

Possibility of Non-enzymatic mRNA Degradation in *E. coli* :

I. Bell-shaped Kinetic Feature

Hyeong-Won Ryu* and Thong-Sung Ko

The Institute of Science & Technology, Mokwon University, Daejeon 302-729, Korea. *E-mail: howryu@mokwon.ac.kr
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Although vigorous research efforts, aimed to understand the mechanism of mRNA degradation that is important in regulation of gene expression, have been directed entirely to RNases to identify putative mRNases since the discovery of the mRNA instability, the elucidation of the mRNA degradation mechanism has been the slowest process among principal gene regulatory processes in *Escherichia coli*.¹⁻³

In respect of shorter half-lives of prokaryotic mRNAs than those of eucaryotic mRNAs,³ a 5'-to-3' directionality of mRNA decay in accordance with the 5'-to-3' directionality of coupled transcription-translation in prokaryotes has been paid special attention.⁴ Formerly, in this line of research works, a putative ribosome-associated 5'-to-3' exoribonuclease (RNase V) had been proposed as an mRNase,⁵ but it is known that such a 5'-to-3' exoribonuclease does not exist in *E. coli*.⁶ Therefore, current models suggest that mRNA degradation is initiated by a certain endoribonuclease(s) (functional inactivation of message) and the subsequent degradation of resulting fragments is carried out by 3'-to-5' exoribonucleases (mass decay).^{1,2}

According to the case of polycistronic *lac* mRNA degradation,⁷ it is initiated near the 5' end and progressed in the net 5'-to-3' direction, and predominant cleavage sites are the phosphodiester bonds between pyrimidine and adenine residues (e.g., U ↓ A). Furthermore, most of the mRNA degradation products in growing *E. coli* have 5'-OH ends.⁸ These specificities are also known to correspond to those of non-enzymatic hydrolysis of RNA,⁹ nonetheless, the possibility of non-enzymatic degradation was neglected. For example, an enzyme, RNase M was proposed as a new mRNase candidate for the characteristic activity.⁸ However, it was failed to identify the structural gene for RNase M.¹⁰

Again, in line of "only RNases degrade (inactivate) mRNA" concept, RNase E which is an essential enzyme carrying out 5S rRNA maturation¹¹ and a 5'-end-dependent endoribonuclease showing the sequence specificity of A+U rich single-stranded regions,¹² and RNase E-based degradosome assembly have attracted so much attention as alternative mRNase candidates.^{2,13} However, RNase E not only shows marked preference for cleaving RNA that carries a monophosphate group at the 5' end rather than a triphosphate or a hydroxyl, but also generates 5'-phosphate ends instead of 5'-OH ends (Fig. 1).^{6,9,12,14,15} In addition, the observation of rapid degradation of *rpsO* mRNA (ribosomal protein S15 mRNA) in a triple mutant (RNase E⁻, PNPase⁻, and RNase

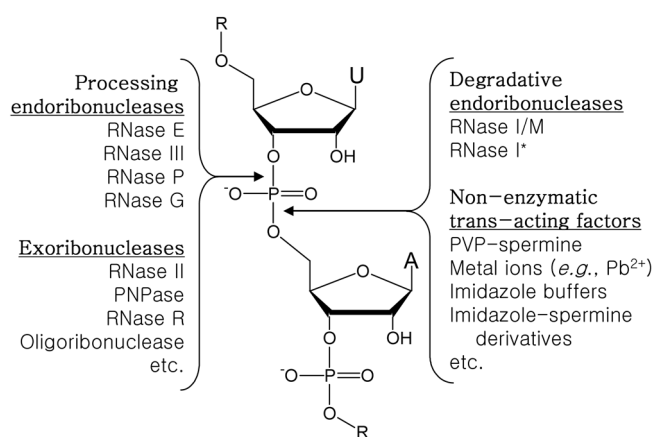


Figure 1. Specificities of *E. coli* RNases and non-enzymatic trans-acting factors in RNA phosphodiester bond cleavage.

E⁻)¹⁶ and of normal half-lives for several mRNAs in cells containing truncated forms of RNase E which preclude degradosome assembly,¹⁷ may indicate that RNase E and the degradosome assembly are irrelevant to the functional decay of mRNA.

Consequently, there is no obvious evidence to support the concept of "only RNases degrade mRNA". Three decades ago, in advance of the discovery of RNase-free self-cleavage activity of RNA,¹⁸ a possibility of non-enzymatic *E. coli* mRNA degradation mediated by protein biosynthetic machinery was proposed by Ko.¹⁹ Recently, it has been discovered that translational pausing can promote endonucleolytic mRNA cleavage near the paused sites of ribosomes even in the absence of known endoribonucleases of *E. coli*.²⁰ This finding led in this field to suppose the possibility that ribosomes may be involved in the endonucleolytic cleavage event.²¹

Results and Discussion

Construction of a simple simulation model. In order to investigate the degradation mechanism of *E. coli* mRNA, we devised a simple simulation model possessed with elementary physicochemical key features of the protein synthetic machinery - mRNA interaction. We adopted the PVP (polyvinylpyrrolidone)-spermidine system,⁹ expected to have only nonspecific noncovalent binding groups instead of specific catalytic groups, as a ribosome-like trans-acting factor, and

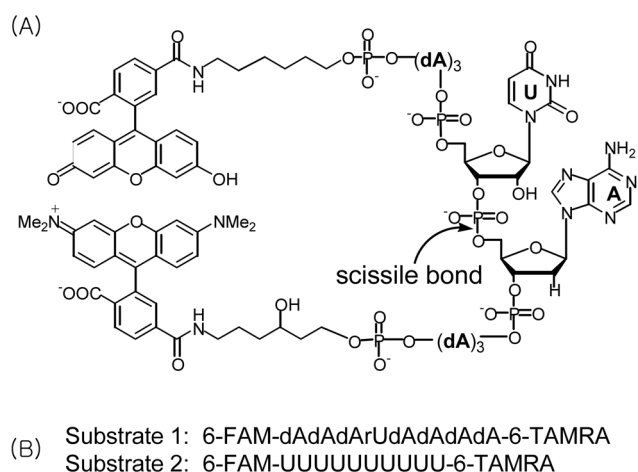


Figure 2. (A) Chemical structure of fluorogenic substrate 1. (B) Nucleotide sequence of substrates, where 6-FAM and 6-TAMRA refer to 6-carboxyfluorescein and 6-carboxytetramethylrhodamine, respectively.

the chimeric oligonucleotide (substrate 1) containing a scissile bond (U ↓ A) as a model of functional mRNA with cis-acting elements, and the decameric U (substrate 2) as a reference of the mRNA model (Fig. 2).

Sequence irrespective bell-shaped curves and hyper-susceptibility of UA sequence in non-enzymatic hydrolysis of oligoribonucleotides. In the hydrolytic cleavage of oligonucleotides catalyzed by the trans-acting PVP-spermidine system, both substrate 1 and substrate 2 showed bell-shaped dependence on the concentration of PVP-spermidine, irrespective of the nucleotide sequence (Fig. 3). Although substrate 1, which has a scissile bond (U ↓ A) for ribonucleolytic cleavage, has 9 times less such cleavable bonds than substrate 2, the measured release rates of fluorescent products of the two substrates in the absence of

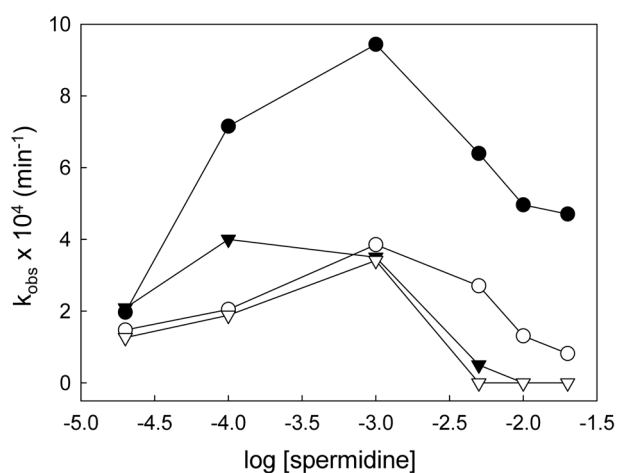


Figure 3. Dependence of the rate constants of hydrolytic cleavage of oligonucleotides on PVP-spermidine concentrations. The reactions were carried out in the presence (0.2%) (●, ▼) and absence (○, ▽) of PVP under the conditions described in the Experimental Section. The substrate concentrations were 100 nM (substrate 1: ●, ○) and 140 nM (substrate 2: ▼, ▽), respectively.

PVP were similar, possibly due to the instability of the UA phosphodiester bond of substrate 1 (uncatalyzed rate constant of the hydrolysis, $k_u = 1.2 \times 10^{-4} \text{ min}^{-1}$) compared to UU of substrate 2 ($k_u = 1.5 \times 10^{-5} \text{ min}^{-1}$).²² Moreover, in the presence of PVP, substrate 1 showed higher reactivity than substrate 2. The bell-shaped curve and the hyper-susceptibility of UA sequence to hydrolytic cleavage can be characteristic features of non-enzymatic RNA degradation.⁹

Protein synthetic machinery-mediated poly(U) degradation and a bell-shaped curve. We revisited the previous report (Kuwano *et al.*, *Proc. Natl. Acad. Sci. USA* 1969, 64, 693) on the degradation of poly(U) mediated by the cell-free protein synthetic machinery of *E. coli*. The data of Figure 4A (Fig. 1e in the original paper)^{5a} which show that the rate of poly(U) degradation is increased by the increase of ribosome concentration, demonstrate that the ribosome component of protein biosynthetic machinery is necessary for the degradation. And, Figure 4B (Fig. 1c in the paper)^{5a} which shows that the rate of poly(U) degradation is inversely proportional to the concentration of 5'-β-γ-methylene-guanosine triphosphate (GMP-PCP), demonstrates that the stabilization of poly(U) from degradation is due to the stalled ribosomes on poly(U) by blocked translocation caused by GMP-PCP, an unhydrolyzable GTP analogue which inhibits G and T factors needed for translation. This observed ribonucleolytic activity led them to propose an mRNAse named as RNase V (5'-to-3' exoribonuclease),⁵ but nobody including them found such an enzyme, *i.e.*, 5'-to-3' exoribonuclease, in *E. coli*.⁶

One of the most naive explanation of the poly(U) degradation activity of ribosomes would be contamination of the ribosome component with certain RNases. In such a case, the degradation reaction should follow enzyme kinetics, showing a linearly increasing rate profile that is directly proportional to the ribosome concentration because of the

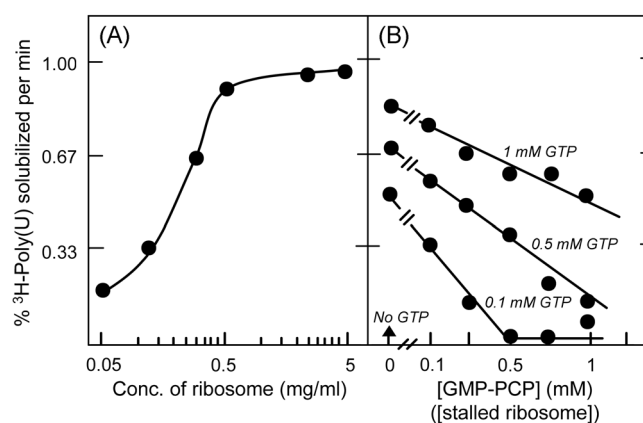


Figure 4. Dependence of poly(U) degradation on the concentration of ribosome (A) and of GMP-PCP (B) mediated by the cell-free protein synthetic machinery of *E. coli*. In panel B, the reactions were carried out in the presence of 2 mg/ml of ribosomes with varying concentrations of GTP, where the increase of GMP-PCP concentration with decreasing GTP corresponds to the increase of ribosome stalling on poly(U) by blocked translocation. The data was transferred from Kuwano *et al.* (1969) without permission of the authors.

contamination. But in our present reexamination of their data (Fig. 1e plus 1c), we find that the degradation reaction kinetics is not that of an enzymatic reaction but that of a non-enzymatic reaction as shown in Figure 4: we can actually get a bell shape when the two panels are combined together (panel A plus panel B in Fig. 4).

Here, we can claim that the degradation reaction belongs to a non-enzymatic reaction instead of an enzymatic reaction. In addition, a study reported that the ribonucleolytic activity of *E. coli* cell-free protein synthetic machinery was insensitive to human placental RNase inhibitor, whereas removal of the 70S ribosome fraction from complete assay mixtures prolonged the half-life of an mRNA.²³ However, the putative RNase assumed to be associated tightly with ribosomes has been neither detected nor identified up to date. Therefore, it may be improbable to ascribe the ribonucleolytic activity observed in Figure 4 to any RNase contamination of the system.

Then, the trans-acting factor responsible for the poly(U) degradation ought to be ribosome itself. So far as we obtained an important clue that the mechanism of functional mRNA degradation can be that of non-enzymatic degradation by protein biosynthetic machinery, in which ribosomes act as a trans-acting factor, we were interested in experimental further verification of the non-enzymatic degradation mechanism. In our simulation model system employed for this purpose, in which PVP-spermidine functions as a trans-acting factor, the non-enzymatic degradation reaction system demonstrated a bell-shaped dependence on the concentration of PVP-spermidine (Fig. 3), just like the above protein synthetic machinery-mediated mRNA (poly(U)) degradation system (Fig. 4).

Effects of antibiotics on mRNA stability can be explained by the present model of non-enzymatic mRNA degradation. The antibiotics that have no influence on RNase action can exert profound effects on mRNA stability *via* their effects on ribosome - mRNA interaction. Puromycin, which strips fully extended mRNA of bound ribosomes, can cause scissile sites (*e.g.*, U ↓ A) to be exposed and the mRNA to be destabilized in hydrolytic environment. On the other hand, the protection of scissile sites from hydrolytic environment by the trans-acting stalled ribosomes, caused by fusidic acid and chloramphenicol which freeze ribosomes

Table 1. Effects of antibiotics on mRNA stability in *E. coli*^a

control	Half-lives (min)			mRNA species
	ribosome release	ribosome freeze		
	puromycin	fusidic acid	chloramphenicol	
2.8	0.8			<i>lac</i> mRNA
1.2	0.6	2.6		total mRNA
5.5	1.0			<i>trp</i> mRNA
2.5		9	18.5	total mRNA
3.5			10	<i>lacZ</i> mRNA

^aData were collected from the previously published reports.⁴

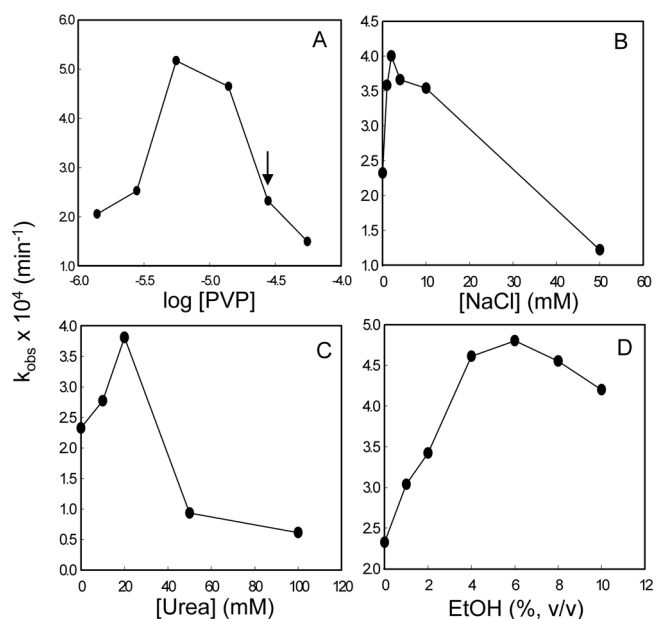


Figure 5. Effect of relieving the excessive binding interaction of the trans-acting PVP-spermidine system with oligonucleotides on its negative catalysis activity. (A) Plot of the rate constants of hydrolytic oligonucleotide-cleavage vs. concentration of PVP. The reaction was performed with substrate 2 (120 nM) in the presence of 1 mM spermidine with increasing PVP concentration under the conditions described in the Experimental Section. Dependence of the hydrolytic cleavage activity of PVP-spermidine on the varying concentrations of sodium chloride (B), urea (C), and ethanol (D), respectively, was examined at the reaction conditions (1 mM spermidine and 27.8 μ M PVP) indicated by the arrow in panel A.

on mRNA, can stabilize the mRNA. Thus the present model of non-enzymatic mRNA degradation can be supported by the observed effects of antibiotics on mRNA stability, and *vice versa* (Table 1).⁴

To verify above assumption further, we examined the effect of relieving the excessive binding interaction of the trans-acting PVP-spermidine system with oligoribonucleotides on its negative catalysis activity (Fig. 5). Expectedly, as the result of the addition of varying concentrations of sodium chloride, urea, and ethanol, respectively, at the reaction condition of the excessive binding interaction (containing 1 mM spermidine and 27.8 μ M PVP) indicated by the arrow in panel A of Figure 5, another bell shape in each case was obtained (panel B-D of Fig. 5).

The observations that even widely spaced translating ribosomes are able to contribute to the mRNA stability and stalled ribosomes are especially effective for protecting mRNA from degradation,³ can not be explained by the current concept that is entirely limited to the ribosomal steric barricade effect to keep off accessible RNases.²⁴ However, our present model, *i.e.*, the mechanism of protein biosynthetic machinery-mediated non-enzymatic degradation of *E. coli* mRNA as also proposed by Ko previously,¹⁹ may provide the answer to the perplexing problem for the role of ribosomes in mRNA stability as discussed above.

The characteristics that the non-enzymatic scissile site,

U ↓ A, is the primary cleavage site of *E. coli* mRNA and the 5'-OH end generated by non-enzymatic degradation reactions is that of most mRNA degradation products of *E. coli*, may also support our suggestion of the mechanism of non-enzymatic (non-RNase) degradation. Here, we can also suppose that the activities, named as RNase M and RNase E which cause cleavage at the U ↓ A site, may probably be those of non-RNase trans-acting factors acting on the mRNA sequence. In conclusion, we suggest the possibility of non-enzymatic (non-RNase) rather than enzymatic (RNase) degradation in functional inactivation of mRNA in *E. coli*.

Experimental Section

The hydrolytic cleavage assay of oligonucleotides was performed with dual-labeled fluorogenic oligonucleotides (Integrated DNA Technology, USA) based on FRET (fluorescence resonance energy transfer).²⁵ Fluorescence emission intensity was measured at 518 nm (slit width 10 nm) upon excitation at 490 nm (slit width 10 nm) using a Perkin-Elmer spectrofluorometer and spectra were recorded with a scan speed of 100 nm/min using a 0.3-cm path length rectangular cell at room temperature. The reactions were carried out with shaking at 37 °C for eight hours in 500 μL of 50 mM Tris buffer, pH 7.5, containing 1 mM EDTA, 100-140 nM substrate, 0-3% PVP (PVP 360, RNase free, Sigma Chemical Co.), and 0-50 mM spermidine (Sigma). To determine rate constants, the maximum fluorescence intensity of product (I_{\max}) was measured with the addition of sufficient RNase A (type 1-AS, Sigma) to cleave all the substrate molecules. The fluorescence intensity of substrate (I_0) was measured prior to the addition of RNase A. From the fluorescence intensity of each sample (I_s), the concentration of product ([P]) was determined from the equation, $[P] = [S]_{\text{total}} \times (\Delta I / \Delta I_{\max})$; here, $[S]_{\text{total}}$ = total substrate concentration, $\Delta I_{\max} = I_{\max} - I_0$, and $\Delta I = I_s - I_0$, respectively. The pseudo first-order rate constants (k_{obs}) were determined from $k_{\text{obs}} = [P] / \Delta t / [S]_{\text{total}}$. The kinetic runs were performed in duplicate (Fig. 5) or triplicate (Fig. 3) and the reproducibility of the data was better than ±7%.

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