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Compartmentalization in a Water-in-Oil Emulsion Repressed the Spontaneous Amplification of RNA by Qβ Replicase†

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ABSTRACT: During RNA replication mediated by Qβ replicase, self-replicating RNAs (RQ RNAs) are amplified without the addition of template RNA. This undesired amplification makes the study of target RNA replication difficult, especially for long RNA such as genomic RNA of Qβ phage. This perhaps is one of the reasons why the precise rate of genomic RNA replication in the presence of host factor Hfq has not been reported *in vitro*. Here, we report a method to repress RQ RNA amplification by compartmentalization of the reaction using a water-in-oil emulsion but maintaining the activity of Qβ replicase. This method allowed us to amplify the phage Qβ genome RNA exponentially without detectable amplification of RQ RNA. Furthermore, we found that the rate constant of genome RNA replication in the exponential phase at the optimum Hfq concentration was approximately 4.6 times larger than that of a previous report, close to *in vivo* data. This result indicates that the replication rate *in vivo* is largely explained by the presence of Hfq. This easy method paves the way for the study of genomic RNA replication without special care for the undesired RQ RNA amplification.

Qβ replicase is an RNA-dependent RNA polymerase originating from RNA phage Qβ (*1*, *2*). The replicase synthesizes the complementary strand RNA from a single-stranded template RNA. Since the synthesized complementary strand (minus strand) can also be a template for replicase, the template RNA amplifies exponentially in the presence of excess replicase (*3*, *4*). Such autocatalytic replication is a characteristic of RNA replication by Qβ replicase. As a template RNA, the replicase recognizes the genomic RNA of Qβ phage and other self-replicating RNAs, such as "6S" (*5*), "variant" (*6*−*8*), or "RQ" (*9*). We have referred to those self-replicating RNAs that are not genomic RNA as "RQ RNA". These RNAs are short (100−300 nt) and originated from several sources as described below.

The replicase is composed of four subunits: the phage genome-derived β-subunit, host-derived translational elongation factors EF-Tu and EF-T, and the ribosomal S1 subunit (*1*). For efficient replication of Qβ genomic RNA, another host factor, Hfq, is required (*10*−*12*). Hfq has been reported to bind to a specific region of genomic RNA and is used to recruit the replicase (*13*).

Genomic RNA of Qβ phage has been amplified exponentially *in vitro* by a highly purified replicase (*3*). Assuming the first-order reaction, the rate constant was 0.024/min per RNA in that experiment (calculated from data in the exponential phase). The rate was much slower than the *in vivo* rate constant (0.3/min) (*14*). The slow replication rate *in vitro* could be due to the lack of Hfq protein, which was not identified at that time. However, to date, there has been no report of exponential amplification in the presence of an optimum concentration of Hfq, and thus the rate constant remains unknown.

The difficulty in replicating genomic RNA exponentially is due to the undesired amplification of RQ RNA, which appears without any template RNA addition. Small RQ RNAs (usually smaller than 220 nt) are amplified extremely rapidly because replication rate is known to correlate with template RNA size (*15*). As an example, the RQ RNA, s222, amplifies 10-fold per minute. In contrast, the replication rate of longer genomic RNA (4200 nt) is predicted to be approximately 20 times slower, resulting in immediate inhibition of genomic RNA replication by the rapidly amplified RQ RNA. We observed almost no genome amplification but an enormous amount of RQ amplification by the replicase prepared by the usual purification procedure (Figure 2, bulk reaction). Similar results were obtained by a commercially available replicase (Epicenter, USA; data not shown). Therefore, to perform exponential replication of genomic RNA, special care is required to avoid RQ RNA amplification.

RQ RNAs were originally seen as spontaneously amplified RNA, without template RNA addition, *in vitro* by Qβ replicase and named "6S", "variant", or RQ RNA (*6*−*8*, *16*). Several origins for RQ RNAs are known, including contamination (*17*, *18*), *de novo* synthesis from NTPs (*19*−*22*), and recombination from several RNA origins (*23*−*26*): Qβ genomic RNA (*27*), rRNA (*9*), and tRNA (*28*, *29*). To avoid RQ RNA amplification, several experimental conditions are required depending on the origin of the RQ RNA. Elimination of RQ RNA requires a highly purified replicase and careful manipulation (*17*) since RQ RNA has been known to originate from the air in laboratories using replicases (*18*). To decrease *de novo* synthesis, a decrease in

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NTP concentration along with enhanced ionic strength is required (*21*). To eliminate any chance of recombination, reaction conditions without any potential RQ source, such as tRNA or rRNA, are required. If all of these conditions are satisfied, RQ RNAs were repressed and genomic RNA amplified exponentially (*3*). However, these conditions restrict possible experiments and hamper the study of genomic RNA replication. For example, if we have to use only highly purified enzymes, an experiment utilizing a crude extract such as a cell-free translation system is impossible; such experiments are important in understanding the mechanism of infection of phages (*30*). Furthermore, if we have to decrease the NTP concentration, we cannot examine replication under physiological conditions. Therefore, we need another method to repress RQ RNA amplification that does not require highly purified enzymes or extreme experimental conditions.

We applied a compartmentalization method using a water-in-oil emulsion to the genomic RNA replication. After optimization, this method allowed us to observe efficient genome replication without RQ amplification keeping the activity of replication. We measured the replication rate of genomic RNA during the exponential phase in the presence of Hfq at the optimal concentration and found that the rate was approximately 4.6 times larger than that of a previous report (*3*).

EXPERIMENTAL PROCEDURES

RNA Replication Reaction. The standard reaction mixture contained 125 mM Tris-HCl (pH 7.6), 5 mM magnesium chloride, 1.25 mM each NTP, 0.1% BSA,[1] 10 nM s222 (Figure 1) or genomic RNA (Figure 2−4), and 240 nM Qβ replicase. After incubation in a test tube or the emulsion, the samples were recovered. Replication product of s222 (Figure 1B) was subjected to electrophoresis on an 8% polyacrylamide gel, followed by SYBR green II (Molecular Probes, USA) staining. Replication product of genomic RNA (Figures 2−4) was subjected to electrophoresis on a 1% agarose gel in TAE buffer, followed by SYBR green II staining.

RNA Preparation. s222 RNA was prepared as previously described (*15*). Genomic RNA of Qβ phage was prepared by *in vitro* transcription from PCR fragments using SKQβ plasmid (*31*) as a template and the following primers: 5′-TCTCTCTAATACGACTCACTATAGGGGGACCCCCTTTAGG-3′ and 5′-CAAACCCGGGAGGAGAGAGGGCAAAGC-3′.

Emulsion Preparation and Sample Recovery. Emulsion preparation was modified from a previous report (*32*). The oil phase contained 4.5% Span80 (Sigma, USA) and 0.5% Tween 80 (Sigma, USA) in light mineral oil (Sigma, USA). The oil phase was saturated by mixing with buffer (150 μL per 1 mL of oil) containing 125 mM Tris-HCl (pH 7.6), 5 mM magnesium chloride, and 0.1% BSA, vortexed briefly, and incubated for 20 min at 37 °C, and the upper phase was collected after centrifugation at 22000*g* for 20 min at 4 °C. The collected saturated or nonsaturated oil (480 μL) was vigorously mixed with 20 μL of reaction solution using a vortex mixer (Vortex-Genie 2; Scientific Industries, USA) for 60 s in a 1.5 mL plastic tube (Watson, Japan). The emulsion was incubated for the indicated time at 37 °C; then 2 μL of 100 mM EDTA and 200 μL of diethyl ether were added and immediately centrifuged at 22000*g* for 20 min at 4 °C.

[1]Abbreviations: PCR, polymerase chain reaction; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; w/o emulsion, water-in-oil emulsion; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; S1, ribosomal protein S1.

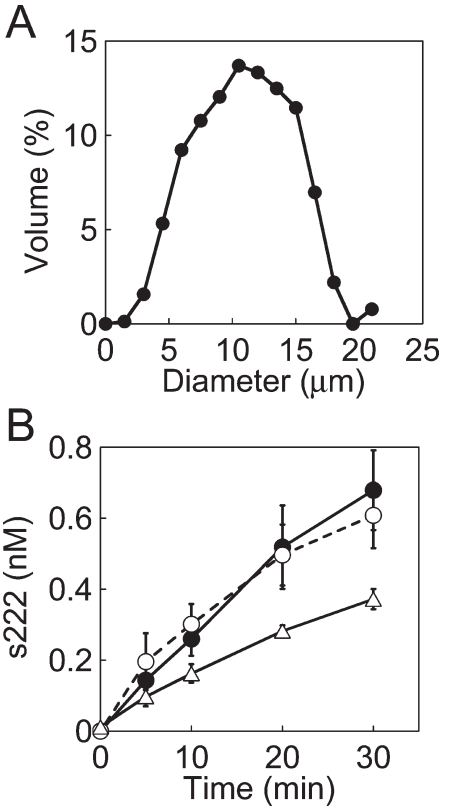

FIGURE 1: (A) Size distribution of the w/o emulsion used in this study. The size was measured by microscopy and assumed that the emulsion was spherical. (B) Replication activity in the emulsion. Replication of an externally added RQ RNA (s222, 10 nM) was measured under usual bulk reaction conditions (closed circles) and in w/o emulsion with (open circles) or without (triangles) the oil saturation. The error bar shows standard deviation ($n = 3$).

The upper phase was discarded, 200 μL of diethyl ether was added, and the emulsion was centrifuged again. The upper phase was discarded, and residual water phase was dried at 4 °C for 15 min.

Purification of Qβ Replicase. The Qβ replicase used in this study was an α-less heterotrimer, purified as follows: *Escherichia coli* BL21(DE3) harboring the plasmids pET-Tu-Ts and pBAD33-rep (*31*) was grown at 30 °C in LB media supplemented with 50 μg/mL ampicillin, 20 μg/mL chloramphenicol, and 1.0 mM lactose until OD_{660} = 1 and then incubated for an additional 3 h with 0.2% arabinose. The collected cell pellet was disrupted by a Multi-Beads Shocker (Yasui Kikai, Japan) in buffer A [50 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 1 mM EDTA, 500 mM NaCl]. The cell lysate was subjected to ammonium sulfate precipitation (0.39 g of ammonium sulfate/mL) and suspended in buffer B (same as buffer A but lacking 100 mM NaCl). After desalting through a PD10 (GE, USA) column, the sample was applied to a Hi Trap Q HP column (GE, USA) and eluted by NaCl gradient (100−400 mM) in buffer B. The fractions containing the heterotrimer were collected and further desalted with another PD10 column, applied to a Hi Trap SP HP column (GE, USA), and eluted by NaCl gradient (100−400 mM). The fractions containing heterotrimer were collected and used as replicase. Result of SDS−PAGE is shown in the Supporting Information (Figure S2). Hfq proteins and S1 subunit were prepared as previously described (*33*, *34*).

RESULTS

One of the possible methods to repress RQ amplification is compartmentalization of the reaction solution. In the case of

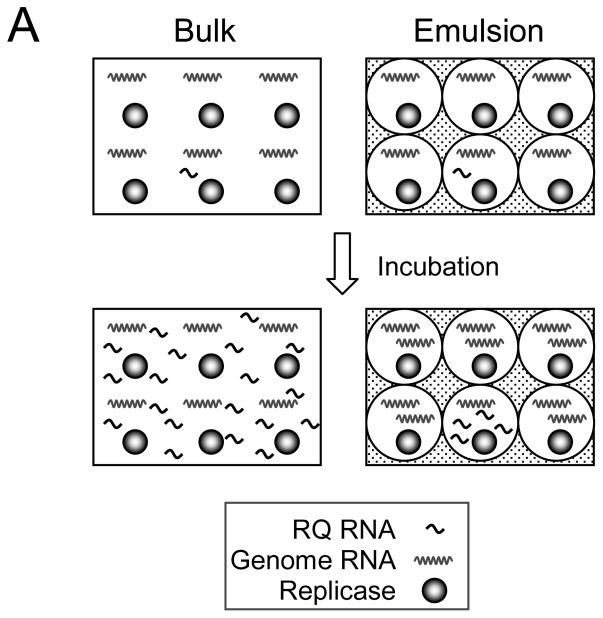

FIGURE 2: (A) Schematic drawing of the compartmentalization effect. In the case of the usual noncompartmentalized bulk reaction, if one molecule of RQ RNA was contaminated, or appeared by *de novo* synthesis or recombination, it was amplified rapidly and became prevalent in the entire reaction solution. If compartmentalized in an emulsion, amplification of the RQ RNA was confined to a compartment, and other compartments were free from RQ RNA after incubation resulting in efficient replication of genomic RNA. (B) Genomic RNA replication in the w/o emulsion. Genomic RNA of Qβ phage was incubated in the standard reaction mixture containing S1 protein (200 nM) and Hfq protein (300 nM). After 30 min incubation at 37 °C in the emulsion and extraction, the replication product was subjected to agarose electropholesis followed by SYBR green II staining. "SS" and "DS" indicate bands corresponding to single- or double-stranded genomic RNA, respectively.

usual noncompartmentalized bulk reactions, even if one molecule of RQ RNA is contaminating or appears by *de novo* synthesis or recombination, it is amplified rapidly and prevails in the entire reaction solution. If amplification of RQ RNA is confined to a compartment and other compartments are free from RQ RNA (Figure 2A), it is no longer possible to adversely affect the reaction, resulting in efficient replication of target RNA.

A water-in-oil (w/o) emulsion was used to form compartments for a specific PCR (*35*−*38*) or *in vitro* molecular evolution experiment (*32*, *35*, *39*). We applied this method to RNA replication by Qβ replicase. We prepared the w/o emulsion by

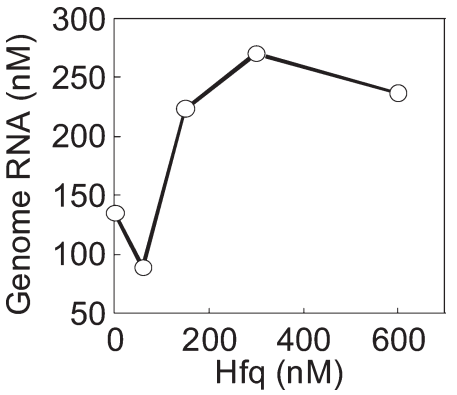

FIGURE 3: Hfq concentration dependency of genomic RNA replication in the w/o emulsion. Genomic RNA replication in the emulsion was measured as described in Figure 2 except for the Hfq concentration. The incubation time was 40 min. Genomic RNA was quantified by measuring band intensity of single- and double-stranded RNA.

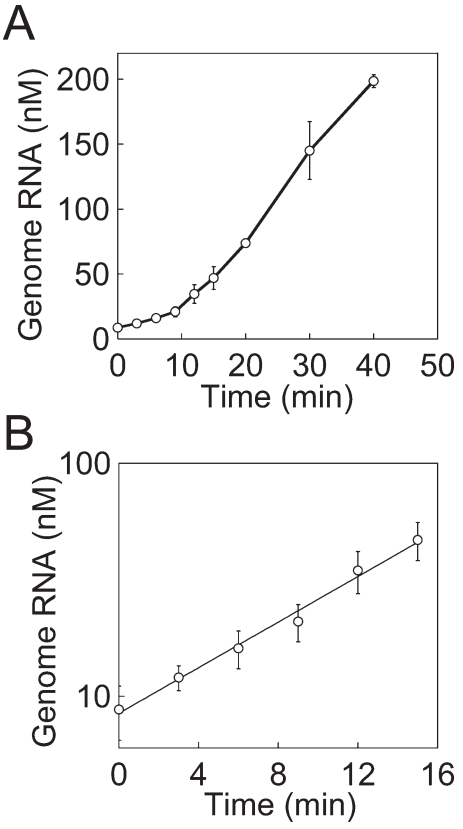

FIGURE 4: Kinetics of genomic RNA replication in the emulsion. (A) Genomic RNA replication in the emulsion was performed as described in Figure 2 except for 480 nM Qβ replicase. Genomic RNA was quantified by measuring band intensity of single- and double-stranded RNA. (B) Exponential phase data (0−15 min) were replotted on a semilogarithmic graph.

mixing the oil phase (mineral oil containing detergents) and water phase (reaction solution) with a vortex mixer. The size distribution was measured under a microscope (Figure 1A).

We compared the replication activity of Qβ replicase in the emulsion to that in a usual bulk reaction. The reaction mixture contained Qβ replicase and the RQ RNA, s222, a derivative of MDV-1(*15*). The mixture was incubated in the emulsion and recovered at high efficiency (> 95%), and the amount of s222 was measured (Figure 1B). When we used the same oil phase as the previous study (*32*), we observed lower rates of RNA replication

(triangles) when compared with the usual bulk reaction (closed circles). One of the possible reasons for the decreased replication rate was leakage of small molecules into the oil phase from the reaction mixture as this oil is known to solubilize water molecules during incubation (*40*). Therefore, we saturated the oil phase with reaction buffer omitting NTPs, replicase, and RNA. When we used the saturated oil phase (open cicles), the replication rate was restored to the same levels as the bulk reaction. These results indicate that replication activity in emulsions using saturated oil is compatible to the usual bulk conditions.

Next, we compared genomic RNA replication in the usual bulk conditions to that in the emulsion. The replication of long template RNAs, such as genomic RNA, was sensitive to RQ RNA amplification because the replication of long templates is slow and easily inhibited by rapidly amplified RQ RNA. We observed almost no amplification of genomic RNA in the bulk conditions as opposed to large amounts of amplified RQ RNA (Figure 2, bulk reaction). Genomic RNA was then amplified after incubation in the w/o emulsion, and RQ RNA was below the limits of detection (Figure 2, emulsion reaction). These results clearly demonstrate the advantages of carrying out the reaction in an emulsion.

Simple inhibition of RQ RNA replication in an emulsion cannot explain this repression fully because s222 replicated normally in the emulsion (Figure 1B). In addition, degradation or irreversible inactivation of RQ RNA during the emulsification process is not the cause of this repression because RQ RNA recovered from the emulsions was amplified successfully in the bulk condition (Supporting Information Figure S3). Part of the repression can be explained by the compartment effect because the number of compartments was much larger than the expected number of RQ RNAs appearing in this experimental condition and time scale. The average size of the emulsion was 10 μm (Figure 1A), corresponding to about 10^7 compartments per 20 μL of reaction solution. The RQ RNA number in this experiment was estimated to be about 100 molecules per 20 μL reaction volume (Supporting Information Figure S1). Initial genomic RNA concentration was 10 nM, corresponding to about 10^{11} molecules per 20 μL reaction volume. These data indicate that most of the compartments contained approximately 10^4 molecules of genomic RNA and no RQ RNA. This result does not mean there was no amplification of RQ RNA. RQ RNA must be amplified in some compartments; however, the frequency of such compartments would be low and thus below the limits of detection as a whole.

The 10 μm size of the emulsion prepared by vortex in this case is suitable for the repression of RQ RNA; larger numbers of RQ RNA would be produced depending on experimental conditions. In such cases, smaller emulsions (1−5 μm) could be prepared by stirring or filtration instead of vortex (*32*, *41*), allowing for this emulsion method to be available for a broad range of experimental conditions.

Using this method, we measured the rate constant of genomic RNA replication at the optimum Hfq concentration. First, we confirmed the necessity of Hfq for genomic replication in the emulsion. We measured genomic RNA concentration after replication at various Hfq concentrations and found that Hfq enhances replication in the emulsion (Figure 3). The effects of Hfq proteins were almost saturated at 300 nM, consistent with previous results (*11*). We analyzed the kinetics of genome replication in emulsions at the optimum Hfq concentration. We observed the exponential phase followed by a linear phase. The rate constant was calculated from the exponential phase assuming first-order kinetics to be 0.11/min, 4.6 times higher than previous data without Hfq addition (0.024/min) (*3*), and closer to *in vivo* data (0.3/min) (*14*).

DISCUSSION

To date, the undesired amplification of RQ RNA was an inhibitory factor in studying genomic RNA replication. We have applied a compartmentalization method using a w/o emulsion, by which the amplification of RQ RNA was repressed and the amplification of genomic RNA was enhanced. This easy method will pave the way for the study of genomic RNA replication in a wide range of experimental conditions without undesired RQ RNA amplification occurring.

Using this method, we found that the rate constant of genomic RNA replication at the optimum Hfq was 4.6 times higher than previous data without Hfq addition (*3*) and closer to *in vivo* data (*14*). This result indicates that the replication rate *in vivo* is largely explained by the presence of Hfq and also suggests that there are other factors to enhance genomic replication *in vivo*.

As another application, Qβ replicase was examined as an RNA amplification tool (*42*) or as a central component for artificial cell models (*34*, *43*−*45*). However, the undesired amplification of RQ RNAs severely diminished the usefulness of Qβ replicase. Although other problems exist, such as template specificity or formation of double strands, this emulsion method has solved one of the major difficulties evident in RNA amplification methods.

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SUPPORTING INFORMATION AVAILABLE

Figures S1−S3 illustrating estimation of RQ RNA number, SDS−PAGE result of the purified replicase, and reamplification experiment of RQ RNA. This material is available free of charge via the Internet at http://pubs.acs.org.

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