Qβ Replicase Discriminates between Legitimate and Illegitimate Templates by Having Different Mechanisms of Initiation*

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The abbreviations used are: nt, nucleotide(s); Qβ RNA (termed so for being Replicable by Qβ replicase), a non-genomic RNA capable of exponential amplification by Qβ replicase; ds, double-stranded; ss, single-stranded; ATA, a urintricarboxylic acid; ITP, inosine 5′-triphosphate.

Qβ replicase, the RNA-directed RNA polymerase of bacteriophage Qβ, amplifies the 4217-nt1-long genomic Qβ RNA and a number of Qβ RNAs, which are usually ≥250 nt in length. The natural source of Qβ RNAs is Qβ phage itself or Qβ phage-infected Escherichia coli cells where these RNAs are formed by recombination from viral and/or cellular RNAs and propagated (1–3). Recently, many new Qβ RNAs have been selected from random (4) or artificially designed (5) sequences, or produced by in vitro RNA recombination (6, 7). The amplification of these RNAs is exponential as long as the enzyme is in molar excess:

The number of RNA molecules doubles in each round of replication, because both the original RNA and its complementary copy are replicase templates. Approximately 10⁵ copies of a single genomic RNA molecule are produced in a Qβ phage-infected E. coli cell in less than 1 h (8). Amplification of small Qβ RNAs is much faster: up to 10ⁱ⁰ copies are produced at room temperature within 10 min in a cell-free system comprised of purified Qβ replicase and all four NTPs (3), and this is the absolute record of the rate of nucleic acid amplification. The high amplification rate allows single Qβ RNA molecules to rapidly produce detectable molecular colonies if they are amplified in a gel (9, 10).

However, Qβ replicase does not amplify most RNAs, including any tested cellular RNAs or genomic RNAs of other viruses. Selection experiments indicated that only a few of the initial diversity of 10¹² unique sequences of 50–77 nt in length are replicable (4), demonstrating a very high degree of template specificity of the enzyme. Now, almost 40 years since its discovery (11), this specificity remains a mystery. Qβ replicase discriminates between templates in the absence of sequence-specific primers or promoters (3), and hence its strategy must be different from those commonly employed by DNA polymerases and DNA-dependent RNA polymerases.

Previous studies identified several structural features of Qβ replicase templates as follows: (i) Oligo(C) cluster, usually CCC, at the 3′ end and the complementary oligo(G) at the 5′ end. No other common sequence elements were detected. The oligo(G) was found to serve as the initiation signal (1, 12–14) with GTP being the initiating nucleotide (1, 13, 15, 16). The role of the 5′-terminal oligo(G) is believed to code for the oligo(C) end and the complementary oligo(G) at the 5′ end of the complementary strand (17). Two C residues are enough (18), but initiation is more efficient with CCC or longer clusters (12). The 3′-most nucleotide of the product strand is A, which is not coded by the template and is added during strand termination. The 3′-terminal A is not copied and is dispensable for replication (19), but increases the efficacy of initiation and serves as a punctuation mark making the penultimate C the preferred initiation site (18). At elevated concentrations of GTP (20) or in the presence of Mn²⁺ ions (16), Qβ replicase can copy heterologous RNAs, including those lacking the 3′-terminal oligo(C). However, even in this case the first nucleotide of the product strand is pppG (1, 16), in agreement with the fact that Qβ replicase can initiate on internal oligo(C) clusters that are not hidden in the secondary structure (18, 21).

(ii) Unpaired 3′-terminal oligo(C), with the 5′-terminal oligo(G) being base-paired elsewhere (5, 22). This requirement can be understood as a means to ensure unrestricted access of Qβ replicase to the initiation site. (iii) Internal 8- to 15-nt-long pyrimidine-rich segments (4, 23). The presence in an RNA of such segments, selectively bound by the protein synthesis factor EF-Tu, one of the four Qβ replicase subunits (24), results in a 10-fold increase of the RNA affinity to the replicase (4, 25).
(iv) Folding of most of the RNA chain into long stable hairpins. The insertion of unstructured segments decreases the template activity of a replicable RNA (26). The stable secondary structure is believed to play an important role in maintaining the single-strandedness of the replicative intermediate, which is vital for the exponential amplification (27).

Obviously, this list of requirements is too restrictive, and it cannot explain the observed degree of the template specificity of the enzyme. Artificial RNAs designed in accordance with the above rules, are poor templates (5, 24). However, they sometimes acquire the ability to replicate upon a number of mutations whose structural significance is not well understood (5). This means that there must be important, not yet identified features that make RNA replicable.

In this work, we investigated template properties of RNAs obtained by cleaving RQ135 RNA (28) into two fragments and compared them with the properties of the intact RQ RNA. Whereas the 3' fragment inherited the initiation oligo(C) cluster, the 5' fragment inherited the 5' terminal oligo(G). Because the two clusters were in separate molecules, we did not expect the exponential amplification and analyzed the ability of the fragments to direct the synthesis of their complementary copies. Unexpectedly we found that, although only the 3' fragment inherited the initiator oligo(C) cluster, the 5' fragment was also capable of template activity allowing Qβ replicase to initiate and elongate. However, whereas the enzyme recognizes the 3' fragment in the same way as it does with the intact RQ RNA, a quite different mechanism is employed for the initiation of RNA synthesis on the 5' fragment and a number of its derivatives, although they meet the structural criteria cited above. The most striking features of the new mechanism are that the initiation can occur without regard to oligo(C) clusters and in the absence of GTP, and does not lead to the formation of a stable replicative complex that is characteristic of typical Qβ replicase templates.

EXPERIMENTAL PROCEDURES

Qβ Replicase and Its Templates—A highly purified enzyme (29) was isolated from Escherichia coli HB101 cells transfected with plasmid pREP, carrying the catalytic (β) replicase subunit downstream the temperature-inducible PR promoter of phage λ (30), using a procedure based on the published protocol (31). The variant of RQ135 RNA used here was prepared by transcription of Smal-digested plasmid pT7RQ135, (−) sequence (32) modified by inserting CGAUCC between positions 52 and 53 of the original RQ135, (−) sequence (28); the template properties of this variant were indistinguishable from those of the authentic RQ135 RNA. The 3' fragment of RQ135 RNA was prepared by transcription with T7 RNA polymerase as described earlier (6). The 11 variants of the 5' fragment were obtained by run-off transcription of a plasmid in which the first 52 nucleotides of the RQ135 sequence were inserted, together with upstream T7 promoter, between sites HindIII and PstI of the plasmid pUC18 multiple cloning sites (6). By cutting the plasmid at restriction sites located downstream of the inserted RQ sequence, eight “nested” variants of the 5' fragment were obtained and designated after the respective restriction sites. Three more variants (Δ fragments) were obtained by deleting from the plasmid a 25-bp-long segment between sites PstI and Smal (Fig. 1A). All transcripts were gel-purified (6). Where indicated, RNAs were oxidized with sodium periodate (33); the extent of the 3'-terminal modification was close to 100%, judging by the inhibition of 3'-OH-dependent RNA recombination and by a streptavidin-induced gel-shift of the RNA biotinylated at the oxidized end (61).

RNA Synthesis—Reactions were carried out during 10 min at 22 °C in 10-μl aliquots containing replicate buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM MgCl₂, 1 mM EDTA), 0.5 μM Qβ replicase (M, 199,956), 0.05 μM RNA template, 1 mM each of ATP, UTP, [α-32P]CTP (50–200 μCi/μmol) and indicated concentration of GTP, and stopped by adding 5 μl of 30 mM EDTA. Templates were pre-melted by heating in 0.2-ml PCR tubes (at a 10× concentration in 0.1 mM Tris-EDTA, pH 8.0) during 2 min in boiling water bath followed by a transfer to ice-cold water. Labeled nucleotides were purchased from Amersham Biosciences.

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 non-denaturing electrophoresis (34) through a 8% polyacrylamide gel containing 10% glycerol, with temperature of the electrode buffer being maintained at 10–12 °C. All RNA bands were revealed by silver staining (35), whereas labeled products were detected by scanning with a Cyclone™ phosphorimaging device (Packard Instrument). Where indicated, reaction products were melted prior to electrophoresis. To this end, phenol- and chloroform-extracted samples were precipitated with ethyl alcohol (following the addition of 10 μg of linear polyacrylamide (36) and NaCl to 0.5 μl); pellets were twice washed with 90% alcohol, dissolved in 10 μl of 0.1 mM Tris-EDTA, pH 8.0, and melted as above.

RESULTS

RNAs Derived from the 5' Portion of RQ RNA and Lacking Its Initiator Sequence Are Qβ Replicase Templates—In the course of studies on RNA recombination between the supplementing 5' and 3' fragments of RQ135 RNA (6, 7) we unexpectedly noted that Qβ replicase can copy each of these fragments, although the initiator oligo(C) cluster was at the 3' fragment only. We prepared eleven 5' fragment variants that were extended at the 3' end with sequences derived from the pUC18 multiple cloning sites (see “Experimental Procedures”). This resulted in an array of RNAs with altered initiation regions (Fig. 1A) as well as altered folding (Fig. 2).

Fig. 1B shows that all the 5' fragment variants can function as Qβ replicase templates, although with varying efficiency. As in the case of the 3' fragment, the product is mainly comprised of a dsRNA consisting of the original template and the labeled complementary copy. This follows from the ability of the labeled material to be completely or partially melted producing a band whose mobility is identical or similar to that of the template (Fig. 1B) and from its resistance to nuclease S1 (see below, Fig. 4D).

Remarkably, the size of the labeled ssRNA released upon melting correlates with template length, indicating that each template, regardless of its 3'-terminal sequence, is copied beginning at or close to its 3' end. This result is unexpected and apparently contradicts the established principles of template recognition by Qβ replicase, according to which the 5' fragment variants lacking oligo(C) at the 3' end should be either not copied at all or copied at elevated GTP concentration, beginning at the internal oligo(C) clusters (18). If the latter were true, copying would give rise to products of a few fixed sizes, 50 nt, 78 nt, and perhaps 85 nt (cf. Fig. 1A; the products might be 1 nt longer if untemplated A was added at termination). The absence of significant initiation from the internal oligo(C) clusters is probably caused by these clusters being hidden in the secondary structure (cf. Fig. 2 and Ref. 21).

Notably, template activities of the 5' fragment variants do not correlate with their 3'-terminal sequences and are independent of the presence of oligo(C) clusters at the 3' end. For example, fragment Smal ending with the canonical sequence CCCCA is a poor template, whereas fragment BamHI ending with AUG and fragment EcoRI ending with AAUU are good templates. Furthermore, although fragments ΔSacI and ΔEcoRI have the 3' termini identical to those of fragments SacI and EcoRI, respectively, the former are much poorer templates than the latter. This might suggest that template activity of the 5' fragment variants is mainly determined by their secondary
requirements are saturated at a very low GTP concentration is needed (20). It would be natural to expect that the better template the lower GTP requirement will be a measure of its quality: the better template the lower GTP concentration is needed (20). A further increase in the concentration of the initiating nucleotide does not help to involve the poorer template in replication.

Plurality of Mechanisms for Copying the 5′ Fragment Variants—The unexpected GTP dependence of the 5′ fragment variants led us to a series of experiments aimed at elucidating the mechanism of Qβ replicase uses for copying these templates. Surprisingly, it turned out that there is no single mechanism common to all the variants; rather, there exist diverse mechanisms whose relative contribution varies from template to template.

At first, we found that 5′ fragment variants differently responded to oxidation with sodium periodate (Fig. 4A), which eliminates the 3′-hydroxyl group (33) thereby preventing the addition of nucleotides to the 3′ end of RNA. For example, copying of the EcoRI fragment was drastically reduced, suggesting that most of the dsRNA product was formed as a result of the 3′-terminal elongation. This inhibition was not caused by traces of periodate or by-products of the oxidation reaction, because the addition of the oxidized preparation did not reduce label incorporation on the native fragment (Fig. 4A). Earlier, Qβ replicase was found to be able of elongating RNAs at the 3′ end to various extents by copying the 3′-proximal region of the template, but the formation of full-sized dsRNAs was never observed (38). In contradistinction to EcoRI, fragment BamHI remained to be efficiently copied upon oxidation, as did RQ135 RNA (6) and Qβ RNA (19), suggesting that in this case much of the synthesis is due to de novo initiation of the complementary copy. At the same time, with both EcoRI and BamHI fragments, oxidation eliminated the bands migrating ahead of dsRNA (Fig. 4A), suggesting that these bands represented incomplete products formed through terminal elongation and, hence, that terminal elongation might contribute to the BamHI copying, too.

Two mechanisms could account for the involvement of the template 3′ hydroxyl in RNA synthesis: (i) elongation of the template at the 3′ end to form a short initiator sequence (e.g. oligo(C) cluster) and then de novo initiation of the complementary copy and (ii) use of the 3′ end as a primer for “snapback” RNA synthesis resulting in a hairpin in which the template and the complementary copy make up opposite sides of the stem and are covalently linked through the loop. The first mechanism is supported by the fact that melting of the products formed on the EcoRI fragment results in enhancement of the
band moving just above the template, i.e. representing a product strand somewhat longer than template (Fig. 4B).

To explore the possibility of the second mechanism, we probed the reaction products with nuclease S1 capable of selective degradation of single-stranded nucleic acids (39). If there existed a loop linking the annealed complementary strands, it would be digested by nuclease S1, and melting of the digested RNA would release a template-sized product. The expected effect was indeed observed (Fig. 4B), indicating that at least part of the dsRNA had the hairpin structure. Although in the case of fragment EcoRI much of dsRNA was still not melted despite the high nuclease concentration used (see “Experimental Procedures”), a notable increase in the mobility of the entire dsRNA band was observed. This suggests that nuclease S1 removes from dsRNA a flexible element, and this is also consistent with the hairpin hypothesis.

Thus, the results suggest that a variety of mechanisms bring about the overall RNA synthesis on the 5’ fragment variants: de novo initiation of the complementary copy (seen with periodate-oxidized templates), 3’-terminal elongation resulting in a hairpin-shaped dsRNA (detected by probing with nuclease S1), and a mixture of the two, in which de novo initiation is preceded by the synthesis of a short initiator sequence at the 3’ end of the template (detected as a longer-than-template product strand that is released from dsRNA upon melting). Although the formation of a hairpin twice the size of the template besides de novo strand initiation is new for Qb replicase, similar activities have been reported for some other positive-strand RNA viruses (reviewed in Ref. 40).

Initiation on the 5’ Fragment Variants Is GTP-independent—All earlier tested Qb replicase templates demonstrated the absolute need in GTP (1). ITP, a GTP analog, was reported to be a substitute for GTP during elongation but not at the initiation step (41). In agreement with previous reports, the 3’ fragment showed no RNA synthesis when GTP was entirely replaced with ITP (Fig. 5A). However, RNA synthesis was observed with most of the 5’ fragment variants, and almost at the same level as in the presence of GTP (cf. Fig. 1B). The only exception was fragment SacI, whose template activity was greatly reduced.

RNA synthesis occurring in the absence of GTP on fragments SacI and EcoRI was suppressed by periodate oxidation of the
templates (Fig. 5B), indicating that this synthesis was due to the 3’-terminal elongation, in which case the initiation step was omitted. Surprisingly, oxidation did not eliminate template activities of fragments BamHI and SmaI. Such a behavior of the SmaI fragment was most unexpected, because, like the 3’-fragment and other typical Qβ replicase templates, it carried the initiator oligo(C) cluster at the 3’ end and should be copied beginning with GTP.

The latter observations suggested that de novo strand initiation could occur in the absence of GTP. To test this possibility directly, we compared the incorporations of [γ-32P]GTP and [α-32P]CTP on the 5’ fragments manifesting the highest template activities. Incorporation of labeled γ-phosphate is a direct means to see which nucleotide is initiating, because the 5’-terminal nucleotide, and only it, retains its 5’-triphosphate group. As a standard, we used RQ135 RNA assuming that all its product strands begin with pppG. Fig. 5C shows that, although [γ-32P]GTP incorporation is readily seen with RQ135 RNA and fragment SacI, it is not detected with fragments BamHI and EcoRI. For fragment EcoRI such a result was expected and could be explained by the fact that most of RNA synthesis was due to the 3’-terminal elongation. However, in the case of fragment BamHI, especially one oxidized with periodate to prevent terminal elongation, this result indicated that de novo initiation did not use GTP, even though GTP was present in the reaction mixture.

Replicative Complexes with the 5’ Fragment Variants Are Unstable—Previous studies have demonstrated that upon initiation the replicative complex becomes resistant to aurintricarboxylic acid (ATA). ATA inhibits RNA synthesis by Qβ replicase (42), presumably by interfering with the binding of RNA templates at the active site of the enzyme (43). However, it does not inhibit elongation of already initiated strands if added 30 s after the onset of poly(G) synthesis on a long poly(C) template, or after the initiation on Qβ RNA in the presence of GTP and ATP that provide for the synthesis of the first 13 nt of the complementary strand (44).

Fig. 6A demonstrates that a similar response to ATA is displayed by RQ135 RNA and its 3’ fragment. ATA does not prevent elongation on these templates nor substantially changes their GTP requirements (cf. Figs. 3 and 6A), even if initiation has been carried out with GTP alone that provides for the synthesis of only 3-nt-long RNA pieces. ATA decreases the amount of products synthesized on RQ RNA and eliminates ssRNA from the products synthesized on the 3’ fragment, presumably, due to the prevention of reinitiation. However, the single-stranded product is still observed in the RQ RNA-templated reaction, suggesting that its generation does not require reinitiation, i.e. it is released in the first round of template copying.

Unexpectedly, the 5’ fragment variants responded quite dif-

Fig. 3. Effect of GTP concentration on RNA synthesis. Band intensities for each template were adjusted by varying the specific radioactivity, exposure time, and signal enhancement and do not reflect the template activity. Arrows indicate template locations.

Fig. 4. Probing the mechanisms for copying the 5’ fragment variants. A, label incorporation in samples containing 0.05 μm native (Nat) and/or periodate-oxidized (Oxi) fragments. B, products synthesized on the indicated templates were either digested with nuclease S1 (lanes S1) or left undigested, and melted (lanes M) or not melted before electrophoresis. GTP concentration was 100 μm. Arrows indicate locations of respective templates.
Two-step reactions were carried out as in a control samples. Concen-
trations at the elongation step; during initiation, concentration of all
reaction components, including GTP, were 25% higher. B, inability of
the 5’ fragment variants to direct elongation in the presence of ATA.
Two-step reactions were carried out as in A; NTPs present during the
initiation step are indicated by A, G, C, or U below the lanes. Concent-
tration of GTP at the elongation step was 1 mM for Sacl and EcoRI
fragments and 100 µM for other fragments. Asterisks indicate ATA-free
control samples.

Paradoxically, the overall affinity of Qβ replicase to the 5’
fragment appeared to be higher than to the 3’ fragment. In a
competitive binding experiment, when the 5’ fragment (BamHI) and the 3’
fragment were simultaneously provided, Qβ replicase preferentially bound
the 5’ fragment (Fig. 7). The results were independent of the presence of Mg2+
and GTP (data not shown).

**Formation of the First Phosphodiester Bonds Stabilizes the Replicative Complexes with RQ RNA or Its 3’ Fragment**—There was a report that in the presence of GTP, Qβ replicase specifically
binds the genomic RNA of phage Qβ resulting in a non-
dissociating complex, in which the bound RNA did not exchange
with excess of non-labeled exogenous Qβ RNA during
20 min at 4°C. Mg2+ could be omitted, suggesting that formation
of phosphodiester bonds was not required (45). Fig. 8A shows that, similarly, Qβ replicase forms a stable complex with
RQ135 RNA if GTP is added without Mg2+. This complex is

**FIG. 6. Stability of replicative complexes in the presence of a urintricarboxylic acid.** A, products synthesized on RQ135 RNA and its 3’ fragment in a two-step process, comprising a 10-min incubation in the presence of GTP and Mg2+ (initiation) followed by the addition of a mixture of the missing NTPs and ATA (1 mM final concentration) and further incubation during 10 min (elongation). Indicated are GTP concentrations at the elongation step; during initiation, concentration of all reaction components, including GTP, were 25% higher. B, inability of the 5’ fragment variants to direct elongation in the presence of ATA. Two-step reactions were carried out as in A; NTPs present during the initiation step are indicated by A, G, C, or U below the lanes. Concentration of GTP at the elongation step was 1 mM for Sacl and EcoRI fragments and 100 µM for other fragments. Asterisks indicate ATA-free control samples.

**FIG. 7. Binding of the 5’ and 3’ fragments to Qβ replicase.** The RNA fragments (0.5 pmol each, individually melted before the reaction) were incubated in 10-µl aliquots with the indicated amounts of replicase during 10 min at 22°C in the replicase buffer lacking Mg2+ and NTP and then loaded on a non-denaturing polyacrylamide gel. After electrophoresis the gel was stained with silver.

**FIG. 8. Formation of stable initiation complexes.** Templates were incubated during 10 min with Qβ replicase in the absence or presence of 100 µM GTP and/or 10 mM MgCl2 as indicated, followed by a 10-min elongation in the presence of 1 mM ATA and missing components. A, the missing components and ATA were added simultaneously as an admixture. B, the missing components were added 3 min after the addition of ATA. Asterisks indicate control samples in which ATA contacted with Qβ replicase before RNA templates.

The above data present evidence for a new type of Qβ replicase
templates, whose properties contrast the earlier established principles of template recognition by Qβ replicase. Among the templates tested, only RQ135 RNA and its 3’ fragment behave as expected of typical Qβ replicase templates: they are copied beginning at the 3’-terminal oligo(C) cluster, their GTP requirements correlate with template strength, no RNA synthesis is observed when GTP is replaced with ITP, and initiation in the presence of GTP results in a stable replicative
complex capable of elongation in the presence of ATA. We term these legitimate templates and legitimate initiation.

The 5' fragment variants (with the exception of fragment SacI, which constitutes a special case discussed below) differ from the legitimate templates in a number of aspects as follows: (i) RNA synthesis on these templates occurs without regard to oligo(C) clusters, whether 3' terminal or internal. (ii) The initiation on these templates is GTP-independent. GTP can be entirely substituted for by ITP; hence, it is dispensable not only for elongation, but for initiation as well. In some cases (fragment EcoRI), these might be because the true initiation step is omitted and RNA synthesis occurs as the 3'-terminal template elongation. However, in other cases (fragments BamHI and SmaI), ITP substitutes for GTP even if the template is oxidized. Earlier, it was reported that ITP could substitute for GTP in the presence of an oligonucleotide primer, which allows Qβ replicase to bypass the initiation step (41). However, no primer was added in this case, and all templates were gel-purified to remove any short oligonucleotides that could have been accumulated as a result of abortive transcription by T7 RNA polymerase (46). (iii) GTP requirements of these templates are exceptionally low, much lower than that of the legitimate templates. Interestingly, the same GTP dependence is observed irrespective of the template 3'-terminal sequence, and whether RNA synthesis predominantly starts as a 3'-terminal elongation (fragment EcoRI) or as de novo initiation (fragments BamHI). This might be either because the initiation on illegitimate templates is mechanistically similar to elongation or because GTP is not used as the initiating nucleotide with any of these templates. (iv) The requirements of these templates for GTP are saturated at approximately the same nucleotide concentration, although their template activities are very dissimilar. This opposes the earlier observation that higher GTP concentrations are needed for less efficient templates (20). Again, this may indicate that nucleotides other than GTP serve for initiation. (v) In some cases (fragment BamHI), new RNA strands do not begin with GTP even if GTP is present in the reaction mixture. This observation suggests that sometimes other NTPs than GTP can serve as initiating nucleotides and provides a rationale for the ability of some templates to be copied without having oligo(C) at the 3' end. (vi) Finally, initiation on these templates does not result in the formation of ATA-resistant complexes, which are characteristic of legitimate Qβ replicase templates, including Qβ RNA (44), RQ135 RNA, and its 3' fragment. Inhibition by ATA is due to the presence of a polymeric fraction that competes with RNA for binding at the enzyme active site (43). The inability of Qβ replicase to elongate on 5' fragment variants indicates that the stability of replicative complexes in this case is much lower than with the legitimate templates. Notably, both the elongation of newly initiated strands (fragment BamHI) and the 3'-terminal elongation of a template (fragment EcoRI) are inhibited by ATA.

As far as the chemical compositions of the replicative complexes (the enzyme, the template RNA, and the nascent oligonucleotide) are essentially identical, we have to conclude that complexes with legitimate templates and those with illegitimate templates differ conformationally. When Qβ replicase initiates on a legitimate template, it undergoes transition into a "closed" conformation, in which the template becomes bound so tightly that it does not dissociate during several minutes, which is greater than the time needed for the copying of the entire genomic RNA of Qβ phage, ~100 s (27). Apparently, it is this transition that specifically requires GTP, which must be present at a higher concentration than is needed for strand elongation. This provides a plausible explanation for the higher GTP requirements of the legitimate templates compared with the 5' fragment variants.

Similar conformational transitions, from an unstable "open" to a highly stable "closed" complex during entering the elongation step, after which RNA synthesis becomes insensitive to ATA (42), were well documented for both viral and cellular DNA-dependent RNA polymerases (46, 47). Formation of the stable complexes is believed to be necessary for RNA polymerases to be able to transcribe long DNA templates without dissociating from them; this is important because RNA polymerases cannot resume synthesis of RNA once they are released from DNA (47). The same may be true of Qβ replicase when it copies legitimate templates. Indeed, Qβ replicase seems to be unable to switch templates, which requires that the enzyme dissociates from one template and resumes RNA synthesis on another (48). The homologous RNA recombination in Qβ phage is a million times less frequent than in poliovirus (49) and is not detectable with the purified Qβ replicase in vitro (6).

Prevention of the premature release might be important for a vital function of Qβ replicase, such as maintaining the single-strandedness of the template and the complementary nascent strand, without which exponential replication is not possible. Indeed, any dissociation of the replicative complex results in collapsing the two strands into a duplex that cannot serve for further replication (27).

It is not clear at present what structural features of legitimate templates determine the Qβ replicase commitment to entering the closed conformation and, ultimately, its template specificity. The predicted secondary structures do not reveal notable differences between legitimate and illegitimate templates, although there is evidence that the enzyme does not always prefer the most stable RNA foldings. Upon binding to the replicase, RNAs might significantly change their structure; the large negative energy of the binding (50) could compensate for the acquisition of less favorable foldings, and the binding of GTP and Mg²⁺ at the active site could provide an additional energy.

The overall affinity cannot be a basis for the discrimination between legitimate and illegitimate templates: Qβ replicase binds the 5' fragment even tighter than the 3' fragment. Also, it shows similar affinities to replicable RNAs and other RNAs (such as most of tRNAs) and only a slightly higher affinity to ssRNA compared with dsRNA (25), on which it cannot initiate (51, 52). Furthermore, as seen from Fig. 2, illegitimate templates can possess features such as the base-paired 5' end, the unpaired 3' end (5, 22), and pyrimidine-rich internal tracts (23, 24) that have been argued to be unique to legitimate templates.

Obviously, further investigations and a more fine analysis are needed to elucidate the structural determinants essential for the legitimate initiation. In this regard, fragment SacI deserves special attention. Its properties are intermediate between those of the legitimate and illegitimate templates. Like RQ135 RNA and its 3' fragment, it is readily copied in the presence of GTP (Fig. 1), is hardly copied in the presence of ITP (Fig. 5A), and its copying requires a relatively high concentration of GTP (Fig. 3) that serves as the initiating nucleotide (Fig. 5C). Yet, like other 5' fragments, it fails to form a stable initiation complex (Fig. 6B). Such an intermediate position may make this fragment a sensitive indicator of structural manipulations that favor or disfavor the legitimate initiation.

In conclusion, we would like to note that the present study provides a set of criteria for distinguishing between the legitimate and illegitimate Qβ replicase templates, each of which can be efficiently copied by the enzyme. This can be especially useful for the analysis of artificial templates designed
for testing specific hypothesis on the mechanism of template recognition (5, 24).

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REFERENCES