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Stabilization of mRNA in an *Escherichia coli* cell-free translation system

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It became clear that mRNA can be stabilized in a cell-free translation system of *Escherichia coli* by hybridization with a small DNA fragment at its 3′ terminus. The stability increased when a small DNA fragment containing a stable hairpin structure with a GAAA loop was used. The enhancement of stabilization was brought about because the hairpin structure is resistant towards the nucleases contained in the translation system. The hairpin structure is effective by stabilizing the added DNA fragment itself towards the nucleases.

Stable DNA hairpin; In vitro translation; mRNA stabilization

1. INTRODUCTION

In vitro RNA-dependent protein-synthesizing systems facilitate elucidation of the processes of translation and isolation of synthesized proteins. However, bacterial cell-free translation systems are inherently contaminated with nucleases [1,2] so that exogeneously added mRNAs are apt to be degraded.

To construct a highly efficient cell-free translation system, we attempted to stabilize mRNAs from nuclease attacks in the system. In bacteria, translation is always coupled with transcription, thus the decay process of mRNAs in bacteria is more complex than that in eukaryotic cells. Recent studies indicate that some of the hairpin structures at the 3′ termini of mRNAs stabilize the mRNAs themselves [3–5]. It is considered that the transcripts degrade from their 3′ termini by 3′ exoribonucleases included in the cell ([6] and A. Spirin, personal communication). We have elucidated that even in vitro the necessary translation system containing partially purified ribosomes and some factors for protein synthesis usually involves 3′-exonuclease activity [7].

Thus, we attempted to render an mRNA resistant towards 3′ exonucleases by protecting the 3′ terminus in an *E. coli* translation system, in which the mRNA was hybridized with a small DNA fragment consisting of a stable hairpin sequence, CGGCGAAAGCCG [8,9] and an additional sequence complementary to the 3′ terminus of the mRNA.

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2. MATERIALS AND METHODS

2.1. *mRNA synthesis*

mRNAs were synthesized by the in vitro transcription system using synthetic DNA templates and T7 RNA polymerase [10]. Primer and template DNAs were synthesized on an Applied Biosystems 381A DNA synthesizer and purified by high-performance liquid chromatography using an M&S PACK C-18 (M&S Co.) or a Wakopak WS-DNA (Wako Pure Chemical Ind. LTD.) column. ^{32}P-labeled RNAs were prepared by incubating the reaction mixture including 50–100 nM of template DNAs, 10–18 μg of T7 RNA polymerase, 0.75–1.25 mM each of unlabeled ATP, CTP, UTP and GTP, and 16–24 μCi [α-^{32}P]UTP (Amersham, approximately 3000 Ci/mmol) for the internal labeling or 4 μCi [γ-^{32}P]GTP (DuPont, 30 Ci/mmol) for the 5′-terminal labeling. Labeled mRNAs were purified by electrophoresis on a 12% polyacrylamide gel containing 7 M urea, followed by elution with a buffer containing 0.3 M sodium acetate, 10 mM magnesium chloride and 10 mM Tris-HCl (pH 7.5). The eluates were dialyzed against the elution buffer. The ^{32}P-labeled mRNAs were precipitated with ethanol and dissolved in 10 mM Tris-HCl (pH 7.5).

2.2. *Assessment of mRNAs stability in in vitro translation systems of* E. coli

E. coli Q13 ribosomes were prepared according to the already published procedure [11], followed by treatment with bentonite [12]. The *E. coli* A19 S100 fraction used for protein synthesis was provided by Drs K. Watanabe and T. Ueda.

^{32}P-labeled mRNAs (0.01–1.7 pmol) were incubated with the *E. coli* 70S ribosomes (5–12 pmol) and the S100 fraction (0.011–0.026 OD_{280} units = 10.8–25.8 μg) in 10 mM Tris-HCl (pH 7.5), 0.1 M ammonium chloride, 10 mM magnesium acetate and 6 mM 2-mercaptoethanol. At a specified time, a portion of the mixture was added to the same volume of dye solution containing 10 M urea, and the fate of the ^{32}P-labeled mRNAs was analyzed by 12% or 20% polyacrylamide gel electrophoresis containing 7 M urea, 0.08 M Tris-phosphate (pH 8.0) and 2 mM EDTA.

3. RESULTS AND DISCUSSION

We designed an mRNA sequence (RNA1) as shown in Fig. 1a, which encodes a sex pheromone, cAD1 of *Streptococcus faecalis* [13] and contains the Shine–Dalgarno sequence AGGA [14,15] and AUG codon, as a model of a prokaryotic mRNA. The model mRNA was

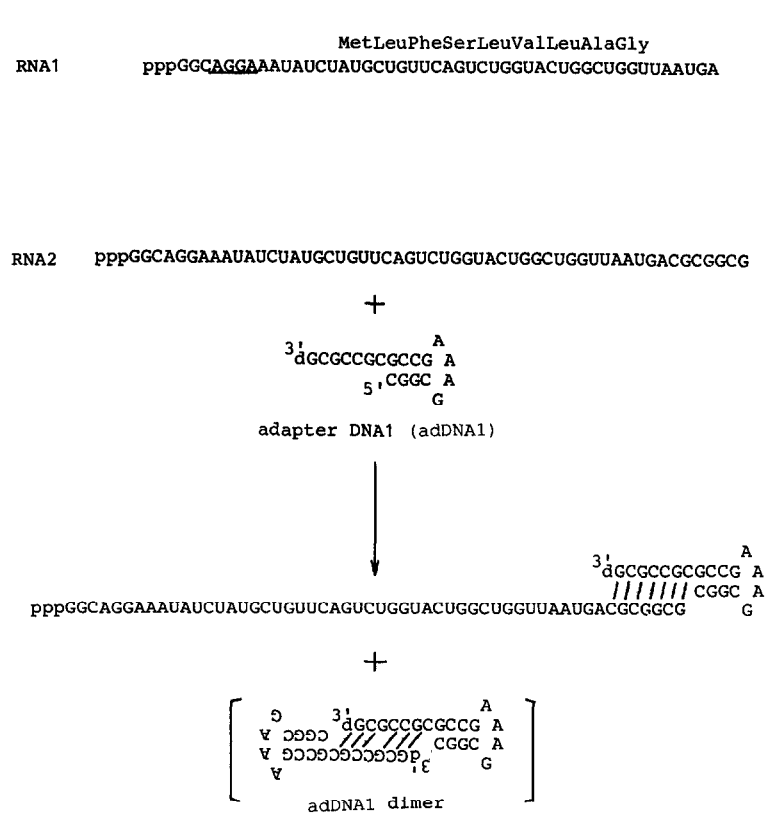

Fig. 1. (a) Model RNA sequence (RNA1) coding for cAD1 peptide sequence added with methionine at the N-terminal. The underlined nucleotides indicate the Shine-Dalgarno sequence. (b) Hybridization of RNA2 with adapter DNA1 (adDNA1) for the stabilization of RNA2 in *E. coli* cell-free translation system. adDNA1 dimer is a speculated structure, possibly formed in excess of added adDNA1.

synthesized by the in vitro transcription system using a synthetic DNA template and T7 RNA polymerase [10], and purified by polyacrylamide gel electrophoresis.

First, mRNAs having the hairpin sequence, CGGCGAAAGCCG, at the 3′ terminus or both the 5′ and 3′ termini were synthesized and their stability was examined in the *E. coli* translation system including the S100 fraction and 70S ribosomes. However, the mRNAs could not be stabilized so much in the system (data not shown).

In prokaryotes, translation is coupled to transcription so that the 3′ terminus of a nascent mRNA is always attached to the parent genomic DNA. To mimic this situation, we attempted to protect the 3′ terminus of the mRNA by hybridizing with a small DNA fragment. RNA2 was designed for this purpose, by adding seven more nucleotides to the 3′ terminus of RNA1 (see Fig. 1b). This is capable of hybridizing the mRNA with a small DNA fragment named adapter DNA (adDNA) which consists of extraordinarily stable hairpin sequence, CGGCGAAAGCCG [8,9] and an additional sequence complementary to the 3′ terminus of RNA2, as shown in Fig. 1b.

The RNA2–adDNA1 hybrid was incubated with the mixture including the S100 fractions and ribosomes of *E. coli* at 37°C for 1 h. As shown in Fig. 2, RNA2 was protected considerably against the nuclease attacks by adding approximately 400 mol equiv. of adDNA1 in the solution (lane 4). In contrast, RNA2 without adDNA1 was digested to a considerable extent in the system, and the main degradation product was found as the band corresponding to RNA fragments which are approximately 10–20 bases shorter than RNA2 (lane 2).

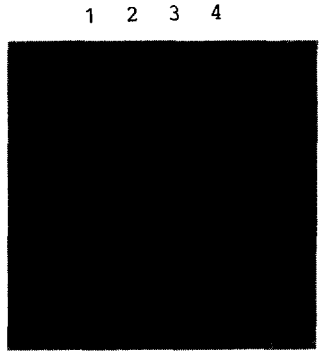

Fig. 2. Effect of adDNA1 on the stabilization of RNA2. Internally ^{32}P-labeled RNA2 (1.7 pmol) was annealed with 356 pmol (lane 3) and 712 pmol (lane 4) of adDNA1 or without adDNA1 (lane 2) in a buffer at 90°C for 3 min. The mixture was incubated with S100 fractions (25.8 mg) and 70S ribosomes (12 pmol) at 37°C for 1 h. Lane 1 is the original labeled RNA2. Each RNA was analyzed by electrophoresis in 12% polyacrylamide gel containing 7 M urea.

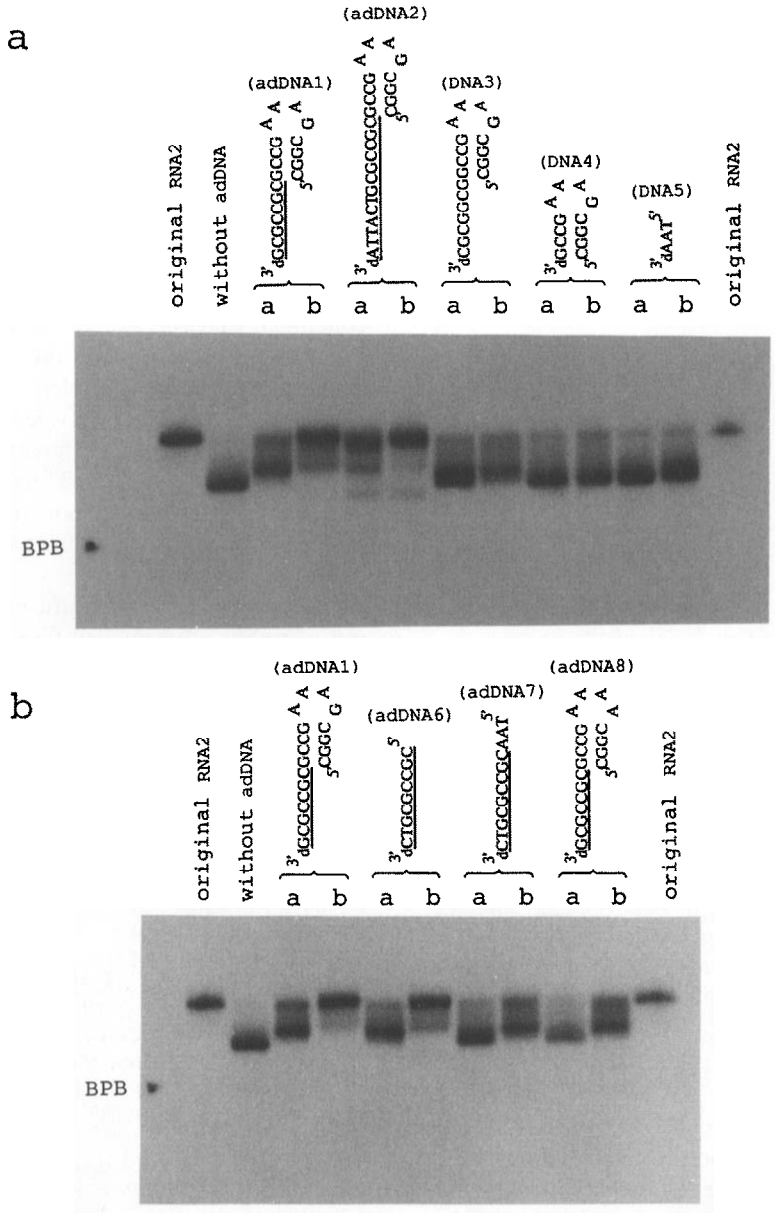

Fig. 3. Stability of RNA2 hybridized with several kinds of DNA fragments in the *E. coli* cell-free translation system. Internally ^{32}P-labeled RNA2 (1.72 pmol) was annealed with 100 mol equiv. (lane a) or 400 mol equiv. (lane b) of several kinds of DNA fragments indicated in the figure or without DNA (after heated at 90°C for 3 min). The hybridized mixture containing RNA2 and adDNAs was incubated with S100 fractions (21.5 μg) and 70S ribosomes (10 pmol) at 37°C for 1 h, and analyzed by electrophoresis in 12% polyacrylamide gel containing 7 M urea. Underlined nucleotides denote the sequences complementary to the 3′ terminus of RNA2.

To confirm the idea that the stabilizing effect of adDNA1 on RNA2 is due to the hybrid formation between them, we examined the stability of RNA2 added by various small DNA fragments containing either a sequence with higher complementarity to the 3′ terminus of RNA2 or unrelated sequences to the RNA2. As shown in Fig. 3a, RNA2 shows a great stability by hybridizing with adDNA2 containing a 13-base sequence complementary to the 3′ terminus of RNA2. In contrast, RNA2 was not stabilized by other DNA fragments (DNA3–5) possessing no complementary sequences to RNA2 (Fig. 3a). Therefore, adDNA may directly stabilize RNA2 by hybridizing with it, but does not serve as an inhibitor towards nucleases in the translation system.

As shown in Fig. 3b, adDNA6 which has a sequence complementary to the 3′ terminus of RNA2 but no hairpin structure shows the stabilization effect, but to a lesser extent than adDNA1. RNA2 was also stabilized to some extent by other DNA fragments having the AAT sequence (adDNA7) or the other hairpin structure, CGGCAAAAGCCG, attached to the 3′ terminus of the sequence complementary to the RNA2 (adDNA8) (Fig. 3b). However, the effects by the DNA

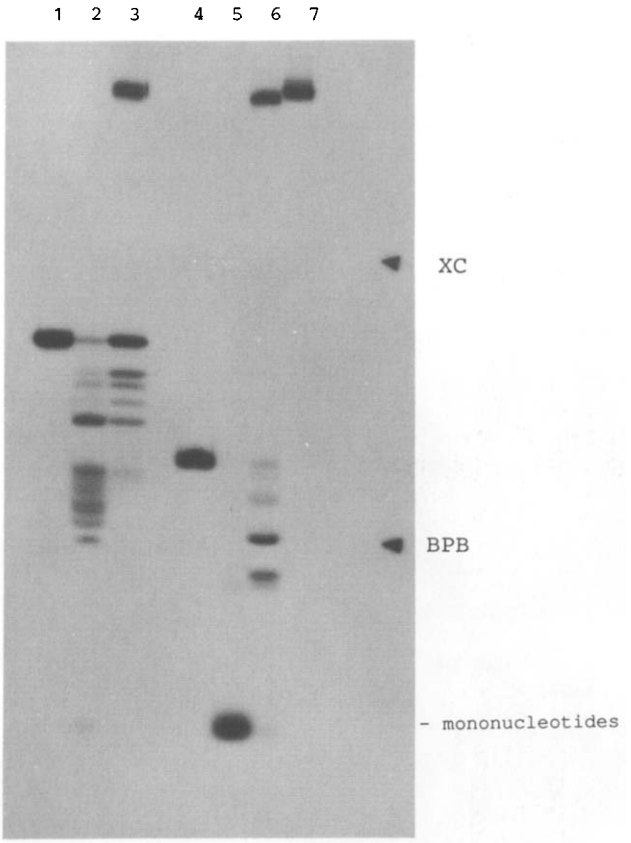

Fig. 4. Stability of adDNAs in the *E. coli* cell-free translation system. Lanes 1, 4 and 7 are original 5′-^{32}P-labeled adDNA1, adDNA6 and ^{32}P-internally labeled RNA2, respectively. Each 5′-labeled adDNA1 (1.2×10^{-3} pmol; lanes 2 and 3) and adDNA6 (6.5×10^{-4} pmol; lanes 5 and 6) was incubated with S100 fractions (10.75 μg) and 70S ribosomes (5 pmol) at 37°C for 1 h in the absence of RNA2 (lanes 2 and 5) or in the presence of internally ^{32}P-labeled RNA2 (lanes 3 and 6). Each mixture was analyzed by electrophoresis in a 20% polyacrylamide gel containing 7 M urea.

fragments were much less than those by adDNA1 or adDNA6. The hairpin structure formed by the CGGCAAAAGCCG sequence (T_m = 74.5°C in 0.1 M NaCl) is known to be less stable than that formed by the CGGCGAAAGCCG sequence (T_m = 89°C) [16,17]. Thus, it is concluded that the most effective adapter DNA is the one which comprises a longer sequence complementary to the 3′ terminus of an mRNA and a thermally stable hairpin structure, such as that formed by the GCGAAAGC sequence [9].

Although the hybridization of RNA2 with adDNAs is very effective against exonucleases, a large amount of adDNAs are required for the complete stabilization of mRNAs. A possible reason would be because adDNA1 forms a dimer structure (see Fig. 1b) due to the high self-complementality. Thus, we synthesized mRNA and adDNA, each possessing low self-complementality, and examined the stabilization effect of the adDNA towards the mRNA. However, the stabilization of the mRNA also requires an excess amount of the adDNA, as in the case of adDNA1 (data not shown).

Another possible reason would be that most of adDNAs are also degraded by nucleases in the system. As shown in Fig. 4, the stability of ^{32}P-labeled adDNA1 and adDNA6 were examined in the system without RNA2 (lanes 2 and 5). adDNA1 was more resistant towards the nucleases than adDNA6, which was completely degraded into mononucleotides (lane 5). Hybridization of DNA fragments with mRNA2 considerably prevents the degradation of the adDNAs themselves (lanes 3 and 6) as well as that of mRNA2. It is thus concluded that the added DNA fragments are also degraded with nucleases, so that an excess amount of them are required for the complete stabilization of mRNAs. The stability difference between adDNA1 and adDNA6 correlates with their different stabilizing effects on RNA2 shown in Fig. 3b. Thus, it is evident that introduction of the stable hairpin structure into the terminus of adDNA enhances the stabilizing effect against the mRNA degradation.

The present method is not only useful for constructing a highly efficient cell-free translation system, but also applicable to the isolation of full-length RNAs from cells, and to the stabilization of antisense oligomers.

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REFERENCES

[1] Deutscher, M.P. (1988) Trends Biochem. Sci. 13, 136–139.
[2] Brawerman, G. (1989) Cell 57, 9–10.
[3] Belasco, J.G., Beatty, J.T., Adams, C.W., Gabain, A.V. and Cohen, S.N. (1985) Cell 40, 171–181.
[4] Newbury, S.F., Smith, N.H., Robinson, E.C., Hiles, I.D. and Higgins, C.F. (1987) Cell 48, 297–310.
[5] Chen, C.-Y.A., Beatty, J.T., Cohen, S.N. and Belasco, J.G. (1988) Cell 52, 609–619.
[6] Gabain, A.v., Belasco, J.G., Schottel, J.L., Chang, A.C.Y. and Cohen, S.N. (1983) Proc. Natl. Acad. Sci. USA 80, 653–657.
[7] Ishikawa, M., Ikebukuro, K., Hirao, I. and Miura, K. (1991) Nucleosides and Nucleotides 10, 1377–1390.
[8] Hirao, I., Nishimura, Y., Naraoka, T., Watanabe, K., Arata, Y. and Miura, K. (1989) Nucleic Acids Res. 17, 2223–2231.
[9] Hirao, I., Nishimura, Y., Tagawa, Y., Watanabe, K. and Miura, K. (1992) Nucleic Acids Res. 20, 3891–3896.
[10] Milligan, J.F., Groebe, D.R., Witherell, G.W. and Uhlenbeck, O.C. (1987) Nucleic Acids Res. 15, 8783–8798.
[11] Ohta, T., Sarkar, S. and Thach, R.F. (1967) Proc. Natl. Acad. Sci. USA 58, 1638–1644.
[12] Fraenkel-Conrat, H., Singer, B. and Tsugita, A. (1961) Virology 14, 54–58.
[13] Mori, M., Sakagami, Y., Narita, M., Isogai, A., Fujino, M., Kitada, C., Craig, R.A., Clewell, D.B. and Suzuki, A. (1985) FEBS Lett. 178, 97–100.
[14] Shine, J. and Dalgarno, L. (1974) Proc. Natl. Acad. Sci. USA 71, 1342–1346.
[15] Shine, J. and Dalgarno, L. (1975) Nature 254, 34–38.
[16] Tanikawa, J., Nishimura, Y., Hirao, I. and Miura, K. (1991) Nucleic Acids Symp. Ser. 25, 47–48.
[17] Hilbers, C.W., Blommers, M.J.J., Ven, F.J.M.v.d., Boom, J.H. and Marel, G.A. (1991) Nucleosides and Nucleotides 10, 61–80.