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Chemically synthesized circular RNAs with phosphoramidate linkages enable rolling circle translation

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Circular RNA without a stop codon enables rolling circle translation. To produce circular RNAs, we carried out one-pot chemical synthesis of circular RNA from RNA fragments with the use of an EDC/HOBt-based chemical ligation reaction. The synthesized circular RNAs acted as translation templates, despite the presence of unnatural phosphoramidate linkages.

In recent years, many types of circular RNAs have been discovered in cells, and attention has focused on elucidating their functions[1]. One of these reported functions is the translation of circular RNAs into proteins[2,3]. Recently, we reported that small circular RNAs excluding stop codons can efficiently synthesize proteins through rolling circle translation (Figure 1)[4,5]. Once the ribosome binds to these circular RNAs, an infinite rolling circle translation reaction is initiated, which results in the production of long, repeating peptides in prokaryotic and eukaryotic systems. Because ribosome binding is the initiation step, it is also the rate-limiting step of mRNA translation; however, it only needs to occur once in the circular RNA system, which results in very efficient protein synthesis when compared with that of linear mRNA. This strongly suggests that the circularization of mRNA is a key strategy for ensuring efficient protein synthesis. However, although circular RNA has many advantages over linear mRNA, the preparation of circular RNA is currently limited by impractical methods. Generally, circular RNA is prepared by a ligation reaction of linear RNA[6,7] or a splicing reaction of pre-mRNA[8-10]. However, such enzymatic methods are costly and unsuitable for large-scale synthesis of circular RNA. Our group and others have reported the use of the chemical ligation reaction via the unnatural phosphoramidate (P-N) linkage for the synthesis of long oligonucleotides[11-13]. Brown and co-workers succeeded in the chemical synthesis of cyclic single-stranded DNAs

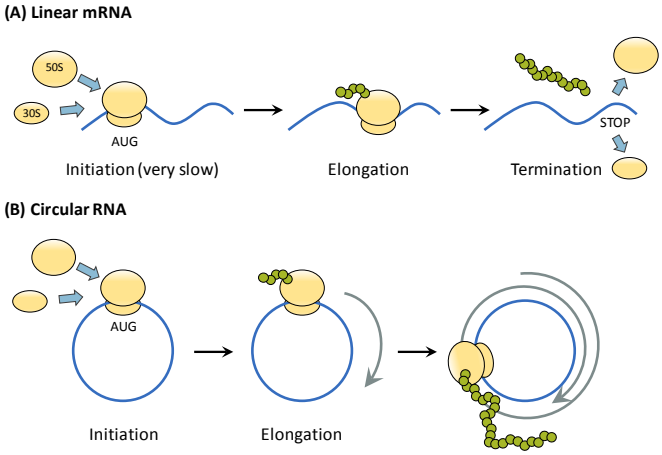

Fig. 1 (A) Translation of Linear mRNA. (B) Rolling circle translation of circular RNA without a stop codon.

using P-N bond formation to mediate the chemical ligation reaction, and these DNAs served as templates for rolling circle amplification (RCA)[14]. These chemical strategies are easily scalable and cost-effective when compared with enzymatic methods. Therefore, in this study, we investigated the chemical synthesis of circular RNAs using EDC/HOBt-based P-N bond formation to mediate the chemical ligation reaction of RNA fragments. There are only a few published examples of chemically synthesized linear mRNA being successfully translated into peptides[15-17], and the resulting peptides were only ten or fewer residues in length. In this paper, we evaluated the rolling circle translation of chemically synthesized circular RNAs with an unnatural phosphoramidate backbone in a cell-free protein synthesis system, and demonstrated the production of long, repeating peptides from such RNAs for the first time.

Circular RNAs with unnatural phosphoramidate (P-N) linkages were designed. These were synthesized by an EDC/HOBt-based chemical ligation reaction between 3'-amino-

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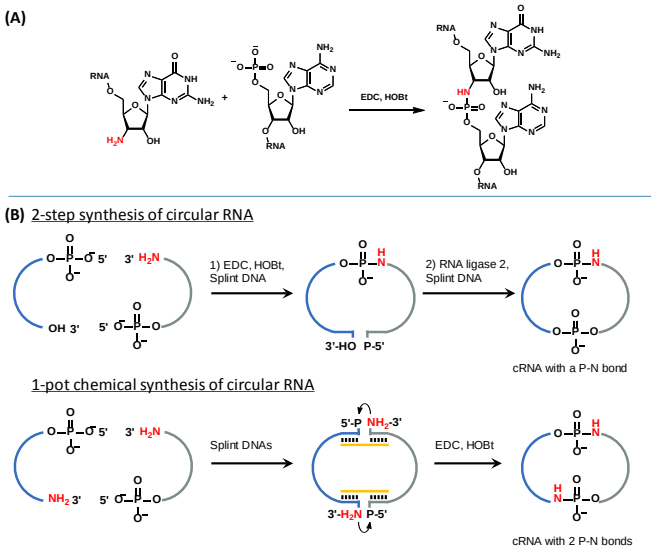

Fig. 2 (A) EDC/HOBt chemical ligation reaction between 3′-amino modified RNA and 5′-phosphorylated RNA. (B) Two-step chemoenzymatic synthesis or one-pot chemical synthesis of circular RNA.

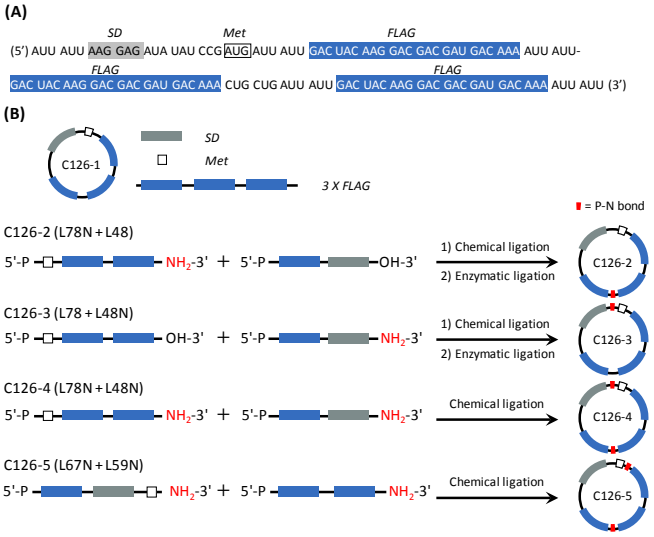

Fig. 3 (A) Sequence of the 126-mer RNA used in this study. (B) Series of 126-mer circular RNAs used in this study.

modified RNA and 5′-phosphorylated RNA (Figure 2A). Two synthetic routes for the construction of circular RNA were tested: (1) two-step chemoenzymatic synthesis and (2) one-pot chemical synthesis (Figure 2B). To determine the effect of P-N bond replacement on the translation efficiency of the circular RNAs, we synthesized circular RNA containing one or two P-N bonds. For the synthesis of the circular RNAs, we first divided a 126-mer linear RNA, which encoded a Shine-Dalgarno (SD) sequence and three FLAG tags, into a 78-mer and 48-mer or a 69-mer and 59-mer (Figure 3A). Each RNA fragment contained a phosphate group at the 5′-end and an amino group or hydroxyl group at the 3′-end (Figure 3B). The 3′-amino group was introduced using 3′-amino modified Guanosine CPG (Scheme S1). All synthesized RNA fragment and splint DNA sequences are presented in Tables S1 and S2.

The chemical ligation reaction of circular RNA was performed in an EDC/HOBt-containing conjugation system in the presence of a complementary DNA strand. During the two-step chemoenzymatic synthesis, the first chemical ligation reaction of the linear 126-mer RNA was completed successfully through P-N bond formation in 0.5 M of EDC/HOBt in a HEPES-NaOH buffer (pH = 8.7) (Figure 4A). Single P-N bond-containing circular RNAs were then synthesized through RNA ligase 2-mediated circularization of the chemically synthesized 126-mer linear RNAs (Figure 4B). The target 126-mer circular RNAs were purified by 8% denaturing PAGE and isolated from the gel using a crush and soak method. Two types of circular RNAs with a single P-N bond at different positions were synthesized, and the overall yields from the two-step reaction were calculated from the intensity of the bands as 9.5% for C126-2 and 13.3% for C126-3. Additionally, the isolation yields were 3.7% for C126-2 and 3.2% for C126-3. One-pot chemical synthesis was also performed using RNA fragments containing phosphate at the 5′-end and an amino group at the 3′-end. During synthesis, the same EDC/HOBt-containing HEPES-NaOH buffer was used. This one-pot chemical ligation reaction was first performed with 5 μM of each RNA fragment, but the ligation reaction only proceeded for C126-4 and not for C126-5 (Figure 5). For the construction of C126-5, only trace amounts of circularized product were detected after 16 h incubation. To improve this low ligation yield, the concentration of each RNA fragment was increased from 5 μM to 20 μM in the reaction solution. Although the ligation yield remained lower than that of C126-4, this increase in RNA concentration was effective. Generally, circularization of single-stranded RNA is performed with low concentrations of oligonucleotides (<1 μM) to avoid undesirable polymerization. In contrast, one-pot chemical circularization appears to preferentially proceed at 20 μM RNA because it comprises an intermolecular reaction of two RNA fragments. This is an advantageous property in terms of large-scale synthesis. Thus, we performed the one-pot chemical circularization reaction with 20 μM RNA and the reaction yields

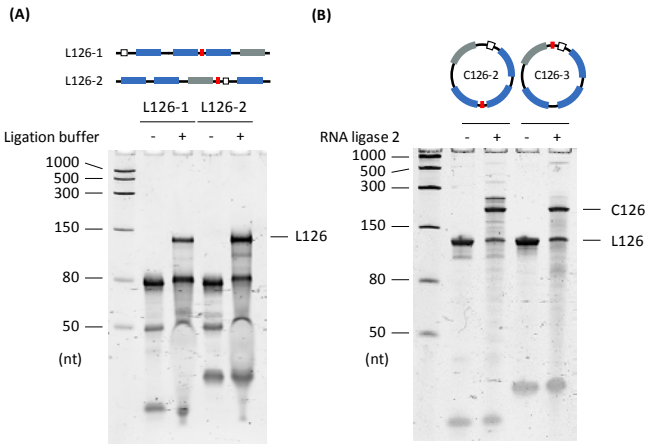

Fig. 4 Eight percent denaturing PAGE analysis of the reaction mixtures (8 M urea, 30 min, 25 mA constant). Visualized by SYBR Green II staining. (A) Chemical synthesis of 126-mer linear RNAs. (B) Enzymatic circularization of 126-mer linear RNAs.

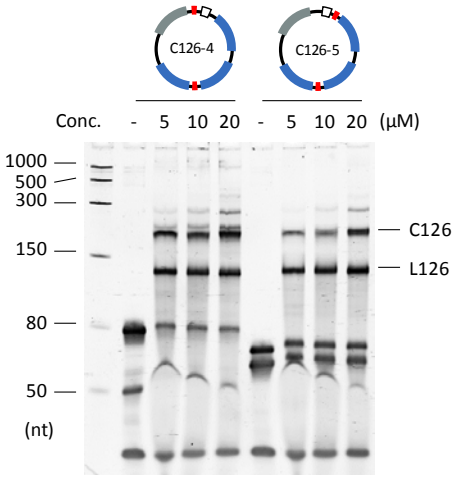

Fig. 5 Eight percent denaturing PAGE analysis of reaction mixtures (8 M urea, 30 min, 25 mA constant). One-pot chemical syntheses of C126-4 and C126-5 were performed at different RNA fragment and splint DNA concentrations. For gel analysis, each loading amount was adjusted by dilution.

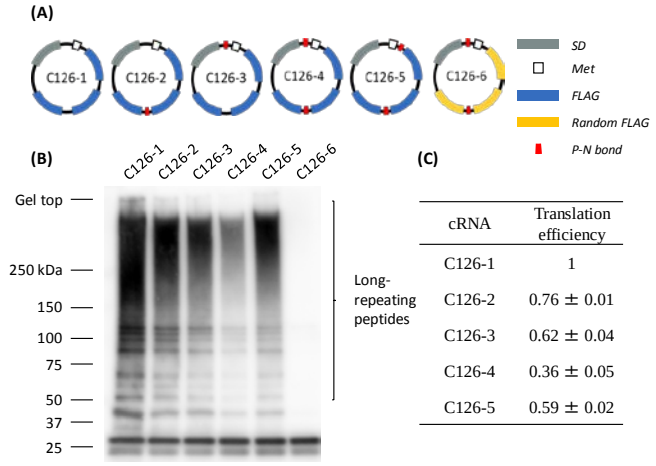

Fig. 6 (A) Series of synthesized circular RNAs. (B) Translation of the chemically synthesized circular RNAs was detected by western blotting using anti-FLAG M2 antibody. (C) Translation efficiency of each circular RNA relative to that of C126-1. Data are presented as the mean ± SEM (n = 3).

calculated from the intensity of the bands were 32.4% for C126-4 and 22.6% for C126-5. The isolation yields were 12.5% for C126-4 and 7.6% for C126-5. After gel purification, we verified the purity of the isolated circular RNAs by RP-HPLC (Figure S1). The circularity of all synthesized RNAs was confirmed by 3′-exonuclease RNase R treatment (Figure S2). We also confirmed the structure of C126-4 by MALDI-TOF/MS analysis after MazF enzyme digestion[18] (Figure S3).

Finally, we initiated translation of these chemically synthesized circular RNAs in a reconstituted *E. coli* cell-free system known as the *PURE* system[19,20]. The translation products were detected by western blotting (Figures 6 and S6) but not detected by coomassie blue staining (Figure S7). C126-6, which contains the same nucleotides as C126-4 but does not encode FLAG peptides, was also chemically synthesized and used for the translation experiment (Figure S4). To assess the effect of P-N bond substitution on translation, we also prepared a natural circular RNA (C126-1) with all P-O bonds according to a previously reported method[4]. The synthesized circular RNA sequences used in this study are summarized in Figure S5. All P-N bond-containing circular RNAs except C126-6 were successfully translated into long, repeating FLAG peptides through rolling circling translation. This is the first report of the translation of an RNA molecule containing unnatural P-N bonds. However, the translation efficiency of the circular RNAs was dependent on the position or number of introduced P-N bonds (Figure 6C). C126-2 with a P-N bond located 42 bases distal from the SD sequence showed the highest translation efficiency (0.76). C126-3 with a P-N bond located within the SD sequence showed lower efficiency (0.62) than C126-2. The introduction of the P-N bond into the SD sequence decreased the translation efficiency more significantly. A similar trend was observed for circular RNAs (C126-4 and C126-5) with two P-N bonds. C126-4 with a P-N bond within the SD sequence showed much lower translation efficiency (0.36) than C126-5 (0.59), which has a P-N bond 19 bases downstream from the SD sequence.

Translation of circular RNA is subdivided into two steps consisting of initiation and elongation. The initiation step is known to be the rate-limiting step. Because the SD sequence is a ribosomal recruitment site[21], P-N bond replacement within this region might disturb ribosomal association on the circular RNAs. In comparison, P-N replacement appears to have a weaker effect on the elongation step. For example, protein products represented by the band at ~40 kDa arise from the ribosome dissociating during circular RNA elongation. However, the amount of these protein products did not increase when a P-N bond was introduced. From these results, we concluded that P-N bond replacement in circular RNAs disturbs the ribosomal binding initiation step of circular RNA translation but not the elongation step.

Conclusions

We succeeded in the one-pot chemical synthesis of a series of 126-mer circular RNAs using an EDC/HOBt-based chemical ligation reaction of RNA fragments. Furthermore, it was found that the chemically synthesized, P-N-modified circular RNAs were translated into long, repeating peptides via rolling circle translation. This is the first report of the production of long, repeating peptides from chemically synthesized RNA molecules. From translation experiments, it was also found that the P-N backbone modification only disturbed ribosome binding during the initiation step but did not affect the elongation step of the translation reaction in a prokaryotic translation system. Although further optimization of the circular RNA sequence is required to improve its translation efficacy, this chemical approach for circular RNA synthesis has potential applications in the development of novel RNA-based protein synthesis techniques and strategies for gene therapy.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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