Cell-free Protein Synthesis Conducted by Template DNA with Repetitive Sequence

Takahiko Nojima,* Hiroshi Kimura, and Teruo Fujii
Center for International Research on MicroMechatronics, Institute of Industrial Science, The University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo 153-8505

(Received March 25, 2008; CL-080318; E-mail: nojima@iis.u-tokyo.ac.jp)

DNA with repetitive sequence of green fluorescent protein (GFP) was prepared by rolling circle amplification with ϕ29 DNA polymerase from a bacterial expression vector carrying a GFP gene. The prepared DNA was subjected to a cell-free coupled transcription–translation system and green fluorescence was observed, which shows that the template with repetitive sequence properly functioned in the reaction. The efficiency of the reaction conducted by the template with repetitive sequence was higher than those by linear template coding single gene or by circular plasmid.

Cell-free translation has become a useful technique in modern biotechnology[1] and attracts much attention from the fields of nanotechnology and nano-biotechnology owing to its ability to convert genetic information to real material in artificial structures.[2] Although the system has been improved for this decade and become a useful tool for protein production, the yield of the reaction remains a matter of improvement. In the present report, DNA template with repetitive sequence was introduced to cell-free coupled transcription–translation system (Figure 1). We expected that such a repetitive structure would shorten the distance required for transcriptional and translational machinery to migrate between reactions, which would streamline the turnover of the machinery, and thereby the yield of the product would be increased.

Template DNA with repetitive sequence (concatemeric DNA) was prepared by multiprimed rolling circle amplification (RCA) catalyzed by ϕ29 DNA polymerase coupled with random hexamer primers (TempliPhi 100 Amplification Kit, GE Healthcare).[3] Experimental conditions are described in Supporting Information.[14] Figure 2 illustrates the structure of the plasmid DNA (3.6 kb) used as the starting material for RCA, carrying a UV-optimized variant of GFP gene.[6] Construction of the plasmid was carried out according to standard genetic engineering. Its construction is described in Supporting Information.[14] RCA was carried out for 1, 2, and 4 h at 30 °C. ϕ29 DNA polymerase is known to have a capacity to perform strand displacement DNA synthesis for more than 70,000 nucleotides without dissociating from the template nucleotides in an isothermal condition.[7] Actually, electrophoretic gel showed that the chain length of the product was more than 10,000 bp (Supporting Information[14]). Because it was difficult to determine the molecular weight of such a concatemeric DNA, the reaction period of RCA was used as a rough guide of chain length of the products in this study.

We also prepared linear template DNA carrying single GFP gene by the standard polymerase chain reaction (PCR).[8] Two primers were set at back-to-back position on the starting plasmid, so that the chain length of the PCR-product was approximately equal to that of the starting plasmid (3.6 kb). The sequences of the primers and the reaction conditions are described in Supporting Information.[14]

Cell-free coupled transcription–translation was carried out in a 100-μL reaction containing T7 RNA polymerase and 1 μg template DNA at 30 °C for 4 h. The reaction was performed with a commercial kit (RTS 100 *E. coli* HY Kit, Roche Diagnostic) according to manufacturer's protocol. After the reaction, the sample was incubated for 12 h at 4 °C for maturation of GFP.[9] Yield of the product was determined by fluorescence intensity of GFP using a fluorescence spectrophotometer (FP-3000, Microtec Nition). Excitation wavelength and emission wavelength were set at 485 and 530 nm, respectively.[10] Fluorescence

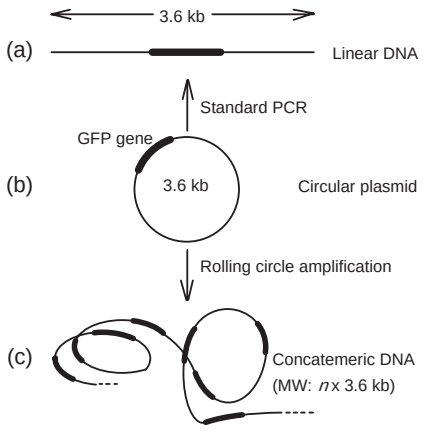

Figure 1. Structures of template DNAs for cell-free coupled transcription–translation. (a) Linear DNA amplified from circular DNA by standard PCR. (b) Circular plasmid used as starting material in the preparation of (a) and (c). Detailed is described in Figure 2. (c) Concatemeric DNA with repetitive sequence prepared by ϕ29 DNA polymerase.

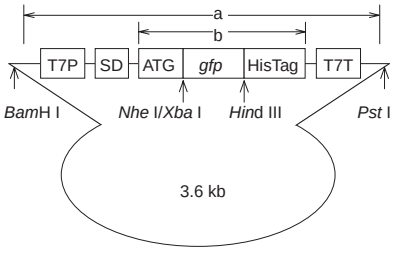

Figure 2. Structure of the circular DNA used in this study as the starting material for the preparation of DNA templates for cell-free coupled transcription–translation. T7P; T7 RNA polymerase-specific promoter, SD: Shine-Dalgarno sequence,[4] ATG; initiation codon, gfp; GFPuv gene, HisTag; hexahistidine-tag,[5] T7T; T7 RNA polymerase-specific terminator. "a" and "b" correspond to transcribed and translated region, respectively.

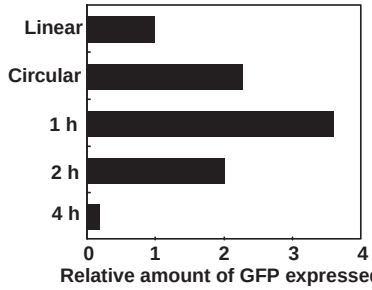

Figure 3. Amount of GFP synthesized in a cell-free coupled transcription–translation system conducted by various kinds of template DNA. "Linear" and "Circular" correspond to PCR-amplified linear template and plasmid DNA, respectively. "1 h," "2 h," and "4 h" are the reaction period for the ϕ29 DNA polymerase-dependent concatemeric DNA synthesis (not the reaction period for protein synthesize).

intensities were normalized: that from linear template was defined as 1.

Figure 3 shows the relative yields of the cell-free protein synthesis conducted by various kinds of template DNA. When the template DNA prepared in 1 h RCA was used, the amount of the protein synthesized was higher than those in which plasmid or linear DNA was used. When the template prepared in 2 h RCA was used, the amount is also higher than that in which linear DNA was used. In each reaction, the number of the GFP gene and the weight of subjected DNA were fixed (2.5×10^8 copy and 1 μg) and the only difference is the structure of the templates: circular, linear, or concatemeric. These results suggest that the concatemeric structure has an ability to enhance the transcription, translation, or both in the cell-free coupled transcription/translation system.

We suggest that when the template with repetitive sequence is used, the distance that RNA polymerase should move between a terminator and the next promoter is shortened, which should streamline the efficiency of turnover of the transcriptional machinery. Since in the cell-free coupled transcription–translation system, the transcribed mRNA is soon served as a template for translation, it is also expected that ribosome and other translational machinery, as well as transcriptional machinery, are localized and condensed near the template DNA, and it enhances total efficiency of coupled transcription–translation. Such a localized condition is also expected to originate molecular crowding environment.[11]

On the other hand, the more the chain length of the template DNA is increased, the more the local concentration of the DNA is increased. It would cause a steric hindrance for transcriptional and translational machinery, and the efficiency of the enhanced protein synthesis is expected to return. Actually, the GFP expression was decreased when the template prepared in 2 h RCA was used, and depressed when that in 4 h RCA was used (Figure 3 "2 h" and "4 h").[12] Further research is necessary to analyze the detailed mechanism on the biochemical reaction involving concatemeric DNA.

In conclusion, the template DNA with repetitive sequence for cell-free coupled transcription–translation reaction was prepared by ϕ29 DNA polymerase. The reaction was successfully progressed and the efficiency was higher than those conducted by plasmid and by PCR-amplified linear DNA.

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10 In a small proportion of transcribed region positioned in a terminus of RCA-synthesized DNA, unlike those of PCR-amplified DNA, GFP-coding region is truncated. However, it has negligible effects on the purity of synthesized GFP because (i) It is known that amino acid (aa) residues 2-232 of a total of 238 aa in the GFP are required for the fluorescence.[13] Synthesized GFP lacking more than 7 aa of its C-terminus is therefore unobserved in fluorescence measurement, and (ii) The possibility that the terminus of RCA-synthesized DNA positions at one of the 6 aa of C-terminus (corresponding to 18 bp in DNA) of GFP gene is 0.5%.
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14 Supporting Information is available electronically on the CSJ-Journal web site; http://www.csj.jp/journals/chem-lett/.

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