

Degradation of Stable RNA: Identification of Degradation Intermediates in M1 RNA-Overexpressing Cells

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Control of RNA degradation is an important way in regulating gene expression.^{1,2} As for stable RNA in bacteria, which is mostly housekeeping RNA, RNA decay generally does not occur during exponential growth.³ However, the cellular level of stable RNA can be modulated upon physiological or environmental changes. It has been reported that some starvation conditions can cause an extensive degradation of stable RNA such as rRNA.³ rRNA decay was also observed under stationary phase.³ For more understanding of global regulation of cellular metabolism through control of stable RNA biogenesis, it is crucial to understand how stable RNA is protected during exponential phase from degradation that takes place under certain conditions. However, the protection mechanisms still remain not understood. Recently, we have reported that M1 RNA, a stable RNA, is rapidly degraded during exponential phase in cells where C5 protein is sequestered by excess truncated M1 RNA.⁴ M1 RNA is the catalytic component of RNase P, a tRNA processing enzyme that generates the mature 5' end of tRNA from its precursor,⁵ while C5 protein is the protein component.⁶ C5 protein functions as a cofactor in the enzyme reaction catalyzed by the RNase P ribonucleoprotein. The instability of M1 RNA under the condition lacking C5 protein for binding to M1 RNA to form RNase P holoenzyme might result from susceptibility to RNase attack. Therefore, the M1 RNA decay process can be a useful model to understand in detail how stable RNA decay is modulated. To examine the M1 RNA degradation pathway, the first thing to do is to identify its intermediates. In the C5 protein-sequestered cells, no apparent intermediates were observed,⁴ possibly because they might be transiently present in a very small amount in the cell. Previously, however, we found that when M1 RNA is overexpressed, degradation intermediates are accumulated.⁷ This intermediate might be the same intermediate in the degradation pathway in the C5 protein-sequestered cells because most of overexpressed M1 RNA would be naked due to a shortage of C5 protein. As an initial attempt to examine the regulation of M1 RNA degradation, in this study, we set to identify the degradation products of M1 RNA observed in M1 RNA-overexpressing cells. To overexpress M1 RNA in the cell, we used plasmid pLM1 which has the *rnpB* transcription unit in it.⁸ Total RNA was prepared from pLM1-containing cells growing in the exponential phase and fractionated on a denaturing polyacrylamide gel. Degrading products that migrated faster than M1 RNA were observed

with a major species of about 300 nts (Fig. 1). The total RNAs were subjected to RACE analysis. For this purpose, first we performed cyclic ligation reactions with two sets of primers sets (93f/116r and 147f/171r) after treating pyrophosphatase or without its treatment. The pyrophosphatase treatment was done because if M1 RNA kept grabbing the intact triphosphate group at the 5' end, the cyclic ligation reaction would not take place without removing the pyrophosphate. Without pyrophosphatase treatment, DNA fragments with the size of about 300 nts were amplified much less than with the enzyme treatment (data not shown), suggesting that the 300-nt RNA species contained the 5' triphosphate. In overall, less RACE products were generated when pyrophosphatase was not used. Table 1 shows that the longest species contained the M1 RNA sequence from +1 to +296. We also observed fragments of +1 to +208, +1 to +166, and +1 to +137-139. These fragments might be derived by 3' degradation from the +296 species. In addition to these +1-containing fragments, +136-139 species digested mostly near position +85 were observed. Therefore, the +136-139 species were likely to be cleaved near position +85 at their 5' regions after 3' degradation proceeded up to

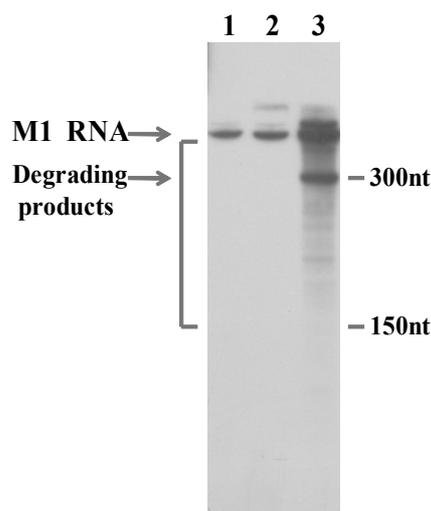


Figure 1. Overexpression of M1 RNA. Total cellular RNAs were prepared from 2×10^7 cells, fractionated on a 5% polyacrylamide gel containing 7 M urea, and subjected to northern blot analysis. For RNA preparation, JM109 cells containing the following plasmids were used: Lane 1, no plasmid; lane 2, pGEM3; lane 3, pLM1.

Table 1. Sequencing analysis of the RACE products.

	Primer pairs	5' end ^a	3' end ^a
Cyclic RACE	93f/116r	+1	+295 (1)
		+1	+139 (2)
		+1	+137 (1)
		+1	+149 (1)
		+2	+137 (1)
		+59	+141 (1)
		+70	+167 (1)
		+75	+139 (1)
		+83	+137 (1)
		+84	+138 (1)
		+86	+137 (1)
		+87	+139 (1)
		+88	+137 (2)
	147f/171r	+1	+296 (1)
		+1	+208 (1)
3' RACE	f+22/E1		+296 (1)
			+295(+A) (1)
			+293(+AAA) (1)
			+239 (1)
			+208 (1)
			+170 (1)
		+144 (1)	

^aThe 5' or 3' ends of M1 RNA degrading intermediates were determined by analyzing the sequencing data of the RACE products. The numbers in double brackets indicate frequency of occurrence.

+137-139. Since most degradation species retained the +1 nucleotide, we performed 3' RACE to further examine the 3' degradation pathway. We found the same +296 and +208 species as in the cyclic ligation, with additional +295(+A), +293(+AAA), +239, +170, and +144 species. This heterogeneity of 3' ends suggests that the +296 species is subjected to 3' trimming. Furthermore, +295(+A) and +293(+AAA) species containing A residues at the 3' end were observed, which were likely to result from 3'-trimming of the +296 species because 3'-trimming is accelerated with A residues at the 3' end added by poly(A) polymerase.⁹ This implies that the +296 species is the first cleavage product. It is not clear yet whether the +296 species was generated by an endoribonuclease or exoribonucleases. However, we predict that the +296 species might be one cleaved by an endoribonuclease, because only shorter, but not longer degradation species were observed. However, the sequence or structure near position +296 is not consistent with the known recognition sites for endoribonucleases, RNase III, RNase E, RNase G, and RNase P.¹⁰⁻¹³ Identification of an RNase responsible for generating the +296 species remains to be demonstrated. From our data, we propose a working model for M1 RNA degradation: M1 RNA is cleaved first by an endoribonuclease and then the cleaved 5' fragment is degraded

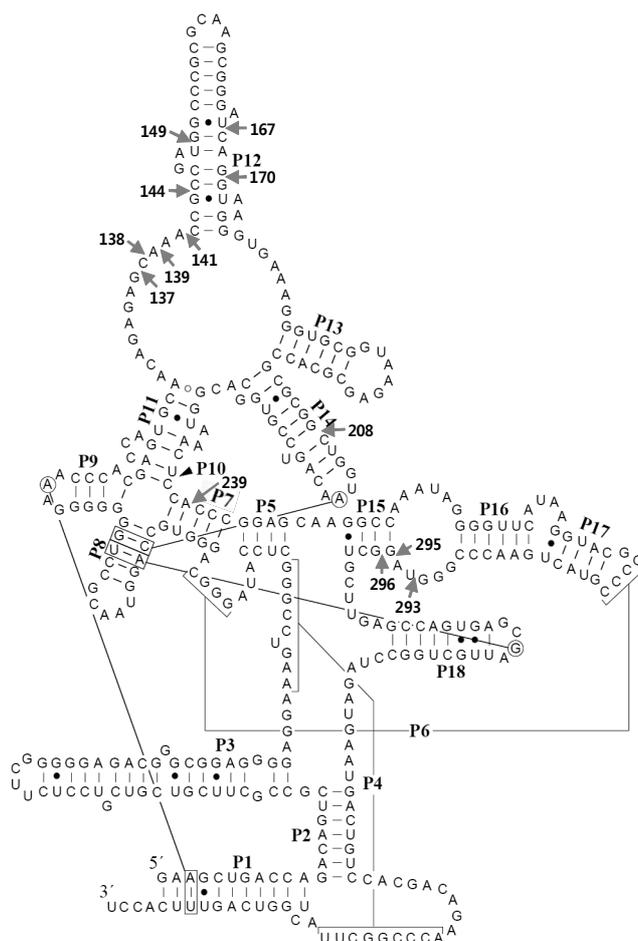


Figure 2. Positions of 3' ends of M1 RNA degradation intermediates in a secondary structure model of M1 RNA.¹⁸ The arrows indicate the 3'-ends of M1 RNA degradation intermediates.

from the 3' end by exoribonucleases. Interestingly, we did not observe the 3' cleaved fragment that would be also generated by the endoribonucleolytic cleavage (Fig. 1). This fragment might be degraded much more rapidly than the 5' fragment. This is possibly because the 3' fragment has monophosphate rather than triphosphate at the 5' end, whose removal is known to a rate-determining step for RNA degradation.^{14,15} Importantly, the identification data of M1 RNA degradation intermediates provided by this study can be used to assess in detail how degradation of stable RNA is regulated upon certain changes of physiological conditions.

Experimental Section

Bacterial strain and plasmid. The *E. coli* K-12 strain JM109 was used for analysis of M1 RNA degradation. The M1 RNA-expressing plasmid used was pLM1,⁸ a derivative of pGEM3, carrying an intact *rnpB* transcription unit.

Preparation of total cellular RNA. *E. coli* cells containing pLM1 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 37 °C to an OD₆₀₀ of 0.5 at 37 °C, as previously described.¹⁶ Total cellular RNA was isolated

from the culture by hot phenol extraction as described previously.¹⁷ Contaminating DNA was removed by adding Turbo DNase (Ambion) to RNA samples according to the manufacturer's instruction.

Northern hybridization. Total RNA was electrophoresed on 5% PAGE gels containing 7 M urea, transferred to Hybond-N+ membranes (Amersham Biosciences) and probed for M1 RNA. The M1 RNA probe was generated from *Hind*III-linearized pLMd23 DNA,⁷ using T7 RNA polymerase and [α -³²P]CTP. Northern blots were visualized by BAS1500 (Fuji) and TINA software (Raytest).

RACE assays. RACE analysis was carried out as previously described,¹⁷ with minor modifications. Briefly, 1 μ g of total cellular RNA was treated with 1 U of tobacco acid pyrophosphatase (TAP) (Epicentre) in a 50 μ L reaction. For intramolecular circularization, the TAP-treated RNA or untreated RNA was then ligated at 17 °C for 16 h with 110 U of T4 RNA ligase (New England Biolabs) in the same reaction volume. Intramolecularly ligated RNA was then reverse-transcribed and PCR-amplified using a One-Step RT-PCR premix kit (Intron) according to the manufacturer's instructions. 3' RACE analysis was carried out similarly to cyclic RACE except that TAP-untreated RNA was ligated with E1 3' adaptor.¹⁸ Primer pairs used for RT-PCR were 93f/116r and 147f/171r, and f+22/E1: 93f, 5' CCA CGA CCA GTG CAA CAG AGA G; 116r, 5' GTT TCC CCC CCA GGC GTT ACC TG; 147f, 5' GTA AGG GTG AAA GGG TGC GGT AAG AG; 171r, 5' CTG ATC CCG CTT GCG CGG GCC ATC; f+22, GCT TCG TCG TCG TCC TCT TC. The E1 sequence was previously described.¹⁷ PCR products were separated on a 5% polyacrylamide gel and those migrating more rapidly than M1 RNA-derived one were purified by gel elution. They were analyzed by DNA sequencing to determine junctions of 5' and

3' ends or 3' ends of RNA after cloning into a T-Blunt vector (Solgent).

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