Biochemical and Biophysical Research Communications **268,** 136–140 (2000)
doi:10.1006/bbrc.2000.2095, available online at http://www.idealibrary.com on IDEAL®

Effects of C5 Protein on *Escherichia coli* RNase P Catalysis with a Precursor tRNA^{Phe} Bearing a Single Mismatch in the Acceptor Stem

Bo Hyun Park, June Hyung Lee, Meehyein Kim, and Younghoon Lee[1]
Department of Chemistry and Center for Molecular Design and Synthesis, Korea Advanced Institute of Science and Technology, Taejon 305-701, Korea

Received December 13, 1999

***Escherichia coli* RNase P, an RNA-processing enzyme that cleaves precursor tRNAs to generate the mature 5′-end, is composed of a catalytic component (M1 RNA) and a protein cofactor (C5 protein). In this study, effects of C5 protein on the RNase P catalysis with a precursor *E. coli* tRNA^{Phe} having a single mismatch in the acceptor stem were examined. This mutant precursor unexpectedly generated upstream cleavage products at the −8 position as well as normal cleavage products at the +1 position. The cleavage at the −8 position was essentially effective only in the presence of C5 protein. Possible secondary structures for cleavage at the −8 position deviate significantly from the structures of the known RNase P substrates, implying that C5 protein can allow the enzyme to broaden the substrate specificity more than previously appreciated.**

Key Words: **RNase P; C5 protein; M1 RNA; substrate mutation; precursor tRNA^{Phe}.**

RNase P is initially characterized as an RNA processing enzyme which removes 5′-flanking sequences from tRNAs to generate the mature 5′ termini (1). In addition to the precursor tRNAs (ptRNAs), natural non-tRNA substrates of RNase P, such as precursors to 4.5S RNA (p4.5S RNA) and tmRNA (transfer-messenger RNA), are found in *Escherichia coli* (2–4). *E. coli* RNase P is responsible for the 5′ maturation of C4 antisense RNAs from bacteriophage P1 and P7 (5) and can cleave bacteriophage ϕ80-induced M3 RNA (6) and turnip yellow mosaic virus (TYMV) RNA (7). RNase P enzymes from diverse organisms have been shown to contain both essential RNA and protein components. The *E. coli* holoenzyme consists of two subunits, a large RNA subunit (M1 RNA, 377 nucleotides), and a small basic protein (C5 protein, 119 amino acids). M1 RNA cleaves its substrate at the correct position in the absence of the protein subunit *in vitro* (8), although both components are essential for the activity of RNase P *in vivo* (9, 10) and for the efficient *in vitro* reactions under physiological conditions (8). Thus, M1 RNA is the catalytic subunit of the holoenzyme. The presence of C5 protein can reduce the dependency of M1 RNA on Mg^{2+} concentration *in vitro* (11). ptRNA molecules serve as the good substrates for the M1 RNA-alone reaction, while p4.5S RNA requires C5 protein as a protein cofactor for its efficient cleavage. Kinetic analysis with p4.5S RNA as a substrate has shown that the presence of C5 protein dramatically affects the K_m value compared to the k_{cat} value, although the k_{cat}/K_m value is similar to that of ptRNAs (12). Thus, the protein cofactor plays a crucial role in the substrate recognition of p4.5S RNA by the RNase P holoenzyme.

C5 protein has been shown to be able to reduce deleterious effects of mutations in the catalytic RNA molecule (13). It has also been shown to correct the aberrant cleavages observed with the RNA molecule alone (14), by possibly participating in the formation of an active conformation of the RNA molecule. Further evidence that C5 protein participates in the formation of the active conformation of M1 RNA could be obtained from a study in which ribozymes were evolved in the presence of C5 protein (15). In the presence of C5 protein, M1 RNA variants were selected, in addition to M1 RNA that was the only ribozyme selected in the absence of C5 protein. These M1 RNA variants did not display RNase P activity in the absence of C5 protein. Therefore, C5 protein plays an essential role in making some defective ribozymes maintain the catalytically active conformation.

To examine whether C5 protein can also alleviate the effect of mutations in substrates on substrate recognition by M1 RNA, we initially introduced a single nucleotide change from C to A at position 67 in *E. coli* ptRNA^{Phe}. This nucleotide change created a G6-A67

[1] To whom correspondence should be addressed. Fax: 82-42-869-2810. E-mail: ylee@sorak.kaist.ac.kr.

0006-291X/00 $35.00

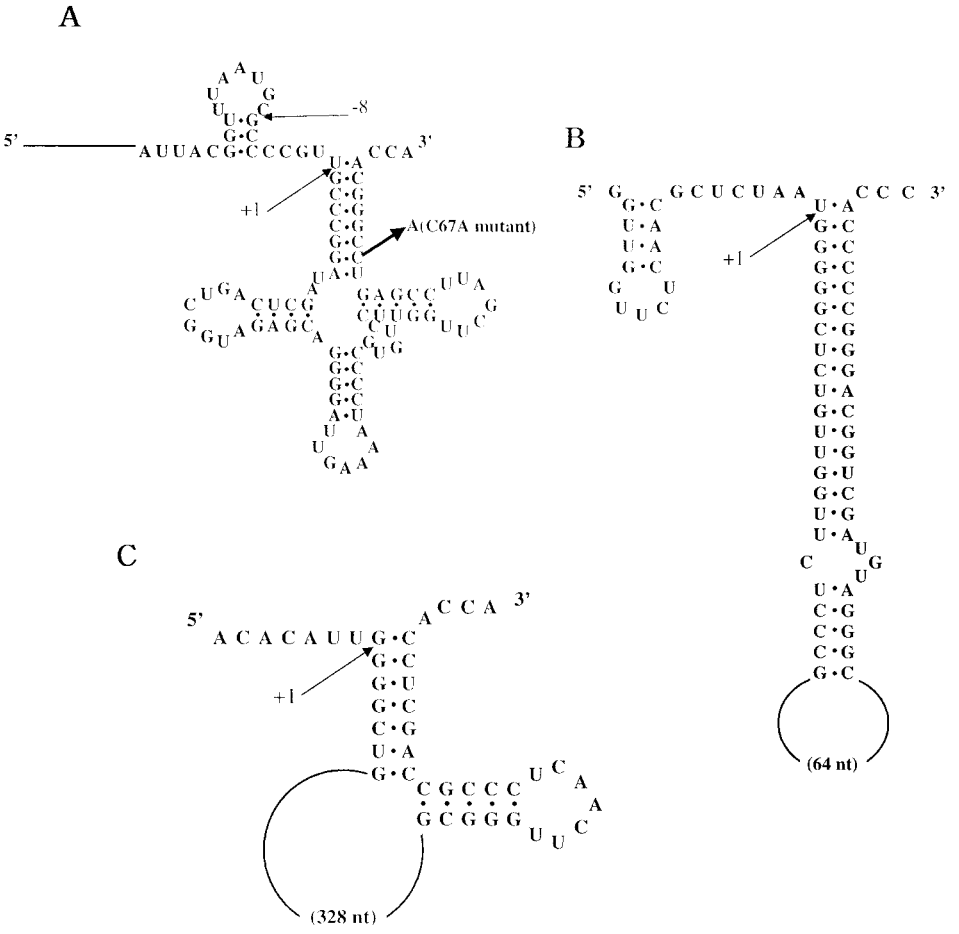

FIG. 1. The secondary structures of some natural substrates for RNase P from *E. coli*. The sites of cleavage by RNase P of each substrate and the base change in ptRNA^{Phe}(C67A) are indicated in thin and thick arrows, respectively. (A) ptRNA^{Phe}. (B) p4.5S RNA. (C) ptm RNA.

mismatch in the acceptor stem. The ptRNA mutant derivative was used as a substrate in the RNase P reaction in the presence or absence of C5 protein. To our surprise, RNase P produced upstream cleavage products at the −8 position, as well as normal cleavage products at the +1 position. Here we report the effects of C5 protein on the upstream and normal cleavage.

MATERIALS AND METHODS

Preparation of substrates. DNA templates for ptRNA^{Phe} and ptRNA^{Phe}(C67A) were obtained by polymerase chain reaction from plasmid pHW2CCA (16) with a pair of primers, SP and 67C, and a second pair of primers, SP and 67A, respectively: primer SP, 5′-ATTTAGGTGACACTATAGAATACAAGCTTGG-3′; primer 67C, 5′-ACAAGCCTGGTGCCCGGACTCGGAA-3′; 67A, 5′-ACAAGCCTGGTGCCCGTACTCGGAA-3′. The RNA substrates were generated by *in vitro* transcription with SP RNA polymerase using the *Bst*NI-cleaved DNA templates. The substrates prepared by the *in vitro* transcription were purified in denaturing 8% polyacrylamide gels 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′ or 3′ terminus of the transcript. To prepare the 5′ end-labeled RNA, RNA was incubated with calf intestinal alkaline phosphatase to hydrolyze the 5′-triphosphate group, which resulted from the *in vitro* transcription reaction. It was labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. Alternatively, the 3′ end-labeled RNA was produced by ligation of [5′-^{32}P]pCp to the 3′ terminus of RNA using T4 RNA ligase.

RNase P assay and measurement of kinetic parameters. M1 RNA and C5 protein were prepared as described (17). M1 RNA and the RNA substrate were separately renatured by heating for 3 min at 80°C in 50 mM Tris–HCl, pH 8.0, and 100 mM NH_4Cl, and by letting the solution cool down 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 with either 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. Aliquots were withdrawn from the reaction mixture at time intervals and mixed with an equal volume of stop solution (200 mM EDTA, pH 8.0, 10 M urea, 0.1% bromophenol blue, 0.1% xylene cyanol). The cleavage products were electrophoresed on 8% denaturing gels containing 8 M urea and quantitated by analyzing the gel with a Molecular Dynamics PhosphorImager. Steady-state turnover measurements of RNase P activity were performed under conditions of excess substrate ([S]/[E] ≥ 5). The concentration of enzyme used was 2 nM. Experiments measuring single turnovers were performed and analyzed exactly as described for the steady-state experiments, except that the enzyme was in excess over substrate ([E]/[S] ≥ 2.5).

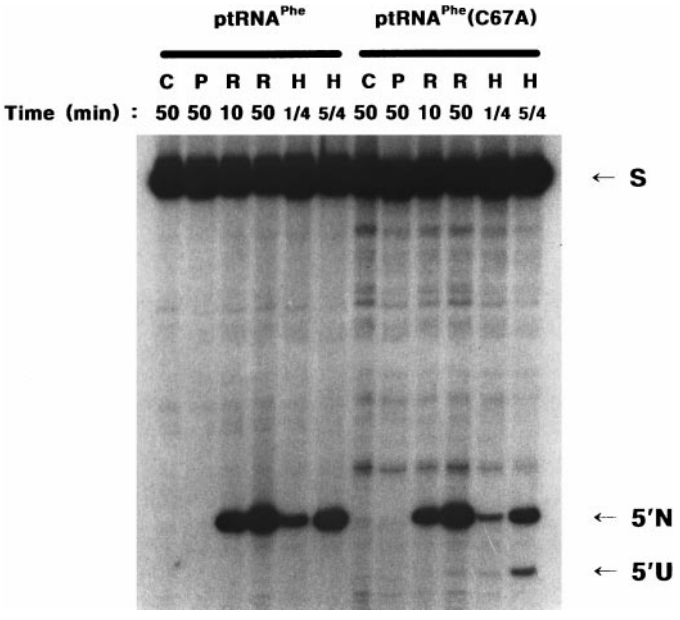

FIG. 2. Steady-state turnover measurements of RNase P activity. The 5′ end-labeled ptRNA^{Phe} or ptRNA^{Phe}(C67A) of 25 nM was incubated in 2 nM enzyme for time intervals indicated above each lane. Lane C, minus enzyme control; lane P, C5 protein 30 nM; lane R, M1 RNA 2 nM; lane H, RNase P holoenzyme 2 nM. The cleavage products are indicated in the right side of the figure: S, substrate; 5′N, the normal cleavage product; 5′U, the upstream cleavage product.

RESULTS

To introduce a minor disruption of the acceptor stem in *E. coli* ptRNA^{Phe}, we displaced C with A at position 67 of ptRNA^{Phe} having the 5′-leader sequence of 102 nucleotides (16). We did this so that the displacement would create a G6-A67 mismatch, thus leaving the five contiguous base pairs in the acceptor stem (Fig. 1). The mutant ptRNA^{Phe}, ptRNA^{Phe}(C67A), and the wild-type ptRNA^{Phe} were used as substrates for RNase P in the condition of steady-state cleavage (Fig. 2). Both ptRNA^{Phe} and ptRNA^{Phe}(C67A) were processed at the +1 position described as the normal cleavage site. The reaction with ptRNA^{Phe}(C67A), however, resulted in additional products that would be generated by cleavage at a site several nucleotides upstream of the normal cleavage site. When compared to the normal cleavage, the upstream cleavage dramatically increased in the presence of C5 protein. In the absence of C5 protein, the ratio of products cleaved at the normal and upstream cleavage is about 110:1; the ratio is reduced to about 5:1 in the presence of C5 protein. Therefore, C5 protein increased the upstream cleavage at least 20-fold more than it did the normal cleavage, indicating that C5 protein is essential for efficient cleavage at the upstream cleavage site. Analysis of the reaction products with a 5′ or a 3′-end showed that the upstream cleavage occurred at position −8 (Fig. 3).

To accurately analyze the effect of C5 protein on the cleavage of ptRNA^{Phe}(C67A) by M1 RNA, multiple turnover kinetic parameters of ptRNA^{Phe}(C67A) and ptRNA^{Phe} were measured and compared (Table 1). The single base change of C to A at position 67 lowered the k_{cat} of the cleavage reaction at the +1 position 3-fold in either the absence or the presence of C5 protein; the K_m values, however, were slightly (1.6-fold) increased in the absence of C5 protein and barely changed in the presence of C5 protein. In the holoenzyme reaction using ptRNA^{Phe}(C67A), the K_m value of the cleavage reaction at the −8 position was almost the same as that at the +1 position, whereas the k_{cat} value is 4-fold lower. On the other hand, it was practically impossible to determine the kinetic parameters of the cleavage reaction at the −8 position due to its much lower cleavage efficiency by M1 RNA alone than that at the +1 position (see Fig. 2). Instead, the RNase P reaction was carried out in the condition of single-turnover cleavage (Fig. 4). Although the data of Fig. 4 did not give the accurate values of k_{cat}/K_m because the reaction was too fast in this cleavage condition, they showed that the activation of cleavage of ptRNA^{Phe}(C67A) at the −8 position by C5 protein was much higher than that at the +1 position. This high activation was comparable to that in the multiple-turnover condition. In the low Mg^{2+} concentration of 2 mM where the single-turnover cleavage was quite slow, the single-turnover kinetic parameter k_{cat}/K_m was mea-

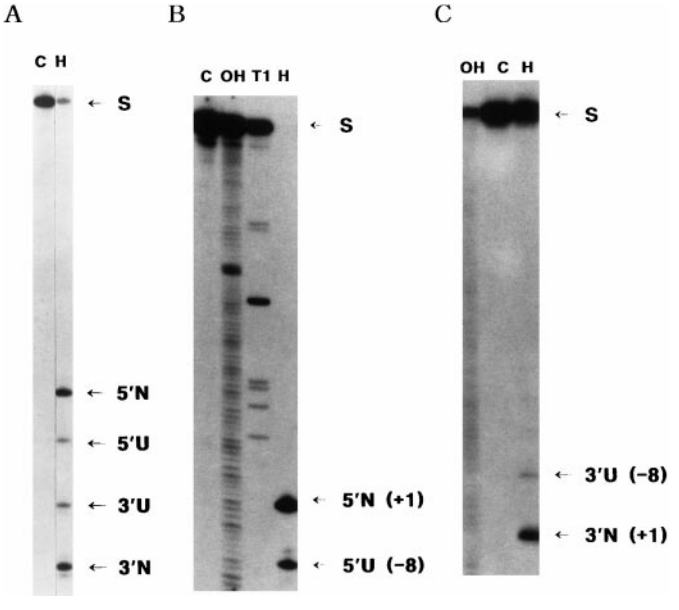

FIG. 3. Cleavage sites of ptRNA^{Phe}(C67A) by RNase P. The internally labeled substrates with [α-^{32}P]CTP (A), the 5′ end-labeled substrates with [γ-^{32}P]ATP (B), and the 3′ end-labeled substrates with [^{32}P]pCp (C) were used. Lanes C and H indicate minus enzyme control and RNase P holoenzyme, respectively. Partial alkaline hydrolysis (denoted by OH) and partial RNase T1 digestion (denoted by T1) of the end-labeled ptRNA^{Phe}(C67A) molecules were used as size markers. The positions of the 5′-cleavage and 3′-cleavage products at the +1 position (for the normal cleavage) and at the −8 position (for the upstream cleavage) are indicated by 5′N, 3′N, 5′U, and 3′U, respectively in the right side of the figures.

TABLE 1

Multiple Turnover Kinetic Parameters of ptRNA^{Phe} and ptRNA^{Phe}(C67A) in Reactions Catalyzed by either M1 RNA or the RNase P Holoenzyme

Substrate	Cleavage site	K_m (μM)		k_{cat} (s^{-1})		k_{cat}/K_m (μM^{-1} s^{-1})	
		M1 RNA	Holoenzyme	M1 RNA	Holoenzyme	M1 RNA	Holoenzyme
ptRNA^{Phe}	+1	0.013	0.034	0.0069	0.34	0.51	10
ptRNA^{Phe}(C67A)	+1	0.021	0.038	0.0020	0.12	0.091	3.0
ptRNA^{Phe}(C67A)	−8	ND	37	ND	0.027	ND	0.73

Note. ND, not determined.

sured (Table 2). The C5 protein increased the k_{cat}/K_m 3.6-fold, 2.8-fold, and 42-fold in the cleavage reactions of ptRNA^{Phe} at the +1 position, ptRNA^{Phe}(C67A) at the +1 position, and ptRNA^{Phe}(C67A) at the −8 position, respectively. Even in the low Mg^{2+} condition, the presence of C5 protein is also 15-fold more effective on the −8 cleavage of ptRNA^{Phe}(C67A) than the +1 cleavage of ptRNA^{Phe}(C67A). Therefore, this high cleavage efficiency of ptRNA^{Phe}(C67A) at the −8 position in the presence of C5 protein resulted from the increase of the overall reaction velocity, not from the decrease of product inhibition.

DISCUSSION

In this study, we have initially examined whether or not C5 protein could alleviate the effect of mutations in substrate on RNase P catalysis by M1 RNA, using a derivative of *E. coli* ptRNA^{Phe}, ptRNA^{Phe}(C67A), having a G6-A67 mismatch in the acceptor stem. It comes as a surprise that RNase P cleaved ptRNA^{Phe}(C67A) to generate upstream cleavage products at the −8 position, as well as normal cleavage products at the +1 position. The increase of the overall reaction on the cleavage of ptRNA^{Phe}(C67A) at the +1 position by C5 protein was about 1.7-fold higher than that with ptRNA^{Phe} (see Table 1). The higher effect of C5 protein on the +1 cleavage of ptRNA^{Phe}(C67A) resulted mainly from the restoration of K_m that was reduced by the substrate mutation. The effect of C5 protein on the −8 cleavage of ptRNA^{Phe}(C67A) is more dramatic (20-fold higher than that on the +1 cleavage), indicating that the −8 cleavage is highly dependent on C5 protein.

It is interesting how RNase P recognizes and cleaves ptRNA^{Phe}(C67A) at the −8 position. There are two possible explanations: (1) RNase P recognizes ptRNA^{Phe} in a single binding mode and cleaves it at the two sites. In a model structure of M1 RNA–ptRNA complex (18, 19), the mature tRNA domain interacts with M1 RNA so that the normal cleavage site resides in close proximity of the active site. Therefore, it is highly unlikely that both the +1 and the −8 positions are oriented to the active site in the same binding mode. (2) The ptRNA^{Phe}(C67A) can exist, in part, in an altered conformation with a stem structure consisting of the base pairing between nucleotides −8 to −5 and nucleotides +69 to +72, and with the 3′-terminal RCCA as in the

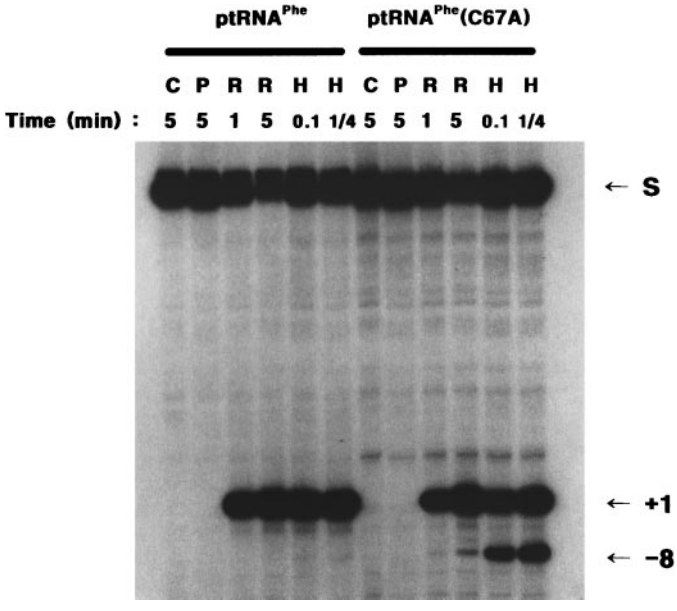

FIG. 4. Single turnover measurements of RNase P activity. The 5′ end-labeled ptRNA^{Phe} or ptRNA^{Phe}(C67A) of 25 nM was incubated in 30 nM enzyme for time intervals indicated above each lane. Lane C, minus enzyme control; lane P, C5 protein 300 nM; lane R, M1 RNA 30 nM; lane H, RNase P holoenzyme 30 nM. The cleavage products are indicated in the right side of the figure: S, substrate; +1, the product of cleavage at the +1 position; −8, the product of cleavage at the −8 position.

TABLE 2

Single Turnover Kinetic Parameters of ptRNA^{Phe} and ptRNA^{Phe}(C67A) in Reactions Catalyzed by either M1 RNA or the RNase P Holoenzyme

Substrate	Cleavage site	k_{cat}/K_m (μM^{-1} s^{-1})	
		M1 RNA	Holoenzyme[a]
ptRNA^{Phe}	+1	0.22	0.79
ptRNA^{Phe}(C67A)	−8	0.064	0.18
ptRNA^{Phe}(C67A)	−8	0.001	0.042

[a] Measured at 2 mM $MgCl_2$.

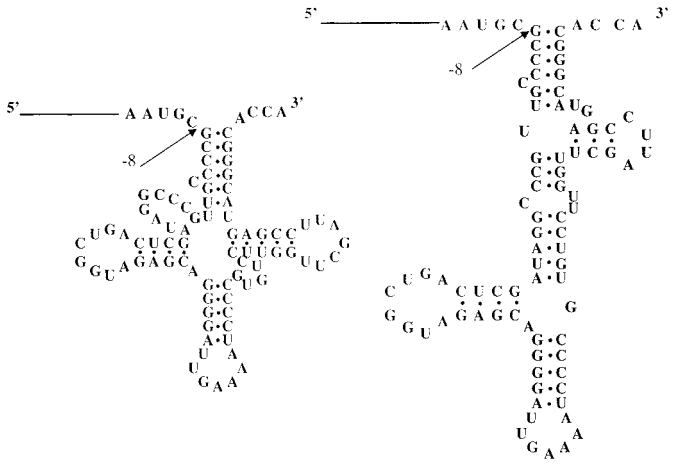

FIG. 5. Two possible secondary structures of ptRNA^{Phe}(C67A) for cleavage at the −8 position. The sites of cleavage by RNase P are indicated in arrows.

acceptor stem. This altered conformation can form by the slippage of the upstream 8 nucleotides into the acceptor stem (two possible secondary structures are shown in Fig. 5). If ptRNA^{Phe}(C67A) takes this conformation, it is likely that cleavage at position −8 by RNase P occurs. However, this conformation might be composed of secondary structures quite different from the structures of the known RNase P substrates. First, the acceptor stem-like structure contains only four contiguous base-pairs or six base-pairs with a single-nucleotide bulge. This short stem structure contrasts with the acceptor stem of 7 base-pairs for cleavage of ptRNA and ptmRNA, or the long stem of 16 base-pairs of p4.5S RNA (Fig. 1). Second, some of the displaced nucleotides from the original acceptor stem can base-pair with nucleotides in the T stem-loop, which could disrupt the T stem-loop. Therefore, the conformation of ptRNA^{Phe}(C67A) for cleavage at the −8 position seems to be quite different from those of the known RNase P substrates since they at least have the long stem structure or the acceptor stem and T stem-loop structure.

The our result that C5 protein is essential for cleavage of ptRNA^{Phe}(C67A) at position −8 may be consistent with the C5 protein-dependency of the RNase P reaction previously shown with pt4.5S RNA as a substrate (12). Even though the secondary structure of 4.5S RNA is different from the common ptRNA structure, p4.5S RNA forms a unique hairpin structure that can mimic the coaxial acceptor stem stacked on the T stem-loop (12). However, the conformation of ptRNA^{Phe}(C67A) for cleavage at position −8 does not resemble the common ptRNA structure or the p4.5S RNA structure. This suggests that C5 protein allows the ribozyme to broaden the substrate specificity more than previously appreciated (1). In this context, it may be worthy of note that the holoenzyme exhibits similar K_m values for the +1 cleavage of ptRNA^{Phe}, the +1 cleavage of ptRNA^{Phe}(C67A), and the −8 cleavage of ptRNA^{Phe}(C67A).

The accurate function of C5 protein *in vivo* is uncertain. Our data showed that a substrate having a conformation much deviated from those of the known RNase P substrates was efficiently cleaved only in the presence of C5 protein *in vitro*. This finding suggests that the RNase P holoenyzme can recognize various RNA molecules as substrates. Besides 5′ maturation of tRNA, 4.5 RNA, and tmRNA, therefore, RNase P may be involved in a wide variety of RNA metabolism within a cell because only the holoenzyme is a functional RNase P enzyme *in vivo*. For example, the 3′-end processing of a leucine-specific suppressor tRNA (20) and the 5′ end-processing of a precursor M1 RNA (21) were reported to depend on RNase P in *E. coli*.

ACKNOWLEDGMENT

This work was supported by a grant from the Korea Science and Engineering Foundation through the Center for Molecular Design and Synthesis.

REFERENCES

1. Frank, D. N., and Pace, N. R. (1998) *Annu. Rev. Biochem.* **67,** 153–180.
2. Bothwell, A. L., Garber, R. L., and Altman, S. (1976) *J. Biol. Chem.* **251,** 7709–7716.
3. Bourgaize, D. B., and Fournier, M. J. (1987) *Nature* **325,** 281–284.
4. Komine, Y., Kitabatake, M., Yokogawa, T., Nishikawa, K., and Inokuchi, H. (1994) *Proc. Natl. Acad. Sci. USA* **73,** 1912–1916.
5. Hartmann, R. K., Heinrich, J., Schlegl, J., and Schuster, H. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 5822–5826.
6. Bothwell, A. L., Stark, B. C., and Altman, S. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 1912–1916.
7. Guerrier-Takada, C., van Belkum, A., Pleij, C. W., and Altman, S. (1988) *Cell* **53,** 267–272.
8. Guerrier-Takada, C., Gardiner, K., Marsh, T., Pace, N., and Altman, S. (1983) *Cell* **35,** 849–857.
9. Schedl, P., and Primakoff, P. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 2091–2095.
10. Sakano, H., Yamada, S., Ikemura, T., Shimura, Y., and Ozaki, H. (1974) *Nucleic Acids Res.* **1,** 355–371.
11. Guerrier-Takada, C., Haydock, K., Allen, L., and Altman, S. (1986) *Biochemistry* **25,** 1509–1515.
12. Peck-Miller, K. A., and Altman, S. (1991) *J. Mol. Biol.* **221,** 1–5.
13. Lumelsky, N., and Altman, S. (1988) *J. Mol. Biol.* **202,** 443–454.
14. Guerrier-Takada, C., Lumelsky, N., and Altman, S. (1989) *Science* **246,** 1578–1584.
15. Kim, J. J., Kilani, A. F., Zhan, X., Altman, S., and Liu, F. (1997) *RNA* **3,** 613–623.
16. Kim, I. Y., and Lee, S. Y. (1990) *Mol. Cells* **1,** 3–7.
17. Park, B. H., Choi, Y. N., Park, J. W., Sim, S., Gil, M. C., Kim, S., Kim, M., and Lee, Y. (1998) *Mol. Cells* **8,** 96–100.
18. Harris, M. E., Nolan, J. M., Malhotra, A., Brown, J. W., Harvey, S. C., and Pace, N. R. (1994) *EMBO J.* **13,** 3953–3963.
19. Harris, M. E., Kazantsev, A. V., Chen, J-L., and Pace, N. R. (1997) *RNA* **3,** 561–576.
20. Nomura, T., and Ishihama, A. (1988) *EMBO J.* **11,** 3539–3545.
21. Lundberg, U., and Altman, S. (1995) *RNA* **1,** 327–334.