*REPLICATION OF VIRAL RNA, XI. SYNTHESIS OF VIRAL "MINUS" STRANDS IN VITRO**

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The replication of viruses containing single-stranded RNA[1] as genetic material is accompanied by the formation, within the host cell, of substantial quantities of "minus" strands which are isolated predominantly in a double-stranded, RNase-resistant form (for references, see Billeter *et al.*[2]). Several lines of evidence indicate that a complex containing "minus" and "plus" strands is an intermediate in viral RNA synthesis.[3–8]

An enzyme preparation, RNA replicase, has been obtained from RNA phage-infected *E. coli*, by Haruna and Spiegelman.[9, 10] A remarkable specificity of the enzyme for the RNA of the inducing virus was demonstrated.[10] This preparation synthesized viral RNA ("plus" strands) *in vitro* when primed with viral "plus" strands.[11] Clearly, it was of considerable interest to determine whether or not "minus" strands were formed during the course of this synthesis. We have analyzed the RNA synthesized *in vitro* by Q_β replicase and have found that in the early phase of synthesis only "minus" strands could be detected, whereas at later times "plus" strands were synthesized. This sequence of events was similar to that found *in vivo*, in phage-infected bacteria.[12]

Materials and Methods.—Q_β RNA: Phage Q_β was obtained from Professor I. Watanabe, Tokyo. It was grown on *E. coli* Q 13, obtained from Dr. S. Spiegelman, and purified by a procedure similar to that described for phage MS2.[13] Q_β differs in its behavior from MS2 inasmuch as it forms a precipitate band during the CsCl density gradient centrifugation; it redissolves on dilution with water. The RNA was extracted by the procedure of Gesteland and Boedtker[14] and stored as a precipitate in 66% ethanol at −70°C. Q_β RNA can be distinguished from MS2 RNA by virtue of its capacity to anneal specifically with denatured double-stranded Q_β RNA but not at all with double-stranded MS2 RNA.[15] For use as a template, Q_β RNA was fractionated on a sucrose gradient, and the peak fractions, corresponding to an $S_{20,w}$ of about 28*S*, were pooled and utilized immediately. As shown in Figure 1, such peak fractions contain no detectable amounts of degraded RNA.

Double-stranded Q_β RNA: This was prepared as described for MS2 RNA,[16] with similar yields.

Q_β replicase: The enzyme preparation used was a gift from Drs. Haruna and Spiegelman. When assayed under the conditions described by Haruna and Spiegelman,[10] 20 μl of the solution (3.1 mg protein/ml) incorporated 0.45 mμmole of UTP into acid-insoluble material, in 20 min at 35°. The same values were obtained both in Dr. Spiegelman's and in our laboratory. The specific template requirement of the Q_β enzyme[10] was confirmed.

Preparation of the product of Q_β replicase: Incubations were carried out under the conditions described by Haruna and Spiegelman.[10] The product was extracted twice with 2 vol of phenol (equilibrated with 0.05 *M* Tris HCl buffer, pH 7.6), and the phenol was removed by three ether extractions. The samples were freed of radioactive nucleotides by dialysis for 20 hr against several changes of 1 × SSC and finally, 0.1 × SSC. Determination of RNase-resistant radioactive RNA was carried out as described previously.[16, 17]

Double isotope specific dilution assay: This assay was used to determine radioactive viral "plus" and "minus" strands in the presence of radioactive nonviral RNA. The radioactive viral RNA is first converted into a double-stranded, RNase-resistant form by annealing with an excess of heat-denatured double-stranded viral RNA.[17] The product is then subjected to the specific dilution assay[3, 17] by heating and reannealing in the presence of an excess of unlabeled "plus"

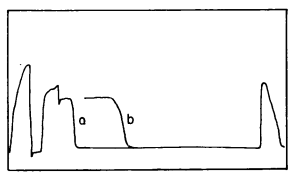

FIG. 1.—Velocity centrifugation of purified Q_βRNA. Q_βRNA was purified by sucrose density centrifugation as described in *Methods*. The densitometer tracings were from photographs taken after (*a*) 8 min, and (*b*) 16 min of centrifugation at 50,740 rpm; $S_{20,w}$ = 27.5*S*.

strands. The radioactive "plus" strands are thereby displaced from the duplex and become RNase-sensitive. Radioactivity due to "minus" strands remains RNase-resistant.

In practice, the assay was carried out by distributing, into a series of tubes, aliquots of a mixture containing the C^{14}-labeled sample to be analyzed, a small quantity of P^{32}-labeled viral "plus" strands (as an internal standard), and unlabeled double-stranded viral RNA in excess of the "plus" strands present in the sample mixture. Increasing amounts of unlabeled viral "plus" strands (from 0 to about a 20-fold excess over the double-stranded RNA) were then added to the tubes. The mixtures were heated, reannealed, and the RNase-resistant P^{32}- and C^{14}-radioactivities were determined. When a mixture of P^{32}-labeled Q_β RNA and the C^{14}-labeled product of Q_β replicase (40-min incubation) was annealed with double-stranded RNA, but without addition of unlabeled Q_β RNA, 85% of the C^{14}-radioactivity and 80% of the P^{32}-radioactivity were converted to an RNase-resistant form. When the reaction was carried out in the additional presence of excess nonlabeled Q_β RNA (Fig. 2), 53% of the C^{14}-radioactivity and only 3% of the P^{32}-radioactivity was resistant to RNase. A plot of the C^{14}-values against the corresponding P^{32}-values gives a straight line, with the slope indicating the fraction of C^{14}-radioactivity in "plus" strands and the intercept giving the fraction of C^{14}-radioactivity in "minus" strands (see *inset*, Fig. 2). The method underestimates "minus" strands by about 5–10%. A detailed description of the assay will be given elsewhere.[18]

Results.—Synthesis of "minus" strands: Q_β replicase was incubated with radioactive nucleoside triphosphates as described.[10] The integrity of the Q_β RNA used as a template was ascertained by ultracentrifugal analysis immediately prior to the experiment (cf. Fig. 1). Samples of the reaction mixture were withdrawn at different times during incubation, purified, and analyzed by the double isotope specific dilution assay. As shown in Table 1 (expt. 1) and Figure 3, no radioactive "plus"

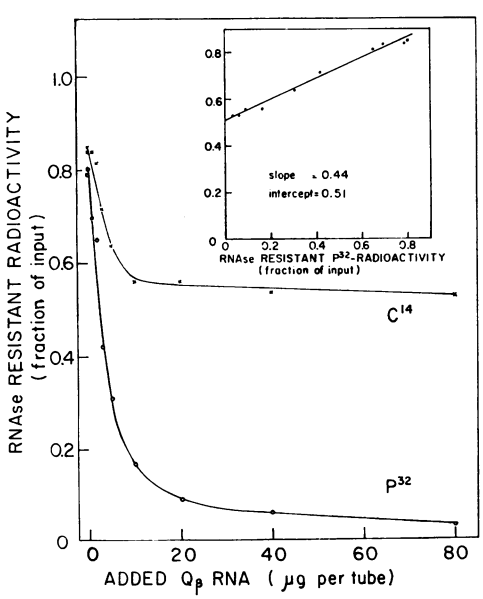

FIG. 2.—Analysis of C^{14}-labeled product of Q_β replicase by the double isotope specific dilution assay. The product was prepared as described in the footnote to Table 1 (expt. 1); incubation time, 40 min. Of the radioactivity 28.5% was RNase-resistant. Each assay tube contained, in 20 μl of 2.5 × SSC, 480 cpm of C^{14}-labeled product, 900 cpm of P^{32}-labeled Q_β RNA (0.1 μg), 5 μg of double-stranded Q_β RNA, and unlabeled Q_β RNA as indicated. After heating and annealing, the RNase-resistant radioactivities were determined, and blank values (RNase-resistant radioactivity after heat denaturation; less than 1% in all cases) were subtracted. The resulting values are expressed as the fraction of input radioactivity. *Inset:* the C^{14}-values are plotted against the corresponding P^{32}-values, in order to determine the fraction of C^{14}-radioactivity in "plus" and "minus" strands (slope and intercept, respectively, of the resulting straight line).

TABLE 1

ANALYSIS OF THE PRODUCT OF $Q\beta$ REPLICASE*

Expt. no.	Incubation time (min)	Radioactivity incorporated (cpm)	RNase-resistant Radioactivity (%) Before phenol	After phenol	"Minus" strands (%)	"Plus" strands (%)
1	3	9,800	13	33	80	Not detected
	7.5	29,400	15	28	76.5	Not detected
	15	65,000	17	21	47	32.5
	40	104,000	21	29	39	39.5
	100	126,000	25	24	34	51.0
2	5	15,350	21	61	80	
	10	37,950	20	33	73	
	20	86,000	21	25	46	
	40	141,000	22	34	35	
	80	177,000	26	33	38	

* The incubations were carried out as described by Haruna and Spiegelman,[10] using unlabeled ATP and C^{14}-labeled UTP, CTP, and GTP, each of specific radioactivity 25,000 cpm/mμmole. In expt. 1, 250 μg of $Q\beta$ replicase and 0.88 μg of purified $Q\beta$ RNA were used; final vol, 0.5 ml. In expt. 2, 250 μg of enzyme were incubated with 5 μg of $Q\beta$ RNA (obtained from Drs. Haruna and Spiegelman); final vol, 0.5 ml. Aliquots were withdrawn at the times indicated and diluted tenfold with SSC (expt. 1) or with 0.15 *M* NaCl (expt. 2). Total acid-insoluble and RNase-resistant radioactivity were determined as described previously.[16, 17] However, pyrophosphate (pH 7.0) and phosphate (pH 6.8) were added (final concentrations, 0.04 and 0.1 *M*, respectively) prior to acid precipitation. The RNA was isolated by the phenol procedure and dialyzed prior to analysis. In expt. 1, "plus" and "minus" strands were determined by the double isotope specific dilution assay. In expt. 2, "minus" strands were determined by heating aliquots of the product with 2 mg/ml of $Q\beta$ RNA in 2.5 × SSC, for 3 min at 120° and 7 min at 85°, and measuring the RNase-resistant radioactivity. The radioactivity of heat-denatured, nonreannealed samples of the replicase product was less than 1%.

strands could be detected within the first 7 min of incubation even though the presence of 5–10 per cent of the radioactivity in "plus" strands would have been clearly detectable. Of the acid-insoluble radioactivity, 75–80 per cent was found in "minus" strands. Failure to account for all of the radioactive RNA as virus-specific material is probably due to incomplete annealing. Radioactive "plus" strands were first detected after 15 min of incubation and their amount exceeded that of "minus" strands at 100 min. An amount of RNA equivalent to that of the added template was synthesized by the twelfth minute. A similar experiment was carried out, using as template $Q\beta$ RNA provided by Drs. Haruna and Spiegelman. This time, only radioactive "minus" strands were assayed for, by heat-denaturing and annealing the product with an excess of unlabeled $Q\beta$ RNA, and then determining the RNase-resistant radioactivity. The results (Table 1, expt. 2) were comparable to those of the first experiment.

Sucrose gradient centrifugation of the labeled product of $Q\beta$ replicase, isolated after 40 min of incubation (Fig. 4), showed that a large fraction of the radioactive RNA sedimented around 27*S*, as does intact viral RNA, in agreement with the report by Haruna and Spiegelman.[19] RNase-resistant RNA sedimented with an $S_{20,w}$ around 14*S*, a value similar to that found for virus-specific, double-stranded RNA formed *in vivo*.[4] Annealing of the gradient fractions with "plus" strands revealed "minus" strands not only in the region corresponding to double-stranded RNA, but also in the 27*S* region.

Discussion.—Earlier work[3–8] has led to the concept that double-stranded RNA (or a complex of "plus" and "minus" strands that gives rise to double-stranded RNA during the isolation procedure[6]) is an intermediate in viral RNA synthesis, with "minus" strands serving as template for the formation of progeny "plus" strands. The hypothesis demanded that "minus" strand synthesis precede the formation of "plus" strands. This has now been shown to be the case, both *in vivo*[12] and *in vitro*.

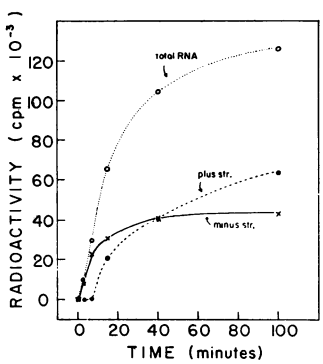

FIG. 3.—Synthesis of "plus" and "minus" strands by Q_β-replicase. The values are calculated from the data of Table 1, expt. 1. O. . .O, Total incorporation of radioactive label into acid-insoluble material; x——x, radioactivity in "minus" strands; ●- - -●, radioactivity in "plus" strands.

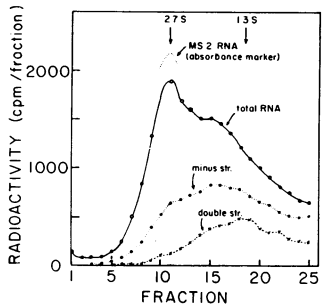

FIG. 4.—Sucrose gradient analysis of the product of Q_β replicase. An aliquot of the Q_β replicase product, described in the legend to Fig. 2, to which 20 A_{260} units of MS2 RNA were added as a marker ($S_{20,w}$ = 27S), was layered on 28 ml of a linear sucrose density gradient (3–20% sucrose in SSC) and centrifuged for 16.5 hr at 4° and 21,000 rpm in the Spinco SW 25.1 rotor. The gradient was syphoned off from the bottom of the tube, and the absorbance at 260 mμ was recorded by means of a flow cell. The fractions were examined for (*a*) acid-insoluble radioactivity (O——O), (*b*) acid-insoluble radioactivity after RNase digestion (x- - -x), and (*c*) radioactive "minus" strands (●. . .●); these were determined by heating an aliquot of each fraction with 167 μg/ml of Q_β RNA in 2.5 × SSC, for 3 min at 120°C and for 2 hr at 85°, and then determining the acid-insoluble radioactivity after RNase digestion. The blank (RNase-resistant radioactivity of the enzymatic product after heat denaturation; less than 1% of the total radioactivity) was subtracted from all values.

Formation of "minus" strands *in vivo* was detectable several minutes prior to that of "plus" strands. Furthermore, the rate of "minus" strand synthesis approached a constant value at about 15 min, while the rate of "plus" strand formation increased up to 22 min after infection, suggesting separate control mechanisms for the two processes.[12]

The present experiments with Q_β replicase showed that in the first minutes of incubation predominantly, if not exclusively, "minus" strands were formed, and that subsequently "plus" strands were synthesized. The second phase of synthesis has been detected by Spiegelman *et al.*[11] with use of infectivity assays. It is remarkable that the major part of the "minus" strands formed early in the *in vitro* reaction occur in an RNase-sensitive form (Table 1) and therefore appear not to be part of a hydrogen-bonded double helix. The sedimentation profile of the "minus" strands (Fig. 4) suggests that free single-stranded "minus" strands may occur.

Replication of viral RNA thus appears to involve two steps: (*a*) synthesis of "minus" strands, with "plus" strands serving as template, and (*b*) synthesis of "plus" strands, with "minus" strands as template. A complex of "plus" and "minus" strands, detected as double-stranded RNA, has been shown to occur as intermediate in viral RNA synthesis *in vivo*.[4, 7, 12] Step (*a*) has been studied *in vitro* by August and his collaborators,[20, 21] who purified a virus-induced polymerase from *E. coli* infected with su-11,[22] a mutant of the RNA phage f_2 which causes the formation of excessive amounts of "minus" strands (in a double-stranded form) in the infected host.[23] Step (*b*) was demonstrated with use of an enzyme-template complex RNA synthetase[24] that synthesized almost exclusively" plus" strands *in vitro*.[17] As

shown in this paper, Haruna and Spiegelman's RNA replicase is capable of carrying out both synthetic steps. By their elegant analysis of RNA phage mutants, Lodish and Zinder[8, 23] have shown that the two steps of RNA replication can be genetically dissociated. It remains to be seen whether the two enzymatic activities can be separated.

Summary.—When incubated with nucleoside triphosphates and Q_β RNA as template, Q_β replicase synthesizes predominantly, if not exclusively, "minus" strands in the early phase of incubation and, later on, mainly "plus" strands. Part but not all of the "minus" strands occur in a double-stranded form.

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[1] Abbreviations: RNA, ribonucleic acid; ATP, GTP, UTP, and CTP, the 5′-triphosphates of adenosine, guanosine, uridine, and cytidine, respectively; RNase A, pancreatic ribonuclease A; Tris, tris(hydroxymethyl)aminomethane; SSC, 0.15 *M* NaCl, 0.015 *M* sodium citrate, pH 7; cpm, counts per minute; A_{260}, absorbance at 260 mμ, 1.0 cm light path; "plus" strands are defined as viral RNA strands of the parental type as opposed to "minus" strands which have the complementary base sequence.

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