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Adaptive Evolution of an Artificial RNA Genome to a Reduced Ribosome Environment

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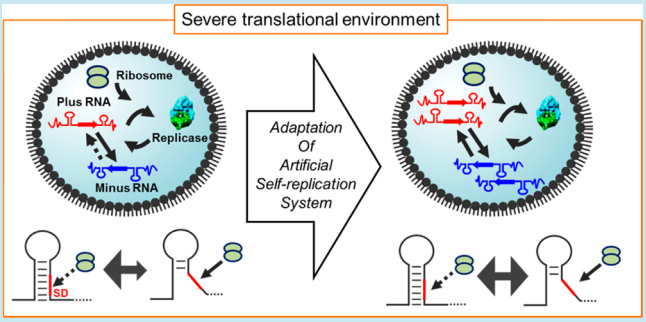
Supporting Information

ABSTRACT: The reconstitution of an artificial system that has the same evolutionary ability as a living thing is a major challenge in the *in vitro* synthetic biology. In this study, we tested the adaptive evolutionary ability of an artificial RNA genome replication system, termed the translation-coupled RNA replication (TcRR) system. In a previous work, we performed a study of the long-term evolution of the genome with an excess amount of ribosome. In this study, we continued the evolution experiment in a reduced-ribosome environment and observed that the mutant genome compensated for the reduced ribosome concentration. This result demonstrated the ability of the TcRR system to adapt and may be a step toward generating living things with evolutionary ability.



KEYWORDS: *adaptation, RNA genome, self-replication, translation, RNA replicase, evolution*

Adaptive evolution is a remarkable characteristic of living things that allows them to survive in various environments by changing gene expression and gene function. The *in vitro* reconstitution of these adaptive abilities from biological molecules, such as polynucleotides and proteins, is a substantial challenge in the interdisciplinary field between chemistry and biology.

Recently, many researchers have attempted to reconstitute the functions of living things from specific molecules to understand the basic principles of function and to develop new technologies.[1−7] The reconstitution of evolutionary abilities requires the replication of genetic information, mutation, and heredity.[8] Currently, two types of self-replication systems with the ability to evolve have been established using polynucleotides and proteins;[9,10] in both, the RNA or DNA replicates through the activity of externally supplied replication enzymes, such as RNA-dependent RNA polymerase or a set of reverse transcriptase, transcriptase, and DNA polymerase enzymes. The evolution of resistance to ethidium bromide and neomycin has been observed by using these systems.[11,12] However, the biggest difference between these artificial self-replication systems and natural living things is the lack of translation machinery, which is needed to translate the information encoded in DNA or RNA into proteins.

To obtain a system more similar to that of living things, we constructed a translation-coupled RNA replication (TcRR) system by combining a single-stranded RNA genome encoding the RNA-dependent RNA polymerase (RNA replicase) and a reconstituted *Escherichia coli* translation system,[13] in which the genomic RNA replicates through the translation of the encoded replication enzyme.[14,15] Furthermore, we have previously reported that the genomic RNA in our system is able to simulate Darwinian evolution by continuously repeating the replication in cell-like compartments.[16] However, this previous study was performed under a rich environment, in which all of the translation components and nutrients were in excess supply. Therefore, the adaptation ability of this system in severe environments is unknown.

Another important property of the TcRR system is that the replication depends on two different but related functions: translation of a replicase and replication by the replicase. This multifunctional replication is a characteristic of natural self-replication systems, such as single-stranded RNA viruses; however, little is known regarding how evolution proceeds and how each function changes through evolution in such a multifunctional genome replication system. The TcRR system, a simple system reconstituted from all defined components, is an ideal experimental model for understanding the evolution of multifunctional genome replication.

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In the previous study, we performed an experimental evolution of the TcRR system with an excess amount of ribosome, and in this study, we used the RNA genome after the previous evolution and extended the evolution experiment under a severe translation environment by reducing the ribosome concentration. We examined (1) whether the artificial genome adapted to the environment and (2) whether the two functions, translation and replication, changed during its evolution.

RESULTS

Evolution Experiment under Reduced Ribosome Conditions. We performed a long-term replication experiment with the TcRR system under severe translation environments. To make a severe translation environment, we chose to reduce the ribosome concentration because the ribosome is the central component of the translation system. The condition investigated is not physiologically relevant, but reducing the ribosome concentration allows us to specifically decrease the translation activity.

The TcRR system is composed of a single-stranded RNA genome (plus RNA) that encodes a catalytic subunit of an RNA-dependent RNA polymerase (Qβ replicase) and a reconstituted *Escherichia coli* translation system (the customized PURE system.[13,14,16] In this system, the subunit of the RNA replication enzyme was translated from the plus RNA and formed the active replicase with other subunits (EF-Tu and Ts) in the translation system. The replicase synthesized the complementary strand to the genome (minus RNA), which was also recognized by the replicase. Thus, the original plus RNA was replicated. We first performed this replication process under reduced ribosome conditions (200 nM) in a cell-like compartment (a water-in-oil emulsion) (Figure 1). After the replication, we recovered and amplified the minus RNA by reverse transcription and PCR to produce cDNA because initially, the replication of genomic RNA was not sufficient to be directly transferred to the next round of replication. Plus RNA was prepared from the cDNA by in vitro transcription and encapsulated with the translation system for the next round of the TcRR. In this system, mutations were spontaneously introduced through replication error; if more active mutant genomes appear, they should dominate the population, resulting in increased minus RNA synthesis.

We repeated the replication cycle with 0.1 nM plus RNA and 200 nM ribosomes for 15 rounds. The starting plus RNA is the RNA clone (Round 128 clone) that was obtained after 128 rounds of evolution at a high ribosome concentration in the previous study.[16] The replicated minus RNA concentration gradually increased after the TcRR and had increased approximately 30-fold by Round 15 (Figure 2A). The ribosome concentration was reduced to 50 nM, and the cycle was repeated for another 15 rounds. The replicated minus RNA concentration dropped once during the ribosome reduction but had increased approximately 2-fold after 15 rounds (Figure 2B).

We chose 8 clones of the plus RNA at Rounds 11, 15, and 30 to investigate possible mutations and then sequenced the clones. The average number of mutations increased constantly as the rounds progressed (Figure 2C, Total). Some of the mutations were common to the clones in the same replication cycle. We considered common mutations that were present in more than 50% of clones as "fixed." The number of "fixed" mutations increased as the number of rounds increased (Figure 2C, Fixed). These results indicated that spontaneous mutations

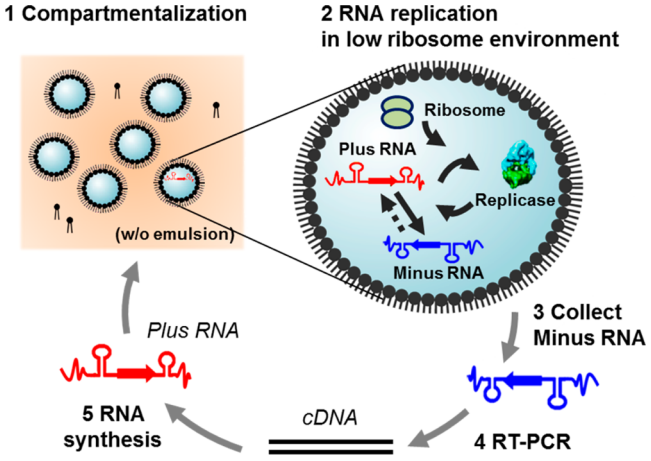

Figure 1. Scheme for the TcRR reaction and the evolution experiment. (1) The genomic plus RNA (0.1 nM) and the reconstituted translation system, including the reduced ribosome concentration (200 nM), was encapsulated into a water-in-oil (w/o) emulsion. (2) After incubation of the emulsion at 37 °C for 4 h, the replicase subunit encoded in plus RNA was translated and formed an active replicase, with the association of the EF-Tu and Ts subunits in the translation system. The replicase synthesized minus RNA using the plus RNA as a template and then replicated plus RNA using the minus RNA as a template. (3) The synthesized minus RNA was recovered from the emulsion, and (4) cDNA was prepared by reverse transcription and PCR (RT-PCR). (5) Plus RNA was prepared by *in vitro* transcription and used for the next round of the TcRR reaction. Spontaneous mutations were introduced during the replication processes, RT-PCR, and *in vitro* transcription.

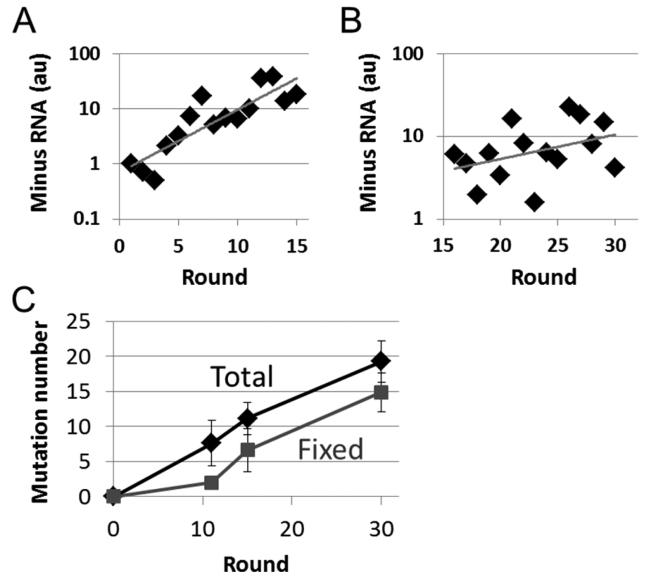

Figure 2. Adaptation of the genome RNA to the reduced ribosome concentrations. (A) The minus RNA concentration after the TcRR reaction with 200 nM ribosome (except for 50 nM, in Round 1). The minus RNA concentration was measured by quantitative RT-PCR and normalized to that of Round 1. (B) The minus RNA concentration after the TcRR reaction of each round with 50 nM ribosome (except for 12.5 nM in Round 18). The ribosome concentration was reduced to 50 nM after Round 15. (C) The average number of mutations. The sequence of eight RNA clones was analyzed for each round. The total number (Total) of sequences and the number of common mutations that were present in more than 50% of clones in the same round (Fixed) were counted. The error bars indicate the standard deviation.

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were introduced throughout the replication cycles and that some of these mutations were selected and then eventually dominated the population. The sequence results and the increase in minus RNA replication indicated that the genomic RNA adapted to the reduced ribosome environment by evolution.

Biochemical Analysis of the RNA Clones. To investigate how the genomic RNA adapted to the reduced ribosome conditions, we examined the biochemical properties of the original genomic RNA and the evolved clones at Rounds 11, 15, and 30. We chose the clone with the highest TcRR activity at each round. The TcRR activities of all the selected clones are shown in Supporting Information Figure S1. The mutations in each clone are shown in Supporting Information Table S1.

We first constructed a mathematical model of the TcRR reaction to quantitatively analyze the minus RNA synthesis in the TcRR reaction. The minus RNA replication comprised two reactions: the translation of the replicase and the synthesis of the minus RNA by the replicase (Figure 3). The translation

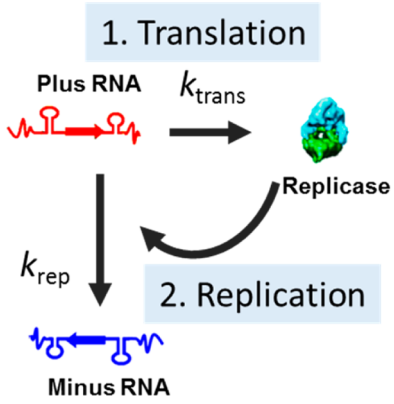

Figure 3. Schematic drawing of the mathematical model of minus RNA synthesis in the TcRR reaction. Minus RNA synthesis in the TcRR system comprised two reactions: the translation of the replicase, and the replication of the minus RNA. In this model, the translation was assumed to be a single-order reaction dependent on the plus RNA concentration, and the replication was assumed to be a single-order reaction dependent on the replicase concentration. The translation and replication activities are represented by the rate constants k_{trans} and k_{rep}, respectively. According to this model, the minus RNA concentration describes the second-order equation of time (see eq 2 in Methods).

reaction was assumed to be a single-order reaction dependent on plus RNA concentration with the rate constant k_{trans}. The replication reaction (minus RNA synthesis) was assumed to be a single-order reaction dependent on the replicase concentration with the rate constant k_{rep}. According to this mathematical model, the replicase and minus RNA concentrations are represented as eqs 1 and 2, respectively (see Methods).

We measured the kinetics of the translation-coupled minus RNA synthesis and analyzed them based on the mathematical model. We performed the TcRR reaction with 0.1 nM plus RNA clones from each round under the reduced ribosome conditions (200 nM) and measured the kinetics of the minus RNA concentrations (dots in Figure 4A). The kinetics fit eq 2 and were consistent with the mathematical model (lines in Figure 4A). We estimated the product of the two rate constants (k_{trans} k_{rep}) from the curve, which represented the TcRR activity

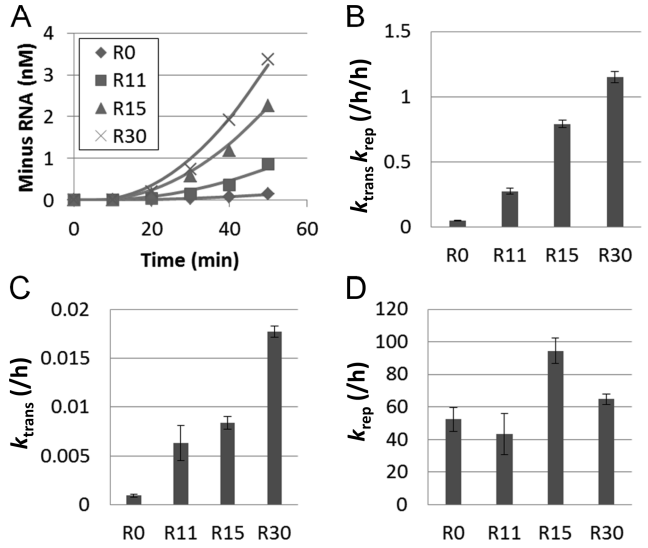

Figure 4. Activities of TcRR, translation, and replication at the lower (200 nM) ribosome concentration. (A) The kinetics data for the minus RNA synthesis in the TcRR reaction at the lower (200 nM) ribosome concentration. The plus RNA clones (10 nM) were incubated with the translation system, and the minus RNA was measured by quantitative RT-PCR. The line shows the result of the curve fit with eq 2 (see Methods). (B) The product of the translation and replication rate constants (k_{trans} k_{rep}). The products represent the TcRR activity and were estimated from the curve fit of A. (C) The translation rate constants (k_{trans}). The translation of each clone was measured at the lower ribosome concentration as described in Method. (D) The replication rate constants (k_{rep}). The replication rate constants were estimated by dividing the product shown in B by the translation rate constants shown in C.

(Figure 4B). The product (k_{trans} k_{rep}) increased as the rounds progressed and finally reached a 23-fold increase at Round 30, supporting the notion that TcRR activity increases during evolution. Increased TcRR activity was also observed in the longer incubation experiments for both the minus and plus RNAs (Supporting Information Figure S2).

Next, we measured the translation rate for each clone and estimated the rate constant (k_{trans}) (Figure 4C). In this experiment, we omitted UTP in the reaction mixture to negate the effect of replication. The translation rate constants increased as the rounds progressed and reached an approximately 19-fold increase at Round 30. This result indicated that the translation activity of the genomic RNA significantly increased during evolution.

Next, we estimated the replication rate constants (k_{rep}) by dividing the product (k_{trans} k_{rep}) in Figure 4B by the translation rate constant (k_{trans}) in Figure 4C. The replication rate constants of all of the clones were similar, although they were slightly increased at Round 15 (Figure 4D). This result indicated that the replication activity did not significantly increase during evolution. This result was further confirmed by another experiment, in which we measured the replication rate constants in the presence of known concentrations of translated replicases under uncoupled conditions (Supporting Information Figure S4A).

The replication rate constants obtained above depend on two activities: the replicase activity as a polymerase encoded in each clone and the RNA activity as a replication template. To separately investigate the replicase activity, we measured the replication using the same RNA template for all of the

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replicases. After translation of the replicase from each RNA clone, as described in the above translation experiment, an aliquot was transferred to a second reaction mixture that included radioisotope-labeled NTPs and a template RNA (s222 RNA), an RNA that is highly replicable by Qβ replicase. This experiment is designed so that the s222 RNA exists in excess compared with the contaminating plus RNAs to avoid the replication of the contaminating RNAs. The replicated s222 (222 nt) was separated by polyacrylamide gel electrophoresis, and the amount of s222 replicated was measured by radioisotope incorporation. To obtain the replicase activity, the replication amounts were normalized to the amount of translated replicase measured as described above. The replicase activities were similar for all of the clones, indicating that the replicase activity did not significantly change during the evolution (Supporting Information Figure S3).

Analysis of the RNA Structure of the Clones. The results from the biochemical analysis of the RNA clones indicated that increases in TcRR activity during evolution were primarily caused by an increase in the translation activity. One possible mechanism of the translation increase is relaxation of the RNA structure; the translation activity is known to highly depend on the looseness of the RNA structure around the Shine-Dalgarno (SD) sequence[17] and the translation initiation site.[18−20] To test this hypothesis, we performed selective 2′-hydroxyl acylation analyzed by primer extension (SHAPE),[21] which we used to estimate the degree of RNA structure relaxation (a more relaxed structure shows higher SHAPE reactivity). We measured the SHAPE reactivity of around SD sequence (Figure 5A) and found that the SHAPE reactivities of the SD sequence (^{216}GGAG^{219}) increased in the evolved clones (R11, R15, and R30 in the dotted square, Figure 5A). The average SHAPE reactivity increased in the evolved clones and reached a 7-fold increase by Round 30 (Figure 5B). We also calculated the ribosome-binding probability using RBS Designer,[39] and we found that the SD-ribosome binding probability increased in a manner similar to the SHAPE reactivity (Figure 5C). These relaxed RNA structures around the SD sequence could partially explain the increased translation activities of the evolved clones.

Translation and Replication Activities at the High Ribosome Concentration. The evolved clones exhibited improved translation and TcRR activities at the lower (200 nM) ribosome concentrations (Figure 4B and C). Next, we examined whether the evolved clones exhibited improved activities at the original and higher (1 μM) ribosome concentrations. We measured the kinetic parameters at the higher ribosome concentration using the same methods described for the lower ribosome concentration experiments. We first measured the kinetics of the minus RNA replication in the TcRR reaction and estimated the product (k_{trans} k_{rep}) by curve fitting with eq 2 (Figure 6A). The product, which represented the TcRR activity, did not increase as the cycles progressed. The TcRR activity remained within 0.5- to 2-fold of the values observed in the original RNA (R0) (Figure 6B). The translation rate constant (k_{trans}) increased significantly as the cycles progressed, similar to the lower ribosome concentration, and exhibited an approximately 10-fold increase at Round 30 (Figure 6C). In contrast, the replication rate constant (k_{rep}) estimated by dividing the product (k_{trans} k_{rep}) with k_{trans} decreased significantly as the cycles progressed (Figure 6D). The k_{rep} results were further confirmed by performing the experiment under uncoupled conditions (Supporting Information Figure S4B). In summary, the translation activity of the evolved RNAs increased, but the replication activity decreased; therefore, the net TcRR activity did not change at the higher ribosome concentration.

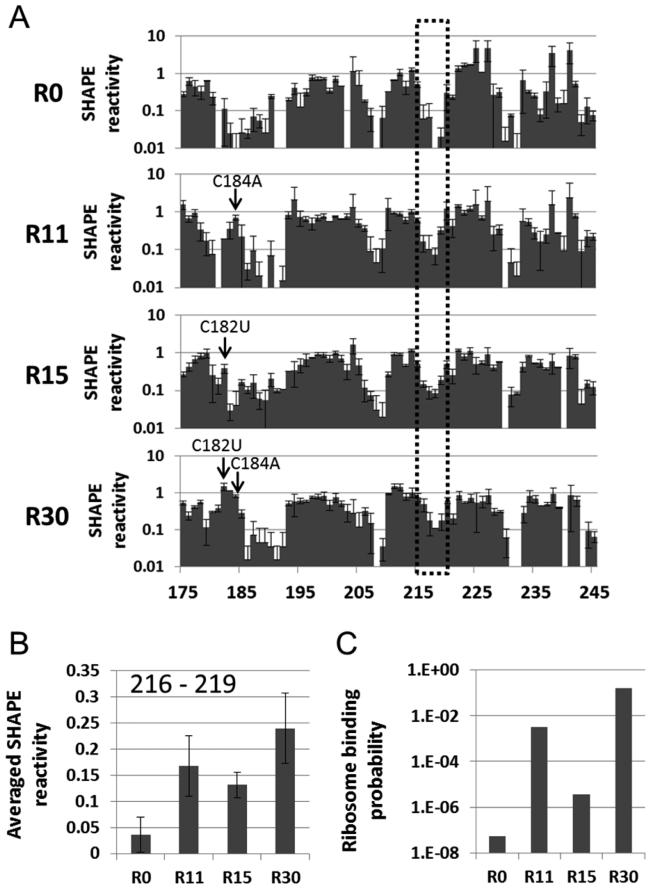

Figure 5. SHAPE reactivity of the clones. Each clone was labeled with structure specific reagents (1-methyl-7-nitroisatoic anhydride), and the label was detected by reverse transcription and analysis of the product length (see Methods). The higher signal (i.e., higher SHAPE reactivity) indicated a relaxed structure. (A) The SHAPE reactivity of the clones around the SD sequence. The SD sequence is shown within the dotted square (216−219). The mutated sites (C184A, C182U) were indicated. The whole sequences and predicted secondary structures are shown in the Supporting Information (Figure S5A and B). (B) The average SHAPE reactivity of the SD sequence. The error bars indicate the standard deviation. (C) The calculated SD-ribosome binding probability. The frequency was calculated by RBS Designer.[39]

DISCUSSION

Adaptation is one of the most remarkable characteristics of living things. In this study, we tested whether the TcRR system, which mimics the replication scheme of the RNA virus, adapted to a new environment (reduced ribosome concentration) via evolution. After 30 rounds of replication, the genomic RNA accumulated mutations and showed increased TcRR activity under the reduced ribosome condition by increasing the translation rate but maintaining the replication rate. The changes in the rate constants represent a clear contrast to the findings in the previous evolution at the higher ribosome concentration,[16] in which the translation rate constant ($k_{translation}$) did not change significantly, but the replication rate constants (k_{rep_plus} and k_{rep_minus}) increased approximately

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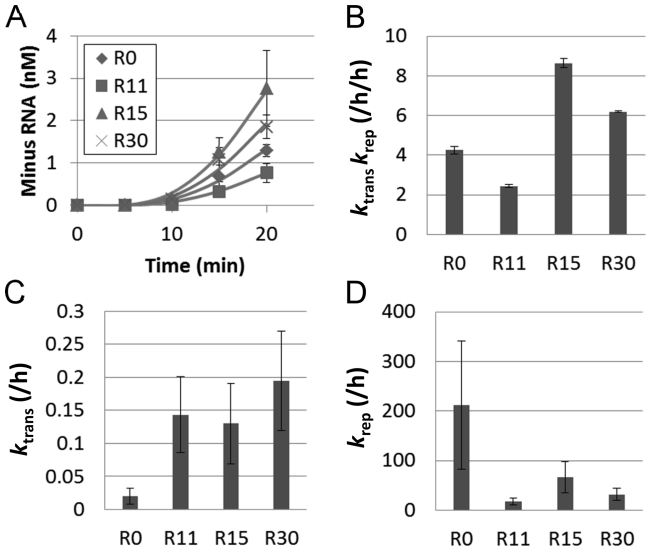

Figure 6. Activities of the TcRR, translation, and replication at the higher (1 μM) ribosome concentration. (A) The kinetics of the minus RNA synthesis in the TcRR reaction at the higher (1 μM) ribosome concentration. Each plus RNA clone (10 nM) was incubated with the translation system, and the minus RNA concentration was measured by quantitative RT-PCR. The line shows the result of the curve fit with eq 2 (see Methods). (B) The product of the translation and replication rate constants (k_{trans} k_{rep}). The products represented the TcRR activity and were estimated from the curve fit of A. (C) The translation rate constants (k_{trans}). The translation of each clone was measured at the higher ribosome concentration as described in Methods. (D) The replication rate constants (k_{rep}). The replication rate constants were estimated by dividing the product shown in B by the translation rate constants shown in C.

2-fold to 3-fold from Round 0 to Round 128. The specific increase in the translation rate under the reduced ribosome condition observed in this study supports the hypothesis that the artificial TcRR system, which was composed of minimal components and did not have complex biological functions, still had some ability to undergo adaptive evolution under the reduced ribosome condition. Thus, the adaptive ability was partially reconstituted in this simple TcRR system, although it is demonstrated only in the limited case of reduced ribosomes, and the adaptation to broader conditions such as different temperatures, salt concentrations, or drug resistance levels remain to be examined. This study is a step forward in constructing an artificial system equipped with the same level of adaptive ability as living things.

The TcRR activity increased primarily because the translation activity increased (Figure 4C). One of the possible mechanisms of the increased translation was the relaxation of the RNA structure around the SD sequence (Figure 5). The relationship between the RNA structure around the SD sequence and translation activity has been extensively investigated. The relaxation of the SD sequence structure facilitated the recruitment of the 30S ribosomal subunit and enhanced the subsequent translational initiation.[19,22,23] This mechanism could explain the increase in translation activity observed in this study.

The SHAPE reactivity increased significantly from R0 to R11, but it remained at the same level from R11 to R30, implying that another mechanism exists that can increase translation. One possible mechanism is the improved codon usage.[24] Other possibilities included improvement of the elongation speed by reducing the number of large or rigid stem structures[25−27] and the decrease in the number of internal SD-like sequences, which has been shown to decrease translation efficiency.[28] These possibilities remain to be examined in future studies.

In this study, we demonstrated the adaptive evolution ability of the TcRR system and also found a limitation of the TcRR system; the increased translation activity of the evolved clones did not result in increased TcRR activity at the higher concentration because of decreased replication activity (Figure 6). This result implied the existence of a trade-off between translation and replication. In addition, this trade-off would limit further evolution of the TcRR reaction. One of the possible mechanisms of this trade-off is competition between the ribosome and the replicase for the genomic RNA and their collision on the genomic RNA.[16] Such a trade-off has also been observed in RNA viruses[29,30] and is considered a general problem for all systems that have a single-stranded RNA genome. The next important challenge for further studies on the evolution of the TcRR system is to overcome this trade-off. One of the possible solutions is to use a switching mechanism from translation to replication[31] or to implement a non-canonical translation system[32] that is utilized by RNA viruses.

One of the important challenges in the field of *in vitro* synthetic biology is the creation of a self-sustaining genome replication system in which all of the components reproduce themselves. A large hurdle to achieving that goal is the reproduction of the components of the translation factors, especially the ribosome, which exists at a high concentration (3 μM)[16] and is the largest complex in the translation system, composed of three RNAs and more than 50 proteins. One possible strategy to overcome this hurdle is the development of a genome replication system that requires fewer ribosomes. This study is a first step for that purpose; the R30 clone obtained in this study acquired the ability to replicate under a lower ribosome concentration (200 nM). Further evolution experiments under reduced ribosome conditions and also with other translation factors would produce a genome that requires minimal translation components to replicate. Such a genome might be an appropriate starting point to construct a self-sustaining genome replication system.

METHODS

Evolution Experiment. The TcRR reaction was performed as described previously.[16] Briefly, the genomic plus RNA (0.1 nM) was mixed with the customized *Escherichia coli* translation system, which was composed of all of the independently purified translation proteins, tRNA, amino acids, and NTPs, and so on. The mixture was encapsulated into a water-in-oil emulsion that was approximately 2 μm in diameter. The evolution experiment was conducted primarily in the same manner as in the previous study, with several modifications described below. The initial plus RNA in this study was the same RNA clone from Round 128 in the previous study, and the ribosome concentration in this study was adjusted to 200 nM (Rounds 1−15, except for Round 1 (50 nM)) or 50 nM (Rounds 16−30, except for Round 18 (12.5 nM)). The incubation was performed for 4 h at 37 °C. After incubation, we added 1 μM ribosome to enhance the recovery of RNA. After centrifugation of the water-droplets, the mixture was treated with 20 mM iodide instead of 10 mM after Round 5. The minus RNA concentration was measured by quantitative PCR

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after reverse transcription (RT-PCR), as described in the previous study.

Sequence Analysis. The genomic RNA was cloned from Rounds 11, 15, and 30, as described previously.[16] Eight clones were obtained from each round, and their sequences were analyzed. The mutations were identified by comparison to the initial genomic RNA (Round 128 clone of the previous study).

TcRR Reaction of the Clones. For the kinetic analysis (Figures 4A and 6A), the TcRR reaction was performed at 37 °C for the indicated times with each clone (10 nM) and at the lower (200 nM) or higher (1 μM) ribosome concentrations without compartmentalization. For the longer incubation experiment (Supporting Information Figure S2), the TcRR reaction was performed at 37 °C for 4 h in the compartment prepared by the water-in-oil emulsion as in the evolution experiment. The minus and plus RNA were measured by the quantitative RT-PCR as described previously.[16]

Translation Activity of the Clones. The TcRR reaction mixture (without UTP) and a reduced (1/10) concentration of methionine including [^{35}S]-methionine was incubated with each plus RNA clone at 37 °C for 2 h at the lower (200 nM) or higher (1 μM) ribosome concentrations. After incubation, the aliquots were subjected to SDS-polyacrylamide gel electrophoresis, followed by autoradiography. The band intensity corresponding to the replicase subunit was quantified by comparing the spot of a known concentration of [^{35}S]-methionine.

Activity of Replicase Encoded in the Clones (Supporting Information Figure S3). The TcRR reaction mixture (without UTP) was incubated with each plus RNA clone (200 nM) at 37 °C for 2 h at the higher (1 μM) ribosome concentration. After incubation, an aliquot was mixed with 9-volume of the replication solution including [^{32}P]-UTP, 125 mM NTPs, 100 nM s222 RNA,[33] 25 μg/mL chloramphenicol, 125 mM Tris-HCl (pH 7.8), 10 mM magnesium chloride, and 0.01% BSA; the solution was further incubated at 37 °C for 30 min. The samples were subjected to 8% polyacrylamide gel electrophoresis and autoradiography. The bands corresponding to the s222 RNA were quantified by comparing the spots of a known amount of [^{32}P]-UTP.

Replication Activity of the Clones under Uncoupled Conditions (Supporting Information Figure S4). The TcRR reaction mixture (without UTP) was incubated with each plus RNA clone at 37 °C for 1 h at the lower (200 nM) or higher (1 μM) ribosome concentrations. After incubation, UTP (final 1.25 mM) and streptomycin (final 30 μg/mL) were added to start the replication and stop the translation, respectively. The solutions were further incubated at 37 °C for 30 min (200 nM ribosome) or 5 mn (1 μM ribosome). The replicated minus RNA concentration was measured by quantitative RT-PCR, as described above.

Mathematical Model of the Translation-Coupled Minus RNA Synthesis. The translation reaction was assumed to be a first-order reaction dependent on the plus RNA concentration with the translation rate constant k_{trans} because we performed the experiment at the higher concentration of ribosome compared with plus RNA. Accordingly, the translated replicase concentration was described as $d[Rep]/dt = k_{trans}[plus\ RNA]$. Solving this equation results in the following equation:

$$[Rep] = K_{trans}[plus\ RNA]t \quad (1)$$

Here, we assumed that plus RNA was constant over time because the increase of plus RNA could be negated under our experimental condition.

The minus RNA synthesis was assumed to be a first-order reaction dependent on the replicase concentration with the replication rate constant k_{rep} because all of the experiments were performed at the higher concentration of plus RNA compared with that of the replicase. Accordingly, the minus RNA replication rate was described as $d[minus\ RNA]/dt = k_{rep}[Rep]$. Solving this equation using eq 1 resulted in the following equation:

$$[minus\ RNA] = \frac{1}{2}k_{rep}k_{trans}[plus\ RNA]t^2 \quad (2)$$

Note that the ribosome dependency is included in the translation rate constant k_{trans} in this model (i.e., k_{trans} is a function of the ribosome concentration).

SHAPE Analysis of RNA Clones. The SHAPE experiment was performed by a two-capillary protocol as described previously[34,35] with the following modifications. The RNAs were prepared by T7 RNA transcription. The RNAs (1 pmol) were equilibrated in 10 μL of folding buffer (buffer solution of the translation system),[16] without some translation components, potassium glutamate, NTPs, or tRNAs, at 37 °C for 20 min and treated with 1/10 vol of 1-methyl-7-nitroisatoic anhydride[36] in DMSO (1M7, 10 mM) or neat DMSO at 37 °C for 70 s. The RNAs were purified with an RNeasy mini kit (Qiagen, Hilden, Germany) and dissolved in 10 μL of sterile water. For the sequencing reactions, unmodified RNA (1 pmol) was added to 9 μL of sterile water. The 2′-*O*-adducts were detected by primer extension with 5′- fluorescently labeled primer (5′-GTCGAATCTCGGGCTGAATG-3′). All of the primers were labeled with VIC or NED (Applied Biosystems, Foster City, CA). VIC was used for the sequencing channel. NED was used for the (+) and (−) reagent channel. Fluorescently labeled primer (3 μL, 0.3 mM) was added to the (+) and (−) 1M7 reactions and sequencing reactions. The primer-template solutions were incubated at 65 °C for 5 min and 37 °C for 1 min and placed on ice. The ddGTP (1 μL, 10 mM) was also added to the sequencing reactions. The primer extension was initiated by the addition of Superscript III enzyme mix[35] (6 μL) and SuperScript III (1 μL, 200 units, Invitrogen) to the (+) and (−) reagent reactions and the sequencing reactions; the solutions were incubation at 45 °C for 2 min, 52 °C for 20 min, and 65 °C for 5 min. After primer extension, each (+) and (−) reaction was combined with the sequencing reaction. The cDNA samples were purified with Performa DTR Gel Filtration Cartridges (EdgeBio systems, Gaithersburg, MD) and resolved on the Genetic Analyzer 3130 (Applied Biosystems). The raw electropherograms examining the fluorescence intensity versus elution time were analyzed using QuShape.[34] The SHAPE reactivities for the primer were normalized using model-free statistics as previously described.[37,38]

■ ASSOCIATED CONTENT

Supporting Information

Additional figures. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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