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# Effects of ribosomes on the kinetics of Qβ replication

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ABSTRACT

**Bacteriophage Qβ utilizes some host cell translation factors during replication. Previously, we constructed a kinetic model that explains replication of long RNA molecules by Qβ replicase. Here, we expanded the previous kinetic model to include the effects of ribosome concentration on RNA replication. The expanded model quantitatively explained single- and double-strand formation kinetics during replication with various ribosome concentrations for two artificial long RNAs. This expanded model and the knowledge obtained in this study provide useful frameworks to understand the precise replication mechanism of Qβ replicase with ribosomes and to design amplifiable RNA genomes in translation-coupling systems.**


## 1. Introduction

The Qβ replicase is an RNA-dependent RNA polymerase that is responsible for replication of the simple positive-sense single-stranded RNA genome of bacteriophage Qβ [1]. The replicase is a heterotetramer composed of a virally encoded β-subunit and three host translational proteins: ribosomal protein S1 and elongation factor Tu (EF-Tu) and Ts. The core enzyme of Qβ replicase is a heterotrimer composed of the β-subunit, EF-Tu, and EF-Ts [2], and the S1 protein is dispensable for synthesis of a positive-strand RNA from a negative-strand RNA template [3–5]. EF-Tu and Ts have been reported to modulate the RNA elongation process [6–8]. The positive-strand genomic RNA is copied by this enzyme into complementary negative-strand RNA [9]. Both the positive and negative strands serve as equally efficient templates in the next round of RNA synthesis. Therefore, in the presence of excess Qβ replicase, some RNAs, such as genomic RNA and small RNAs called RQ RNAs (Replicable by Qβ replicase, e.g., MDV-1 and RQ 135) [10,11], are replicated exponentially in an autocatalytic manner [10,12]. Due to this unique characteristic, there have been many attempts to apply Qβ replicase in several applications, such as RNA amplification [13], RNA virus detection [14], detection of RNA recombination [15], a model system of Darwinian evolution [16], a biological example of a hypercycle [17], and a gene replication component of an artificial cell model [18–22].

In contrast to small RQ RNAs and the Qβ phage genome, most artificial long RNAs, the sequences of which encode proteins, are not replicated exponentially by Qβ replicase due to hybridization of the template with the newly synthesized complementary strand. The double-stranded RNA (dsRNA) thus formed cannot be used as a template for the next round of replication [23–29]. Therefore, if more than half of the newly synthesized RNA hybridizes with template RNA, the RNA does not show exponential replication [30]. This dsRNA formation hinders the broader application of the replicase. A greater understanding of the mechanism underlying dsRNA formation and the development of means of inhibiting dsRNA formation during RNA replication are crucial for the broader application of Qβ replicase.

The mechanism of dsRNA formation has been studied using small RQ RNAs (usually 70–220 nt in length). The kinetics of dsRNA formation for long RNA, however, has not been quantitatively analyzed due to the lack of kinetic modeling studies. Previously, we constructed a kinetic model that explains replication of long RNA molecules by Qβ replicase [31]. However, this model did not include the effects of the ribosome and S1 ribosomal subunit concentrations on Qβ RNA replication, which have been reported to reduce the ratio of dsRNA to total synthesized RNA during Qβ RNA replication [32,33]. To understand Qβ RNA replication under physiologically relevant conditions (i.e., in the presence of

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ribosomes), it was necessary to expand this kinetic model to include the effects of ribosome concentration on RNA replication.

In the present study, the previous kinetic model was expanded to include the effects of ribosome concentrations on single-stranded RNA (ssRNA) and dsRNA synthesis during Qβ RNA replication. Furthermore, all kinetic parameters were measured using two artificial template RNAs consisting of different fragments of Qβ genomic RNA [31].

## 2. Experimental procedures

### 2.1. Plasmid construction and preparation of template RNAs

The plasmids pUC-MDV(−)β(+), pUC-MDV(+)β(−), and pUC-U1 were prepared previously [21,31,34]. These plasmids were used to produce MDV(−)β(+), MDV(+)β(−), and U1 RNA, respectively. The β-subunit was encoded between short replicable RNA in MDV(−)β(+) (2125 nt). In U1 (3035 nt), the fragment of Qβ phage genome (1404–2352) was embedded in MDV(−)β(+). MDV(+)β(−) is the complementary strand of MDV(−)β(+). The plasmid pUC-U1(−) was used to produce U1(−) RNA, which is the complementary strand of U1, and was constructed by insertion of the cDNA fragment of the complementary Qβ phage genome (1404–2352) into pUC-MDV(+)β(−). The complementary cDNA fragment of pUC-U1 was prepared by PCR using primer 1 (5′-CCTCGAGATCTCCTCTGGTCCTCAATCCGCGTG-3′) and primer 2 (5′-TGCTGTCTTAGACATGCATTTC-3′) with the plasmid SKQβ as the template. This fragment was ligated with the fragment amplified by PCR using primer 3 (5′-ATGTCTAAGACAGCATCTTCGCGTAACTC-3′) and primer 4 (5′-GAGGAGATCTCGAGGCCTGCTAG-3′) with the plasmid pUC-MDV(+)β(−) as the template using an In-Fusion PCR cloning kit (Takara, Shiga, Japan) according to the manufacturer's instructions. Template RNAs were prepared by transcription using T7 polymerase as described previously [31].

### 2.2. RNA replication

The replication reaction was carried out at 37 °C by adding template RNA (100 nM) and purified Qβ replicase (10 nM) [35] (fraction of active Qβ replicase: 0.16 [31,34]) to the amino acid-free cell-free translation system (lab-made PURE system) [22,36,37]. The PURE system contained Mg $(OAc)_2$ (16 mM), potassium glutamate (70 mM), spermidine (0.375 mM), dithiothreitol (6 mM), ATP (3.75 mM), GTP (2.5 mM), CTP (1.25 mM), UTP (1.25 mM), creatine phosphate (25 mM), 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) (100 mM, pH 7.6), and translation components of *Escherichia coli*. The amounts of synthesized RNA were measured by incorporation of [$^{32}$P]-UTP (PerkinElmer, Waltham, MA) during synthesis and determination of the band intensity on dried agarose gels after electrophoresis in TBE buffer at 4 °C as described previously [31]. Radiolabeled newly synthesized RNAs were detected by autoradiography (Typhoon FLA 7000; GE Healthcare, Little Chalfont, UK) and quantified using ImageQuant software (GE Healthcare) (Fig. S1). As reported previously [31], the production of positive-strand RNA was ignored because externally added positive-strand RNA was present in excess over newly synthesized negative-strand RNA (added positive-strand RNA/newly synthesized RNA molar ratio ≈10; see Figs. 2a and S3), and therefore all replicase molecules were expected to bind to the positive-strand RNA to synthesize negative-strand RNA.

### 2.3. Preparation of ribosomal protein S1

A plasmid encoding S1 protein (pET21aS1) was constructed. For overproduction of S1 protein, *E. coli* JM109 (DE3) cells harboring pET21aS1 were grown in a 5-L-scale fermenter at 37 °C. Cell samples (50 g) were resuspended in buffer A (50 mM HEPES–KOH, pH 7.6, 10 mM $MgCl_2$, 7 mM 2-mercaptoethanol) and lysed using a Multi-Beads Shocker (Yasuikikai, Osaka, Japan). The supernatant was mixed with ammonium sulfate to 60% saturation. Precipitated proteins were collected by centrifugation and dissolved in buffer A. The samples was dialyzed twice against buffer A and loaded onto a HiTrapQ column (GE Healthcare). The column was eluted with a linear gradient of NaCl (0–1 M). Fractions containing S1 protein were pooled and concentrated. The sample was loaded onto a Superdex 200 pg size exclusion column (GE Healthcare) equilibrated with buffer B (50 mM HEPES-KOH, pH 7.6, 100 mM KCl, 10 mM $MgCl_2$, 7 mM 2-mercaptoethanol). Protein purity was confirmed by SDS–PAGE.

### 2.4. Filter binding assay

Ribosome–RNA interaction was evaluated by the filter binding method as described previously [38]. [$^{32}$P]-UTP-labeled MDV(−)β(+), MDV(+)β(−), U1, and U1(−) were prepared by in vitro transcription. The labeled RNAs (50 nM) were incubated with ribosomes in binding buffer consisting of 10 mM Tris–HCl, 10 mM $MgCl_2$, 60 mM $NH_4Cl$, and 6 mM 2-mercaptoethanol for 30 min at 37 °C. Aliquots were loaded onto nitrocellulose filters (0.45 μm pore size; Whatman, Dassel, Germany) that had been soaked in the binding buffer. The filters were washed 5 times with 2 ml of binding buffer. Radioactivity retained on the filters was measured with a liquid scintillation counter (LSC-5100; Aloka, Tokyo, Japan).

### 2.5. Kinetic model

We constructed a new kinetic model that includes the effects of ribosome concentration on kinetic parameters based on our Qβ RNA replication model reported previously [31]. In this model (Fig. 1), it was assumed that positive-strand RNAs and negative-strand RNAs bind to the ribosome, and this binding affects the rates of RNA replication and hybridization. Assuming that the binding of replicase to RNA is in equilibrium, the concentrations of negative-strand RNA in ssRNA or dsRNA can be written as in Eqs. 1 and 2 (see Supplementary information for derivation).

When $k_{col} \neq 0$,

$$[(-)\mathrm{RNA_t}] = \frac{(1 - e^{-k_{col}[(+)\mathrm{RNA_t}]t})k_{ss}[\mathrm{Rep_t}]}{k_{col}\,[(+)\mathrm{RNA_t}]}\,A, \tag{1}$$

$$[\mathrm{DS_t}] = (k_{ss} + k_{ds})\,[\mathrm{Rep_t}]\,t - (-)\mathrm{RNA}(t), \tag{2}$$

where $A = \dfrac{[(+)\mathrm{RNA_t}]}{K_{rib} + [(+)\mathrm{RNA_t}]}$.

When $k_{col} = 0$,

$$[(-)\mathrm{RNA_t}] = k_{ss}[\mathrm{Rep_t}]\,t \tag{1′}$$

$$[\mathrm{DS_t}] = k_{ds}\,[\mathrm{Rep_t}]\,t. \tag{2′}$$

Note that except for Fig. 3b, the experiments were carried out in the presence of excess positive-strand RNA and so "*A*" could be approximated to 1.

Here, $k_{ss}$, $k_{ds}$, and $k_{col}$ are functions of several parameters and ribosome concentration as follows:

$$k_{ss} = \frac{k_{ss1} + \dfrac{[\mathrm{Rib_t}]}{K_4}\,k_{ss2}}{1 + \dfrac{[\mathrm{Rib_t}]}{K_4}} \tag{3}$$

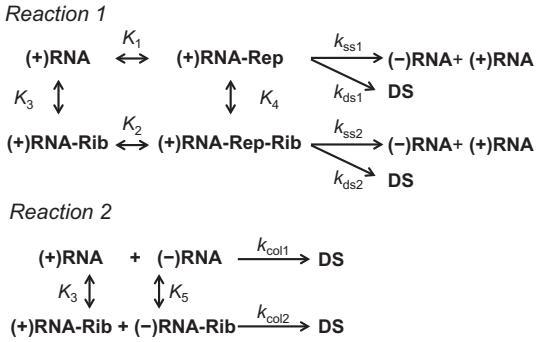


**Fig. 1.** Kinetic model of Qβ RNA replication with ribosomes. Positive-strand RNA–ribosome complex ((+)RNA–Rib); Positive-strand RNA-replicase–ribosome complex ((+)RNA-Rep–Rib); Negative-strand RNA–ribosome complex ((−)RNA–Rib). Ribosome binding to RNA or RNA-replicase complex was assumed to be in equilibrium with dissociation rate constants $K_3$ and $K_4$, respectively. Similarly, binding of replicase to RNA or RNA–ribosome complex was assumed to be in equilibrium with dissociation rate constants $K_1$ and $K_2$, respectively. In Reaction 1, single-stranded negative-strand RNA was synthesized from (+)RNA-Rep or (+)RNA-Rep–Rib with rate constants of $k_{ss1}$ or $k_{ss2}$, respectively. The dsRNAs were synthesized from (+)RNA-Rep or (+)RNA-Rep-Rib at $k_{ds1}$ or $k_{ds2}$, respectively. In Reaction 2, single-stranded newly synthesized negative-strand RNAs hybridized with positive-strand RNAs with a rate constant $k_{col1}$ to form dsRNA. The ribosome-bound forms ((+)RNA-Rib and (−)RNA-Rib) hybridized with a rate constant $k_{col2}$.

$$k_{ds} = \frac{k_{ds1} + \frac{[Rib_t]}{K_4} k_{ds2}}{1 + \frac{[Rib_t]}{K_4}} \quad (4)$$

$$k_{col} = \frac{[Rib_t]}{K_4 + [Rib_t]} k_{col2} \quad (5)$$

Eqs. (3)–(5) were used for curve fitting in Fig. 4a–c, respectively.

From equations S1′ and S2′, the rates of total RNA synthesis, which are the sums of the rates of ssRNA and dsRNA synthesis, can be written as follows (see Supplemental information):

$$\frac{d([(-)RNA_t] + [DS_t])}{dt} = \frac{(k_{ss} + k_{ds})[(+)RNA_t][Rep_t]}{K_{rib} + [(+)RNA_t]}, \quad (6)$$

where $K_{rib} = \frac{K_1(1 + \frac{[Rib_t]}{K_3})}{1 + \frac{[Rib_t]}{K_4}}$.

This equation was used to fit the data in Fig. 3b.

All curve fitting procedures were performed using OriginPro (OriginLab, Northampton, MA) and other calculations were performed with Mathematica (Wolfram Research, Champaign, IL).

## 3. Results

### 3.1. Kinetic model of double-stranded RNA formation taking into consideration the effects of ribosome concentration

The ribosome is an important factor influencing both RNA synthesis rate and dsRNA formation by Qβ replicase [33], although the kinetic mechanisms underlying these actions have not yet been elucidated. Therefore, we attempted to expand our previously constructed kinetic model [31] to include the effects of ribosomes (Fig. 1).

In Reaction 1, the binding steps of positive-strand RNA ((+)RNA) and positive-strand RNA-replicase complex ((+)RNA-Rep) to the ribosome were assumed to be in equilibrium with dissociation constants $K_3$ and $K_4$, respectively. The dissociation constant of the binding process of replicase to positive-strand RNA–ribosome complex ((+)RNA-Rib) was also set as $K_2$. Different rate constants were assigned for ssRNA and dsRNA synthesis from positive-strand RNA-replicase and positive-strand RNA-replicase–ribosome complexes ($k_{ss1}$, $k_{ds1}$, $k_{ss2}$, and $k_{ds2}$, respectively).

In Reaction 2, it was assumed that the binding steps of ribosomes to positive- and negative-strand RNA are in equilibrium with dissociation constants $K_3$ and $K_5$, respectively, because filter binding assay [38] indicated that ribosomes bound to both positive- and negative-strand RNAs, with dissociation constants of 180–510 nM (Fig. 2 and Table 1). Different rate constants, $k_{col1}$ and $k_{col2}$, were assigned for the rates of hybridization between free RNAs ((+)RNA and (−)RNA), ribosome-bound RNAs ((+)RNA-Rib and (−)RNA-Rib), respectively.

For simplicity, we included only two collision pathways in our model, i.e., between (+)RNA and (−)RNA and between (+)RNA-Rib and (−)RNA-Rib (Fig. 1). This is reasonable because experiments were performed with a ribosome concentration higher than the dissociation constant between ribosome and RNA, where almost all RNA was assumed to be in the ribosome-bound form.

Based on these reaction schemes, the newly synthesized ssRNA and dsRNA were formulated as in the previous model [31] (Eqs. (1) and (2)) except that parameters $k_{ss}$, $k_{ds}$, and $k_{col}$, which were constants in the previous model, became functions of the ribosome concentration and some rate constants in this model (see Eqs. (3)–(5) in Section 2).

### 3.2. Measurement of RNA replication kinetics

The effects of ribosomes on ssRNA and dsRNA synthesis were evaluated using this model. Similar to our previous study [31], experiments were performed using two positive-strand RNA tem-

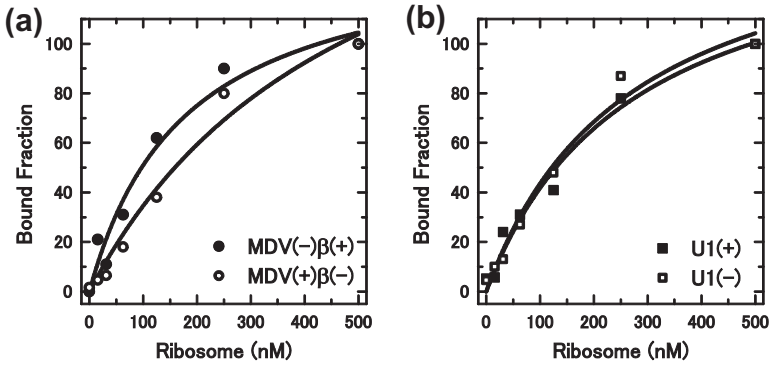


**Fig. 2.** Nitrocellulose filter binding assay. Radioisotope-labeled RNAs (MDV(−)β(+), MDV(+)β(−), U1(+), and U1 (−)) were prepared by in vitro transcription using T7 RNA polymerase and purified using RNeasy columns (Qiagen). The labeled RNAs were incubated for 30 min at 37 °C with ribosomes at various concentrations. (a) MDV(−)β(+) (closed circles) and MDV(+)β(−) (open circles) (b) U1(+) (closed squares) and U1 (−) (open squares).

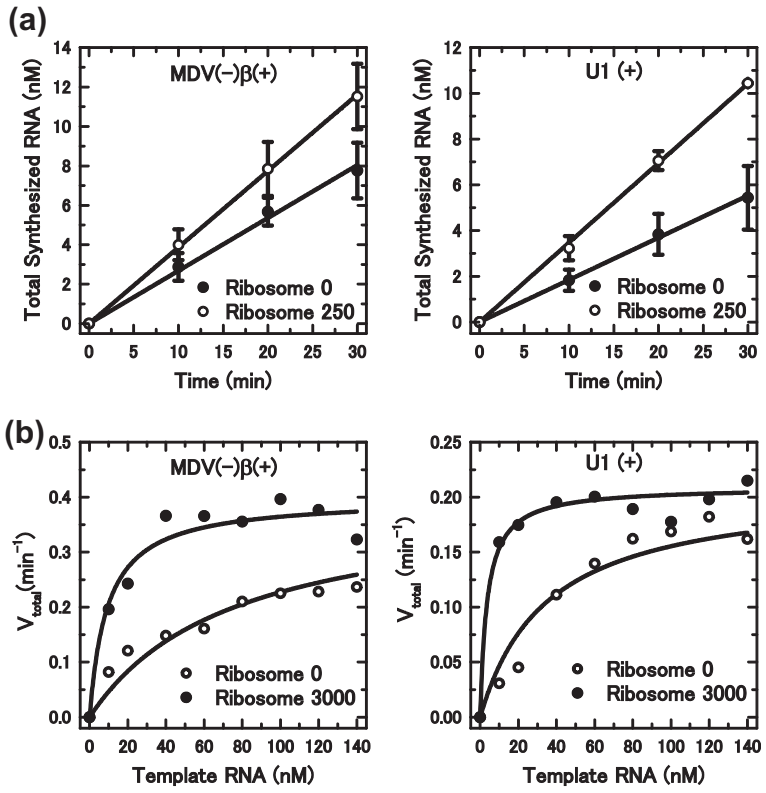


**Fig. 3.** RNA synthesis of MDV(−)β(+) and U1 in the presence or absence of ribosomes. Replication with Qβ replicase was carried out in an amino acid-free cell-free translation system with MDV(−)β(+) or U1 at the indicated ribosome concentrations. (a) Time course of total RNA (ss + dsRNA) synthesis in the absence or presence of 250 nM ribosomes (template RNA (100 nM)/Qβ replicase (10 nM) molar ratio = 10). (b) Effects of template Plus RNA concentration on the rate of total RNA synthesis ($V_{total}$) in the absence or presence of 3000 nM ribosomes (Qβ replicase: 10 nM). Experimental data are shown as dots and fitting results to the kinetic model are shown as lines. The error bars indicate the standard deviation of two independent measurements.

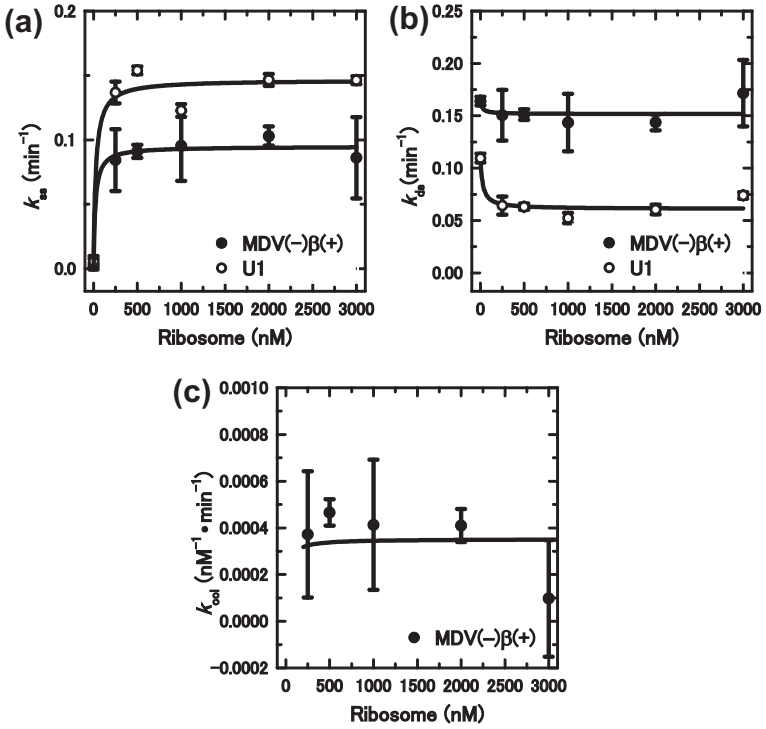


**Fig. 4.** Effects of ribosome concentration on the values of $k_{ss}$, $k_{ds}$, and $k_{col1}$. The values of $k_{ss}$ (a), $k_{ds}$ (b), and $k_{col}$ (c) were estimated by curve fitting of the kinetic data shown in Fig. S2 (template RNA (100 nM)/Qβ replicase (10 nM) molar ratio = 10, Ribosome (>250 nM)/template RNA (100 nM) molar ratio >2.5) with Eqs. 1 and 2, and then plotted against ribosome concentrations for MDV(−)β(+) (closed circles) and U1 (open circles). These data were further fitted with Eqs. (3)–(5), respectively (lines). Error bars indicate the standard error. Some error bars are smaller than the respective symbols.

**Table 1**
Kinetic parameters of the kinetic model considering the effects of ribosome concentration.

| | MDV(−)β(+) | U1 |
|---|---|---|
| $k_{ss1}$ ($min^{-1}$) | $(3.3 \pm 9.4) \times 10^{-3}$ | $(6.1 \pm 11) \times 10^{-3}$ |
| $k_{ss2}$ ($min^{-1}$) | $(9.5 \pm 0.4) \times 10^{-2}$ | $(1.5 \pm 0.05) \times 10^{-1}$ |
| $k_{ds1}$ ($min^{-1}$) | $(1.6 \pm 0.1) \times 10^{-1}$ | $(1.1 \pm 0.1) \times 10^{-1}$ |
| $k_{ds2}$ ($min^{-1}$) | $(1.5 \pm 0.04) \times 10^{-1}$ | $(6.1 \pm 0.5) \times 10^{-2}$ |
| $k_{col2}$ ($nM^{-1}$ $min^{-1}$) | $(3.6 \pm 0.7) \times 10^{-4}$ | n.d. |
| $K_1$ (=$K_{rib0}$) (nM) | $(7.5 \pm 1.1) \times 10$ | $(3.5 \pm 0.5) \times 10$ |
| $K_2$ (nM) | $8.8 \pm 4.9$ | $3.4 \pm 2.4$ |
| $K_3$ (nM) | $(1.8 \pm 0.6) \times 10^2$ | $(2.7 \pm 0.8) \times 10^2$ |
| $K_4$ (nM) | $(2.1 \pm 0.9) \times 10$ | $(2.6 \pm 1.6) \times 10$ |
| $K_5$ (nM) | $(5.1 \pm 2.0) \times 10^2$ | $(2.7 \pm 0.7) \times 10^2$ |
| $K_{rib3000}$ (nM) | $9.3 \pm 2.5$ | $3.6 \pm 2.0$ |

The values are means ± standard errors. The values of $K_2$ and $K_4$ were calculated using the experimentally determined kinetic parameters. The value of $k_{col2}$ was obtained from the $k_{col1}$ value at 3000 nM ribosomes. n.d. not determined.

plates, MDV(−)β(+) or U1, in the presence of an excess of positive-strand RNA over replicase, but ribosome concentration was varied from the absence to 3000 nM ribosomes (Fig. S2). The total (−)RNA, the sum of ssRNA and dsRNA forms, increased linearly regardless of ribosome concentration, but the rate in the absence of ribosomes (Fig. 3a) was lower than that in their presence (see Fig. 3a for 250 nM ribosomes; see Fig. S3 for 500–3000 nM ribosomes). This enhancing effect of the ribosomes on RNA synthesis was consistent with the results reported previously [33]. We examined whether this effect was due to an increase in the affinity of RNA to replicase (i.e., lower $K_2$ than $K_1$) or an increase in the maximum rate of RNA synthesis (i.e., higher $k_{ss2} + k_{ds2}$ than $k_{ss1} + k_{ds1}$). The rates of total RNA synthesis ($V_{total}$) were measured in the absence of ribosomes and at 3000 nM ribosomes with various template RNA concentrations ranging from 10 to 140 nM (Fig. 3b). At 3000 nM ribosomes, total RNA synthesis reached the maximum rate ($V_{max}$) with template RNA concentration greater than 40 nM for both MDV(−)(+) and U1. On the other hand, in the absence of ribosomes, a template RNA concentration of more than 100 nM was required to reach the maximum rate for both MDV(−)(+) and U1. These results suggested that the presence of ribosomes increases the affinity of positive-strand RNA to replicase for both template RNAs. In addition, in the case of MDV(−)(+) RNA, the maximum rate of total RNA synthesis ($V_{max}$) at a ribosome concentration of 3000 nM was higher than that in the absence of ribosomes (Fig. 3b), suggesting that the presence of ribosomes also increases the maximum rate of RNA synthesis.

To quantitatively evaluate the effect of ribosomes, we analyzed the time courses of ssRNA and dsRNA synthesis in the presence of various ribosome concentrations. The values of $k_{ss}$, $k_{ds}$, and $k_{col}$ at each ribosome concentration were estimated by fitting the time course data with Eqs. 1 and 2 (Fig. S2), and are plotted against ribosome concentrations in Fig. 4. Here, the parameters $k_{ss}$, $k_{ds}$, and $k_{col}$ are functions of $k_{ss1,2}$, $k_{ds1,2}$, $k_{col2}$, $K_{3,4}$, and ribosome concentration (see Eqs. (3)–(5)), and represent the overall rate constants for ssRNA and dsRNA synthesis in Reaction 1 and dsRNA synthesis in Reaction 2, respectively. The value of $k_{ss}$ was much smaller in the absence of ribosomes than at concentrations above 250 nM (Fig. 4a). The values of $k_{ds}$ for MDV(−)β(+) remained almost within the same range in the absence to 3000 nM ribosomes (Fig. 4b). On the other hand, the $k_{ds}$ value of U1 was about twofold larger in the absence of ribosomes than at concentrations over 250 nM. These results indicated that ssRNA synthesis was specifically promoted by the presence of ribosomes for both template RNAs. The values of $k_{col}$ of MDV(−)β(+) were almost constant at ribosome concentrations of 250–3000 nM, but $k_{col}$ could not be determined in the absence of ribosomes because the amount of ssRNA present was too small (Fig. 4c). The values of $k_{col}$ of U1 were too small to be determined. The parameters of the kinetic model shown in Fig. 1, i.e., $k_{ss1}$, $k_{ss2}$, $k_{ds1}$, $k_{ds2}$, and $k_{col2}$, were estimated by fitting these data to Eqs. (3)–(5) (Table 1). The values of $K_2$ and $K_4$ were calculated from the kinetic parameters determined according to Eqs. S8 and S20 (see Supplemental Information). These parameter values quantitatively illustrated the effects of ribosomes. For example, the rate constant of ssRNA synthesis was about 20–30-fold higher in the ribosome-bound form ($k_{ss2}$) than in the unbound form ($k_{ss1}$) for both MDV(−)β(+) and U1 (Table 1 and Fig. 4a). The effects of ribosomes on the rate constants of dsRNA synthesis were dependent on template RNA; the rate constant of MDV(−)β(+) in ribosome-bound form ($k_{ds2}$) was almost equivalent to that of the unbound form ($k_{ds1}$), whereas the rate constant of U1 was about twofold smaller than that of the unbound form (Fig. 4b).

### 3.3. Qβ RNA replication with S1

In addition to the ribosome, ribosomal protein S1 has been reported to increase the proportion of ssRNA in newly synthesized RNA during Qβ RNA replication [32]. Therefore, we examined whether S1 can increase the ratio of ssRNA to total synthesized RNA during Qβ RNA replication in the same fashion as the ribosome. In the absence of ribosomes, newly synthesized ssRNA was hardly detected during Qβ RNA replication (Fig. S2). However,

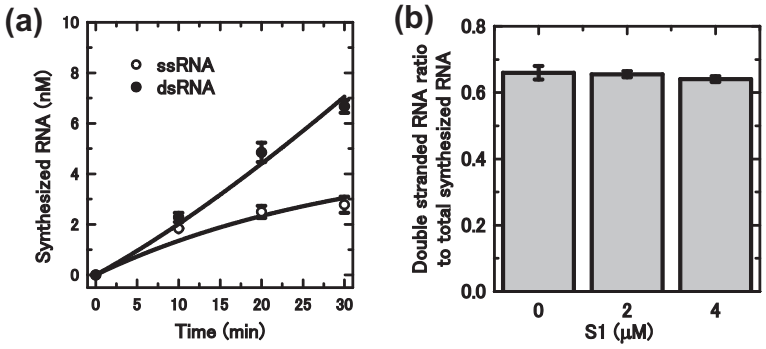


**Fig. 5.** Effects of S1 on the double-stranded RNA synthesis during Qβ RNA replication. RNA replication with 10 nM Qβ replicase was carried out in an amino acid-free cell-free translation system (ribosome concentration: in the absence or presence of 3000 nM ribosomes) with 100 nM MDV(−)β(+) RNA (template RNA (100 nM)/Qβ replicase (10 nM) molar ratio = 10). (a) Time course of ssRNA (open circles) or dsRNA (closed circles) synthesis (S1 concentration: 2000 nM). Experimental data are shown as dots and fitting results to the kinetic model are shown as lines. The error bars indicate the standard deviation of two independent measurements. (b) The ratio of double-stranded RNA to total synthesized RNA at various S1 concentrations (ribosome concentration: 3000 nM). The error bars indicate the standard deviation of two independent measurements.

addition of S1 protein to the ribosome-free cell-free translation system resulted in an increase in the proportion of ssRNA in the product RNA of Qβ RNA replication in the same manner as observed in the presence of ribosomes (Fig. 5a). On the other hand, addition of S1 to the cell-free translation system (ribosome concentration 3000 nM) did not alter the ratio of dsRNA to total synthesized RNA (Fig. 5b), suggesting that the effect of ribosomes can be sufficiently explained by the effect of the S1 subunit.

## 4. Discussion

The present study was performed to expand our previous kinetic model to include the effects of ribosome concentration on RNA replication. This model could quantitatively explain the dynamics of dsRNA formation during Qβ RNA replication of long RNAs in the presence of ribosomes. This is the first study to quantitatively examine the effects of the presence of ribosomes on dsRNA formation during Qβ RNA replication, and it yielded three major findings.

First, the presence of ribosomes enhances the maximum rate of total RNA synthesis ($V_{total}$) by Qβ replicase (Fig. 3b and Table 1). In this study, template RNA was present in excess over Qβ replicase. Therefore, this enhancement can be explained by enhanced elongation rate of RNA replication by ribosomes. During the elongation process of RNA replication, the replicase is known to pause just after synthesis of RNA regions that form hairpin structures [39]. It has also been reported that the ribosome itself has helicase activity [40]. Moreover, ribosomal protein S1 has been reported to be able to unwind double-stranded RNA [41]. Taken together, the enhancement of replication rate by the presence of ribosomes may be attributed to resolution of the hairpin structure at these pause sites by helicase activity. Another possible explanation is that the ribosome accesses the secondary structure of template RNA before replicase reaches the site and unwinds the secondary structure. This may facilitate the progress of RNA replication by Qβ replicase. However, further studies are required to validate these hypotheses.

Second, the presence of ribosomes increases the affinity of Qβ replicase for template RNA. The Qβ phage genome has two internal sites called the S-site and the M-site, and the interaction of replicase with the M-site, mediated by ribosomal protein S1, is important for template activity [42]. The template RNAs used in this study had the sequence of the M-site, and under our experiment conditions, S1 could be supplied by ribosomes in the reaction buffer. Therefore, the binding of S1 to the M-sites of template RNAs may have contributed to the increased affinity of Qβ replicase for template RNA.

Third, by insertion of Qβ genomic sequence, the rates of dsRNA synthesis during Qβ RNA replication in the presence of ribosomes ($k_{ds2}$) was reduced by about 60% compared to $k_{ds1}$. This result suggests that the presence of ribosomes suppresses duplex RNA formation in the positive-strand RNA-replicase complex. Due to the insertion of Qβ genomic sequence, the secondary structure of U1 could be different from that of MDV(−)β(+). This change in secondary structure may cause rearrangement of intermolecular hybridization and thus contribute to dsRNA formation. Taken together, these observations suggest that the ribosome may be able to resolve the intermolecular hybridization between U1 complementary RNA strands by its helicase activity and thus promote the release of ssRNA.

Our results suggested that the effects of ribosomes on the kinetics of Qβ replication can be sufficiently explained by ribosomal protein S1 (Fig. 5). S1 is also part of a subunit of Qβ replicase. Therefore, it is possible that replicase (Rep) and ribosomes (Rib) first interact with each other (Rep–Rib) and subsequently form a complex with RNA ((+)RNA-Rep–Rib), which was ignored to simplify our model (Fig. 1). The introduction of this process between replicase and ribosome does not significantly alter the fitting results or the estimated parameter values although the values of $K_2$ and $K_4$ could not be estimated.

In the present study, we constructed a kinetic model that can quantitatively explain the effects of ribosome concentration on Qβ RNA replication. However, the mechanism underlying the reduction in ratio of dsRNA to total synthesized RNA remains unclear. The secondary structure of RNA have been reported to affect dsRNA synthesis during Qβ RNA replication [43]. Insertion of Qβ genomic RNA sequence is expected to cause the secondary structure of U1 to be different from that of MDV(−)β(+). Therefore, it is necessary to determine the types of changes in RNA secondary structure that affect dsRNA synthesis by Qβ replicase. This will be helpful for the design of replicable RNAs encoding useful genes by Qβ replicase and enable reconstruction of artificial cell models using RNA as genetic information.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.11.018.

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