. (B) C5 protein levels in different strains were analyzed by anti-C5 protein antibody. pG and p12 indicate cells containing pGEM as a control vector and pLMdP12 that expresses P12-deleted M1 RNA, respectively.

P12-deleted M1 RNA or high expression of C5 protein.

Then we examined whether C5 protein could be sequestered by overexpressed P-12 deleted M1 RNA to generate free M1 RNA in MG1655 or GW10. If C5 protein is sequestered as in JM109 cells containing pLMdP12, the cellular RNase P activity would be decreased and precursor tRNAs are accumulated. Therefore, we assessed the sequestration of C5 protein in MG

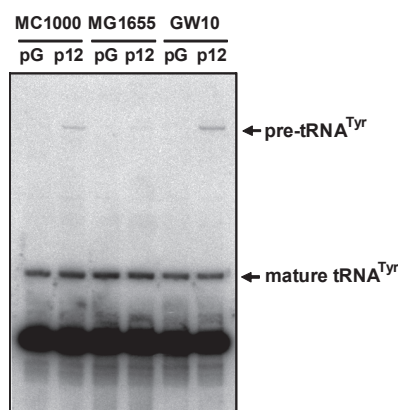


Figure 4. Effects of C5 protein sequestration on RNase P activity in MG1655 and GW10 cells. Total cellular RNAs prepared from MG1655 or GW10 cells containing pLMdP12 were subjected to northern blot analysis. The membrane was probed with an anti-tRNA^{Tyr} oligonucleotide. pG and p12 indicate cells containing pGEM as a control vector and pLMdP12 that expresses P12-deleted M1 RNA, respectively.

1655 and GW10 by analyzing the accumulation of tRNA^{Tyr} precursor (Fig. 4). Both cells containing pLMdP12 accumulated the tRNA precursor as did JM109, while cells containing the vector plasmid pGEM3 did not. This data suggests that C5 protein is sequestered by overexpressed P12-deleted M1 RNA derivative in MG1655 or GW10 and that the ineffectiveness of overexpressed P12-deleted M1 RNA on the M1 RNA stability would result from the defection of some RNase activities involved in degradation of protein-free M1 RNA.

We compared genotypes of MG1655, GW10, JM109, MC1000, and DH5 α . We found that MG1655 and W3110 (the parental strain from which GW10 was derived) have the *rph-1* genotype resulting from a C-terminal frameshift mutation in *rph* encoding RNase PH, an exoribonuclease,²¹ while JM109, MC1000, and DH5 α have no mutation in the *rph* locus. The *rph-1* mutation severely reduces RNase PH activity and has a polar effect on expression of the downstream *pyrE* gene, reducing PyrE activity and causing partial pyrimidine starvation when mutant cells are grown in the minimal medium.²¹ The mutant RNase PH protein expressed by *rph-1* has a little smaller size than the wild type enzyme, with the C-terminal sequence of ESNPL instead of the normal GIESIVATQKAALAN sequence. The shorter size of the mutant enzyme is due to the frameshift caused by a one-base-pair deletion of the first base (G) of codon 223 (Fig. 5).

To examine whether RNase PH is involved in degradation of protein-free M1 RNA, we constructed an RNase PH expression vector, pAC-rph by cloning the *rph* gene into the pACYC 184 vector. Then we analyzed M1 RNA stability in cells containing both pLMdP12 and pAC-rph. The presence of pAC-rph, not the control vector pACYC184, reduced M1 RNA stability in the presence of pLMdP12. This reduction of M1 RNA stability was not observed with the control plasmid pGEM3 (Fig. 6). Therefore, it is likely that protein-free M1 RNA generated by the sequestration of C5 protein with overexpressed P12-deleted M1 RNA might be degraded by RNase PH. Since RNase PH is an exoribonuclease, this result suggests that degradation of

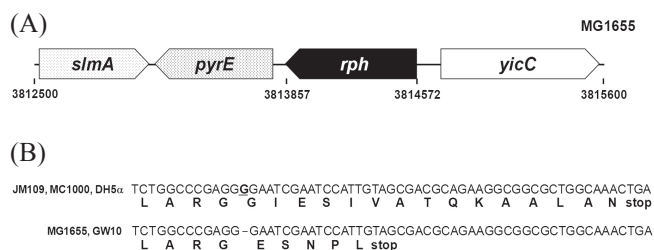


Figure 5. The *rph-1* mutation. (A) Schematic representation of the locus of *rph* and its transcription unit in strain MG1655. (B) The *rph-1* gene generates a truncated RNase PH at the C-terminal region. The single deleted nucleotide of *rph-1* is underlined in bold in the wild type *rph* sequence.

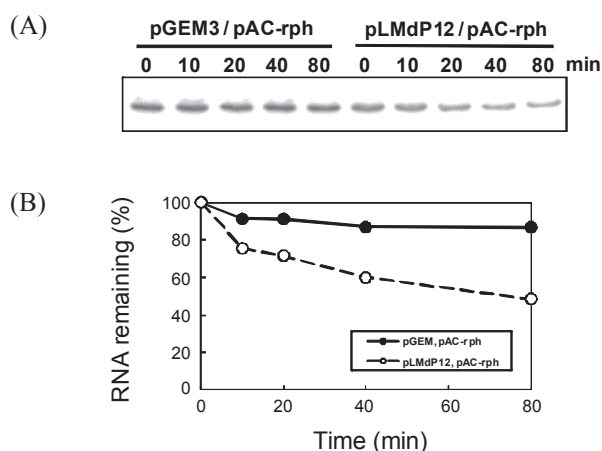


Figure 6. Restoration of instability of free M1 RNA in MG1655 by plasmid pAC-rph carrying the intact *rph* gene. Total cellular RNA was analyzed as in Fig. 1. pACYC184 was used as a control vector.

protein-free M1 RNA may start from the 3' end and that C5 protein may protect this degradation somehow although C5 protein does not seem to directly interact with the 3' end of M1 RNA.^{22,23} Furthermore, our data imply that RNase PH might be a key exoribonuclease that participates in turnover of protein-free M1 RNA in the cell. However, it does not seem to be RNase PH alone that is responsible for degradation of protein-free M1 RNA because the supply of RNase PH by a multicopy plasmid in MG1655 and GW10 did not fully recover the degradation sensitivity of protein-free M1 RNA to the level of JM109 or MC1000. Therefore, additional factors seem to be involved in maintaining the M1 RNA stability although they need to be demonstrated.

Experimental Section

Bacterial strains and plasmids. The *E. coli* K-12 strains in this study are JM109 [F^+ *proA*⁺ *proB*⁺ *lacIq* Δ (*lacZ*) *M15* Δ (*lac proAB*) *supE44* *hadR17* *recA1* *gyrA* *thi-1* *endA1* *relA1* *e14-lamda*],²⁴ MG1655 [F^- *rfb50* *rph-1*],²⁵ DH5 α [*supE44* *lacU169* Δ (*lacZ*) *M15* *hadR17* *recA1* *endA1* *gyrA96* *thi-1* *relA1*],²⁶ MC 1000 [F^- *lamda* *araD139* Δ (*ara-leu*) *7697* Δ (*lacIY*) *74* *galU* *galK* *rpsL*],²⁷ GW10 [*rng*⁺ *zce-726::Tn10Tc^r*],²⁰ GW11 [*rng::cat* *zce-726::Tn10Tc^r Cm^r*],²⁰ and GW20 [*ams1* *zce-726::Tn10Tc^r*].²⁰

Plasmid pLMdP12 was a derivative of the pGEM3 vector, which was capable of overexpressing a truncated M1 RNA.²⁸ pAC-rph, was constructed by cloning the PCR product containing the *rph* sequence into the *NcoI-EcoRI* sites of the pACYC184 vector, which can co-exist with pGEM3.²⁹ The *rph* sequence-containing PCR product carried the sequence spanning the region of -200 to +918 relative to the *rph* transcription start site. This PCR fragment was amplified using primers pAC-rph1 (5'-CATGC CATGGCGCATTTCCCAGGTTGCGCTC-3') and pAC-rph2 (5'-GGAATTCGATCGCGCCCGG-TATTAAG CAG -3').

Total cellular RNA preparation. JM109 cells containing plasmids were grown overnight (37 °C) in LB containing ampicillin (50 mg/mL), diluted 1:100 in the same medium, and grown to an A₆₀₀ of approximately 0.5 at 37 °C. For RNA stability assays, rifampicin (150 mg/mL) was added to the cultures to stop transcription. Cultures were then analyzed at different time intervals. In case of temperature sensitive mutant GW20 strain, cells were grown overnight at 30 °C, diluted (1:100) into the fresh media, and grown to an A₆₀₀ of 0.5. Cultures were incubated at 42 °C for 10 min before the rifampicin treatment. Total cellular RNA was prepared by hot phenol extraction, as described previously.³⁰

Northern blot analysis. Northern blot analysis of RNA samples were performed, as described previously.³¹ The probes used for the M1 RNA detection were an anti-M1 RNA riboprobe,²⁸ or a stem P12-specific oligonucleotide probe.¹⁷ tRNA^{Tyr}, 6S RNA, and 5S RNA were also analyzed with antisense oligonucleotide probes.¹⁷ The stem-specific probe hybridized with intact M1 RNA, but not with P12-deleted M1 RNA.

Western blot analysis. The *E. coli* cells containing plasmids were grown in LB containing ampicillin (50 μ g/mL) at 37 °C to an A₆₀₀ of about 0.5. Cells were centrifuged down at 10,000 g for 10 min. After harvesting the cells, the cell pellet was suspended in cold phosphate buffered saline (pH 7.2) containing 50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF, 200 μ M sodium orthovanadate, 1 mM PMSF, and protease inhibitor (CompleteTM; Roche) for sonication. The soluble fraction of the sonicated cells was resolved on a 15% SDS-polyacrylamide gel and electrophoretically transferred to a nitrocellulose membrane (Amersham). Protein bands were immunostained with an anti C5 protein antibody, and were visualized by using an Enhanced Chemi-Luminescence Kit (Amersham).

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