*REPLICATION OF VIRAL RNA, XVI. ENZYMATIC SYNTHESIS OF INFECTIOUS VIRAL RNA WITH NONINFECTIOUS Q_β MINUS STRANDS AS TEMPLATE**

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Earlier studies on the replication of the RNA[1] of RNA phages, both *in vivo* and *in vitro*, provided evidence that a viral minus strand, i.e., an RNA strand complementary to the one found in the phage particle (the plus strand) was formed early in replication and served as template for the synthesis of progeny RNA (for references, see ref. 2). Spiegelman and his colleagues[3, 4] purified a viral RNA polymerase from *E. coli* infected with the RNA phage Q_β and showed that this enzyme preparation (Q_β replicase) utilized infectious Q_β RNA as template for the synthesis of more infectious viral RNA.[4, 5] On the basis of their studies they suggested that minus strands were neither formed nor required in this reaction.[6, 7] However, a reinvestigation of the Q_β replicase system in this laboratory[8] clearly showed that in the early phase of the *in vitro* reaction, minus strands were synthesized almost exclusively, while plus strands were detected only after several minutes of incubation. More recent experiments by Spiegelman and his collaborators led these authors to similar conclusions.[9, 10]

While it is commonly held that minus strands occur as part of a double-stranded helix, hydrogen bonded to plus strands, we have suggested that the replicating complex may, in its native state, contain the template and the nascent product in a predominantly single-stranded[11] state (i.e., not extensively hydrogen-bonded to each other), possibly held together by the enzyme or by short hydrogen bonded regions or both. Following treatment with phenol or sodium dodecylsulfate, the structure apparently rearranges to become largely double-helical.[12, 13] It is relevant to note in this connection that, whereas species of double-stranded Q_β RNA ("replicative form," "replicative intermediate") extracted from Q_β-infected *E. coli* are not able to prime Q_β replicase *in vitro*,[9, 14] the same material after heat denaturation is an excellent template for the enzyme.[14] Under these conditions, synthesis of the product is directed preferentially by the minus strand in the first minutes of incubation.

The above observations made it desirable to study the behavior of isolated minus strands, and we therefore undertook the isolation and purification of Q_β minus strands.[15] We now show that, on incubation with Q_β replicase and nucleoside triphosphates, noninfectious minus strands direct a rapid synthesis of infectious viral RNA in substantial excess over the added template.

Materials and Methods.—Q_β replicase: The enzyme was prepared essentially according to Spiegelman and collaborators,[3, 4] including the CsCl and sucrose-density gradient centrifugations. The latter was carried out as detailed earlier.[13] The specific activity of the enzyme used in these experiments was 15 (mμmoles UMP incorporated/mg/min). No nuclease activity was detected using the criteria previously described.[13] Contamination of the enzyme preparation by virus was less than 2500 PFU/mg.

TABLE 1. *Infectivity of different viral RNA preparations before and after heating.*

RNA	Infectious units (PFU $\times 10^{-6}/\mu g$) Not heated	Heated 90 sec at 100°
1. Q_β plus strands	1650	1200
2. Q_β minus strands (step 6)	<0.001	0.19
3. Double-stranded Q_β RNA	<0.001	130

Aliquots containing about 0.06 μg of RNA in 0.2 ml of 0.003 *M* EDTA were assayed for infectivity either directly or after heating.

Phage RNA: Labeled and unlabeled phage were prepared[8, 16] and the RNA was extracted as described elsewhere.[8, 17]

Partially double-stranded viral RNA: Partially double-stranded MS2 and Q_β RNA were prepared as described earlier.[14] These preparations contained both "replicative form," presumed to be a double-helix containing a plus and a minus strand,[16, 18–20] and "replicative intermediate," described as a double-stranded core with tails of nascent plus strands attached.[18, 21] They will be designated as "double-stranded RNA" for the sake of simplicity. After digestion with RNase under standard conditions,[22] 78% of the Q_β preparation and 67% of the MS2 preparation remained acid-insoluble. After heat denaturation, more than half of the MS2 and one third of the Q_β preparation sedimented as expected for full-length viral RNA (27*S* and 30*S*, respectively).

Q_β minus strands: Minus strands were prepared as previously described,[15] with addition of one further purification step. A step 5 preparation (52 μg RNA) was dialyzed against 0.5 mM EDTA–0.1% sodium dodecylsulfate (SDS) for 12 hr at 4°, concentrated to about 0.15 ml by lyophilization, and centrifuged through a 5–23% linear sucrose gradient in 0.05 *M* Tris-HCl (pH 7.6)–0.1% SDS for 120 min, at 4° and 65,000 rpm in the Spinco SW65 rotor. The leading fractions of the 30*S* band containing the single-stranded RNA were pooled, dialyzed for 16 hr against 3 changes of 0.5 *M* EDTA, pH 7, and concentrated by lyophilization (step 6 preparation). No infectious RNA was found in this preparation (Table 1, expt. 2; see also Table 3), although a specific infectivity 10^{-5} that of Q_β plus strands could have been detected. However, since residual plus strands were separated from single minus strands (steps 5 and 6) after conversion into double-stranded form through self-annealing, some double-stranded RNA containing infectious plus strands might still contaminate the minus strand preparation. This was indeed the case but the contamination was small. After heating samples under conditions known to denature double-stranded Q_β RNA without substantial loss of infectivity of the plus strands (Table 1, expts. 1 and 3), some infectivity (about 10^{-4} that of plus strands) appeared in the step 6 preparation (Table 1, expt. 2).

Incubations with Q_β replicase: Incubations were carried out at 37°, with $MgCl_2$, 12.8 mM; Tris-HCl buffer, pH 7.4, 84 mM; ATP, UTP, GTP, and CTP (one of which had P^{32} label), each 0.8 mM; replicase 0.1 mg/ml, and template, added as specified. Acid-insoluble radioactive RNA and RNase-resistant labeled RNA were determined as described earlier.[22, 23]

Determination of infectious RNA: Samples (15 μl) were mixed with 1 μl of SDS (1.5%) and 1 μl of pronase (previously incubated for 2 hr at 37°; 7.5 mg/ml in 0.15 *M* EDTA, pH 7). An aliquot (10 μl) was drawn into a 10-μl micropipette and incubated for 30 min at 35°.[4] The pronase-digested sample (containing 0.05–0.1 μg of RNA) was mixed with 3 mM EDTA, pH 7.0 (0.55 ml) and then added to 0.55 ml of *E. coli* K12W6 spheroplasts. Purified RNA preparations were assayed without the pronase digestion. The preparation of spheroplasts and the assay of RNA infectivity was carried out as described by Strauss.[24] One μg of Q_β RNA gave rise to 10^8–10^9 PFU.

Determination of base composition: P^{32}-labeled RNA (20 μg, 20,000 cpm) and 0.25 mg of ribosomal RNA, added as carrier, were digested for 20 hr at 37° in 0.2 *M* acetate buffer, pH 4.5 (0.2 ml) with heat-treated Takadiastase[25] (15 units of RNase activity). After adding 10 μl of 1.5 *N* HCl, aliquots (4000 cpm) were spotted on Whatman 3 MM paper strips (4 × 96 cm) and the nucleotides were separated by electrophoresis (3 hr at

42 v/cm) in a mixture of acetic acid, pyridine, and H_2O (10:1:89), pH 3.5. The spots were cut out and the radioactivity was determined by scintillation counting in Liquifluor (Packard Instrument Co.).

Other materials: Heated Takadiastase was a gift of Mr. H. Schwam of this department. Other reagents were obtained from the sources indicated earlier.[13, 14, 23]

Results.—Properties of purified minus strands: The electrophoretic pattern, the sedimentation profile, and the annealing behavior of purified minus strands have been described.[15] With H^3-labeled MS2 RNA as a density marker (ρ= 1.626 gm/cm^3),[26] Q_β plus strands were estimated to have a buoyant density of 1.627, and Q_β minus strands 1.617 gm/cm^3. The base composition of Q_β plus and minus strands, given in Table 2, is in good agreement with the assumption that the complementarity relationship postulated for the two strands of a DNA double-helix is also applicable to the two viral strands capable of forming a hydrogen-bonded RNA double-helix.[27]

TABLE 2. *Nucleotide composition of P^{32}-labeled Q_β plus and Q_β minus strands.*

Nucleotide	P^{32}-Q_β plus strand (Moles %)	P^{32}-Q_β minus strand (Moles %)
AMP	22.9 ± 0.2*	29.1 ± 0.1
UMP	29.2 ± 0.1	22.8 ± 0.1
GMP	24.1 ± 0.0	23.9 ± 0.1
CMP	23.8 ± 0.1	24.2 ± 0.1

P^{32}-labeled Q_β minus strands (step 6) were prepared as described, from *E. coli* infected with Q_β and labeled from 0 to 40 min with P^{32}-phosphate (40 mc/l).[24]

* Standard error of the mean $\bar{x} = \sqrt{\frac{\Sigma(x - \bar{x})^2}{N(N-1)}}$. Three analyses were carried out on each preparation.

Minus strands as template for Q_β replicase: Purified noninfectious Q_β minus strands promoted vigorous synthesis of RNA from the onset of incubation and infectious units appeared within two minutes (Table 3, expt. 1, see also Fig. 2). After four minutes, the amount of RNA synthesized (Table 3, col. 3) was equivalent to three times the input. The infectious units increased from 0 (<60) to 2.7×10^6 and 18.3×10^6 PFU, respectively, after 4 and 15 minutes. No infectivity appeared when nucleoside triphosphates or minus strands were omitted (expts. 2 and 3). Assuming that the specific infectivity of newly synthesized Q_β RNA and natural Q_β RNA is similar, the infectivities in experiment 1 (calculated from Table 3, expt. 4, time 0) correspond to 0.014 μg of Q_β RNA, i.e., an amount equivalent to about half the template added, after 4 minutes, and to 0.095 μg, i.e., 3 times the input after 15 minutes of incubation. The greater discrepancy between total and infectious RNA at earlier than at later times of incubation may be due to the presence of a relatively larger proportion of incomplete strands early in incubation.

In marked contrast with the above results, the incorporation of nucleotides directed by plus strands (Table 3, expt. 4) lagged behind that promoted by minus strands. Only a fraction (about one fifth) of the amount of RNA added as template was synthesized during the first two minutes. Moreover, as noted by Mills *et al.*,[9] in the early phase of incubation there was a disappearance rather than an increase of infectivity, possibly due to involvement of the added template

TABLE 3. *Synthesis of infectious RNA by Q_β replicase with either Q_β plus or Q_β minus strands as template.*

	(1) Time of incubation (min)	(2) P^{32}-UMP incorporated (cpm/10 μl)	(3) RNA synthesized (μg/10 μl)	(4) Infectious Units (PFU $\times 10^{-6}$/10 μl) Total	Net synthesis
1. Q_β minus strands (step 6) ($0.03 \mu g/10 \mu l$)	0	0	0	0*	0
	2	2900	0.064	1.6	1.6
	4	4020	0.089	2.7	2.7
	6	4850	0.11	3.2	3.2
	10	8600	0.19	11.4	11.4
	15	13,000	0.29	18.3	18.3
2. Q_β minus strands (step 6) ($0.03 \mu g/10 \mu l$). No nucleoside triphosphates	0	—	—	(0.001)	—
	15	—	—	0†	—
3. No template	0	—	—	(0.003)	—
	6	—	—	(0.0005)	—
	15	—	—	0†	—
4. Q_β plus strands (0.038 μg/10 μl)	0	0	0	7.3	0
	2	280	0.0064	5.0	−2.3
	4	900	0.020	3.7	−3.6
	6	2470	0.057	6.2	−1.1
	10	5100	0.11	13.0	5.7
	15	9200	0.20	24.0	16.7

The standard reaction mixtures (0.12 ml) contained, per 10 μl, 1 μg of Q_β replicase. UTP was P^{32}-labeled (spec. radioactivity, 54,000 cpm/mμmole). At the times indicated, aliquots (15 μl) were withdrawn for the determination of acid-insoluble radioactivity and infectious RNA. Each value in col. 2 was corrected for the blank (25 cpm). One mμmole of UMP corresponds to 1.16 μg of Q_β plus strands or 1.54 μg of Q_β minus strands (free acid). A value of 1.2 μg RNA per mμmole of UMP was used throughout to calculate the amount of RNA synthesized (col. 3), since in all but the early incubations with Q_β plus strands the product consisted predominantly of plus strands.[14] All values were recalculated for 10-μl aliquots. Expts. 1 and 4 were carried out in parallel. The values in parentheses were too low to be statistically significant.

* <60 PFU.

† <100 PFU.

in a replicating complex. Even after 15 minutes less RNA and fewer infectious units were synthesized with plus than with minus strands.

The template activity of our minus strand preparation is not due to either (*a*) Q_β plus strand or (*b*) double-stranded Q_β RNA contaminants. Besides the fact that contamination by infectious plus strands, either in a single or a double-stranded form, is extremely low (specific infectivity relative to that of plus strands, less than 10^{-5} and 10^{-4}, respectively), possibility (*a*) is ruled out by the finding (Table 3) that with plus strands there was an increase of infectivity only after six minutes of incubation. Possibility (*b*) is ruled out by our previous observation[14] that double-stranded Q_β RNA does not stimulate nucleotide incorporation by Q_β replicase during the first 30 minutes of incubation and by the present finding (Table 4) that only a minute amount, if any, of infectious units was produced (expt. 2) unless the double-stranded RNA was heat-denatured (expt. 3). MS2 minus strands apparently do not serve as template for Q_β replicase since denatured, double-stranded MS2 RNA did not promote the formation of infectious RNA (Table 4, expt. 4).

Dependence of RNA synthesis on template concentration: As seen in Figure 1,

TABLE 4. *Template activity of different RNA preparations in the Q_β replicase system.*

	Time of incubation (min)	UMP incorporated (cpm/10 μl)	Total infectious units (PFU $\times$ 10^{-6}/10 μl)
1. Q_β minus strands (step 6) (0.03 μg/10 μl)	0	0	0*
	5	4250	2.8
	15	10,050	19.0
2. Double-stranded Q_β RNA (0.04 μg/10 μl)	0	0	(0.0003)
	5	0	(0.001)
	15	0	(0.03)
3. Denatured double-stranded Q_β RNA (0.04 μg/10 μl)	0	0	0.39
	15	7500	18.2
4. Denatured double-stranded MS2 RNA (0.04 μg/10 μl)	0	0	3.2
	15	324	2.6

The incubation mixtures (0.08 ml) had the composition indicated in the *Methods* section. Template was added as indicated. Double-stranded RNA dissolved in 0.5 mM EDTA, pH 7, was denatured by heating at 100° for 90 sec. At the times indicated, aliquots were removed for the determination of infectious units (15 μl) and acid-insoluble radioactivity (5 μl). A blank (90 cpm) was subtracted from each value in col. 2. One thousand cpm are equivalent to 0.035 μg RNA. One μg of Q_β RNA gave 3.3×10^8 PFU in the infectivity assay. The values in parentheses were too low to be statistically significant.

* <100 PFU.

minus strands were a more effective template than plus strands at any concentration tested. Moreover, whereas the enzyme system was saturated by plus strands at a level of about 0.11 μg of RNA/μg of protein, there was no indication of even incipient saturation with 0.13 μg of minus strands/μg of protein irrespective of the assay used, whether RNA synthesis or appearance of infectious units. At high template concentrations, incorporation was about seven times greater with minus than with plus strands. Since saturation was not reached with minus strands, the actual difference in V_{max} may be even greater. An approximate calculation shows that the number of template molecules (either plus or minus strands) added to the enzyme incubation was less than the number of enzyme molecules estimated to be present.[28]

The results of Figure 1 are compatible with the assumption that the enzyme preparation has a small number of sites specific for plus, and a larger number specific for minus strands, perhaps associated with two different polypeptide chains. If this were true, the incorporation resulting from short-time incubations with both plus and minus strands (the former at saturating concentrations) should be additive. Table 5 shows that this was indeed the case.

Time required for the synthesis of a plus strand: The *in vitro* system directed by minus strands is ideally suited to determine the time of synthesis of an infectious RNA strand. As the initial infectivity is nil, the completion of a

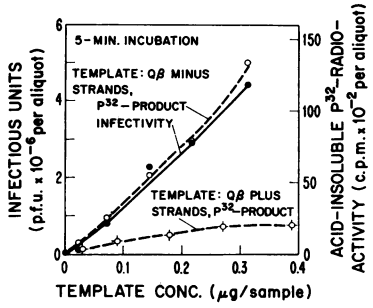

FIG. 1.—Template activity of Q_β plus and minus strands at different concentrations. Standard incubation mixtures (24 μl), containing the amount of template indicated, were incubated for 5 min at 37°. The specific radioactivity of the P^{32}-UTP was 39,500 cpm/mμmole.

TABLE 5. *Additive effect of Q_β plus and Q_β minus strands in promoting nucleotide incorporation by Q_β replicase.*

Template Added		
Plus strand (μg)	Minus strand (μg)	UMP incorporated (cpm)
0.5	—	373
0.75	—	425
—	0.024	231
—	0.072	720
0.5	0.024	657
0.5	0.072	1071

Standard assay mixtures (24 μl) contained the amount of template indicated. After a 5-min incubation at 37°, the acid-insoluble radioactivity was determined. The results are the average of duplicate determinations. The specific radioactivity of the GTP-C^{14} was 5000 cpm/mμmole.

small number of RNA molecules is readily detectable. In Figure 2 the synthesis of infectious units at 37° is plotted as a function of the incubation time. Whereas no infectious units were present up to 90 seconds, infectious RNA equivalent to about 10^7 PFU was generated within the next 20 seconds. This marks the completion of the first crop of progeny RNA molecules. Assuming that the association of enzyme and template was largely completed during the two-minute preincubation in the absence of three nucleoside triphosphates, the time required for the completion of a plus strand (3000 nucleotides) is about 100 seconds and phosphodiester bonds would be formed at the rate of 3000/100 = 30 per second at 37°. The value greatly exceeds that observed for *in vitro* RNA synthesis by RNA polymerase directed by T4 or T7 DNA (2.5 and 7 phosphodiester bonds/sec, respectively)[29, 30] but may still be less than that occurring *in vivo*.

Discussion.—Since the single minus strand[11] efficiently promotes the final step in viral RNA replication, i.e., the synthesis of infectious plus strands, it meets an important requirement for a functional intermediate in the process. Such a requirement is not met by species of double-stranded RNA isolated from infected cells.

The finding that purified minus strands are biologically competent and yet not infectious shows that lack of infectivity is an intrinsic property of minus strands and not a consequence of damage suffered during purification or even during synthesis and sojourn in the host. A reason for the lack of infectivity can easily be given. Initiation of infection in the host requires a virus-specific

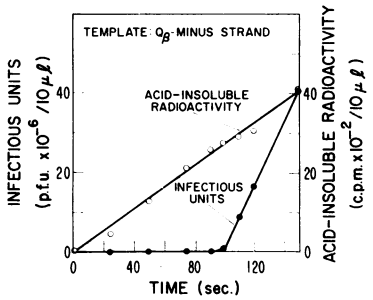

FIG. 2.—Determination of the time required for completion of an infectious RNA strand. Q_β minus strands (0.96 μg), Q_β replicase (15 μg), ATP (0.12 μmoles), and $MgCl_2$ (1.92 μmoles) in 0.1 ml of 0.126 *M* Tris-HCl, pH 7.6, were incubated for 2 min at 37°. The reaction was initiated by the addition of GTP, CTP, and C^{14}-UTP (0.12 μmoles each, in 50 μl). The final reaction mixture had the usual composition. At the times indicated, 15 μl of the mixture were pipetted into tubes containing 1 μl of 1.5% SDS, mixed, and cooled. Infectivity and acid-insoluble radioactivity were determined as described in the *Methods* section. The specific activity of the C^{14}-UTP was 34,000 cpm/mμmole. One μg of Q_β RNA gave 7.5 × 10 PFU.

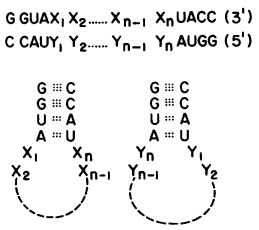

FIG. 3.—Two hypothetical RNA strands which (*a*) are commentary to each other and (*b*) share common sequences at the ends. Each of these strands can assume the "amphora" configuration shown at the bottom of the figure. (*X*,*Y*), any set of complementary bases.

RNA polymerase (synthetase or replicase) coded for by the plus [31–33] but not by the minus strand. The minus strand in fact appears to be incapable of directing the *in vitro* synthesis of acid-insoluble polypeptides.[34] The fact that the minus strands were obtained from *E. coli* infected with Q_β phage proves the assumption that biologically competent minus strands (either free or complexed) are formed during replication *in vivo*. The remarkable template specificity of Q_β replicase,[3] thought to recognize only Q_β plus strands among natural RNA's, has now been extended to a second species of RNA, the Q_β minus strand. If plus and minus strands had the end sequences in common, as in the model of Figure 3, Q_β replicase could recognize both strands with one recognition site. Such a relationship between the two strands implies terminal self-complementarity in each plus and minus strand, as in the "amphora" model proposed for other reasons for the Q_β plus strand.[35] We have looked for double-helical regions in purified Q_β RNA subjected to annealing conditions but have failed to find significant amounts of RNase-resistant RNA (less than 0.2%),[36] and we feel that if such regions existed they would not comprise more than ten base pairs. However, since ten nucleotides might be sufficient to specify a recognition sequence we cannot eliminate the model on the basis of our findings. On the other hand, we have presented evidence which is compatible with the assumption that the recognition sequences of plus and minus strands differ and that the replicase preparation contains different amounts of two specific binding sites, a small number for plus and a large number for minus strands. These sites may be associated with different proteins or protein subunits. The finding of Lodish and Zinder[37] that synthesis of plus and minus strands can be dissociated in certain temperature-sensitive mutants of phage f2 can be explained within the framework of this hypothesis.

Summary.—Purified, single Q_β minus strands are inherently noninfectious and effectively promote the synthesis of infectious viral RNA by Q_β replicase *in vitro*.

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[1] Definitions and abbreviations are as in previous work.[13]

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[28] The specific activity of our $Q\beta$ replicase preparation, purified according to Pace and Spiegelman,[4] was 60 (mμmoles of total nucleotide incorporated per min at 37°), i.e., somewhat higher than that reported from Spiegelman's laboratory.[4, 9, 35] The electrophoretically homogeneous $Q\beta$ enzyme described by August and his colleagues (personal communication) has a corresponding value of 1000. We may therefore estimate our preparation to be about 5% pure. Assuming that the enzyme has a molecular weight[4] of about 10^6, 2.5 μg of the preparation would contain $\frac{0.05 \times 2.5 \times 10^{-6} \times 6 \times 10^{23}}{10^6} = 7.5 \times 10^{11}$ molecules of enzyme. This amount of enzyme is not saturated by the addition of $\frac{0.3 \times 10^{-6} \times 6 \times 10^{23}}{10^6} = 1.8 \times 10^{11}$ molecules of minus strands.
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