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Minimal requirements for template recognition by bacteriophage Qβ replicase: Approach to general RNA-dependent RNA synthesis

(protein–nucleic acid interaction/oligonucleotides/RNA replication)

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ABSTRACT Any oligo- or polynucleotide able to offer a C-C-C-sequence at the 3′-terminus and a second C-C-C-sequence in a defined steric position to Qβ replicase is an efficient template. Corresponding chemical modifications convert non-template RNAs to template RNAs.

The small *Escherichia coli* bacteriophage Qβ induces an enzyme, Qβ replicase, which is responsible for the replication of the phage RNA. The enzyme consists of one virus-specified polypeptide (1, 2) and three host polypeptides. The host proteins are the protein synthesis elongation factors T_u and T_s (3) and the ribosomal protein S_1 (4).

The phage replicase shows a very high template specificity for the complementary plus (virion) and minus strands of the homologous viral RNA (5). Unrelated viral RNAs and most other naturally occurring RNAs do not serve as templates. Despite its capacity for discriminating between Qβ-specific RNAs and all other naturally occurring RNAs, Qβ replicase accepts poly(C) and C-containing random copolymers (6) as well as a variety of so called "6S" RNAs (7). At first glance, this fact seems to be a paradoxical one.

The minimal requirements for RNA template recognition by Qβ replicase are the subject of this paper. We demonstrate that two clusters of cytidine residues in a defined steric position trigger the initiation of RNA synthesis by Qβ replicase.

MATERIALS AND METHODS

Isolation of Phage Qβ Replicase. Phage Qβ replicase was purified and assayed as described by Kamen (8).

Nucleotides. γ-^{32}P-Labeled ribonucleoside triphosphates were prepared by the method of Glynn and Chappel (9). The other labeled ribonucleoside triphosphates were purchased from Amersham Buchler, Braunschweig.

Primer-Dependent Polynucleotide Phosphorylase. Conversion of commercial polynucleotide phosphorylase from *Micrococcus luteus* (Boehringer Mannheim GmbH, Mannheim) to oligonucleotide primer dependence was achieved by treatment with *N*-ethylmaleimide in the presence of 1 M guanidine hydrochloride as described by Letendre and Singer (10). The resulting enzyme preparation was used directly for the elongation reactions of oligo- and polynucleotides described below, without removal of the guanidine hydrochloride.

Preparation of Homopolymers. Poly(U), poly(C), and poly(A) were synthesized from the corresponding nucleoside diphosphates (Waldhof, Mannheim) using purified polynucleotide phosphorylase isolated from *E. coli* K 12 Hfr (11).

Preparation of $(Cp)_nC$ Oligomers. Poly(C) was degraded to a mixture of oligomers by limited hydrolysis with piperidine (6–8 min at 100° in 10% piperidine) (12). The oligomers were fractionated on a QAE-Sephadex A-25 column (1.5 × 70 cm) by elution with a linear gradient, 0.3–0.7 M NaCl, in 0.05 M Tris·HCl, pH 7.5 (one 2000 ml reservoir of each buffer). This gradient ensures the complete resolution of the oligomers up to $(Cp)_{13}$, whereas the oligomers $(Cp)_{14}$ to $(Cp)_{19}$ were only partially resolved. The peak fractions were desalted by Sephadex G-10 filtration. The 3′-terminal phosphates were removed by digestion with human semen phosphatase (kindly provided by Dr. Biebricher). $(Ap)_nA$ and $(Up)_nU$ oligomers were prepared by the same method.

Preparation of $(Up)_7(Cp)_nC$ and $(Ap)_7(Cp)_nC$ Oligomers. Primer-dependent polynucleotide phosphorylase (see above) was used to add a block of C-residues to each oligonucleotide primer $(Up)_6U$ or $(Ap)_6A$. The reaction conditions used were approximately those of Martin *et al.* (13). A typical incubation mixture contained in 3 ml: 0.2 M glycine buffer (pH 9.2), 30 mM CDP, 0.6 M NaCl, 5 mM Mg^{++}, about 200 A_{260} units oligonucleotide, and 0.7 mg of polynucleotide phosphorylase. Incubation was at 37° for 4–6 hr. The block copolymer products were fractionated on QAE-Sephadex A-25 columns as described above, after the addition of oligonucleotide primer as an internal marker. Although the peaks could usually be identified simply by counting from the primer peak the identification was confirmed by complete alkaline hydrolysis of a portion of each of the first several peaks and determination of the Up (or Ap):Cp:C ratio (14).

Preparation of $(Cp)_4(Up)_5(Cp)_nC$ Oligomers. $(Cp)_4(Up)_4U$ was obtained by adding a block of U-residues to the primer $(Cp)_3C$ and separating the products on a QAE-Sephadex A-25 column as described above. For the addition of C-residues to the primer oligonucleotide $(Cp)_4(Up)_4U$ and the subsequent separation and identification of the products $(Cp)_4(Up)_5(Cp)_nC$, the procedures described above were followed.

Preparation of Polynucleotides $(Ap)_m(Cp)_nC$ and $(Up)_m(Cp)_nC$ ($m \gg n$). Poly(A) or poly(U) were treated with semen phosphatase to remove any terminal 3′-phosphates present. Then a block of C-residues was added by using primer-dependent polynucleotide phosphorylase. The incubation mixture contained in 100 μl: 0.1 M Tris·HCl (pH 8.2), 30 mM CDP, 10 mM Mg^{++}, 80 μg of poly(A) [or poly(U)] and 20 μg of polynucleotide phosphorylase. The reaction was run at 37° for 3–12 hr and then stopped by the addition of sodium dodecyl sulfate and a drop of chloroform. The polynucleotides were isolated by chromatography on Sephadex G-50 columns.

RESULTS

In addition to Qβ-specific RNAs, Qβ replicase accepts poly(C) and C-rich copolymers as templates. Evidently the model templates also have to fulfill all requirements for ini-

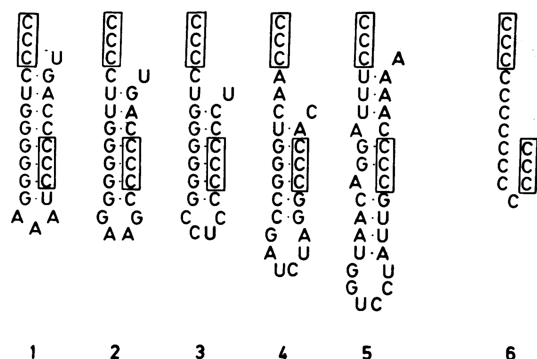

FIG. 1. Nucleotide sequences of the 3′-termini of RNAs acting as templates for Qβ replicase. 1: Qβ (−) strand (15); 2: Midivariant (+) strand (16); 3: Midivariant (−) strand (16); 4 and 5: (+) and (−) strand of a "6S" RNA (W. Schaffner and C. Weissmann, personal communication); 6: Poly (C).

tiation of RNA synthesis. The 3′-ends of all template RNAs sequenced so far terminate with a sequence of at least three C-residues (Fig. 1). A chemical modification of this 3′-terminal C-C-C-sequence leads to a loss of template activity (17), indicating the importance of this C-cluster. Several other viral RNAs, such as those of phages MS2, f2, and R17, and tobacco mosaic virus, which also terminate with a C-cluster at the 3′-end, are inactive as templates. Therefore this sequence cannot be the only requirement for template recognition by Qβ-replicase. Consequently one (or more) additional nucleotide sequences must be involved in the initiation mechanism. Taking into account the poly(C) activity, this additional requirement can only be fulfilled by C-nucleotides.

Examination of template RNAs of known sequence reveals a striking feature. As shown in Fig. 1, all sequences have in common a C-cluster at a defined distance from the 3′-terminus. In order to demonstrate the importance of this internal C-C-C-sequence in the recognition process we prepared several oligonucleotides with defined sequences and investigated their template activity.

$(Cp)_nC$-oligonucleotides

Using the idea of two C-clusters cooperating in the recognition process, one can predict the minimum chain length of C-oligomers acting as templates. As can be estimated from Fig. 1, $(Cp)_{13}C$ should be this critical chain length. In the experiment of Fig. 2 the oligo(C)s ranging from CpC to $(Cp)_{18}C$ were assayed for template activity. No activity is observed for the oligonucleotides up to the chain length 13 (curve I). Within the limited range of $(Cp)_{13}C$ to $(Cp)_{17}C$ a template activity is reached comparable to that of poly(C). At higher oligonucleotide concentration a limited GMP-incorporation directed by the short-chain oligomers $(Cp)_6C$ to $(Cp)_{12}C$ is found (curve II), probably caused by a cooperative action of these oligonucleotides. However the sharp increase of template activity at the chain length 13 is independent of the oligonucleotide concentration.

$(Cp)_4(Up)_5(Cp)_nC$ oligonucleotides

In order to assay more "realistic" nucleotide sequences we prepared oligonucleotides in which the two C-clusters are linked by a U-U-U-U-U-sequence. In the experiment of Fig. 3 the template activity of the oligonucleotides $(Cp)_4(Up)_5(Cp)_nC$ was determined as a function of n. Beginning with n = 4, corresponding to an overall chain length of

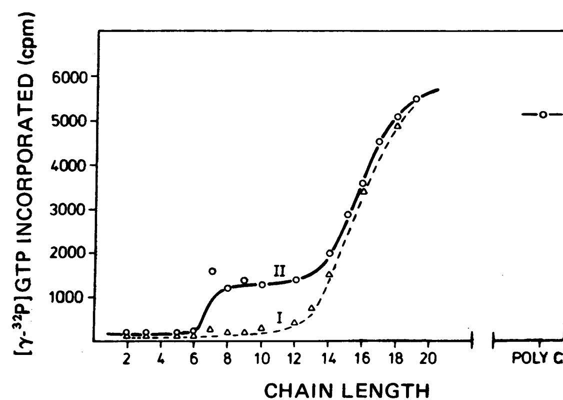

FIG. 2. Template activity of $(Cp)_nC$ oligomers as a function of chain length. The incubation mixture (50 μl) contained 50 mM Tris·HCl (pH 7.5), 10% (v/v) glycerol, 0.1 mM dithiothreitol, 10 mM $MgCl_2$, 0.05 mM [γ-^{32}P]GTP (specific activity 500 Ci/mol), 4 μg of Qβ replicase, and 2.5 μM $(Cp)_nC$ (curve I) or 25 μM $(Cp)_nC$ (curve II), as indicated. Incubation was at 30° for 5 min. A 15 μl aliquot was then applied to DEAE-cellulose paper (Whatman DE 81). The oligonucleotide product was separated from [γ-^{32}P]GTP and $^{32}PP_i$ by electrophoresis in 7% (v/v) formic acid for 10 hr at 15 V/cm. The oligonucleotide products were cut out and their radioactivities were measured by liquid scintillation counting.

14, these model compounds direct the incorporation of AMP and GMP with steeply increasing efficiency. The following evidence can be offered for the actual synthesis of the complementary oligonucleotide $ppp(Gp)_8(Ap)_5(Gp)_3G$ when $(Cp)_4(Up)_5(Cp)_7C$ is used as template: *(a)* omission of GTP from the incubation mixture completely suppressed the incorporation of AMP. *(b)* In a double label experiment using [^{3}H]ATP and [α-^{32}P]GTP the molar ratio of incorporation was found to be 1 AMP:1.9 GMP. *(c)* A nearest neighbor analysis with [α-^{32}P]GTP yields a dinucleotide frequency of 92% GpG and 8% ApG.

$(Ap)_7(Cp)_{14-16}C$ and $(Up)_7(Cp)_{12-14}C$ oligonucleotides

The experiments described so far elucidated the minimal requirements for template activity. We now can ask whether

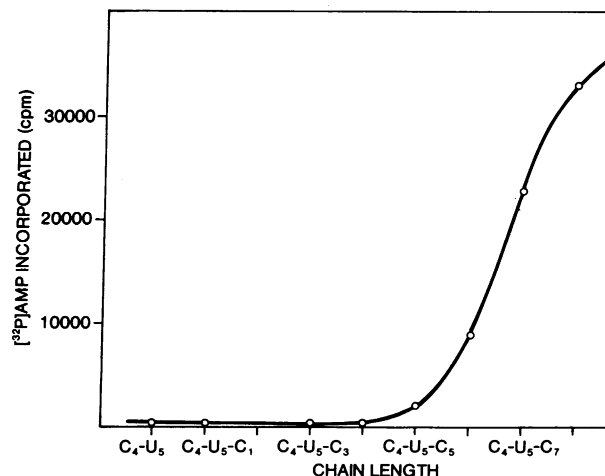

FIG. 3. Template activity of $(Cp)_4(Up)_5(Cp)_nC$ oligomers as a function of chain length. The incubation mixture (50 μl) contained 50 mM Tris·HCl (pH 7.5), 10% glycerol, 0.1 mM dithiothreitol, 10 mM $MgCl_2$, 0.05 mM GTP, 0.05 mM [α-^{32}P]ATP (specific activity 500 Ci/mol), 4 μg of Qβ replicase, and 10 μM oligonucleotide, as indicated. Incubation was at 30° for 5 min. Incorporated radioactivity was measured as described in Fig. 2.

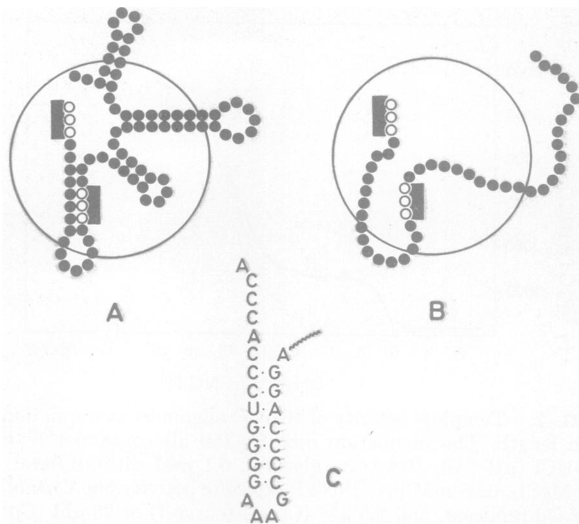

FIG. 4. Model of template recognition of phage RNA replicases. (A) templates with fixed tertiary structure; (B) random copolymers; (C) 3′-terminal sequence of MS2 (−) strand (20). For details see *Discussion*.

Qβ replicase is able to read through the initiation sequence into any nucleotide sequence. As model templates we chose $(Ap)_7(Cp)_{14-16}C$ and $(Up)_7(Cp)_{12-14}C$. Table 1 shows that these oligonucleotides efficiently direct the incorporation of UMP and AMP, respectively. When account is taken of the different base compositions our model templates (Table 1) turned out to be templates as effective as poly(C).

$(Ap)_m(Cp)_nC$ and $(Up)_m(Cp)_nC$ polynucleotides

Although poly(A) and poly(U) are completely inactive as templates for Qβ-replicase, these polymers became excellent templates after being linked with an initiation sequence at their 3′-terminus (Table 2). These experiments suggest that any polynucleotide linked to an initiation sequence can serve as template for Qβ-replicase.

DISCUSSION

Our experiments with oligonucleotides demonstrate quite clearly that a C-cluster at the 3′-end along with a second C-cluster a defined distance from the 3′-terminus triggers initiation of RNA synthesis by Qβ replicase. All template RNAs sequenced so far (Fig. 1) fulfill this minimal requirement in their 3′-end regions. The only exception is Qβ (+) strand RNA. Remarkably, this RNA cannot be replicated by Qβ replicase alone. The presence of at least one further protein factor (6) is necessary for template activity. Nucleotide sequences of Qβ (+) RNA fragments recovered after nuclease treatment from the replicase binding complex with Qβ (+) RNA are not common to the other template RNAs. It follows that these binding sequences are involved in other biological functions [e.g., repressor action of Qβ replicase (18)] and are not necessary to fulfill the minimal requirements for template activity.

Table 1. Template activity of synthetic oligonucleotides

Oligonucleotide	pmol NMP incorporated
$(Cp)_{17}C$	550 (GMP)
$(Ap)_6A$	<1 (UMP)
$(Ap)_7(Cp)_{14-16}C$	395 (UMP)
$(Up)_6U$	<1 (AMP)
$(Up)_7(Cp)_{12-14}C$	195 (AMP)
$(Cp)_4(Up)_5(Cp)_7C$	205 (AMP)

The incubation mixture (100 μl) contained 50 mM Tris·HCl (pH 7.5), 10% glycerol, 0.1 mM dithiothreitol, 10 mM $MgCl_2$, 0.05 mM nucleoside triphosphates (GTP and UTP or GTP and ATP), one of which was labeled with ^{14}C (specific activity 50 Ci/mol), 4 μg of Qβ replicase, and 10 μM oligonucleotide, as indicated. Incubation was at 30° for 10 min. Incorporated radioactivity was determined as described in Fig. 2.

Table 2. Template activity of polynucleotides

Polynucleotide	pmol NMP incorporated (complete incubation mixture)	pmol NMP incorporated (incubation mixture without GTP)
Poly(C)	4500 (GMP)	—
Poly(A)	<5 (UMP)	—
$(Ap)_m(Cp)_nC$	3800 (UMP)	<5 (UMP)
Poly(U)	<5 (AMP)	—
$(Up)_m(Cp)_nC$	1200 (AMP)	<5 (AMP)

The incubation mixture (100 μl) contained 50 mM Tris·HCl (pH 7.5), 10% glycerol, 0.1 mM dithiothreitol, 10 mM $MgCl_2$, 0.2 mM nucleoside triphosphates (GTP and UTP or GTP and ATP), one of which was labeled with ^{14}C (specific activity 5 Ci/mol), 2.7 μg of Qβ replicase, and 1 μM polynucleotide, as indicated. Incubation was at 30° for 10 min. Incorporation was measured by the Millipore filter technique.

The proposed model of template recognition is able to explain a paradoxical property of Qβ replicase, namely, that it is extremely specific against naturally occurring RNAs and yet accepts C-containing random copolymers. Only those sequences able to offer the two C-clusters in the correct steric position can act as templates. Since naturally occurring RNAs have in general a fixed tertiary structure this mechanism efficiently discriminates between templates and nontemplates. On the other hand, RNA sequences with little or no tertiary structure, allowing more flexibility, can nearly always fulfill the initiation conditions, if they have a C-cluster at the 3′-end and a second C-cluster somewhere further in.

Recently it was shown that Qβ replicase generates *de novo* an apparently unlimited variety of self-replicating RNA structures (19). Since no long and complicated nucleotide sequences are necessary for template recognition, a large number of RNAs can indeed fulfill the minimal requirements and can serve as active templates.

A model illustrating these points is shown in Fig. 4.

Furthermore, our model can be modified to explain the specificity of phage MS2, f2, or R17 replicases as well. In analogy to Qβ replicase these replicases accept poly(C) as active template (21) and all cognate RNAs also contain two C-C-C-clusters in a defined steric position. As can be seen from the known 3′-terminus of MS2(−)-strand (Fig. 4), the steric position of the internal C-C-C-cluster is different from that of Qβ-active templates. This displacement could cause the lack of cross-activity of replicases and RNA templates of group I phages (Qβ) and group III phages (MS2, R17, f2, etc.).

It should be possible to convert any desired RNA into a template for Qβ replicase in a way analogous to that described for poly(U) and poly(A). In principle, it should even be possible to modify any RNA to become a self-replicating species.

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