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Functional Circularity of Legitimate Qβ Replicase Templates

Victor I. Ugarov and Alexander B. Chetverin*

Institute of Protein Research of the Russian Academy of Sciences, Pushchino, Moscow Region, 142290 Russia

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Qβ replicase (RNA-directed RNA polymerase of bacteriophage Qβ) exponentially amplifies certain RNAs *in vitro*. Previous studies have shown that Qβ replicase can initiate and elongate on a variety of RNAs; however, only a minute fraction of them are recognized as 'legitimate' templates. Guanosine 5′-triphosphate (GTP)-dependent initiation on a legitimate template generates a stable replicative complex capable of elongation in the presence of aurintricarboxylic acid, a powerful inhibitor of RNA–protein interactions. On the contrary, initiation on an illegitimate template is GTP independent and does not result in the aurintricarboxylic-acid-resistant replicative complex. This article demonstrates that the 3′ and 5′ termini of a legitimate template cooperate during and after the initiation step. Breach of the cooperation by dividing the template into fragments or by introducing point mutations at the 5′ terminus reduces the rate and the yield of initiation, increases the GTP requirement, decreases the overall rate of template copying, and destabilizes the postinitiation replicative complex. These results revive the old idea of a functional circularity of legitimate Qβ replicase templates and complement the increasing body of evidence that functional circularity may be a common property of RNA templates directing the synthesis of either RNA or protein molecules.

Keywords: RNA replicase; replicative intermediate; closed conformation; interacting termini; processive synthesis

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Introduction

Qβ replicase, the RNA-directed RNA polymerase of bacteriophage Qβ,[1–3] manifests a unique ability to amplify RNA molecules *in vitro*, producing up to 10^{10} copies of RNA template within 10 min.[3] Similar to DNA amplification in the polymerase chain reaction (PCR),[4] the RNA amplification is exponential as long as the enzyme is in molar excess over template: the number of RNA molecules increases by a factor of 2 in each round of replication because both the original RNA and its complementary copy can serve as replicase templates.[5] Importantly, the template and its complementary copy do not form a duplex and are released single-stranded (ss) after completion of a replication round.[6] Therefore, unlike PCR, which requires that the reaction medium is periodically heated to melt the double-stranded (ds) DNA product, the Qβ replicase reaction is isothermal, and each next round of replication begins immediately after completion of the previous one. Also, unlike PCR, Qβ replicase displays strict amplification selectivity. Only the so-called replicable RNAs are amplified: the genomic RNA of Qβ phage and short-chained RQ RNAs, a special class of RNAs that are rarely generated *in vivo* or *in vitro* by virtue of sequence recombination and point mutations[3], cellular RNAs and even the genomic RNAs of related RNA phages, for example, phage MS2,[7] are not amplified.

*Corresponding author. E-mail address: alexch@vega.protres.ru.

Abbreviations used: PCR, polymerase chain reaction; RQ RNA (termed so for being *R*eplicable by *Q*β replicase), a non-genomic RNA capable of exponential amplification by Qβ replicase; RQ135 RNA, an RQ RNA whose most abundant subspecies is 135 nt long; nt, nucleotide(s); NTP, ribonucleoside 5′-triphosphate; ds, double-stranded; ss, single-stranded; ATA, aurintricarboxylic acid; DLU, digital light units; PAGE, polyacrylamide gel electrophoresis.

After more than 40 years since the discovery of Qβ replicase,[5] the mechanism of template recognition by this enzyme remains elusive. It appears to be of a very peculiar nature. In contrast to common DNA or RNA polymerases, Qβ replicase does not utilize sequence-specific primers or promoters, and replicable RNAs share no sequence similarity except for the 5′-terminal GGG and 3′-terminal CCC clusters. The gross affinity to a template cannot be a basis for the observed RNA discrimination either, because Qβ replicase demonstrates similar affinities to replicable RNAs and other, including cellular, RNAs (such as most tRNAs) and only a slightly higher affinity to ssRNAs compared with dsRNAs,[8] on which it cannot initiate at all.[9,10] Furthermore, recently, we found that Qβ replicase can copy a range of RNAs whose initiation (3′-terminal) regions comprise diverse sequences. In each case, a full-sized complementary strand was produced.[11] This means that each of these RNAs can serve as a Qβ replicase template and suggests that Qβ replicase can copy RNAs in an almost indiscriminative manner.

The apparent paradox (the strict selectivity in RNA amplification *versus* the lack of selectivity in template copying) was understood upon a detailed comparison of properties of an RQ RNA and its 5′ and 3′ fragments obtained by dividing the RNA sequence at an internal site. It was found that there are two classes of Qβ replicase templates, designated as legitimate and illegitimate, readily distinguishable by a set of criteria, the most important of which are the guanosine 5′-triphosphate (GTP) requirement and the stability of the replicative complex.[11]

Copying of legitimate templates is strictly dependent on GTP, which is absolutely required at the initiation step, although it can be replaced by inosine 5′-triphosphate (ITP) during elongation. The GTP-driven initiation on a legitimate template results in a stable replicative complex that is capable of elongation in the presence of aurintricarboxylic acid (ATA),[11] a potent inhibitor of protein–RNA interactions,[12] which completely blocks RNA synthesis if added before initiation.[13,14] The terms *replicable* and *legitimate* are not equivalent. Although all replicable RNAs are legitimate templates, the class of legitimate templates is broader. According to the above criteria, the 3′ fragment is also a legitimate template, even though it is not replicable (not capable of exponential amplification).[11]

On the other hand, the 5′ fragment and a number of its derivatives with varied 3′-terminal sequences are illegitimate templates: they are readily copied when GTP is entirely replaced with ITP, and they do not form the ATA-resistant replicative complex. ATA completely blocks the elongation of initiated strands even if initiation has occurred in the presence of a combination of ribonucleoside 5′-triphosphates (NTPs) sufficient to synthesize rather long (up to 7 nt) RNA stretches ('primers') and even if the template possesses a CCCC cluster at its 3′ end. Moreover, even in the latter case, copying of the template, including the initiation step, does not require GTP.[11]

These results indicated that the mechanisms of initiation on the legitimate and illegitimate templates are fundamentally different. In particular, the GTP-dependent initiation on a legitimate template drives the enzyme into a 'closed' conformation, which is never attained with illegitimate templates. The resistance to ATA means that, in the closed conformation, the enzyme can synthesize the full-sized complementary copy without dissociation from its template; that is, it becomes highly processive. This property may be important for keeping the complementary template and nascent strand unpaired, a prerequisite of exponential RNA amplification.

These findings can be explained by assuming that Qβ replicase recognizes templates in a two-step process. At the first step ('tasting'), Qβ replicase nonselectively binds with the 3′ end of an RNA template, whether legitimate or not and irrespective of the presence of oligo(C) cluster and, if the complex lives long enough, begins to copy it. At the second step ('swallowing'), condensation of two or more GTP molecules on a legitimate template triggers the Qβ replicase transition into the closed conformation in which the template and the nascent strand remain stably bound during the entire replication round. Initiation on an illegitimate template cannot trigger this transition, and the enzyme:RNA complex remains unstable. As a result, the template and nascent strand either prematurely dissociate (are 'spitted out') or collapse into a duplex. Any of these alternatives results in early cessation of RNA synthesis and, hence, makes the exponential amplification impossible.

Understanding of the structural distinctions between legitimate and illegitimate Qβ replicase templates would help uncover the mechanism of template recognition and, ultimately, should provide for a rational design of RNA molecules capable of extremely high rates of amplification *in vitro*. In this article, we demonstrate that, in contrast to illegitimate templates, the 5′ terminus of a legitimate template cooperates with the 3′ terminus during initiation and contributes to the stability of the replicative complex after initiation. Dissociation of the molecule into 5′-terminal and 3′-terminal fragments and even point mutations at the 5′ terminus reduce the rate of the initiation step and the overall rate of template copying, increase the GTP requirement, and destabilize the postinitiation complex. The results suggest that cooperation between the opposite 5′ and 3′ ends is an important distinguishing feature of legitimate Qβ replicase templates.

Results

The 5′-terminal portion of RQ RNA promotes copying of its 3′-terminal portion

In our experiments, we used a 139-nt-long derivative[11] of RQ135^{-1} RNA, one of the most efficient Qβ replicase templates,[15] and its 75-nt-long 5′-

terminal and 109-nt-long 3′-terminal fragments that supplement each other to the entire RNA sequence and contain foreign extensions at the truncated ends.[16] Putative secondary structures of the 139-nt-long RQ RNA (which will be further denoted as RQ135 RNA for simplicity) and its hybridized fragments are shown in Fig. 1, and those of separate fragments are given elsewhere.[11]

Of the two fragments, the 5′ fragment possesses the properties of an illegitimate template.[11] Properties of the 3′ fragment closely resemble those of a legitimate template by formal criteria (in particular, by the ability to form an ATA-resistant postinitiation complex),[11] although it is not capable of exponential amplification.[16]

The early kinetics of RNA synthesis on the 3′ fragment was found to be ≈90 times slower than that on the intact RQ135 RNA (Fig. 2; cf., black and blue lines with filled symbols). The higher rate of RNA synthesis on the unbroken RNA was not due to the exponential amplification of this template, because these data were obtained within time

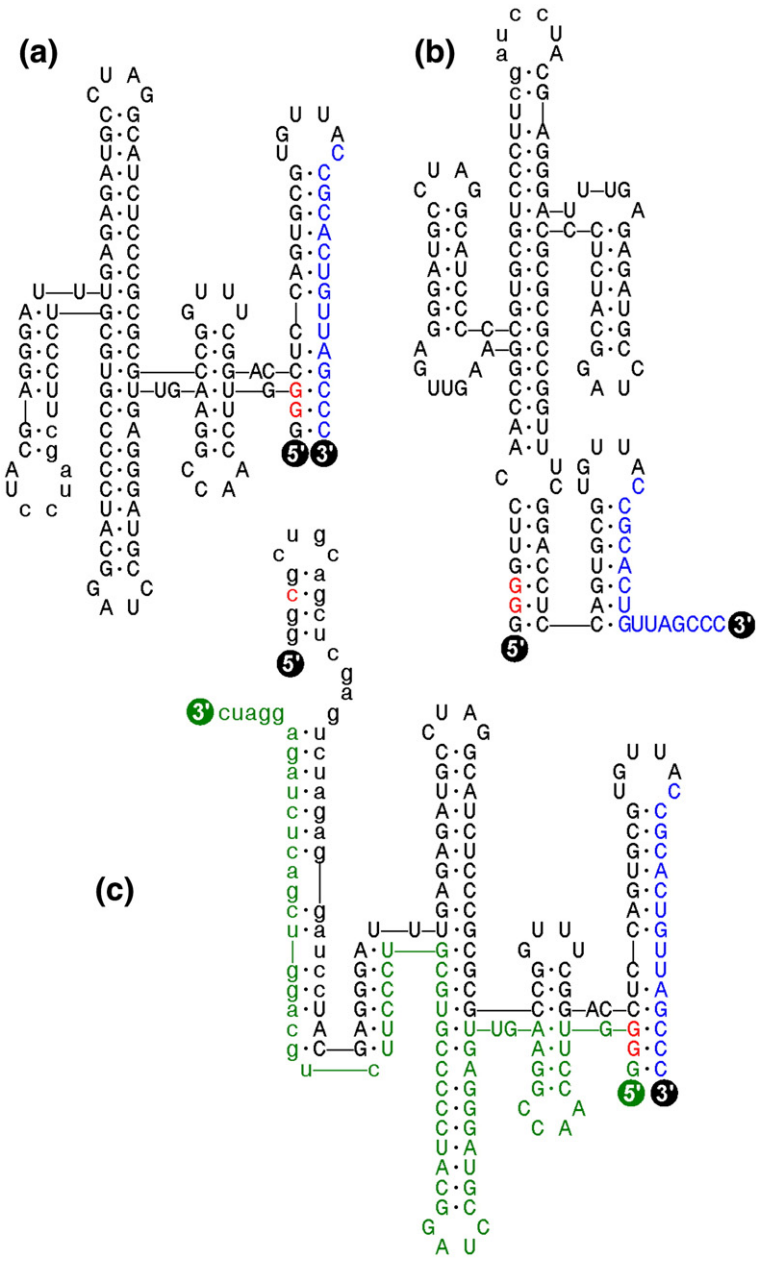


Fig. 1. Putative secondary structures of RQ135 RNA and of a hybrid molecule formed by its 3′ and 5′ fragments. (a) A model accounting for the results of ribonuclease probing.[15] (b) A model generated by program RNA mfold 3.2 [http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/rna-form1.cgi] based on a minimum energy algorithm.[17,18] (c) A model of interacting 3′ fragment (black symbols) and 5′ fragment (green symbols) patterned upon model (a). Bases not present in the wild-type RQ135 RNA are shown with lowercase letters. Blue symbols indicate the 3′-terminal segment responding to ribonucleases as a double helix.[15] Red symbols indicate the 5′-terminal bases subjected to site-directed mutagenesis.

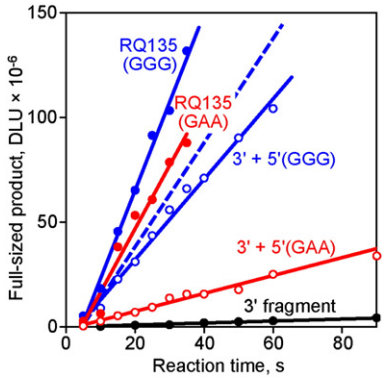


Fig. 2. Initial kinetics of RNA synthesis on the unbroken RQ135 RNA, with the wild-type (GGG, blue line with filled symbols) or mutant (GAA, red line with filled symbols) 5′ terminus, and on its 3′ fragment, alone (black line with filled symbols) or hybridized with the 5′ fragment possessing the wild-type (GGG, blue line with unfilled symbols) or mutant (GAA, red line with unfilled symbols) 5′ terminus. Each line represents the least-squares fit for linear equation ($y=mx+b$). The broken line was obtained from the blue line with unfilled symbols by multiplying it with 42/34, a ratio of the number of G residues (directing the incorporation of [^{32}P]CMP) in the RQ135 RNA and its 3′ fragment, respectively. The ordinate displays amounts of the full-sized product strands [expressed in digital light units (DLU)] in PAGE patterns shown in Supplementary Fig. S3. In this experiment, 1 pmol of incorporated [^{32}P]CMP corresponded to approximately 20×10^6 DLU.

intervals that permitted no more than a single round of replication to occur. The lower template activity of the 3′ fragment was not unexpected, because it lacks a large portion of the RQ135 RNA molecule.

Surprisingly, the rate of RNA synthesis on the 3′ fragment drastically (≈30-fold) increased upon its hybridization with the 5′ fragment (Fig. 2, blue line with unfilled symbols) and becomes similar to the rate of RNA synthesis on the intact RQ135 RNA. The similarity appears even greater on a molar, rather than on the mass, scale, that is, when the relative sizes of the 3′ fragment and RQ135 RNA (and, hence, of their respective product strands) are taken into account (Fig. 2, broken line). The observed enhancement of the 3′ fragment copying is entirely due to the formation of a 3′ fragment:5′ fragment hybrid molecule. Upon annealing under conditions used in this work, the 3′ fragment becomes entirely involved in a hybrid with the 5′ fragment; neither the free 3′ fragment nor its dimer (which is formed upon annealing of the fragment in the absence of the 5′ fragment) could be detected (see Supplementary Fig. S1). Furthermore, self-annealing of the 3′ fragment produces no effect on its copying (Supplementary Fig. S2).

A predicted secondary structure of the hybrid molecule composed of the 3′ and 5′ fragments (Fig. 1c) is similar to that of the RQ135 RNA (Fig. 1a); the only differences are that the 3′ and 5′ portions are not covalently linked and that additional sequences are present in the internal part of the hybrid molecule, which is remote from the 3′ end of the 3′ fragment (the initiation site). Hence, the 3′ fragment may become a better template upon hybridization with the 5′ fragment because, within the hybrid molecule, its initiation region acquires nearly the same conformation as it has within the native fold of RQ135 RNA. An alternative explanation might be that some structural features of the 5′ fragment itself directly contribute to the template recognition by Qβ replicase.

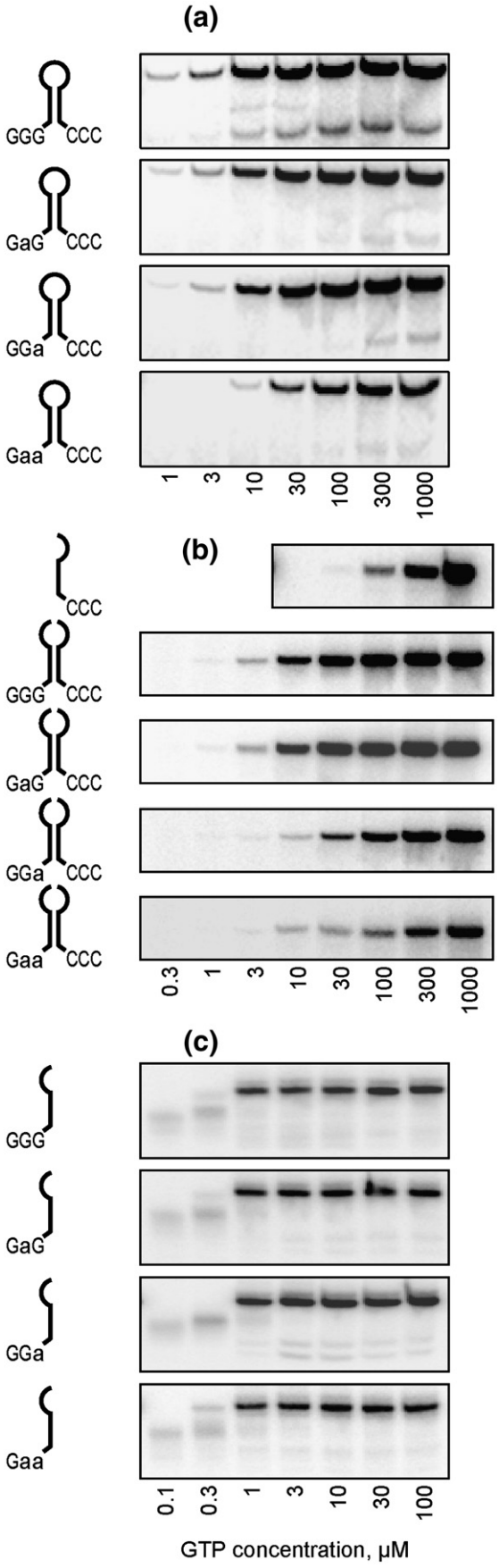
Mutations at the 5′ end decrease the rate of RQ RNA copying and diminish the effect of the 5′-terminal portion

The following experiments demonstrate that the latter alternative is preferable. Using a site-directed mutagenesis procedure, we introduced G→A point mutations at position 2 or 3 (or both) of the 5′ fragment (the reasons for making this substitutions are discussed below). Each of the mutations reduced the rate of copying the hybridized 3′ fragment (not shown), with the double mutant having the greatest effect: it decreased the rate of RNA synthesis ≈6-fold (Fig. 2; cf., blue and red lines with unfilled symbols). Thus, the two purine-for-purine substitutions eliminated most of the stimulating effect of the 5′ fragment on the 3′ fragment copying. Same mutations introduced into the native RQ135 RNA sequence also impaired its template properties, although to a lesser extent, ≈1.5-fold (Fig. 2; cf., blue and red lines with filled symbols), probably because the unbroken strand has less freedom to adopt alternative structures.

Mutations at the 5′ end increase the GTP requirement of legitimate templates

Figure 3 shows how copying of the unbroken RQ135 RNA and its fragments, both wild type and mutant, depends on the concentration of GTP, the initiator nucleotide for legitimate templates.

In these experiments, RNA synthesis on each of the templates was carried out for a fixed time period (10 min). To prevent multiple rounds of RNA synthesis on the replicable RQ135 RNA, it was copied in two steps. At the first step, it was incubated with Qβ replicase in the presence of GTP and Mg^{2+}, and this resulted in the initiation of a product strand. Then, the three missing NTPs [ATP, cytidine 5′-triphosphate (CTP), and uridine 5′-triphosphate (UTP)] were added along with ATA, and incubation was continued for an additional 10 min, which allowed a nascent strand to be elongated to the full-sized complementary copy of the template. ATA does not interfere with strand elongation but prevents reinitiation on the legitimate Qβ replicase templates[11] by blocking RNA–protein interactions.[12] Copying of the 3′ fragment (either in the presence or in the absence of the 5′ fragment) was performed similarly. In this case, the purpose of the addition of ATA was to prevent simultaneous copying of the 5′



fragment whenever it was present, in order to make the complementary copy of the 3′ fragment be the only synthesis product (copying of the fragment mixture in the absence of ATA is shown in Supplementary Fig. S4). The 5′ fragment, an illegitimate template that is incapable of forming an ATA-resistant replicative complex,[11] was copied in one step, in the presence of all four NTPs and in the absence of ATA.

It is believed that GTP requirement is a measure of quality of a Qβ replicase template: the more efficient the template is, the lower GTP concentration is needed for its copying.[19] This indeed holds true for the wild-type RQ135 RNA and its 3′ fragment: a 30-fold lower GTP concentration is required to copy the former compared with the latter (cf., top gels in Fig. 3a and b). The wild-type 5′ fragment, an illegitimate template, does not obey this rule. Its GTP requirement is the lowest and is fully satisfied at 1 μM (top gel in Fig. 3c), in agreement with earlier observations.[11]

Hybridization with the wild-type 5′ fragment reduced the GTP requirement of the 3′ fragment about 10-fold, whereas the A-for-G substitutions at the 5′ end of the 5′ fragment had reverse effects (Fig. 3b), in accord with their effects on the rate of the 3′ fragment copying (Fig. 2). Interestingly, mutation at position 3 tended to have a greater effect than that at position 2. Same mutations similarly affected the GTP requirement of the unbroken RQ135 RNA (Fig. 3a). It looks as if mutations at the 5′ terminus decrease the affinity of the active site of the enzyme for GTP.

In contrast to their effects on RQ135 RNA and its 3′ fragment, the 5′-terminal mutations did not affect the GTP requirement of the 5′ fragment itself (Fig. 3c).

Mutations at the 5′ end decrease the rate and yield of initiation

Insomuch as GTP is the initiator nucleotide for legitimate Qβ replicase templates, the GTP requirement of a template is thought to reflect requirements of the initiation step.[19] The results shown in Fig. 4 support this view. In these experiments, time course of the initiation of new strands on the wild-type

Fig. 3. Effects of 5′-terminal point mutations on GTP dependence of RNA synthesis on (a) RQ135 RNA; (b) its 3′ fragment, alone or hybridized with the 5′ fragment; and (c) the 5′ fragment. The schemes on the left show location of the mutations (indicated with lowercase letters). Synthesis in (a) and (b) was performed in two steps: a 10-min incubation in the reaction mixture containing GTP (initiation) followed by the addition of a mixture of the missing rNTPs and ATA (to 1 mM final concentration) and further incubation for 10 min (elongation). Indicated are the final GTP concentrations at the elongation step; during initiation, the concentration of all reaction components including GTP was 25% higher. Synthesis in (c) was performed in one step (see Materials and Methods), with the GTP concentration being varied as indicated.

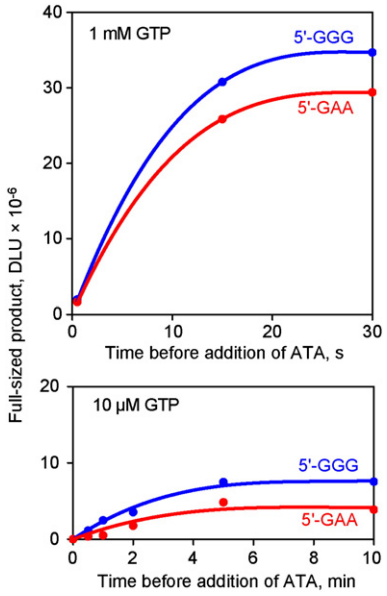


Fig. 4. Time course of the initiation of RNA synthesis on RQ135 RNA with the wild-type (GGG, blue lines) or mutant (GAA, red lines) 5′ terminus at two different GTP concentrations. Synthesis was performed in two steps (see legend to Fig. 3), with the initiation time being varied and the GTP concentration fixed as indicated.

RQ135 RNA and its 5′-terminal point mutants was monitored as follows. After incubation of a template with Qβ replicase in the presence of GTP and Mg^{2+} for the indicated time intervals, the initiation was stopped by the addition of a mixture of ATA and the three missing rNTPs. The initiated strands were allowed to be elongated for 10 min, the reaction products were resolved by electrophoresis through a polyacrylamide gel, and the amount of the full-sized product strand was measured.

It is seen that decreasing the GTP concentration reduced the rate of initiation, as well as the maximal yield of the replicative complex capable of elongation. Moreover, A-for-G substitutions at the 5′ end of the RNA template also reduced the rate and yield of initiation, and this effect was more pronounced at a lower GTP concentration (Fig. 4).

Mutations at the 5′ end decrease stability of the postinitiation replicative complex

As was found previously, initiation on legitimate templates produces a stable replicative complex capable of elongating nascent strands in the presence of ATA.[11] In the next series of experiments, we checked if the 5′-terminal mutations affected stability of the postinitiation replicative complex formed on RQ135 RNA and its 3′ fragment hybridized to the 5′ fragment. To this end, the replication complex was formed by 10-min incubation of Qβ replicase and RNA in the presence of GTP and Mg^{2+}, then ATA was added to prevent further initiation, and decay of the complex was stopped by the addition, at the indicated time points, of the three missing rNTPs. The residual amount of the complex was measured by determining the amount of the full-sized product strands produced during the subsequent incubation of the samples for 10 min.

Figure 5a shows that the half-life of a complex involving the wild-type (5′-GGG) RQ135 RNA was ≈3 min at room temperature (22 °C), which is much longer than the time needed for a one-round copying of this RNA at the same temperature (≈30 s, data not shown). Point mutations at the 5′ end reduced the half-life, down to ≈1 min for a complex involving the double mutant (5′-GAA). Plotting the data in a semilogarithmic scale did not produce straight lines as would be expected of a simple exponential decay. However, we used this kind of presentation as it made the alterations in the decay kinetics induced by mutations easier to observe (Fig. 5b).

It is seen that the 5′-terminal mutations did not appreciably affect the yield of the replicative complex at the high GTP concentration used for initiation (1 mM). However, each of the point mutations significantly increased the rate of the complex decay as revealed by a greater slope of the respective curve at each time point. Mutations at positions 2 and 3 had cumulative effects and, as with the GTP dependence, the A-for-G substitution at position 3 (mutant 5′-GGA) greater destabilized the complex than did the same substitution at position 2 (mutant 5′-GAG, Fig. 5b). In contrast to the unbroken RQ135 RNA, the same mutations did not affect the stability of the complex formed by its hybridized 3′ and 5′ fragments (Fig. 5d, colored lines).

At present, we cannot explain why the decay does not follow the simple exponential kinetics. This is not because of an incomplete inhibition of reinitiation by ATA, in which case the net decay would slow down as the rate of decomposition of the replicative complex approaches the rate of its re-formation. Figure 4 demonstrates that, at the concentration used (1 mM), ATA completely, irreversibly, and almost instantly inhibited the initiation of RNA synthesis. That is why we were able to stop initiation at chosen time points and to measure the initiation rate. There can be a number of other possibilities. For example, the initiation complexes could have different stabilities if they involve one, two, or more replicase molecules. Alternatively, the heterogeneity of complexes could be induced by ATA itself (e.g., due to a variable ATA-to-replicase stoichiometry). Regardless of the source of the observed nonlinearity of the decay kinetics, the plots of Fig. 5 reflect real differences in the stability of postinitiation complexes formed with various RNA mutants.

Mutation at the 5′ end can improve a bad template

In contrast to typical Qβ replicase templates that begin with 5′-GGG, the 3′ fragment studied here begins with 5′-GGC. We therefore wondered if template characteristics of the 3′ fragment could be improved by introducing a G-for-C substitution at

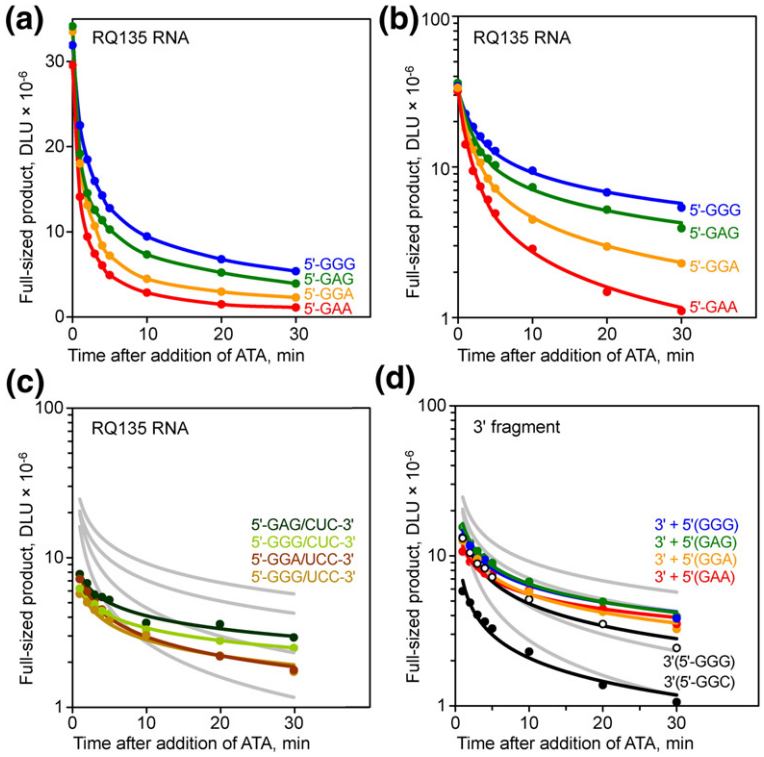


Fig. 5. Time course of decay in the presence of ATA of replicative complexes formed on the unbroken RQ135 RNA (a–c) or on its 3′ fragment (d). Complexes were generated by a 10-min initiation in the presence of GTP. Decay was initiated by the addition of ATA. After the indicated time intervals, a mixture of the missing rNTPs was added, and incubation was continued for 10 min (elongation). The final concentration of each rNTP and ATA was 1 mM. The amounts of full-sized RNAs produced by the residual elongation-competent postinitiation complexes are plotted against time on a linear (a) or exponential (b–d) scale. (a and b) The 5′ terminus of RQ135 RNA was of the wild type (GGG) or carried the indicated mutations. (c) The 3′ terminus of RQ135 RNA carried the indicated mutations, with the 5′ terminus either being of wild type or carrying complementary mutations, as indicated in legends of matching colors. (d) Decay of the complexes formed by the 3′ fragment alone (black lines), whose 5′ terminus was wild type (GGC, filled symbols) or mutant (GGG, unfilled symbols), or by the 3′ fragment hybridized with the 5′ fragment (colored lines), which was of the wild type or carried 5′-terminal mutations, as indicated in legends of matching colors. Gray lines in (c) and (d) are replicas of the lines from (b). Each line represents the least-squares fit through points by using the power equation ($y = ce^{bx}$).

position 3. Figure 5d (black lines) shows that such a single point mutation increased the yield of initiation on the lone 3′ fragment (cf., initial amounts of the complexes) to approximately the same level as did hybridization of the 3′ fragment with the 5′ fragment (colored lines). At the same time, this mutation did not change the rate of the complex decay in the presence of ATA.

Diverse effects of mutations at the 3′ and 5′ ends of legitimate template

As far as the complementary 5′ and 3′ termini of RQ135 RNA can potentially base pair with each other (see, e.g., Fig. 1a), we explored a possibility that the detrimental effects of the 5′-terminal mutations could be compensated for by complementary mutations at the 3′ terminus. To this end, we introduced a C→U mutation at position − 2 or − 3 from the 3′ end, or both, into mutant RQ135 RNAs having the 5′-GAG, 5′-GGA, or 5′-GAA sequence, respectively.

Figure 6 shows that these mutations had no compensatory effect on template activities of the 5′-terminal mutants measured in a single-round assay, in which initiation in the presence of GTP and Mg^{2+} was followed by elongation in the presence of ATA.

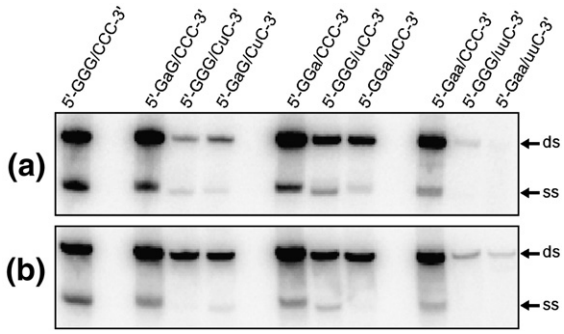


Fig. 6. Products of RNA synthesis on RQ135 RNA, whose 5′ and 3′ trinucleotides (separated by a slash) were of the wild type or contained mutations indicated with lowercase letters. Synthesis was performed in two steps (see legend to Fig. 3). Initiation was performed in the presence of GTP (a) or GTP and ATP (b), whose final concentration was 1 mM each. Arrows indicate the bands corresponding to a ds and ss product.

Moreover, the 5′- and 3′-terminal mutations aggravate each other, and the template carrying two mutations at each of the termini displayed almost no template activity. In contrast to the 5′ terminus, wherein position 3 was more mutation sensitive than position 2, the U-for-C substitution at position − 3 had a weaker effect than at position − 2 (Fig. 6a). However, the two 3′-terminal positions became equally sensitive to mutations if ATP was present in the initiation mixture along with GTP (Fig. 6b). Thus, unlike the 5′-terminal mutations whose effects seem to be not related to copying the 5′-terminal sequence (which occurs late in elongation), the greater effect of the U-for-C substitution at position − 2 than at position − 3 merely reflects the inability of Qβ replicase to create, in a template-directed fashion, the first internucleotide bond when initiation is carried out in the presence of GTP only.

A further distinction between mutations at the opposite ends of RQ135 RNA is that they differently affect formation of the replicative complex and its stability. The 5′-terminal mutations have moderate effects on the complex formation, which are most apparent at low GTP concentrations (Figs. 3a and 4), but these mutations significantly destabilize the complex once it has been formed (Fig. 5b). On the contrary, the 3′-terminal mutations produce more severe detrimental effects on the complex formation than do the 5′-terminal mutations (Fig. 6), but they do not significantly affect the complex stability (Fig. 5c). Moreover, they somewhat compensate for the destabilizing effects of the 5′-terminal mutations: although the initial amounts of the replicative complexes formed by the double 5′-GAG/CUC-3′ and 5′-GGA/UCC-3′ mutants are much smaller than those formed by templates carrying only the 5′-terminal mutations, the former complexes decay at a lower rate than the latter (cf., Fig. 5c and b).

Discussion

Cooperation between the 5′ and 3′ termini of Qβ replicase templates

This article provides evidence that the 5′ and 3′ termini of a legitimate Qβ replicase template cooperate with each other at, and subsequent to, the initiation step. This cooperation does not require that the two termini belong to the same RNA strand. It can be observed even if the template is fragmented, provided that the fragments are held together by noncovalent interactions. However, the mode of cooperation depends on whether the template is intact or not. Whereas sensitivity of the unbroken RQ135 RNA to the 5′-terminal point mutations increases after initiation, which is reflected by a more rapid decay of the replicative complex, a hybrid molecule composed of the supplementing 5′ and 3′ fragments of RQ135 RNA becomes insensitive to the 5′-terminal mutations after initiation. The loss of sensitivity may be due to dissociation of the 5′ fragment, or at least displacement of its 5′-terminal segment, from the replicative complex after initiation. Indeed, despite the very different initial amounts of replicative complexes formed by the 3′ fragment when it is present alone or is hybridized to the 5′ fragment, whether wild type or mutant, the rates of decay of the complexes in the presence ATA are virtually identical (Fig. 5d).

Forty years ago, Spiegelman *et al.* observed a drastic drop in the activity of another legitimate Qβ replicase template, the genomic RNA of phage Qβ, upon its fragmentation into two halves. From these observations, they reasoned that the recognition mechanism involves more than the 'beginning' (3′-terminal) sequence and that Qβ replicase may sense whether a template is intact or not by examining both RNA ends for the proper sequences. They proposed the term *functional circularity* to designate the putative ability of a template to present to replicase its 5′ end, in addition to the 3′ end, at the initiation step.[20,21] This conclusion was later disputed by Schwyzer *et al.* who showed that fragments substantially less than the intact Qβ RNA served as templates for the synthesis of complementary copies.[22] Later, it was shown that copying of the positive Qβ RNA strand, whose length is 4217 nt, requires long-range RNA–RNA interactions between the 3′ end and an internal segment lying up to 1500 nt upstream,[23,24] to which replicase binds with a high affinity.[25,26] Therefore, simultaneous interaction of Qβ replicase with the two sites was thought to be the mechanism for template recognition. Hence, fragmented Qβ RNA might lack template activity in the experiments of Spiegelman's laboratory because the two recognition elements occurred in different fragments.

However, such a recognition mechanism could not be applied to every Qβ replicase template. Thus, no specific replicase binding to an internal site was observed for the negative Qβ RNA strand.[27,28] Moreover, in a number of aspects, recognition of the positive strand of Qβ RNA is exceptional. Among a variety of known Qβ replicase templates, recognition of only this RNA requires the participation of ribosomal protein S1 and protein Hfq, each of which is involved in the communication between the 3′ end and the internal recognition site.[25–27] The purpose of these interactions seems to expose the 3′ end, which, unlike the 3′ end of other templates, is otherwise hidden in the structure of the positive Qβ RNA strand.[29] Such a complex recognition pattern may serve as an intertypic segregation mechanism ensuring that Qβ replicase will not amplify the genomes of other RNA coliphages.[1–3]

The existence of the above mechanism does not exclude the possibility that once exposed, the 3′ end of the positive Qβ RNA strand becomes available for interaction, either direct or through replicase, with the 5′ end. The ability of Qβ replicase to interact with both the 3′- and 5′-terminal structures was documented for the negative Qβ RNA strand whose recognition is not complicated by the participation of proteins S1 and Hfq.[27,28] Moreover, deletion of a hairpin near the 5′ end resulted in suppression of the

initiation of RNA synthesis at the 3′ end of this template.[28] Finally, whenever tested, 5′-terminal structures of Qβ replicase templates manifested higher affinities for the enzyme than did the 3′ terminus,[11,27,30] consistent with a hypothesis that Qβ replicase initiates at the 3′ end while bound to the 5′ end. It is therefore possible that cooperation between the 3′ and 5′ termini is a common property of legitimate Qβ replicase templates.

In the present work, functional circularity, as defined by Spiegelman *et al.*, was demonstrated for three structurally distinct RNAs: (1) intact RQ135 RNA, (2) RQ135 RNA that has been cleaved into two fragments (the hybrid between the 3′ and 5′ fragments), and (3) 3′ fragment. These templates manifested functional circularity in a number of tests, such as the early kinetics of RNA synthesis, the GTP dependence, the rate of initiation, and the stability of the postinitiation complex. Although two of the three templates are not replicable, one has a broken sugar-phosphate backbone and one lacks a half of the RQ sequence; each of these RNAs is a legitimate template according to earlier established criteria.[11] We therefore conclude that functional circularity is a common feature of legitimate Qβ replicase templates, whether replicable or not.

Secondary structure of Qβ replicase templates

Spiegelman *et al.* proposed an 'amphora model' to describe the structure of Qβ replicase templates. They reasoned that a template molecule could form a circle if it possessed complementary terminal sequences capable of base-pairing, and the replicase could then recognize the resulting 'region of double strandedness,'[20,21] a structure now commonly termed *panhandle*. This idea seemingly gained support from subsequent findings that both the positive and negative strands of Qβ RNA, as well as of other replicable Qβ replicase templates, are flanked by oligo(C) and oligo(G) sequences at the 3′ and 5′ ends, respectively.[1–3] However, the length of the putative panhandle (3 to 4 base pairs) would be too small to secure the circle termini.

Weissmann *et al.* gave another explanation for the complementarity of terminal sequences. They argued that, as far as the 3′-terminal oligo(C) is required for initiation, then the 5′ terminus of the positive strand must always be oligo(G) in order that oligo(C) can be synthesized at the 3′ end of the complementary negative strand.[31] Furthermore, a minimum energy algorithm[17,18] predicts that, with a few exceptions, the termini are not base-paired in the secondary structure of RQ RNA. Instead, the 3′-terminal oligo(C) remains ss, whereas the 5′-terminal oligo(G) is base-paired elsewhere,[32,33] as shown in Fig. 1b. The single-strandedness of the 3′ terminus was proposed to be a general prerequisite for replication.[30,32–34]

However, the algorithm used for the prediction of RNA secondary structures[18] does not account for non-Watson–Crick interactions and for the presence of Mg^{2+} ions, which are absolutely required for RNA replication, and assumes that the monovalent cation salt concentration is 1 M, at which RQ RNAs cannot replicate.[35,36] Therefore, the predicted structures may be functionally irrelevant. Indeed, probing of RQ RNAs with ribonucleases that are sensitive to the conformation of the sugar-phosphate backbone strongly suggests that, whenever tested, the 3′-terminal oligo(C) is in a helical structure.[15,37] Moreover, probing of the RQ135 RNA studied here indicated that the entire 15-nt-long 3′-terminal segment (depicted with blue symbols in Fig. 1) is resistant to the single-strand-specific nuclease from mung beans but is attacked by ribonuclease V_1 from cobra venom, which is specific to the A form of RNA helix.[38] These observations could be accounted for by a model for the secondary structure of RQ135 RNA[15] reproduced in Fig. 1a. A distinguishing feature of this model is an imperfect double helix jointly produced by the base-paired termini and an adjacent hairpin, reminiscent of the helix formed by the TΨC hairpin and the aminoacyl acceptor stem of tRNA.[39] Such a terminal helix could potentially be formed by both the positive and negative strands of every efficient Qβ replicase template, including the genomic RNA of phage Qβ, and might perform two related functions: (i) to serve as an analog of 'panhandle,' which might circularize the template even in the absence of Qβ replicase, and (ii) to serve as a specific structure recognizable by replicase.[3]

The terminal helix model perfectly accounts for the present findings that initiation of RNA synthesis is worsened by mutations G→A at positions 2 and 3 of RQ135 RNA and is improved by mutation C→G at position 3 of its 3′ fragment. It was therefore interesting to check whether mutations C→U at positions − 2 and − 3 (from the 3′ end) would compensate for the G→A mutations at positions 2 and 3, respectively, by restoring base-pairing of the RQ135 RNA termini. The negative answer to this question (Fig. 6) does not disprove the model; it only suggests that Qβ replicase might recognize more than just the helical backbone. For example, Qβ replicase might explore the CCC/GGG helix from its minor and/or major groove, as restriction endonucleases do when they bind to their specific dsDNA sites.[40,41] Direct crystallographic studies of a Qβ replicase:RQ RNA complex would give a definite answer, but no crystals of Qβ replicase were obtained as yet.

Single-strandedness of the replicative intermediate

Whether the terminal helix model is correct or not, the present data suggest that simultaneous interaction of Qβ replicase with both the 3′ and 5′ termini, that is, functional circularity of the template, is a prerequisite for legitimate initiation. Each of the secondary structure models discussed above and shown in Fig. 1a and b is compatible with this feature of a legitimate template, as in each case the 3′ and 5′ termini are not far from each other and,

hence, might simultaneously be bound at the replicase active site. Initiation results in a transition of the enzyme into the closed conformation, which distinguishes the relatively narrow class of legitimate templates from a variety of illegitimate templates.[11] The present data further indicate that the termini may remain to be bound by the replicase after such a transition, as long as the 5′-terminal mutations affect the stability of postinitiation replicative complexes. This finding suggests that the template circularity might perform one more function — to help keep the template and the nascent strand ss, which is vital for the exponential amplification.

As yet, the ability of the replicative intermediate to remain ss has not received satisfactory explanation. Weissmann *et al*. pointed out that the exponential RNA synthesis can only occur if two requirements that seem to be mutually exclusive are observed.[6] The synthesis of a complementary strand requires that it is base-paired with its template, but the two strands are to be nonpaired to provide for further replication, because a dsRNA cannot serve as a Qβ replicase template. To solve this paradox, they suggested that shortly after the incorporation of a nucleotide into the nascent strand, the hydrogen bonds binding it with the template are disrupted by the replicase as by a zipper and, once separated, the single strands are stabilized by intramolecular hydrogen bonds.

A correlation between strong secondary structure and template efficacy was indeed observed.[42–44] However, the intramolecular secondary structure cannot, by itself, prevent the complementary strands from collapsing into a duplex, given the far higher stability of the perfect double helix and the very high local concentration of complementary strands at the replicase active site. Indeed, any agent denaturing Qβ replicase, such as phenol, sodium dodecyl sulfate, and pronase, results in annealing of the strands, suggesting that that important role belongs to the protein.[6]

Weissmann *et al*.[6] and then Robertson[45] noted that if Qβ replicase could permanently bind the 3′ end of the template and the 5′ end of the synthesized strand, the strands would produce a 'butterfly structure' composed of two loops incapable of intertwining as needed to form a long double helix. However, similar topological constraints would exist if, instead of being bound with replicase at its 3′ end, the template was circularized through termini secured by either a terminal helix or another replicase molecule. Unlike the butterfly structure capable of accommodating only one enzyme molecule, such a circle would allow a number of replicases to copy the same template, as previously reported.[46,47] Of course, only unbroken circles could maintain the replicative intermediate ss. We would like to note that template circularity might also help in solving a similar problem of prevention of the hydrogen bonding of the template and product strands during RNA replication in the hypothetical RNA world.[48]

Functional circularity of RNA templates

Although inspired by studies on Qβ phage replicase,[20] the idea of functional circularity of linear RNA templates gained initial support from observations made on other viruses. First, circular RNA molecules were observed by the electron microscopy of an alphavirus possessing a nonsegmented, positive-strand RNA genome[49] and two negative-strand RNA viruses, a bunyavirus possessing a tripartite genome,[50,51] and defective interfering RNAs of a paramyxovirus possessing a nonsegmented genome.[52] It was also confirmed that circular structures are maintained due to noncovalent RNA–RNA interactions.[49,51,52] Later, the 5′ and 3′ termini of each of the bunyavirus RNA segments were found to be mutually complementary and form up to 30 base-pair-long ds panhandles.[53] Similar RNA circles held by terminal panhandles, although less stable than in bunyaviruses, were demonstrated for the influenza virus, an orthomyxovirus also possessing a multipartite negative-strand RNA genome.[54] However, for most RNA viruses, the circularity of their genomes could not be established by the electron microscopy. Moreover, even when circular RNAs were shown to exist, it remained unclear whether the circularity relates to template functions of a viral genome.

Recently, there is accumulating evidence that a cooperation between the 5′ and 3′ termini is required for replication and/or transcription of the genomic RNA in a variety of viruses, whose genome is composed of positive-strand, nonsegmented RNA (picornaviruses,[55–57] flaviviruses,[58,59] alphaviruses,[60] coronaviruses,[61] yeast narnavirus[62]); positive-strand, segmented RNA (brome mosaic virus[63]); negative-strand, segmented RNA (orthomyxoviruses,[64,65] bunyaviruses[66]); and even dsRNA (rotaviruses[67]). This cooperation can be achieved either by direct RNA–RNA interactions (bunyaviruses,[53] orthomyxoviruses,[54] flaviviruses,[58,68,69] rotaviruses[70], some picornaviruses[57]) or through a protein bridge that connects the termini (other picornaviruses,[55,56] alphaviruses,[60] coronaviruses[71]). Furthermore, the 5′-to-3′-terminal cooperation proved important for the translation of some viral RNAs[72,73] and probably contributes to the translation of most eukaryotic mRNAs, which may form a circle[74–78] due to bridging the 5′-terminal cap and the 3′-terminal poly(A) tail by the initiation factors eIF4G and eIF4E, and a poly(A)-binding protein.[79–81] This article provides further ground for the concept that functional circularity is a common property of RNA templates.

Materials and Methods

Qβ replicase and its templates

A highly purified Qβ replicase[82] was isolated from *Escherichia coli* HB101 cells transfected with plasmid

pREP, carrying the catalytic (β) replicase subunit downstream of the temperature-inducible P_R promoter of phage λ,[83] using a procedure based on the published protocol.[84] In this study, we used the previously described modified RQ135 RNA,[11] the BamHI variant[11] of its 5′ fragment[16] and the 3′ fragment,[16] which were prepared by runoff transcription of appropriate plasmids with T7 RNA polymerase and purified using polyacrylamide gel electrophoresis (PAGE) as described.[16]

Mutant RNAs were prepared by runoff transcription of plasmids constructed as follows. Point mutations were introduced by carrying out PCR using as templates the plasmids carrying cDNAs for the modified RQ135 RNA,[11] its 5′ or 3′ fragment,[16] and the primer pairs listed in Table 1. Then, the same reverse primer and forward primer 5′-CTGCAGGCATGCAAGCTTAATACGACT-3′ were used to extend the first PCR product upstream from site HindIII (underlined) in a second PCR, except for RQ135 sequences mutated at the 3′ end only that did not need to be extended (cf., Table 1). The resulting products were digested at sites HindIII and either SmaI or PstI (underlined in the sequences of reverse primers, see Table 1) and ligated into pUC18 between the same sites. Before transcription, plasmids were digested at site SmaI (RQ135 sequences mutated at the 5′ end only and the 3′ fragment), PstI (other RQ135 mutants), or BamHI (5′ fragment sequences).

RNA synthesis

Unless otherwise indicated, reactions were carried out at 22 °C in 10-μl aliquots containing replicase buffer [10 mM Tris–HCl, pH 8.0, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid (EDTA)], 0.5 μM Qβ replicase (M_r=199,958), 0.05 μM RNA template, and 1 mM each of ATP, GTP, UTP, and [α-^{32}P]CTP (50–200 mCi/mmol, Amersham Biosciences) and stopped by the addition of 5 μl of 50 mM EDTA. Individual templates were premelted by heating in 0.2-ml PCR tubes (at a 10× concentration in 0.1 mM Tris–EDTA, pH 8.0) for 2 min in boiling water bath followed by a quick transfer to ice-cold water. The 3′ fragment:5′ fragment hybrid was obtained by preparing in a 0.2-ml PCR tube a mixture containing 0.1 μM 3′ fragment and 0.2 μM 5′ fragment in a 2× Mg^{2+}-less replicase buffer, incubating the tube for 2 min in a beaker containing 50 ml of boiling water, and then placing the beaker on a bench until the water cooled down to 40 °C.

Product analysis

Samples were consecutively extracted with 10 μl of phenol and 10 μl of chloroform, mixed with 4.5 μl of sample buffer containing 50% glycerol, and subjected to nondenaturing PAGE[85] through an 8% polyacrylamide gel containing 10% glycerol, with temperature of the electrode buffer maintained at 10–12 °C. The labeled products were detected by scanning with a Cyclone™ storage phosphor system and quantified by measuring the intensity of RNA bands on 16-bit TIFF images (like those shown in Supplementary Fig. S3) using the OptiQuant™ Image Analysis Software (Packard Instrument Company, now part of PerkinElmer, Inc.). The product amount was expressed in DLU (as defined in the OptiQuant™ Image Analysis Software Operation Man-

Table 1. Primers used to introduce point mutations by PCR

Mutant	Forward primer	Reverse primer
RQ135 RNA		
5′-GaG/CCC-3′	5′-AAGCTTAATACGACTCACTATAGaGTTCCAACCGGAAG-3′	5′-CCGCGGATATCGATCCCGGGCTAACAGTG-3′
5′-GGa/CCC-3′	5′-AAGCTTAATACGACTCACTATAGGaGTTCCAACCGGAAG-3′	5′-CCGCGGATATCGATCCCGGGCTAACAGTG-3′
5′-Gaa/CCC-3′	5′-AAGCTTAATACGACTCACTATAGaaGTTCCAACCGGAAG-3′	5′-CCGCGGATATCGATCCCGGGCTAACAGTG-3′
5′-GGG/CuC-3′	5′-CTGCAGGCATGCAAGCTTAATACGACT-3	5′-AACAGCTGCAGGaCTAACAGTGCGGTAACACGC-3′
5′-GGG/uCC-3′	5′-CTGCAGGCATGCAAGCTTAATACGACT-3	5′-AACAGCTGCAGGaGCTAACAGTGCGGTAACACGC-3′
5′-GGG/uuC-3′	5′-CTGCAGGCATGCAAGCTTAATACGACT-3	5′-AACAGCTGCAGGaaCTAACAGTGCGGTAACACGC-3′
5′-GaG/CuC-3′	5′-AAGCTTAATACGACTCACTATAGaGGTTCCAACCGGAAG-3′	5′-AACAGCTGCAGGaCTAACAGTGCGGTAACACGC-3′
5′-GGa/uCC-3′	5′-AAGCTTAATACGACTCACTATAGGaGTTCCAACCGGAAG-3′	5′-AACAGCTGCAGGaGCTAACAGTGCGGTAACACGC-3′
5′-Gaa/uuC-3′	5′-AAGCTTAATACGACTCACTATAGaaGTTCCAACCGGAAG-3′	5′-AACAGCTGCAGGaaCTAACAGTGCGGTAACACGC-3′
5′ fragment		
5′-GaG	5′-AAGCTTAATACGACTCACTATAGaGGTTCCAACCGGAAG-3′	5′-AGACTCGAGCTGCAGAAGGGACGCACG-3′
5′-GGa	5′-AAGCTTAATACGACTCACTATAGGaGTTCCAACCGGAAG-3′	5′-AGACTCGAGCTGCAGAAGGGACGCACG-3′
5′-Gaa	5′-AAGCTTAATACGACTCACTATAGaaGTTCCAACCGGAAG-3′	5′-AGACTCGAGCTGCAGAAGGGACGCACG-3′
3′ fragment		
5′-GGg	5′-AAGCTTAATACGACTCACTATAGGgGCTGCAGCTCGAGTCTAG-3	5′-CCGCGGATATCGATCCCGGGCTAACAGTG-3′

Point mutations are indicated in lowercase letters. Restriction sites at which a PCR product was digested before ligation with a plasmid vector are underlined.

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2008.03.074

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