Towards the construction of a DNA genome replication system for an artificial cell

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Abstract

In vitro constitution of biological functions is a useful strategy to understand the physicochemical principles underlying biological functions (Luisi and Stano, 2013). To date, various cellular functions have been artificially constituted, including self-replication of genetic information, a gene expression, and so on. As a self-replication system of genetic information, our group has reconstituted an RNA genome replication system coupled with translation (Ichihashi, et al. 2013). However, all living organisms have the DNA genomes, which replicate with DNA replicases translated from the genomes via mRNA transcription. A plan for the construction of such a DNA replication system coupled with transcription and translation has been proposed using rolling-circle-type replication scheme approximately 10 years ago (Fig.1, Forster and Church, 2006), although it has not been realized yet. In this study, we attempted to constitute the transcription-and translation-coupled DNA replication (TTcDR) system and perform an evolution experiment to make the system recursive. The aim of the present work is to show a method of the construction of a DNA genome replication system which leads to the *in vitro* construction of an artificial cell.

We prepared a circular DNA encoding phi29 DNA polymerase gene under control of T7 promoter. The circular DNA was mixed with the reconstituted cell-free translation system derived from *Escherichia coli* (Shimizu, et al. 2001), T7 RNA polymerase and RNase inhibitor. To perform rolling-circle replication, we also added dNTPs, a random oligo DNA as a primer, and yeast pyrophosphatase in the mixture (Dean, et al. 2001). We first attempted to optimize the concentrations of several components (T7 RNA polymerase, RNase inhibitor, dNTPs, random DNA oligo, yeast pyrophosphatase, NTPs and tRNA). We found that the optimum concentrations of NTPs, tRNA, T7RNA polymerase and RNase inhibitor are in narrow ranges, indicating that these components are inhibitory to the TTcDR reaction at high concentrations. At the optimized concentrations of all components, the replication product DNA increased approximately 100-fold compared to original conditions. The kinetics of DNA replication showed a concave curve, suggesting that DNA replication accelerated due to the increasing phi29 DNA polymerase over time (Sakatani et al, 2015). This is consistent with the expected kinetics of the TTcDR reaction.

A shortcoming of this TTcDR system is the lack of recursiveness. The initial template DNA is circular, whereas the product is a linear concatemer. To make this system recursive, the linear DNA product must be circularized. We next attempted to circularize the product DNA using Cre recombinase, which has been reported to circularize the linear DNA produced by phi29 DNA polymerase (Huovinen et al, 2011), as proposed previously (Forster and Church, 2006). We added Cre recombinase into the optimized TTcDR system but found that Cre recombinase significantly inhibits the DNA replication catalyzed by phi29 DNA polymerase. This result indicates that Cre recombinase and phi29 polymerase does not work simultaneously. Therefore, to make this DNA replication system recursive, we have to add Cre recombinase after replication and remove it before the next round of replication.

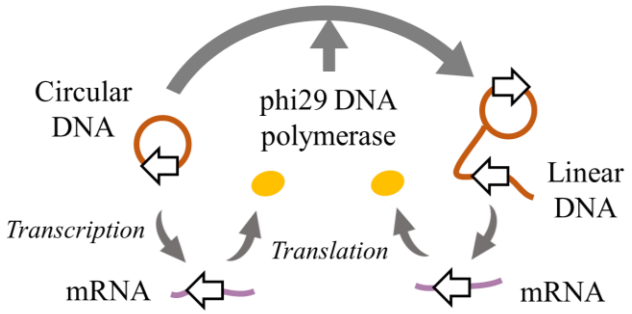

Figure 1. Schema of the TTcDR system. This system consists of a circular DNA encoding phi29 DNA polymerase gene, T7 RNA polymerase, and a reconstituted translation system. T7 RNA polymerase transcribes mRNA from the circular DNA and phi29 DNA polymerase is translated. The polymerase initiates DNA polymerization to produce a long linear DNA. The replication product is further used as a template for transcription to produce phi29 DNA polymerase.

To establish a more automatic recursive replication, which would be suitable for an artificial cell, we next attempted to develop a Cre-resistant phi29 polymerase by using evolutionary engineering. We first established a cycle to repeat the TTcDR reaction in the presence of Cre recombinase (Fig. 2). This cycle consisted of four stages. (i) Encapsulation: the TTcDR system was encapsulated in a water-in-oil emulsion with Cre recombinase. A water droplet was expected to contain less than one DNA molecule. (ii) DNA replication: the emulsion was incubated for the TTcDR reaction. (iii) DNA recovery: the droplets were collected and the product linear DNA was amplified by PCR. (iv) Circularization: after converting the both ends of the DNA into sticky ends, the liner DNA was circularized by ligation. The new circular DNA was then re-encapsulated into the emulsion for the next round of the replication cycle. The product DNA concentration was measured using quantitative PCR. In this system, mutations are introduced into the DNA through polymerization error. If a mutant DNA that encodes Cre-resistant phi29 polymerase appears, it should dominate the population. We repeated this cycle for 40 times and found that the average replication ability of the DNA population increased gradually even in the presence of increasing amount of Cre recombinase. This indicated that more Cre-resistant mutant DNAs were obtained.

In this study, we constructed the TTcDR system and obtained mutants of the DNA genome, the replication of which is tolerant to Cre recombinase. These results provide a step toward the *in vitro* construction of an artificial cell containing a recursive replication system of a DNA genome.

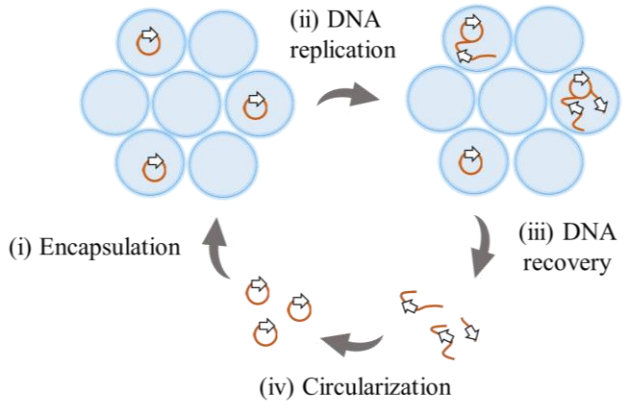

Figure 2. Cycle of the TTcDR for evolution experiment. (i) Encapsulation: the TTcDR system is encapsulated in a water-in-oil emulsion with Cre recombinase. (ii) DNA replication: incubation for the TTcDR reaction. (iii) DNA recovery: the droplets are collected and the product DNA was amplified. (iv) Circularization: the product linear DNA is circularized by ligation.

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