Continuous Translation Reaction

DOI: 10.1002/anie.201302044

Rolling Circle Amplification in a Prokaryotic Translation System Using Small Circular RNA**

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Rolling circle amplification (RCA) is an isothermal, enzymatic process mediated by a specific group of DNA polymerases in which tandemly repeated single-stranded (ss) DNA molecules are synthesized on a short circular ssDNA molecule (Figure 1 a).[1] The reaction produces long nucleic acids because the circular template in essence represents a template of infinite length. Following its discovery, RCA was initially used for the ultrasensitive detection of DNA.[1] More recently, the technique has also been used to detect target molecules other than nucleic acids, such as small molecules and proteins.[1] In RCA, circular ssDNA molecules as short as 13 nucleotides (nt) in length have been shown to act as substrates for polymerases.[2] One of the key features of RCA is that its mechanism not only provides access to long repeating oligonucleotides but that it also provides enhanced levels of production over a given period of time. The synthesis of oligonucleotides by RCA is effectively promoted by the avoidance of the multiple association and dissociation processes between the polymerase and the template.[3]

In 1998, Perriman and Ares demonstrated that circular RNA molecules could be translated in vivo in *E. coli*.[4] The principle aim of their study was the construction of a system to produce repeating protein sequences in *E. coli*. A long chain of multimeric green fluorescent protein (GFP) was produced in *E. coli* when an RNA circle of 795 nt with an infinite open reading frame (ORF) was formed in the cell, although they suggested that the repeating GFP did not emit fluorescence.[4a] In this pioneering study, it was concluded that the circular RNA provided a smaller amount of product from the translation process than linear RNA, because initiation was less efficient on the circular RNA than on the linear RNA.[4a] In this particular report, however, they did not compare the amount of protein production when the continuous protein synthesis occurred. In principle, when the

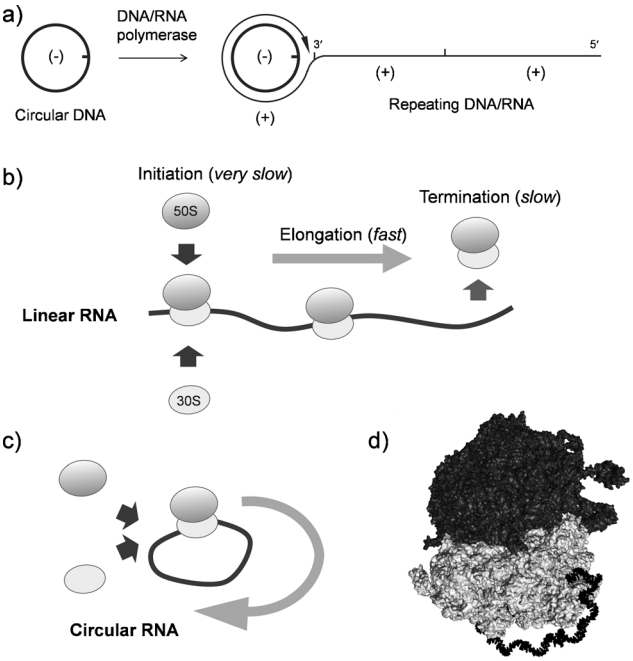

Figure 1. Continuous translation reaction on a small circular RNA. a) Concept of rolling circle DNA synthesis.[1–3] A repeating DNA sequence complementary to the template is produced by polymerase enzymes. b, c) Conceptual diagrams of translation reactions on linear (b) and on circular RNAs (c).[5] Only RNAs and ribosomes are shown. RNA is depicted as a string and the 30S and 50S subunits of the ribosome are depicted as light gray and dark gray ovals, respectively. On the linear RNA, a ribosome detaches from RNA when it encounters a termination codon. The ribosome then reenters the subsequent initiation–elongation–termination cycle. On circular RNA with an infinite reading frame, the ribosome continuously circles the molecule following an initiation process, which leads to the production of a long repeating peptide. d) Computer modeling by MacroModel software showing that an *E. coli* ribosome binds a circular RNA of 126 nt.[8c] The 50S and 30S subunit of a ribosome are shown in dark gray and light gray, respectively.

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[**] H.A. and Y.I. were supported financially by the Ministry of Education, Culture, Sports, Science, and Technology of Japan (MEXT). This work was also supported by a Grant-in-Aid for Scientific Research on Priority Areas, "Lifesurveyor", from MEXT. We are grateful to the Support Unit for Bio-Material Analysis, RIKEN BSI Research Resources Center, for help with the MS analysis.

Supporting information for this article (including experimental details) is available on the WWW under http://dx.doi.org/10.1002/anie.201302044.

translation reaction is subdivided into several steps, including initiation, elongation, termination, and ribosome recycling, initiation represents the rate-limiting step (Figure 1b).[5] Based on this understanding, it was envisaged that a translation reaction conducted on circular RNA would provide a more efficient means of peptide production than the same reaction conducted on the corresponding linear template, in the same way that the RCA reaction can be enhanced by greatly reducing the frequency of the re-initiation process.[3] Herein we demonstrate that peptide synthesis in the translational system can be promoted by a mechanism similar to that of RCA (Figure 1c).

We designed and synthesized a series of small circular RNA molecules of 84 to 252 nt (Figure 2). First, we prepared four RNA circles of different sizes (i.e. 84, 126, 168, and 252 nt) to determine the minimum length of circular RNA required to induce the continuous translation (Figure 2a,b). These circles shared the same ribosomal binding sequence which is known as the Shine–Dalgarno (SD) sequence and the start codon (Figure 3).[6] They also contained multiple 24 nt sequences that coded FLAG peptides. The FLAG octapeptide Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys is a protein tag that can be added to a protein for detection and purification of the expressed protein.[7] As their precursors, two linear RNA units composed of 84 and 126 nt and named L84 and L126, respectively, were constructed (Figures 2 and 3). The minimum length of the RNA circles was set as 84 nt. We estimated that a circular RNA would need to be longer than approximately 70 nt to bind effectively to a prokaryotic ribosome, taking the circular structure into account, because 30–35 nt of the mRNA are known to be covered by the ribosome during the binding process (Figure 1d).[8] For the synthesis, the linear RNA precursors were first obtained by ligating two or three chemically synthesized RNA fragments using T4 DNA ligase (see Table S1 and Figure S1 in the Supporting Information). These linear RNAs were circularized using T4 DNA ligase in a monomeric and dimeric manner (Figure 2).

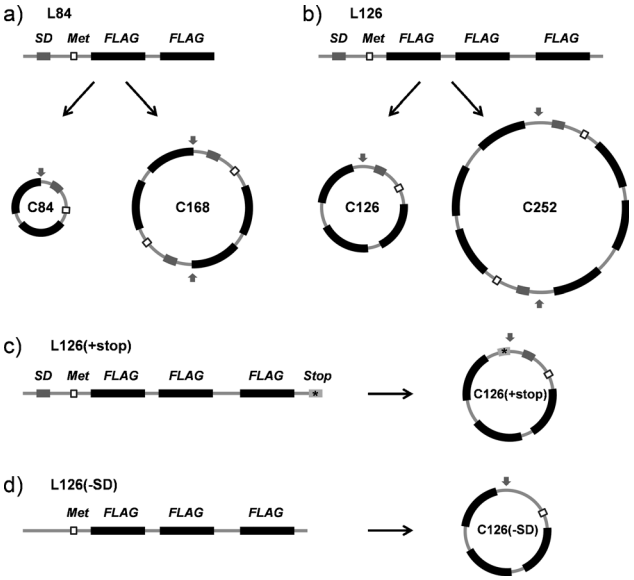

Figure 2. Schematic diagram for the construction of circular RNAs. The linear RNA precursors were circularized using T4 DNA ligase and DNA oligomer templates. Thick arrows on the circles denote the site of ligation. a, b) Construction of circular RNAs to investigate the effect of size on the translation process. Linear 84 nt RNA (L84) (a) and linear 126 nt RNA (L126) (b) were circularized. Two circular RNAs that were produced by being ligated in a monomeric and dimeric manner were isolated from each of the linear RNAs: C84, a monomeric circle of L84; C126, a monomeric circle of L126; C168, a dimeric circle of L84; and C252, a dimeric circle of L126. c) A circular RNA containing stop codons. Linear 132 nt RNA [L126(+stop)] was circularized to form C126(+stop). d) A circular RNA lacking the Shine–Dalgarno sequence. Linear 126 nt RNA [L126(−SD)] was circularized to form C126(−SD).

Once these circular RNA molecules were prepared, they were compared in the translation reaction using a reconstituted *E. coli* cell-free system known as the PURE system

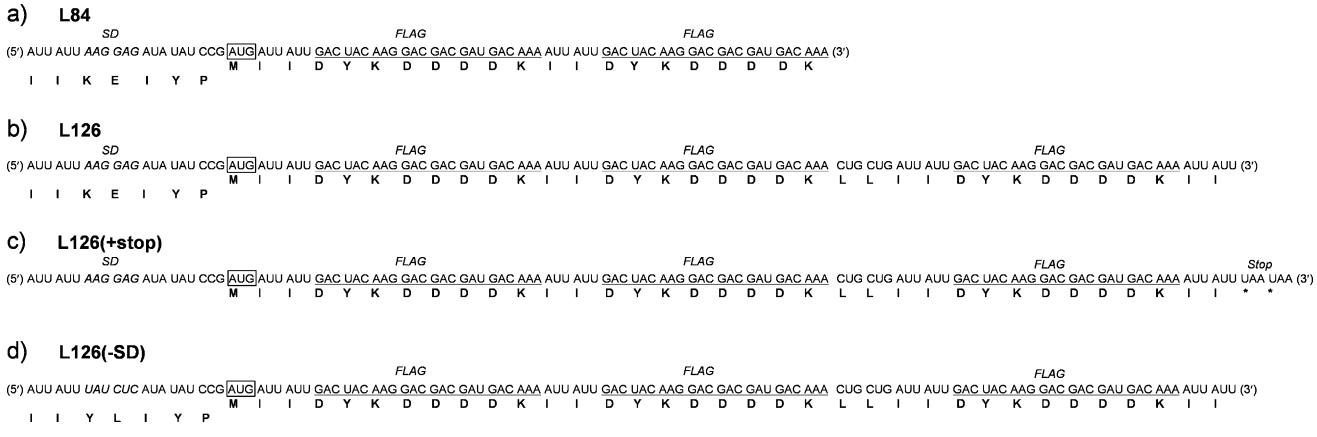

Figure 3. RNA sequences used in this study. The coding regions of the FLAG peptide have been underlined. The Shine–Dalgarno sequence (SD) is shown in italic text. The initiation codon (AUG) is shown in the box. The corresponding amino acid sequences are shown under the nucleotides. When L84 (a), L126 (b), and L126(−SD) (d) are circularized [that is, they are converted to C84, C126 and C126(−SD), respectively], the peptide sequences in the second line can be expressed continuously following the first line, and the translation should then return to the first line. L126 (b) represents a 1.5-fold elongation of L84 (a) through the attachment of a sequence containing one more FLAG code. L126(+stop); (c) has an additional 6 nt sequence of stop codons (UAA UAA) relative to L126 (b) at its 3′-end. The SD sequence AAG GAG was changed to UAU CUC for L126(−SD) (d).

(Figures 2a,b and 4).[9] The kinetic parameters of the protein synthesis reaction have already been determined in a cell-free system and the rates of the initiation and elongation processes in this system were reported to be an order of magnitude lower than the corresponding in vivo rates.[5b] All of the components were rigorously regulated in the PURE system, and it has been reported that the stability of RNA can be significantly improved because of the lack of degradation enzymes.[9] Following incubation, the reaction mixture was subjected to SDS-PAGE and western blot analysis. As shown in Figure 4, the peptides produced from the linear RNAs appeared to be determined by the length of the template RNA (Figure S5). For the circular RNAs, ladder bands were observed for all of the RNA circles that reached to the top of the gel, except for C84 (Figure 4). Based on the occurrence of these bands, the continuous translation reactions were judged to have occurred (Figure S6).[4a] Of the three circles evaluated, C126 appeared to produce the largest amount of peptides (Figure 4). However, in the *E. coli* S30 extract no difference was observed in the amount of translation product produced by C126 and C252, although C126 also seemed to produce more product than C168 in this system (Figure S7). For the smallest RNA circle, C84, only a faint band below 20 kDa was detected (Figure 4). The possibility of the circular molecule C84 being translated itself was also considered because no nicking reactions appeared to have occurred in the translation mixture during the two-hour incubation period, based on northern blot analysis (Figure S8). These results therefore indicated that although C84 was translated, no continuous translation reaction occurred. This result was attributed to the interaction between the SD sequence and the 3′-end of the 16S rRNA of the translating ribosome.[10] We supposed that this interaction would have induced severe strain on the RNA in the smallest C84 circle, leading to the release of the ribosome from the RNA.

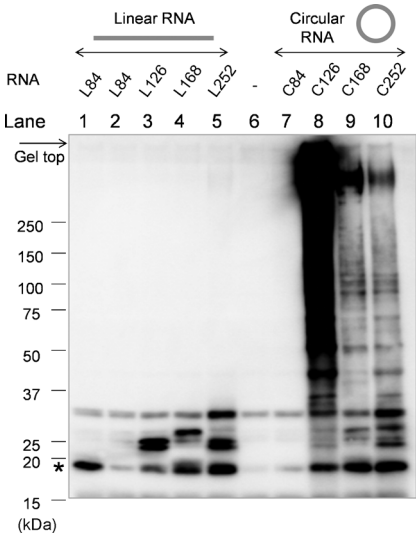

Figure 4. Western blot analysis of cell-free translation on circular RNA templates of different sizes. Linear and circular RNAs of different lengths were compared. Lane 1: L84 (10 μM); lane 2: L84 (1 μM); lane 3: L126 (1 μM); lane 4: L168 (0.5 μM); lane 5: L252 (0.5 μM); lane 6: no RNA; lane 7: C84 (1 μM); lane 8: C126 (1 μM); lane 9: C168 (0.5 μM); and lane 10: C252 (0.5 μM). After the reaction mixtures had been incubated at 37°C for 2 h, a 1 μL sample of each was analyzed by SDS-PAGE using a 10–20% Tris-glycine gel. The peptides were then transfered to a PVDF membrane. Anti-FLAG M2 monoclonal antibody and anti-mouse IgG peroxidase conjugated antibody were used to visualize the blot.

The translation reactions on the circular RNAs were also analyzed by single-molecule imaging (Figures S9 and S10). Fluorescently labeled amino acids were incorporated into the peptide by addition of the charged tRNAs to a cell-free translation mixture composed of *E. coli* S30 extract. The resulting fluorescence resonance energy transfer (FRET) between the two amino acids was observed (Figure S9). The elongation rate was observed to be slower on C168 than on the other circular RNAs, though they were almost the same for C84, C126, C252, and C126(+stop) (Figure S10). This result might explain why lesser amounts of product seemed to be produced from C168 than from C126 according to the western blot analysis (Figure 4 and Figure S7).

We then proceeded to verify our hypothesis that the process of continuous peptide synthesis on a circular RNA gave more product than the same procedure conducted on the corresponding linear RNA template (Figure 5). A linear control sequence defined as L126(+stop) was designed and synthesized as a control sequence for C126 through the addition of termination codons to the 3′-end of L126 (Figures 2c and 3c, and Figures S1 and S2 in the Supporting Information). It was envisaged that the linear precursor L126 would be inappropriate as a control sequence, because bacterial ribosomes are known to stall at the 3′-end of mRNA if it lacks a termination codon.[11] These RNAs were compared in the translation reaction using the PURE system, which included several release factors (Figure 5a).[9] A reduction in peptide synthesis appeared to occur following

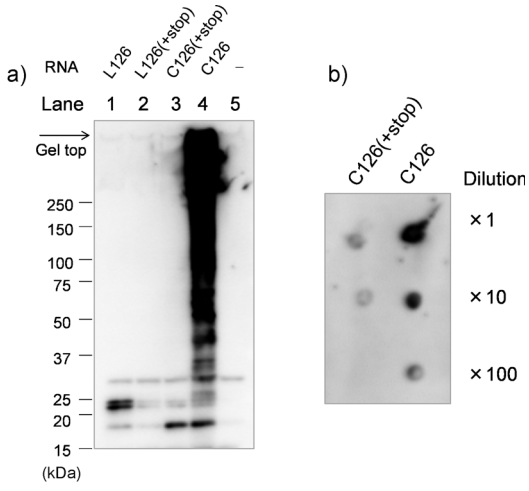

Figure 5. Translation reaction on a circular RNA with stop codons. a) Western blot analysis of the reaction. Lane 1: L126 (1 μM); lane 2: L126 (+stop) (1 μM); lane 3: C126(+stop) (1 μM); lane 4: C126 (1 μM); and lane 5: no RNA. b) Semiquantitative dot blot assay for the reaction shown in (a). Dots from serial 10-fold dilutions of the reaction mixture were compared for the two circular RNA templates C126 and C126(+stop).

the attachment of stop codons to the end of the linear RNA, based on a comparison of the band intensities produced form L126 and from L126(+stop), with this result being contrary to our expectations (lanes 1 and 2 in Figure 5a). To estimate the efficiency of the peptide synthesis, the amounts of peptide produced by C126 were compared with the amounts produced by C126(+stop), which was used in this instance as a model for the linear template, using the dot blot method (Figure 5b). The results clearly indicated that C126 produced a hundred-fold more product than C126(+stop) (Figure 5b). This difference was explained by the notion that a repeating unit of peptide would be produced when the initiation occurred on the circular RNA C126, whereas only one unit of peptide would be produced following the initiation on the circular RNA C126(+stop). Based on these experiments, it was concluded that the circular RNA containing an infinite ORF was a more efficient template for peptide synthesis than its counterpart with a finite ORF, because the latter required a higher frequency of sequential termination, recycling of the ribosome, and the inclusion of a re-initiation processes for the ribosome.

We then designed an experiment to determine whether the initiation process would still affect peptide production in the continuous translation of the 126 nt circular RNA. The SD sequence was complementary to the 3′-end of the 16S rRNA of the 30S ribosome and assisted in the recruitment of a ribosome to mRNA to initiate the translation reaction.[6] The SD sequences within the L126/C126 RNAs were mutated to sequences that would not bind to the 16S rRNA, and their translation reactions were subsequently compared [L126-(−SD)/C126(−SD); Figures 2d, 3d, and 6].[12] Only a minor reduction was observed in the amount of product formed on C126(−SD), which represented 60% of the total density on C126 at 60 min (Figure 6a). In contrast, L126(−SD) was judged to be a particularly poor template for peptide synthesis compared with L126 (Figure 6b).[4a] The data in Figure 6 indicated that the initiation process remained the dominant factor in determining the total amount of peptide produced in our system on the small circular template, based on the minor reduction in the amount of peptide produced from C126-(−SD) compared to that from C126. In contrast to the effect encountered in the linear template, however, the effect in the circular template was not definitive. This difference can also be explained in terms of the nature of the circular RNA, which can theoretically produce infinite repeats of coded peptide from a single initiation, whereas a greater frequency of initiation would be required on a linear template for the ribosome to produce peptide.

In conclusion, we demonstrated that circular RNA with an infinite ORF was translated in the *E. coli* translation system in a manner that was similar to the RCA reaction with polymerases. The circular RNA molecule produced a hundredfold more product than its counterpart within a given period of time. Using this method, the tandem repeat protein motifs found in a significant number of biologically important proteins can be synthesized.[13] During the preparation of polyproteins, the proper folding of the molecule appears to be of critical importance to the success of the process.[4a] The insertion of an appropriate linker sequence between the units could therefore assist in the folding of the polyprotein.[14]

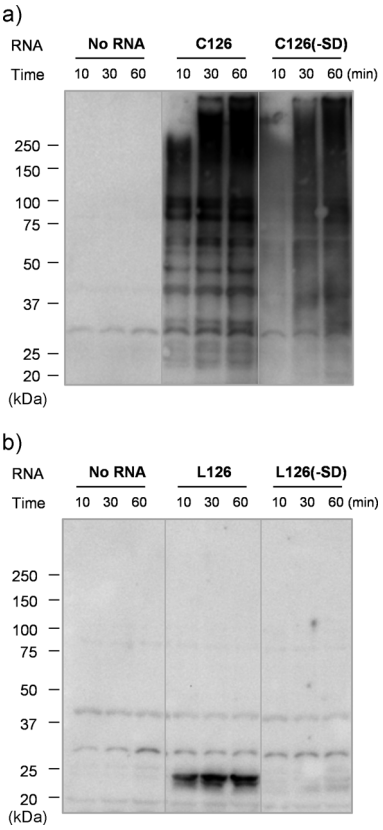

Figure 6. Western blot analyses of the translation reaction on a circular RNA without the Shine–Dalgarno (SD) sequence. Circular 126 nt RNAs with or without the SD sequence are compared in (a). The linear 126 nt RNAs are compared in (b) as a control experiment. The exposure times required to visualize the blots were 5 s for (a) and 60 s for (b).

Received: March 11, 2013
Published online: May 28, 2013

Keywords: cyclization · mRNA · protein expression · RNA structures · synthetic biology

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