resembles the theoretical curve R the better the more points on curve A are used (the smaller the segments are). The method is generally applicable.

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RNA Replication: Required Intermediates and the Dissociation of Template, Product, and Qβ Replicase†

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ABSTRACT: Replication complexes containing only one molecule of Qβ replicase and one strand of midivariant RNA (MDV-1 RNA) template were prepared by incubating the replicase with an excess of MDV-1 (−) RNA. In the presence of excess minus strands, these monoenzyme replication complexes were shown to synthesize essentially pure MDV-1 (+) RNA in both the first and second cycles of replication. When an equivalent concentration of mutant MDV-1 (−) RNA was added to this reaction before completion of the first cycle of replication, only wild-type MDV-1 (+) RNA was produced in the first cycle, but both mutant and wild-type MDV-1 (+) RNA were produced in the second cycle of replication. These results demonstrate that a monoenzyme complex is competent to synthesize RNA and, therefore, that a multienzyme replication complex is not a necessary intermediate of replication. The data also imply that after the completion of chain elongation, the product strand is released from the replication complex and that the template and the replicase then dissociate.

This paper concerns several unresolved questions about the mechanism of RNA replication. The interest in RNA replication, catalyzed by the discovery of single-stranded RNA bacterial viruses (Loeb & Zinder, 1961), was dominated by a model derived from the known mechanism of replication of single-stranded DNA bacteriophages. Thus, it was assumed that the first step in RNA replication would be the synthesis of a strand complementary to the viral RNA. This would result in the formation of double-stranded intermediates (designated RF-I), which would then be converted into multistranded intermediates (designated RF-II), consisting of duplexes generating nascent viral "plus" strands. In this model, both RF-I and RF-II are required intermediates, with RF-I

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preceding RF-II and, further, every RF-II would contain one complete copy of both the plus and the "minus" strands.

Supporting evidence for this model was apparently found in the detection of ribonuclease-resistant structures in infected cells (Kelly & Sinsheimer, 1964; Kaerner & Hoffmann-Berling, 1964; Weissmann et al., 1964; Erikson et al., 1964; Ammann et al., 1964; Nonoyama & Ikeda, 1964) and in vitro (Mills et al., 1966; Bishop et al., 1967b). However, doubts arose when these structures were examined with respect to their composition and their origin. Some of the difficulties encountered (Spiegelman et al., 1968; Weissmann et al., 1968) were as follows. (1) If the infected cell extracts or in vitro reactions were treated with ribonuclease prior to deproteinization, no ribonuclease-resistant structures were found. (2) When isolated, neither the double- nor the multistranded complexes were able to infect protoplasts or serve as templates for Qβ replicase. However, heating these structures to temperatures required to melt them restored both activities. (3) Contrary to the expectation from the DNA model, the multistranded structures did not contain complete copies of both plus and minus strands but, rather, consisted of a complete copy of the template and incomplete copies of its complement.

The first two findings implied that the ribonuclease-resistant structures did not preexist, as such, but were artifacts created during the phenol deproteinization procedure commonly employed in their isolation (Feix et al., 1967). Thus, although they might provide useful information by virtue of being derivatives of the true replicative intermediates, the hydrogen-bonded complexes played no role in the replication process. The third finding showed that the particular model derived from ϕX-174 DNA duplication was not applicable to RNA replication.

We therefore avoided the RF-I and RF-II nomenclature (Mills et al., 1966; Bishop et al., 1967b) in favor of the more neutral designations of "HS" for the double-stranded structures and "FS" for the multistranded structures. This gave credit to the investigators who had characterized these structures (Hofschneider and Franklin) and, at the same time, deferred any commitments as to their replicative roles.

The finding that free negative strands appeared first in reactions templated by plus strands, along with the demonstration that free negative strands are excellent templates for Qβ replicase (Feix et al., 1968), led to the proposal of a five-intermediate mechanism for RNA replication (Spiegelman et al., 1968; Weissmann et al., 1968). In this model, the original viral strand serves as template for the synthesis of a minus strand, forming a two-stranded complex. Before the first minus strand is completed, another replicase molecule starts a new minus strand product on the same template, leading to the appearance of multistranded structures. During synthesis, template and product strands are essentially single stranded. Ultimately, free minus strands are released and the process is repeated with these strands serving as templates. The resulting complexes contain a complete minus strand and partially polymerized plus strands, which are completed and ultimately released as mature infectious strands. This model accounted for the array of structures that had been observed and for the order of their appearance. It also explained the kinetics of the appearance of the minus strands, followed by the appearance of free plus strands (Spiegelman et al., 1968).

However, this mechanism raises certain questions. The discarded DNA model contained no ambiguities about the multistranded intermediates—they were mandatory and could be generated by complexes containing a single replicase molecule. In the five-intermediate model, the formation of multistranded complexes requires the participation of more than one enzyme molecule per complex, but it is by no means certain that a multistranded complex is required for the completion of the replicative cycle. To settle this issue, we designed and performed experiments that answered the following questions. Are the multistranded structures required intermediates in the replicative process? Can a single replicase molecule complete the entire replicative cycle, and in particular, can it dissociate from the complex?

To determine whether a multienzyme complex is a necessary intermediate, we performed experiments under conditions of template excess, in which only monoenzyme complexes were present. Nucleotide sequence analysis of the product RNA was employed to detect the recycling of replicase during and after strand completion. The template used was midivariant RNA (MDV-1 RNA), which consists of two complementary chains (Kacian et al., 1972), both of which serve as excellent templates for Qβ replicase. The fact that the complete nucleotide sequences of MDV-1 (+) RNA and MDV-1 (−) RNA are known (Mills et al., 1973) makes them particularly useful for these experiments.

The results demonstrate that a single replicase molecule can carry out a complete cycle of replication and that multistranded structures are not required intermediates in RNA replication. These experiments also suggest that, at the end of each replicative cycle, the complex releases the product RNA before the replicase dissociates from the template.

Materials and Methods

Qβ replicase was isolated from Qβ bacteriophage-infected *Escherichia coli* Q13 by the procedure of Eoyang & August (1971), with the hydroxylapatite step omitted. [α-^{32}P]-Ribonucleoside triphosphates were obtained from ICN. Ribonuclease T_1 was purchased from Calbiochem, as was yeast RNA, which was purified by phenol extraction prior to use. Pancreatic ribonuclease A was obtained from the Worthington Biochemical Corp. Poly(cytidylic acid) (6–13 S) was purchased from P-L Biochemicals, Inc., and proteinase K was purchased from EM Laboratories, Inc.

MDV-1 RNA, originally isolated from a Qβ replicase reaction that contained no exogenous template (Kacian et al., 1972), was synthesized and isolated as previously described (Kramer et al., 1974). "Mutant MDV-1 RNA" is a triply mutated molecule that was the predominant species in the 20th serial transfer tube of a Darwinian selection experiment (Kramer et al., 1974). The mutant MDV-1 RNA used in these experiments was synthesized and isolated under the same conditions as was the wild-type MDV-1 RNA.

Separation of the complementary strands of wild-type MDV-1 RNA and of mutant MDV-1 RNA was achieved by polyacrylamide slab gel electrophoresis of the RNA in the presence of magnesium ions (Mills et al., 1978).

Nucleotide sequence analysis of [^{32}P]MDV-1 RNA was accomplished by two-dimensional, high-voltage electrophoresis of its ribonuclease T_1 digestion products. Digestions were performed by dissolving the [^{32}P]RNA in 50–100 μL of 500 μM ethylenediaminetetraacetic acid (EDTA), 20 mM triethylammonium carbonate (pH 7), 200 units of ribonuclease T_1/mL, and 300 μg of yeast RNA/mL, followed by incubation for 150 min at 37 °C. The resulting digest was lyophilized and then analyzed by the methods of Sanger and his colleagues (1965), with an altered first dimension buffer (C. Woese, personal communication).

Monoenzyme replication complexes were formed by the incubation of Qβ replicase in the presence of a fivefold excess

of MDV-1 RNA template. Since not all of the replicase in the preparation we used was active, the template-to-replicase ratio was effectively higher. The complexes were formed at 37 °C in 12 mM $MgCl_2$ and 84 mM Tris-HCl (pH 7.4) in the presence of GTP and ATP (Billeter et al., 1969). Since CTP and UTP were not present, only the first few nucleotides at the 5′ end of the product strand were synthesized. Elongation was blocked where the first pyrimidine nucleotide was required, halting all complexes at the same point. The subsequent addition of CTP and UTP removed the elongation block, permitting the monoenzyme complexes to continue replication.

Replicase activity after formation of monoenzyme replication complexes was determined in a 1-mL reaction in which the concentrations of the components were 12 mM $MgCl_2$, 84 mM Tris-HCl (pH 7.4), 200 μM ATP, 100 μM [α-^{32}P]GTP, 1 μg of Qβ replicase/mL, and 10 μg of MDV-1 RNA/mL. Prior to its use as template, the RNA was incubated in water at 100 °C for 1 min, followed by quick-freezing in a dry ice–methanol bath. This reaction was incubated for 4 min at 37 °C to permit the monoenzyme replication complexes to form. Four hundred microliters was transferred to a tube that contained 800 ng of polycytidine (time 0), and incubation was continued at 37 °C. At various times after the addition of the polycytidine, samples were taken from the original reaction and from the one containing polycytidine. Each sample was mixed with 3 mL of cold 5% trichloroacetic acid. At 12 and 12.5 min, respectively, CTP and UTP were added (each to 200 μM final concentration) to the reaction containing just MDV-1 RNA template and to the reaction that also contained polycytidine. Sampling continued after these additions. The amount of labeled GTP incorporated in each sample was determined by trichloroacetic acid precipitation. Nearest-neighbor analysis by alkaline hydrolysis (Bock, 1967) and thin-layer chromatography (Randerath & Randerath, 1967) were used to detect the synthesis of polyguanosine.

The recycling of the replicase and the fate of the product strand were observed in a reaction in which monoenzyme replication complexes were formed by incubating 2.5 μg of MDV-1 (−) RNA and 0.5 μg of Qβ replicase for 4 min at 37 °C in 200 μL that contained 12 mM $MgCl_2$, 84 mM Tris-HCl (pH 7.4), 125 μM ATP, and 100 μM GTP. The ratio of template RNA to active replicase was so high that, when all of the complexes were formed, there were 500 uncomplexed strands for every complexed strand. No radioactive ribonucleoside trisphosphate was present during complex formation, and thus the 5′ end of the product strands synthesized during formation of the complexes was not labeled. After complex formation, 4 nmol of [α-^{32}P]GTP was added. The elongation block was removed 15 s later by the addition of CTP and UTP (each to 125 μM final concentration), and incubation was continued at 37 °C. Thus, the remaining portion of the product of the first cycle of replication was labeled as it was synthesized. Only the product of subsequent cycles of replication was labeled at its 5′ end. Samples were taken at various times after removal of the elongation block and terminated by quick-freezing. Each sample was brought to 200 μL containing 400 mM NaCl, 17 mM EDTA–NaOH (pH 7), and 50 μg of yeast RNA/mL. The RNA in each sample was then isolated as previously described (Kramer et al., 1974). Each RNA was suspended in 100 μL of 97% formamide, 3 mM EDTA, 10 mM sodium phosphate (pH 7) and then melted for 1 min at 100 °C and quick-frozen. It was then analyzed by polyacrylamide gel electrophoresis (Bishop et al., 1967a), and the full-sized product RNA was recovered from the gel for nucleotide sequence analysis.

In the fingerprint pattern of the full-sized product RNA, the presence of the labeled 5′-end nucleotide, pppGp, signals the presence of RNA strands that were completed during the second cycle of replication. The concurrent appearance of oligonucleotides unique to MDV-1 (−) RNA would indicate that the replicase used the MDV-1 (+) RNA synthesized in the first cycle of replication as template in the second cycle of replication.

The fate of the template strand was determined in a similar reaction in which monoenzyme replication complexes were formed by incubating 1.24 μg of wild-type MDV-1 (−) RNA and 0.5 μg of Qβ replicase for 3 min at 37 °C, and then for 1 min at 4 °C, in 100 μL that contained 12 mM $MgCl_2$, 84 mM Tris-HCl (pH 7.4), 248 μM ATP, and 99 μM GTP. CTP was then added (348 μM final concentration) and incubation was continued for 20 min at 4 °C, allowing polymerization to proceed to the 28th nucleotide, where the first uridine is required. The ratio of template RNA to active replicase was such that there were 300 uncomplexed strands for every complexed strand. The reaction mixture was then combined with 98 μL of a solution containing 1.25 μg of mutant MDV-1 (−) RNA, 30 μM [α-^{32}P]GTP, and the same concentration of $MgCl_2$ and Tris-HCl, at 4 °C. Thus, the pool of uncomplexed template in the reaction was altered so that it contained approximately equal amounts of mutant and wild-type MDV-1 (−) RNA. One minute later, the elongation block was removed by the addition of UTP (125 μM final concentration), and incubation was continued at 37 °C. Thus, the remaining portion of the product (after the 28th nucleotide) of the first cycle of replication was labeled as it was synthesized. Only the product of subsequent cycles of replication was labeled at its 5′ end. As in the previous experiment, samples were taken at various times and the RNA was isolated and sequenced as described above.

The elongation block was moved further toward the 3′ end of the product of the first replicative cycle so that two ribonuclease T_1 oligonucleotides, pppGp and ACCCCCCGp, located at the 5′ end of the product strand, would only be labeled in the product of the second cycle of replication. The concurrent appearance of the oligonucleotides unique to mutant MDV-1 (+) RNA would indicate that mutant MDV-1 (−) RNA that was not used in the first cycle of replication was used as template in the second cycle.

This experiment was repeated with mutant MDV-1 (−) RNA serving as the template for the formation of the blocked monoenzyme replication complexes and with wild-type MDV-1 (−) RNA serving as the RNA added just before removal of the elongation block.

The relative template activity of the mutant and wild-type MDV-1 (−) RNA preparations that were used in these experiments was determined in a reaction in which monoenzyme replication complexes were formed by incubating 0.62 μg of mutant MDV-1 (−) RNA, 0.62 μg of wild-type MDV-1 (−) RNA, and 0.5 μg of Qβ replicase for 2 min at 37 °C in 100 μL containing 12 mM $MgCl_2$, 84 mM Tris-HCl (pH 7.4), 250 μM ATP, and 100 μM [α-^{32}P]GTP. The relative amounts of the two RNA types were the same as in the previous experiment, but they were mixed prior to complex formation. The elongation block was removed by the addition of CTP and UTP (each to 250 μM final concentration). Incubation was continued for 4 min at 37 °C, and the reaction was then terminated by quick-freezing. The full-sized product RNA was isolated and sequenced as described above. The relative template activity of the two types of RNA was calculated by

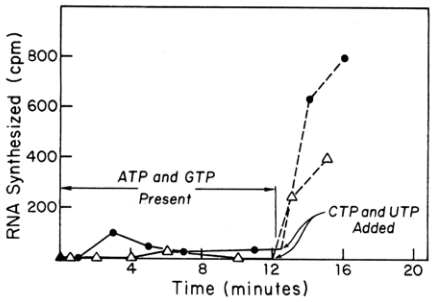

FIGURE 1: Replicase activity after formation of monoenzyme replication complexes. The complexes were formed in the presence of ATP and [α-^{32}P]GTP. Before removal of the elongation block, the reaction was split into two parts (time 0). One part received polycytidine, which will direct any available replicase to synthesize a polyguanosine product (Eikhom & Spiegelman, 1967). Circles show the synthesis in the reaction containing only MDV-1 RNA. Triangles show the synthesis in the reaction that contained both MDV-1 RNA and polycytidine. The lack of polyguanosine synthesis prior to the addition of CTP and UTP indicates that no free replicase remained after the formation of the monoenzyme replication complexes.

comparing the molar recovery of the unique wild-type oligonucleotide and the two unique mutant oligonucleotides with the total moles of product RNA recovered. The mutant MDV-1 (−) RNA had exactly twice the template activity of the wild-type MDV-1 (−) RNA.

Results

Formation of Monoenzyme Replication Complexes. Electrophoretic analyses of replication complexes indicate that when there is an excess of Qβ replicase over MDV-1 RNA template, the predominant intermediate is a multienzyme complex. As the level of replicase is reduced, the number of multienzyme complexes decreases, and becomes undetectable when equivalent amounts of template RNA and replicase are present. Therefore, in the experiments presented here, monoenzyme replication complexes were formed by the incubation of Qβ replicase with greater than a fivefold excess of MDV-1 RNA template. The presence of just the purine ribonucleoside triphosphates during incubation at 37 °C allows these monoenzyme complexes to initiate synthesis and to polymerize the few nucleotides that precede the first pyrimidine (Billeter et al., 1969). During the incubation, all of the active replicase molecules reach this point. When this "elongation block" is removed by the addition of the pyrimidine ribonucleoside triphosphates, chain elongation continues.

Characterization of Monoenzyme Replication Complexes. To see that no uncomplexed active replicase is present after formation of the blocked monoenzyme replication complexes, we observed the kinetics of incorporation of a reaction containing an excess of MDV-1 RNA template (Figure 1). After the initial incubation with ATP and [α-^{32}P]GTP, there was no further incorporation before the elongation block was removed. No additional synthesis was detected when polycytidine, which will direct Qβ replicase to synthesize polyguanosine (Eikhom & Spiegelman, 1967), was added to the reaction. Yet, when the elongation block was removed, synthesis continued in both the presence and absence of polycytidine, and the synthesis of polyguanosine could be detected in the reaction containing polycytidine. Thus, just before removal of the elongation block, these reactions contained virtually no uncomplexed active replicase. These results also confirm the processive nature of the Qβ replicase reaction: the enzyme cannot transfer its activity to another template strand when the product strand is incomplete.

To demonstrate that the replicase contained in a monoenzyme replication complex is able to complete more than one cycle of replication, we prepared a reaction in which the product of the first cycle of replication could be distinguished from the product of the second and subsequent cycles of replication by nucleotide sequence analysis. Blocked monoenzyme replication complexes were prepared using pure MDV-1 (−) RNA and unlabeled ATP and GTP. [α-^{32}P]GTP was added when the elongation block was removed. This reaction was then sampled at various times, and the ^{32}P-labeled product RNA was isolated and analyzed by polyacrylamide gel electrophoresis. The full-length ^{32}P-labeled product RNA from the different samples was eluted from the gels and subjected to nucleotide sequence analysis.

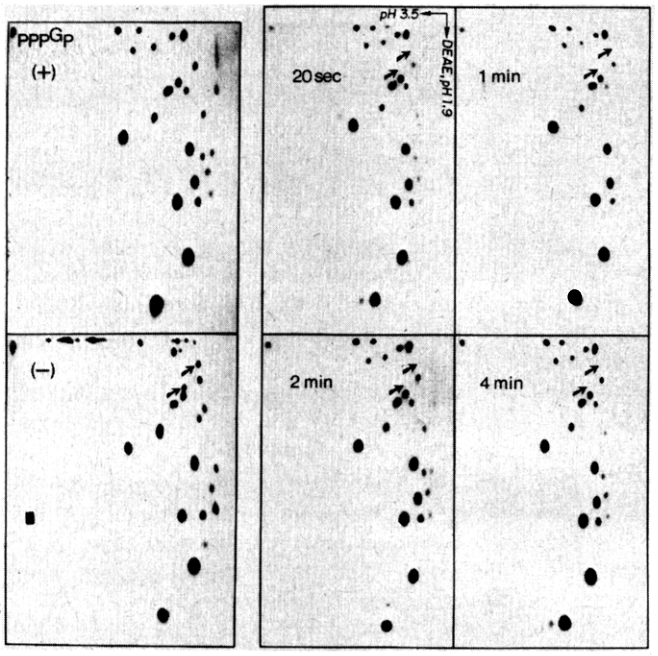

FIGURE 2: Ribonuclease T_1 fingerprint patterns of the product RNA synthesized at various times by monoenzyme replication complexes formed in the presence of an excess of MDV-1 (−) RNA template. The fingerprint patterns of the full-length ^{32}P-labeled product RNA present in the reaction at different times are shown alongside control fingerprint patterns of MDV-1 (+) RNA and MDV-1 (−) RNA. The position of the pppGp nucleotide is indicated in the MDV-1 (+) RNA control. Arrows indicate the positions of two unique MDV-1 (−) RNA oligonucleotides. Unique MDV-1 (−) RNA oligonucleotides do not appear in the later patterns, indicating that virtually no MDV-1 (−) RNA was synthesized in the second cycle of replication.

Figure 2 shows the results of this experiment. The fingerprint pattern of the full-length RNA present in the earliest sample contained very little of the 5′-terminal nucleotide, pppGp. This nucleotide was not labeled in the product of the first cycle, because [α-^{32}P]GTP was not present when the 5′ end of the first-cycle product was polymerized. Thus, the RNA in this fingerprint pattern was primarily the product of the first replicative cycle. The relative increase in the amount of the 5′-terminal nucleotide in the later fingerprint patterns signals the accumulation of full-length product RNA synthesized in subsequent cycles of replication. At 20, 60, 120, and 240 s, the proportion of the product RNA that was synthesized in the second and subsequent cycles was 35, 50, 60, and 80%, respectively. This result demonstrates that monoenzyme complexes are able to complete more than one cycle of replication.

Fate of the Replication Complex Components after Completion of Chain Elongation. It was also possible for us to determine the temporal order of the events that occur after the completion of chain elongation. The fingerprint pattern

of the full-length RNA present in the earliest sample (shown in Figure 2) establishes that, as expected, the product of the first replicative cycle was MDV-1 (+) RNA. Furthermore, the fingerprint patterns from the later samples show that virtually no MDV-1 (−) RNA was produced in the second cycle of replication. Since the amount of MDV-1 (+) RNA synthesized in the first cycle of replication was less than 1% of the amount of MDV-1 (−) RNA that was present in the reaction, no detectable amounts of MDV-1 (−) RNA would be expected to be synthesized in the second cycle if the product strands became part of the pool of available template RNAs. The absence of MDV-1 (−) RNA in the second cycle of synthesis indicates that the product strands acted as if they were simply part of the template pool. This shows that the replicase did not preferentially use the product strand as template in the second cycle of replication.

We performed the next experiment to see if, after the completion of chain elongation, the replicase remains bound to the template strand and then reuses that template in the next cycle of replication. As in the last experiment, blocked monoenzyme replication complexes were formed with MDV-1 (−) RNA template. However, in this experiment, only UTP was absent during the initial incubation, so that the elongation block was located closer to the 3′ end of the product strand. At the time of addition of UTP and [α-^{32}P]GTP, mutant MDV-1 (−) RNA, which differs from the wild-type RNA at three positions in its sequence (Kramer et al., 1974), was also added to the reaction. The amount of mutant MDV-1 (−) RNA added was approximately equal to the amount of wild-type MDV-1 (−) RNA present. Thus, when the elongation block was removed, the monoenzyme replication complexes in the reaction contained only wild-type MDV-1 (−) RNA template, but the RNA that was not complexed with replicase was a mixture of mutant and wild-type MDV-1 (−) RNA. As in the previous experiment, samples were taken at various times after removal of the elongation block and the full-length, ^{32}P-labeled product RNA that they contained was isolated and subjected to nucleotide sequence analysis.

The results are shown in Figure 3. In this experiment there were two 5′-end oligonucleotides that were not labeled in the first cycle of replication, pppGp, as before, and ACCCCCCGp. Neither of these oligonucleotides was present in the fingerprint pattern obtained from the earliest sample, which indicates that only the first cycle of replication had been completed by that time. The important differences between the fingerprint pattern of the mutant MDV-1 (−) RNA and that of the wild-type MDV-1 (−) RNA are that the mutant fingerprint pattern contains two unique oligonucleotides not found in the wild-type pattern and that the wild-type pattern contains one unique oligonucleotide not found in the mutant pattern. The fingerprint pattern of the earliest sample showed that the product of the first cycle of replication was exclusively wild-type MDV-1 (+) RNA, as expected.

If the replicase remained bound to the template strand and then used it again as template in the second cycle of replication, the product would continue to be wild-type MDV-1 (+) RNA, despite the presence of mutant template in the reaction. If, on the other hand, the replicase dissociates from the template, as we have already shown it does from the product, then it should select a new template for the next cycle of replication from the available pool of uncomplexed mutant and wild-type MDV-1 (−) RNA.

The fingerprint patterns of the full-length RNA in the later samples showed the presence of the two 5′-end oligonucleotides, pppGp and ACCCCCCGp, that signal the completion of RNA

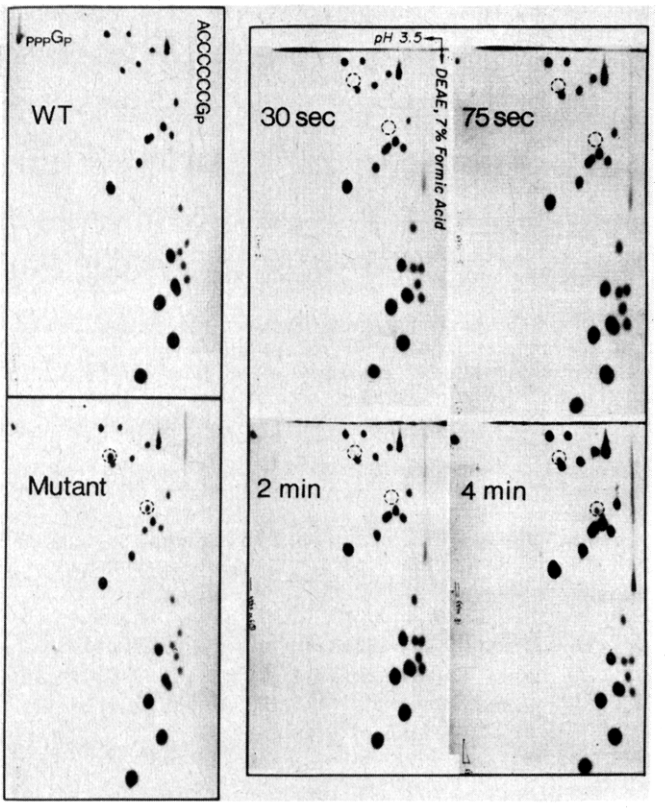

FIGURE 3: Ribonuclease T_1 fingerprint patterns of the product RNA synthesized at various times after the addition of mutant MDV-1 (−) RNA template. The fingerprint patterns of the full-length, ^{32}P-labeled product RNA present in the reaction at different times are shown alongside control patterns of wild-type MDV-1 (+) RNA and mutant MDV-1 (+) RNA. The positions of the pppGp nucleotide (spot) and the ACCCCCCGp oligonucleotide (streak) are indicated in the wild-type MDV-1 (+) RNA control pattern. Circles indicate the positions of the two unique mutant oligonucleotides.

strands synthesized in the second cycle of replication. At 75, 120, and 240 s, the proportion of the product RNA that was synthesized in the second cyle was 20, 34, and 42%, respectively. The appearance of the unique mutant oligonucleotides in these patterns showed that mutant, as well as wild-type, MDV-1 (−) RNA was used as template in the second cycle of replication. This experiment was repeated with the roles of the mutant and wild-type RNAs reversed. Wild-type MDV-1 (−) RNA was added after formation of monoenzyme replication complexes with mutant MDV-1 (−) RNA. Mutant MDV-1 (+) RNA was the exclusive product of the first cycle of replication and both wild-type and mutant MDV-1 (+) RNA were synthesized in the second replicative cycle. These results demonstrate that, after the completion of chain elongation, the replicase is able to dissociate from the template, as well as from the product strand, before choosing another template RNA.

Template Selection after Completion of Chain Elongation. In these experiments, we were also able to see whether the replicase prefers to use the same template strand in consecutive cycles of replication. If the replicase has no tendency to reuse a template strand, then mutant MDV-1 (−) RNA would have been chosen just as frequently as wild-type MDV-1 (−) RNA for use as template in the second cycle of replication. The relative amounts of mutant and wild-type MDV-1 (−) RNA template that were used in the second cycle of replication in these experiments was determined by measuring the relative amounts of the two types of MDV-1 (+) RNA produced.

The proportions of mutant and wild-type MDV-1 (+) RNA produced in the second cycle of replication were readily es-

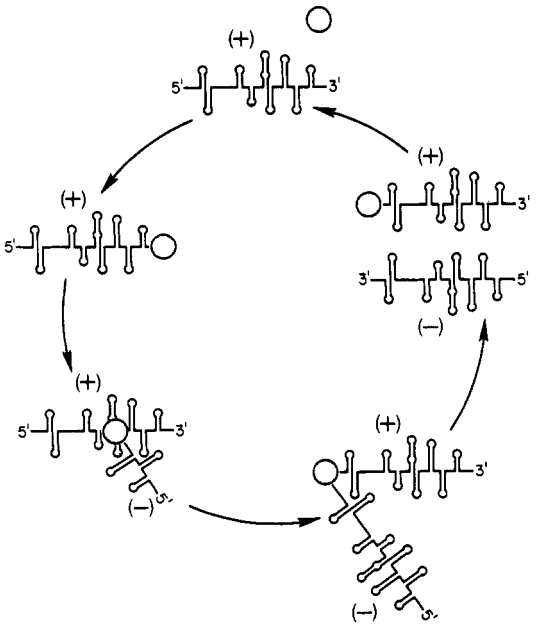

FIGURE 4: Replicative cycle. This scheme illustrates the formation and dissociation of the replication complex. A circle represents the replicase, a (+) denotes the template strand, and a (−) denotes the complementary product strand. This figure does not show the binding of the replicase to the middle of the template, prior to chain initiation.

tablished from the fingerprint patterns. For example, in the experiment in which mutant MDV-1 (−) RNA was added after wild-type MDV-1 (−) RNA, a comparison of the molar amounts of the unique mutant oligonucleotides to the molar amounts of the 5′-end oligonucleotides (which are common to both mutant and wild-type MDV-1 (+) RNA) yielded the percentage of the strands made during the second cycle that was mutant MDV-1 (+) RNA. To better reflect the way the replicase chose its template for the second replicative cycle, we corrected these percentages for the relative template activity of the mutant and of the wild-type MDV-1 (−) RNA used in these experiments.

The results of these measurements and calculations indicate that when mutant RNA was added after wild-type RNA, only 24% of the second cycle product was mutant, rather than the expected 50%. In the experiment where wild-type RNA was added after mutant RNA, only 37% of the second cycle product was wild type. Thus, the template type that was initially present in the replication complexes was used more frequently in the second cycle of replication. These results imply that, after each cycle of replication, the replicase often reassociates with the template strand it has just used.

Discussion

Monoenzyme replication complexes can be formed by using an excess of template RNA. The results of the polycytidine experiment indicate that the replicase in the complex cannot transfer its activity to another template when the synthesis of the product strand is initiated but not completed, confirming the processive nature of the Qβ replicase reaction.

The subsequent experiments show that the replicase contained in the complex is able to complete more than one cycle of replication. Therefore, a multienzyme complex is not a required intermediate for replication.

Replication apparently proceeds in the same fashion with one replicase molecule on a strand of template RNA as with more than one. In particular, a second replicase molecule is not required for the release of the product strand, nor is the presence of more than one replicase necessary to prevent the reassociation of the template and product strands within the replication complex. Reassociation is probably prevented by the formation of secondary structure in the product and template strands during chain elongation (Mills et al., 1978).

The results also suggest a particular scheme for the dissociation of the replication complex after the completion of chain elongation. It has been shown that, at the end of the replicative cycle, the replicase does not retain the product strand for use as template in the next cycle of replication. It has been shown that although the replicase can select any uncomplexed RNA for its next template at the end of each replicative cycle, it sometimes reuses the same template strand. In contrast, it ignores the product strand it has just synthesized. From these observations we infer that the product strand is released from the replication complex before the replicase and the template strand are able to dissociate.

Figure 4 shows a schematic presentation of RNA replication which incorporates the details that have been inferred from these results: the replicase binds a strand of template RNA to form the complex; synthesis of the product strand is initiated and chain elongation occurs; after the completion of chain elongation, the product strand is released from the replication complex; the replicase is then able to dissociate from the template strand and begin a new replicative cycle by binding a template strand.

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Effect of Estrogen on Preprolactin Messenger Ribonucleic Acid Sequences†

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ABSTRACT: A complementary DNA (cDNA) probe has been synthesized for rat preprolactin messenger RNA (mRNA). Following preparative gel electrophoresis, the cDNA was characterized and estimated to be ~75% pure. The reverse transcript is estimated to be greater than 1000 nucleotides long and, therefore, is a full-length copy of the preprolactin mRNA. It is a faithful transcript, as evidenced by back-hybridization with the mRNA. By use of the cDNA as a hybridization probe, the levels of preprolactin mRNA in the pituitary of male and female rats were monitored and found to increase following estrogen treatment. Increases in preprolactin mRNA activity, assayed by in vitro translation in the wheat germ system, were paralleled by increases in hybridization to the cDNA probe after estrogen treatment.

Prolactin synthesis in the rat pituitary is responsive to estrogens (Gersten & Baker, 1970; Lu et al., 1971; Maurer & Gorski, 1977). Preprolactin messenger RNA (pPRL mRNA) from rat pituitaries and its translation product have been partially characterized (Maurer et al., 1976, 1977; McKean & Maurer, 1978). Induced prolactin synthesis is closely paralleled by corresponding increases in pPRL mRNA activity (Stone et al., 1977). Whether this represents an increase in the number of pPRL mRNA molecules or the liberation of a preexisting, formerly untranslatable pool of pPRL mRNA has yet to be demonstrated. The use of molecular hybridization allows the quantitation of specific mRNA within a total RNA population. This approach has shown that increases in translatable mRNA following hormone stimulation are accompanied by increased hybridization to a cDNA probe (McKnight et al., 1975; Shapiro & Baker, 1977; Harris et al., 1975; Evans et al., 1978; Martial et al., 1977; Matusik & Rosen, 1978). We have synthesized a cDNA probe to rat pPRL mRNA and used it to monitor pPRL mRNA levels in the pituitary of estrogen-induced male and female rats. The number of pPRL mRNA sequences within the pituitary increases after estrogen treatment.

Experimental Procedure

Preparation of cDNA. Essentially the method of Kacian & Myers (1976) was used to synthesize cDNA in the initial experiments. Each reaction volume was 50 μL and contained the following final concentrations: 50 mM Tris-HCl, pH 8.3, 8 mM $MgCl_2$, 50 mM KCl, 1 mM dithioerythritol, 0.2 mM dATP, 0.2 mM dGTP, 0.2 mM dTTP, 0.2 mM [^{3}H]dCTP (2.5 Ci/mmol) or 0.2 mM [^{32}P]dCTP (15 Ci/mmol), 4 mM sodium pyrophosphate, 25 μg/mL oligo(dT)$_{12-18}$, 30–60 μg/mL mRNA, and 60 units/mL reverse transcriptase. For preparative syntheses, the following modifications were made: 0.015 mM [^{32}P]dCTP (50 Ci/mol) and 320 units/mL reverse transcriptase.

Reactions were initiated by the addition of enzyme and allowed to incubate for 75 min at 37 °C. The microliters of 1 N NaOH were then added, and the mixture was incubated at 68 °C for 30 min. After neutralization with acetic acid, the solution was extracted with 2 volumes of phenol–chloroform–isoamyl alcohol (100:98:2). The aqueous phase was removed and the organic phase reextracted with 0.5 volume of sterile water. The aqueous phases were pooled and chromatographed on Sephadex G-100 to remove unreacted [^{32}P]dCTP from the cDNA. To the eluted cDNA pool was added 2.5 volumes of 80% EtOH–20% 0.2 M NaCl, and the solution was placed at −20 °C overnight. The cDNA was pelleted by centrifugation, redissolved in 100 μL of sterile water, and stored at −20 °C.

Preparative Gel Electrophoresis. Typically, 100 μL of cDNA in sterile water was combined with 50 μL of a solution containing 0.2% sodium dodecyl sulfate ($NaDodSO_4$), 100 mM EDTA, 50% glycerol, 0.2 mg/mL bromophenol blue (BPB), and 0.6X Peacock's buffer (Peacock & Dingman, 1968). The mixture was heated in a boiling water bath for 2 min and applied to a 4%:10% composite acrylamide slab gel (Peacock & Dingman, 1968). Electrophoresis was begun at 50 V and continued until the BPB dye had migrated approximately 1 cm into the 4% gel. The remainder of the run was carried out at 120 V, until the bromophenol blue had migrated 12 cm. The gel was then covered with saran wrap

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