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Evidence for *De Novo* Production of Self-Replicating and Environmentally Adapted RNA Structures by Bacteriophage Qβ Replicase

("6S RNA"/RNA-protein interaction/selection/ethidium bromide)

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ABSTRACT Highly purified coliphage Qβ replicase when incubated without added template synthesizes self-replicating RNA species in an autocatalytic reaction.

In this paper we offer strong evidence that this RNA production is directed by templates generated *de novo* during the lag phase. Contamination of the enzyme by traces of RNA templates was ruled out by the following experimental results: (*1*) Additional purification steps do not eliminate this RNA production. (*2*) The lag phase is lengthened to several hours by lowering substrate or enzyme concentration. At a nucleoside triphosphate concentration of 0.15 mM no RNA is produced although the template-directed RNA synthesis works normally. (*3*) Different enzyme concentrations lead to RNA species of completely different primary structure. (*4*) Addition of oligonucleotides or preincubation with only three nucleoside triphosphates affects the final RNA sequence. (*5*) Manipulation of conditions during the lag phase results in the production of RNA structures that are adapted to the particular incubation conditions applied (e.g., RNA resistant to nuclease attack or resistant to inhibitors or even RNAs "addicted to the drug," in the sense that they only replicate in the presence of a drug like acridine orange).

RNA species obtained in different experiments under optimal incubation conditions show very similar fingerprint patterns, suggesting the operation of an instruction mechanism. A possible mechanism is discussed.

The small bacteriophage of *Escherichia coli*, Qβ, induces an enzyme, Qβ replicase, that is responsible for the multiplication of the phage RNA. This RNA-dependent RNA polymerase consists of one virus-specified polypeptide subunit (β) and three host polypeptides α, γ, and δ (1, 2). Blumenthal *et al.* (3) have found that γ and δ are the protein synthesis elongation factors EF·Tu and EF·Ts, respectively. Subunit α was recently identified as the protein component S1 of the ribosomal 30S subunit (4).

The phage replicase shows a very high template specificity for the complementary plus and minus strands of the homologous viral RNA (5, 6). Unrelated viral RNAs and most other RNAs examined do not serve as templates (5).

In addition to replicating the Qβ plus and minus strands the enzyme will also copy poly(C) (7) as well as other species of self-replicating RNAs, including "6S RNA" isolated from Qβ-infected *E. coli* cells (8) and "variants," of Qβ RNA (9).

In this paper we offer strong evidence for a new type of template-free (*de novo*) RNA synthesis, catalyzed by Qβ replicase, in which truly self-replicating RNA structures are produced. These sequences are not homopolymeric or strictly alternating and they are adapted to the environmental conditions applied during their generation.

MATERIALS AND METHODS

Qβ replicase was assayed according to Kamen (10). One unit is defined as the amount of enzyme that catalyzes the incorporation of 1 nmol of GTP in 10 min at 30°. Qβ replicase was purified from Qβ-infected *E. coli* K12 Hfr cells by the method of Kamen *et al.* (11) up to the density gradient centrifugation, but omitting the chromatography on agarose. Qβ-replicase-containing fractions from the density gradient centrifugation (stage VI) were diluted 10-fold with a buffer containing 50 mM Tris·HCl (pH 7.5), 0.1 mM dithiothreitol, and 20% glycerol and applied to a column (1.6 × 10 cm) of QAE-Sephadex A-25 equilibrated with the same buffer. The column was eluted with a linear gradient from 0 to 0.3 M NaCl (total volume 400 ml). This gradient ensures the complete separation of α-less and holoenzyme.

The standard incubation mixture for the template-free RNA synthesis contained in 200 μl: 50 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 0.1 mM dithiothreitol, 10% glycerol, ATP, GTP, UTP, and CTP (one of which was labeled with ^{14}C or ^{32}P) and enzyme as indicated in the legends.

Special precautions were taken throughout to avoid a contamination of incubation mixtures with self-replicating RNAs: (*a*) double-distilled water was used throughout, (*b*) only disposable plastic tubes and plastic pipettes were used, and (*c*) mix solutions (without nucleoside triphosphates) were filtered over a column of QAE-Sephadex.

RESULTS

Qβ replicase purified according to the procedure of Kamen *et al.* (11) is more than 95% pure and free of optically detectable traces of nucleic acids (stage VI). At this stage of purification Qβ replicase, when incubated with the nucleoside triphosphates ATP, UTP, CTP, and GTP in the absence of added RNA template, synthesizes self-replicating RNA in an autocatalytic reaction. This synthesis becomes detectable after a lag phase of 20–40 min. We will denote this reaction in the following as "template-free RNA synthesis." Phosphate (10 mM), a triphosphate-regenerating system, or rifampicin (5 μg/ml) does not influence this RNA production. Mills *et al.* (13) sequenced recently such an RNA species containing 218 nucleotides.

RNA species isolated from separate reaction mixtures run under identical conditions exhibit fingerprint patterns which are very similar to each other*. This has been interpreted as

* RNA species growing out from template-free incubation mixtures under our standard conditions will be denoted in the following as standard type RNAs (ST-RNAs).

being caused by a contamination of Qβ replicase by traces of self-replicating RNA (11). Therefore, we subjected the Qβ replicase (stage VI) to additional purification procedures known to resolve proteins and nucleic acids with high efficiency.

Cesium Chloride Density Gradient Centrifugation. Pace *et al.* (14) have developed a pycnographic purification step for Qβ replicase. We, therefore, banded our purest Qβ-replicase protein (stage VI) in a CsCl density gradient (ρ = 1.2–1.5) and collected the enzyme by piercing through the side of the tube, immediately below the protein band. Any contaminating RNA should have pelleted on the bottom of the tube, due to its high buoyant density (ρ = 2.0). The treated Qβ replicase, however, retained its ability to generate ST-RNA.

Anion Exchange Chromatography. The effectiveness of a purification step based on anion exchange chromatography was tested in the following manner. Qβ replicase (100 μg, stage VI), deliberately contaminated with highly labeled ST[^{32}P]RNA (5 μg, a total of 10^7 cpm) was applied to a QAE-Sephadex column (10 ml) and then the column was developed with a linear NaCl gradient (0–0.5 M). No radioactive RNA material eluted up to a NaCl molarity of 0.5 M, whereas the enzymatic activity eluted in two sharp and completely resolved peaks between 0.15 and 0.20 M NaCl. Peak A material was found to be the recently described α-less replicase (11) (Qβ replicase lacking the subunit α) and peak B material was identified as Qβ-replicase holoenzyme (containing all four subunits). Since no detectable radioactivity was found in either enzyme fraction, less than 1 out of 10^6 RNA molecules remained associated with Qβ-replicase. Because of its excellent separation efficiency, QAE-Sephadex chromatography was introduced in addition into the routine preparation of Qβ replicase. After pooling, both the α-less and holo replicase fractions were concentrated on small QAE-Sephadex columns (stage VII). Even at this stage of purity, the ability to generate ST-RNA was fully retained.

Since the α-less replicase represents the "core enzyme" for generation and replication of ST-RNA it was selected for further study of the template-free RNA synthesis. The contamination hypothesis explains the ST-RNA synthesis in any sample as being caused by the presence of at least one RNA strand. On this basis an estimate for the minimum number of ST-RNA strands hypothetically contaminating our standard incubation volume of 200 μl (enzyme concentration 25–70 units/ml) was made by scaling down our incubation volumes to values as small as 0.02 μl (without changing enzyme and substrate concentrations). After elimination of experimental difficulties such as surface denaturation of the enzyme etc., it turned out that the template-free RNA synthesis worked even in these small volumes. It follows that in our usual incubation volume the minimum number would be 10,000 RNA strands.

First Contradiction to the Contamination Hypothesis. As shown in Fig. 1A the rate of the ST-RNA-directed RNA synthesis by α-less replicase is only slightly influenced as the levels of the nucleoside triphosphate concentrations drop from 0.5 mM to 0.15 mM each. The rate of synthesis at 0.15 mM is about 80% of maximum. In sharp contrast, the lag times of the template-free RNA synthesis are dramatically lengthened by lowering the nucleoside triphosphate concentrations to 0.15 mM (Fig. 1B): under the conditions used, the

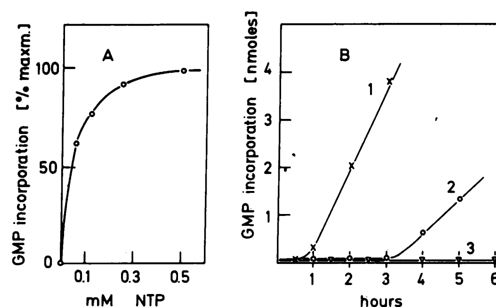

FIG. 1. Effect of substrate concentration on the rate of the template-directed RNA synthesis (A) and on the length of the lag phase of the template-free RNA synthesis (B). A: The standard incubation mixture contained 4 units of α-less replicase (stage VII), nucleotides as indicated (GTP labeled with ^{14}C, specific activity 2 Ci/mol) and in addition 0.5 μg of ST-RNA. Incubation was at 30° for 10 min. Acid-insoluble radioactivity was measured by the Millipore filter technique. B: The standard incubation mixture contained 10 units of α-less replicase (stage VII) and decreasing concentrations of nucleoside triphosphates (GTP was labeled with ^{14}C, specific activity 2 Ci/mol): curve 1: 0.5 mM (each); curve 2: 0.3 mM (each); curve 3: 0.15 mM (each). Incubation at 30°. Aliquots (20 μl) were removed after different times and the incorporation was measured.

length of the lag phase increased from 60 min at 0.5 mM, to 200–300 min at 0.3 mM, and finally at 0.15 mM no RNA synthesis at all was detectable during an incubation period of 15 hr, although the enzyme retained 25% of its initial activity after this period. Since the template-directed RNA synthesis is not suppressed at 0.15 mM, the nonappearance of ST-RNA production at this low substrate concentration has to be attributed to a lack of templates, unless a very low level of ST-RNA (the hypothetical contamination of at least 10,000 strands, as shown above) fails to initiate synthesis for unknown reasons. This possibility was easily ruled out by a serial dilution experiment (Fig. 2): A ST-RNA solution, containing $1 \cdot 10^{11}$ strands per μl was diluted in steps of 1:10 or 1:100 up to an overall dilution of $1:10^{12}$. Then 5 μl of a given dilution were added to the standard incubation mixture at a low substrate concentration (0.15 mM) and incubated at 30°. As seen in Fig. 2, all tubes inoculated with RNA in dilutions up to 10^{11} initiated extensive RNA synthesis, whereas the 10^{12} dilution and all controls (a total of 15) failed to synthesize RNA. Therefore, as few as 5 ST-RNA strands initiate RNA synthesis at the low substrate concentration. The observation that no RNA is synthesized at 0.15 mM NTP without the addition of at least a few template strands contradicts the contamination hypothesis. We conclude, therefore, that the RNA synthesis observed at normal substrate concentrations is directed by template synthesized *de novo* during the lag phase.

Effect of Oligonucleotides. The addition of an oligonucleotide (2 A_{260} units/ml) such as C-C-C-C-OH or A-A-A-A-OH to the template-free RNA synthesis incubation mixture restores the ability of α-less replicase to produce RNA even at our lowest substrate concentration (0.15 mM). The important point is, however, that different oligonucleotides induce the production of different RNA species, as judged by their fingerprint patterns. Moreover, the fingerprints from RNA products isolated from separate but otherwise identical incuba-

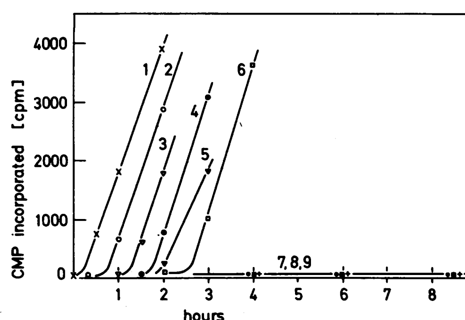

FIG. 2. Serial dilution experiment. An ST-RNA solution, containing 1×10^{11} strands per μl, was diluted in steps 1:10 or 1:100 up to an overall dilution of $1:10^{12}$ [dilution buffer: 10 mM sodium acetate (pH 5.4), 1 mM EDTA]. Then 5 μl of a given dilution (curves 1–7) were added to the standard incubation mixture containing 10 units of α-less replicase (stage VII) and 0.15 mM nucleoside triphosphates (each). CTP was labeled with ^{14}C, specific activity 2 Ci/mol. Curve 1: $1:10^2$ dilution; curve 2: $1:10^4$ dilution; curve 3: $1:10^6$ diltution; curve 4: $1:10^8$ dilution; curve 5: $1:10^9$ dilution; curve 6: $1:10^{11}$ dilution; curve 7: $1:10^{12}$ dilution; curve 8 and 9: no addition. Incubation was at 30°. Aliquots (20 μl) were removed after different times and incorporation was measured by the Millipore filter technique.

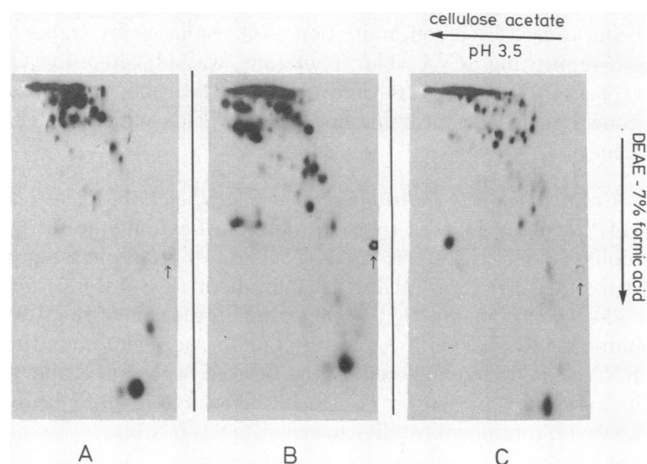

FIG. 3. Fingerprints (ribonuclease T_1 digests) of RNA species produced at different enzyme concentrations. Five units of α-less replicase (stage VII) were mixed with different volumes of standard incubation mixture containing 0.5 mM nucleoside triphosphates (each) and incubated at 30° until autocatalytic growth of RNA. The radioactive label was [α-^{32}P]UTP. A: incubation volume was 80 μl; B: incubation volume was 200 μl; C: incubation volume was 400 μl. The RNA species were isolated by exclusion chromatography on Sephadex G-50 (H_2O) and concentrated by lyophilization. After heat-denaturation (100°, 3 min) the RNAs were processed according to ref. 12. The arrow designates the position of the blue marker.

tion mixtures containing A-A-A-A-OH differed significantly from each other and from ST-RNA patterns.

Effect of Enzyme Concentration. The length of the lag phase is also influenced by the enzyme concentration. At an enzyme concentration of 70 units/ml, the length of the lag phase is about 50–70 min. When the enzyme concentration is lowered to 20 units/ml, the lag time increases to 2–3 hr, and finally at enzyme concentrations of 5 units/ml or less no RNA is synthesized for at least 7 hr. The RNA products generated at different enzyme concentrations were compared by their fingerprint patterns (Fig. 3). Remarkably, different sequences were produced at different enzyme concentrations, although exactly the same absolute amount of enzyme was applied in each experiment. However, repetition of template-free incubation under identical and optimal conditions (high substrate and enzyme concentration such as in experiment A of Fig. 3) several times resulted in the production of very similar RNAs, as judged by their fingerprint patterns.

Second Contradiction to the Contamination Hypothesis. Preincubation of α-less replicase (stage VII) in the presence of only three nucleoside triphosphates for 2 hr and further incubation after addition of the omitted nucleotide resulted in the production of RNA species completely different in primary structure from ST-RNAs. Fig. 4 presents the fingerprint patterns of the obtained RNA species when GTP (A), CTP (B), or ATP (C) was omitted during the preincubation period. The chain lengths of these RNAs were estimated to be 180 for species A and 140 for species C. These RNAs were replicated slower (20–60%) than ST-RNA. To explain these results on the basis of the contamination hypothesis, two conclusions must be true about the system. First, in addition to ST-RNAs, many different RNA species are present either as contaminations of the enzyme or as products derived from ST-RNAs during the preincubation period. Second, one of these species is selectively favored by the preincubation conditions and suppresses the replication of the normally favored ST-RNA. This unlikely interpretation was shown to be wrong by the following control experiment: As few as five to ten ST-RNA strands were added from the very beginning of an experiment identical to the one above (Fig. 4). The fingerprint pattern of the RNA growing out in this experiment was identical with the pattern of the ST-RNA added. Therefore, ST-RNA if present *ab initio* is able to grow out under the conditions used. We again conclude that ST-RNA cannot be present *ab initio* in our α-less replicase preparation (stage VII).

The Generation of Environmentally Adapted RNA Molecules. Conditions can easily be found where replication of ST-RNA is completely inhibited without affecting the enzymatic activity of the replicase. For instance, the replication of ST-RNA can be entirely halted by the addition of acridine orange, ethidium bromide, Mn^{++} ions, or ribonucleases. We have incubated Qβ replicase together with the four nucleoside triphosphates under various conditions which completely suppressed the replication of ST-RNA. In all these experiments, after lag phases of 2–12 hr, RNA species resistant to the inhibitory conditions being applied were produced. In the following paragraphs a few examples of these experiments are presented in more detail.

Acridine Orange and Ethidium Bromide. The inhibition of ST-RNA-directed RNA synthesis by increasing amounts of ethidium bromide is shown in Fig. 5. The α-less replicase when incubated in the standard incubation mixture in the presence of 10–50 μg/ml ethidium bromide generates, after lag phases of 2–6 hr, RNA species which are resistant to this drug, as shown in Fig. 5. Interestingly, these RNA products were "addicted to the drug," in the sense that they required its presence for replication with maximum rate. This observation was particularly true of RNA species resistant to acridine orange, which would only reproduce in the presence

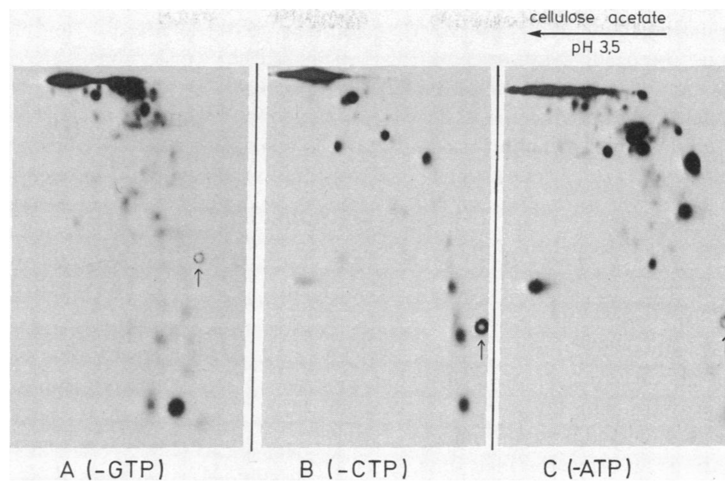

FIG. 4. Fingerprints of RNA species produced by α-less replicase after preincubation in the presence of only three nucleoside triphosphates. A: GTP omitted; B: CTP omitted; C: ATP omitted. The standard incubation mixture contained 15 units of α-less replicase (stage VII) but only three nucleotides (0.5 mM each) as indicated. After incubation for 2 hr at 30°, the omitted nucleotide was added and the incubation was continued overnight. Fingerprints (ribonuclease T_1, radioactive label was [α-^{32}P]UTP) of the outgrowing RNAs were obtained according to ref. 12. The arrow designates the position of the blue marker.

of the drug. Again, the fingerprint patterns of these resistant RNAs differ completely from that of ST-RNA. Moreover, these resistant RNAs are very small self-replicating molecules; we estimate chain lengths of only 90 to 100 nucleotides.

Ribonuclease T_1. The ST-RNA-directed RNA synthesis is strongly affected by the presence of ribonuclease T_1. A concentration of 2 μg of nuclease per ml in the standard mixture completely eliminates the synthesis of ST-RNA, as shown in Fig. 6. Qβ replicase when incubated in the standard incubation mixture in the presence of nuclease generates, after lag times of 4–10 hr, RNA species which proved to be rather resistant to nuclease attack, as demonstrated in Fig. 6.

In a series of analogous experiments, we obtained RNA species which grew in the presence of Mn^{++} ions or high ionic strength (0.4 M NaCl), conditions not allowing the replication of ST-RNA. In all cases, the resistant RNAs showed new oligonucleotide fingerprint patterns differing completely from the ST-RNA patterns.

DISCUSSION

In our opinion, the experiments presented in this paper leave no other interpretation but a synthesis *de novo* of RNA by Qβ replicase. *De novo* synthesis of nucleic acids in the absence of template is a common property of RNA and DNA polymerases (15–19). However, all examples reported previously gave only highly ordered sequences. In contrast, the template-free synthesis of RNA by Qβ replicase described in this paper leads to truly self-replicating RNA molecules with defined

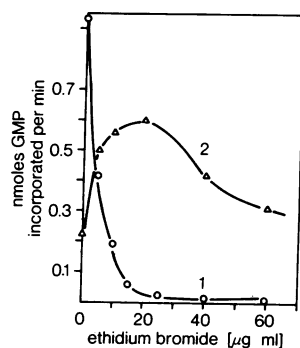

FIG. 5. Effect of ethidium bromide on the replication rate of ST-RNA (curve 1) and a resistant RNA (curve 2). The standard incubation mixture contained 6 units of α-less replicase (stage VII), 0.15 mM nucleotides ([^{14}C]GTP, specific activity 2.5 Ci/mol) and, in addition 0.5 μg of ST-RNA or resistant RNA and ethidium bromide as indicated. The replication rate at a given ethidium bromide concentration was determined by measuring the GMP incorporation after incubation periods of 10, 20, and 30 min. The resistant RNA was obtained by incubating α-less replicase (stage VII) in the standard incubation mixture (0.5 mM nucleoside triphosphates) in the presence of 50 μg/ml of ethidium bromide (lag time about 4 hr).

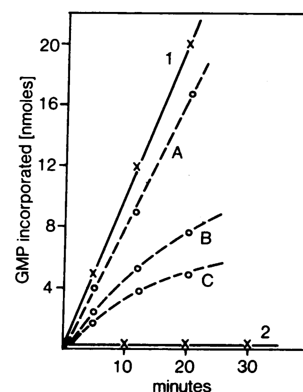

FIG. 6. Kinetics of RNA synthesis directed by ST-RNA (curves 1 and 2) or a "T_1-resistant" RNA (curves A, B, and C) in the absence or presence of ribonuclease T_1. The standard incubation mixture (without dithiothreitol) contained 6 units of α-less replicase (stage VII), 0.15 mM nucleotides ([^{14}C]GTP, specific activity 2.5 Ci/mol) and in addition 0.5 μg of ST-RNA or resistant RNA. Ribonuclease T_1 was added as follows: curve 1 and A: no ribonuclease T_1; curve 2 and B: 2 μg/ml of ribonuclease T_1; curve C: 4 μg/ml of ribonuclease T_1. The resistant RNA was obtained by incubating Qβ replicase (stage VII) in the standard incubation mixture (0.5 mM nucleoside triphosphates, but without dithiothreitol) in the presence of 2 μg/ml of ribonuclease T_1 for 8 hr at 30°.

and nonrepetitive structures. A puzzling fact is the observation that under optimal conditions (high substrate and enzyme concentration)—but only at these—the products formed in separate but otherwise identical template-free RNA synthesis experiments give very similar fingerprint patterns. On the other hand, these RNA products have various replication kinetics, which indicates that the sequences cannot be identical. Moreover, the interpretation of the fingerprint patterns is hampered by the fact that usually more than half of the radioactive label remains in the core material, even though heat-denatured ST-RNAs were used. Nonetheless, these similarities of the fingerprints demand some sort of instruction upon the outcome of the RNA sequence. How this can be achieved in the absence of a template for RNA molecules as long as 200 nucleotides is very difficult to imagine, but there are several observations favoring the idea that Qβ-replicase protein itself could exert this influence.

This enzyme contains several subunits with specific RNA recognition sites. The viral subunit β is responsible for the template specificity of the enzyme, since the analogous RNA replicases containing the same host subunits but a different viral subunit exhibit a different template specificity. Another subunit of Qβ replicase, the protein synthesis elongation factor EF·Tu, forms specific ternary complexes with GTP and aminoacylated tRNAs (20) and ensures the proper binding of the tRNA to the ribosome. Possibly the subunits β and EF·Tu favor the outcome of nucleotide sequences containing the elements of recognition. Studying the sequence of one ST-RNA published by Mills *et al.* (13), we were impressed by the high abundance of the nucleotide sequence UUCG and its complement CGAA. UUCG appears as often as seven times in this RNA and is located in the unpaired regions of the molecule. Remarkably, this sequence UUCG is common to most tRNAs (as TΨCG in the "TΨC loop") and involved in the binding process of tRNA to the ribosome (21–23), which is controlled by EF·Tu. Assuming a random polymerization to oligomers for the first phase of the *de novo* synthesis as postulated for other polymerases (24), Qβ replicase would probably make a preferential use of certain oligonucleotide sequences for the final assembly of RNA molecules. Such a discrimination would limit the number of possible sequences. Moreover, during the replication phase the produced sequences are subjected to a strong selection. First, only self-replicating RNAs (plus and minus strands are recognized by the enzyme) can multiply. The more specific the recognition mechanism the higher is this sort of selection pressure. Second, from several self-replicating RNAs produced simultaneously during the lag phase, the fastest replicating species outgrows its competitors. These ideas might explain the similarity of RNAs produced under the optimal conditions and would also predict the experimental conditions resulting in the *de novo* synthesis of new RNA types: (*1*) Suppression of ST-RNA growth (experiments of Figs. 5 and 6) or (*2*) a supply of changed oligonucleotide sequences, e.g., caused by the omission of one nucleoside triphosphate in the preincubation phase (experiments of Fig. 4) or by the external addition of oligonucleotides (see *Results*).

As shown by our experiments, oligonucleotides influence the outcome of the final RNA species. Though the presence of a contaminating template in our enzyme preparation was ruled out, the possibility of a contamination by an oligonucleotide still exists. This possibility might be another basis to explain the similarities of RNA species produced under optimal conditions.

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