*THE MECHANISM AND DIRECTION OF RNA SYNTHESIS TEMPLATED BY FREE MINUS STRANDS OF A "LITTLE" VARIANT OF Qβ-RNA**

BY D. R. MILLS,† DAVID H. L. BISHOP,‡ AND S. SPIEGELMAN

DEPARTMENT OF MICROBIOLOGY, UNIVERSITY OF ILLINOIS, URBANA

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Kinetic analysis of the reaction mediated by purified[1] Qβ-replicase[2] which yields infectious[3, 4] RNA has provided[5, 6] data consistent with the following pathway of synthesis:

$$\text{Template Q}\beta\text{-RNA} \rightarrow \text{HS} \rightarrow \text{FS} \rightarrow \text{product RNA.}$$

Here, HS are RNA complexes containing template and early product and correspond electrophoretically[5] and by sedimentation analysis[7, 8] to structures found *in vivo* by Francke and Hofschneider.[9] The FS resemble the structures identified by Franklin[10] in cells infected with R-17 bacteriophage. A "pulse-chase" experiment[11] showed that the FS structures were the immediate physical precursors of mature 28*S* Qβ-RNA, thus offering further support for the above sequence of events.

Ribosidetriphosphates containing P^{32} in the β and γ positions were used[12] to label 5′-terminal ends, and GTP was found at the 5′-terminus of completed plus and minus strands. The data further demonstrated that synthesis of the *minus* strand is initiated by complementary copying of the 3′-terminus of the plus-strand template and involves a 5′ to 3′ polymerization. Finally, when β,γ,P^{32}-labeled UTP, ATP, or CTP were used, label was found resident only in the FS structures. Since these structures are presumed to contain nascent positives, it was suggested that the synthesis of plus strands involves a 3′ to 5′ polymerization.

The experiments thus far described have dealt exclusively with the *in vitro* synthesis of normal Qβ-RNA. We have previously shown[13] that it is possible to select variant RNA molecules which can multiply some 15 times faster than the parental ones. The detailed mechanism of variant replication poses a problem with its own intrinsic interest. In addition, these variants are only 17 per cent the size of parental type and, as such, provide inherent advantages as experimental objects for the analysis of various aspects of the replicative mechanism. Because of their smaller size all components, including the HS and FS complexes, move readily into the gel on electrophoresis and are easily separated. An additional advantage is that the ratio of terminal to total nucleotides is seven times greater than in the parental variety. Finally, it is comparatively easy to isolate quantities of pure plus and minus strands.

Feix *et al.*[14] recently demonstrated that the negative strand of Qβ serves as an excellent template for the Qβ-replicase. We have confirmed this interesting observation with Qβ-RNA,[15] and the present paper shows that it is also the case for the little variant. In addition, we show that the same sort of complexes (HS and FS) appear in the reaction mixtures containing minus template as are

observed with positive templates. Finally, and more surprisingly, the data indicate that the direction of synthesis of plus strands is 5′ to 3′ when the enzyme is employing *free negative* variant strands as templates.

Materials and Methods.—(a) *Enzyme, substrates, and assays:* Purification of Qβ-replicase through the stages of cesium chloride and sucrose sedimentation, assay for enzyme activity under standard conditions, and liquid scintillation counting of acid-precipitable product on membrane filters have been detailed elsewhere.[4] H^3-labeled ATP (1 mc/0.26 μmole) and H^3-labeled CTP (1 mc/μmole) were purchased from Schwarz BioResearch. Synthesis of P^{32}-β,γ-labeled UTP and GTP (each at 1×10^{10} cpm/μmole) has been detailed previously.[12]

(b) *Synthesis and isolation of minus strands:* A 25-fold standard reaction (6.25 ml) containing 1 mc H^3-labeled CTP was incubated with enzyme (1.50 mg) and variant RNA (5 μg) at 35° for 30 min. The reaction was terminated with sodium dodecyl sulfate to a final concentration of 0.2% (SDS), adjusted to 0.4 *M* NaCl, and extracted at 4° for 15 min with the phenol-cresol mixture described previously.[16] The aqueous phase was mixed with 2 vol of alcohol and left at −20° for 60 min. After centrifugation, the precipitate, containing RNA and almost all the triphosphates, was dried and suspended in a minimum volume (*ca.* 0.4 ml) of gel elution (GE) buffer (0.4 *M* NaCl, 0.01 *M* tris, 3.0 mM EDTA, pH 7.4), and sucrose crystals were added. The mixture was then loaded on a 90 × 0.7-cm column of Sephadex G-25 (coarse grade) and eluted in GE buffer containing 0.1% SDS through a 1-μl micropipette (Kensington Scientific Corp.) at a rate of 1 ml per 5 min. Separation of RNA from triphosphates was monitored by measuring the total and acid-insoluble radioactivity of each 1-ml fraction. In order to further reduce the amount of radioactive triphosphates, the fractions containing RNA were pooled, diluted to 0.2 *M* NaCl, 10 μmoles of unlabeled UTP added, and the mixture was made exactly 60% with regard to ethanol. After standing *at 0°* for 2 hr, the RNA was freed from residual triphosphates by centrifugation at 4° and 10,000 rpm in the Servall HB rotor for 60 min. Subsequently, the RNA was dissolved in 0.1 ml of one-quarter GE buffer and subjected to 90 min electrophoresis at 10 ma and 50 v on *bis*-acrylamide cross-linked *preswollen* 3.6% polyacrylamide gels.[16] Pairs of 0.5-mm slices were eluted overnight at 4° with 1 ml GE buffer, and the content of eluted material was subsequently determined by counting 4-μl aliquots. The profile of eluted material was similar to the profile shown in Figure 2*E*. Peak fractions from the single-stranded variant region were pooled, filtered from gel debris by passage through a membrane filter, precipitated with alcohol, and dissolved in 0.01 *M* tris, 0.001 *M* EDTA, pH 7.4, and stored at −70°C. That the single-stranded RNA contained only free plus strands was demonstrated by the inability of the preparation to self-anneal at 65° for 60 min in 0.4 *M* NaCl to give an HS structure, and also its similar inability to anneal to Qβ viral RNA.

In order to prepare minus strands, the eluants of the peak HS and FS regions of the gel were pooled, filtered, and alcohol-precipitated as described above. The RNA was then suspended in 1.0 ml of 0.003 *M* EDTA, pH 7.0, heated at 100° for 2 min, quick-cooled in a methanol-dry ice bath, and made 0.4 *M* with respect to NaCl. From the specific activity of the H^3-labeled variant RNA (1×10^5 cpm/μg) the amount of *purified 28S* Qβ viral RNA to be added was calculated such that the microgram ratio of Qβ-RNA to total variant RNA was 30 to 1. After addition of this amount of Qβ viral RNA, the mixture was annealed in 0.4 *M* NaCl at 65° for 60 min, cooled and diluted to 0.2 *M* NaCl, and layered over two 30-ml, 20–5% linear gradients of sucrose in 0.2 *M* NaCl, 0.01 *M* tris, 0.001 *M* EDTA, pH 7.4, at 8° for 18 hr. Comparison of the distribution of radioactivity between this annealed sample and an equivalent parallel annealed variant *plus* strand determined the position of the variant minus-Qβ hybrid and confirmed the lack of minus contamination of the purified plus strands. The fractions containing the hybrid were pooled and alcohol-precipitated. After centrifugation, the RNA was melted and quick-cooled as described above, and the minus strands freed from Qβ-RNA by electrophoresis through preswollen 3.6% polyacrylamide gels. The minus strands were then eluted from the gels with GE buffer, filtered, and alcohol-precipitated. In order to remove

any remaining Qβ fragments or contaminating variant plus strands, a sample of the RNA was self-annealed in 0.4 *N* NaCl at 65° for 60 min and subjected to electrophoresis to detect HS complexes. No more were observed after self-annealing than were present in the initial preparation (see Fig. 1*A*). The yield of minus strands having a specific activity of 1×10^5 cpm/μg was about 5 μg.

(c) *Enzyme reactions, preparation of samples for electrophoresis, and electrophoretic conditions:* Tenfold standard reaction mixtures (2.5 ml) were prepared containing 20 μmoles $MgCl_2$, 200 μmoles tris-HCl buffer, pH 7.4, 1 mg enzyme, 2.0 μmoles of two unlabeled triphosphates, 2.0 μmoles of H^3-labeled ATP (0.25 mc/μmole final), 0.50 μmole of P^{32}-β,γ-labeled triphosphate (GTP or UTP), and 2.0 μg of variant minus strands. The H^3-ATP was included to determine net RNA synthesis and the ratio of terminal β,γ-labeled nucleotide to total RNA. The mixture was incubated at 33° after 0.50 ml had been removed as a zero time sample. At 45 sec another 0.50 ml was removed, and 5.4 μmoles of unlabeled triphosphate (GTP or UTP) were added in an attempt to chase any incorporated β,γ-labeled triphosphate. Additional 0.50-ml samples were removed at 65, 85, and 300 sec. Each sample was mixed with SDS (0.2% final concentration) and phenol-extracted in 0.4 *M* NaCl. Separation from radioactive triphosphates was achieved as described above by passage through Sephadex and 60% ethanol precipitation in the presence of 10 μmoles unlabeled UTP. Ethanol precipitation was repeated *until* the acid-soluble radioactivity was less than half the acid-insoluble value. Finally, each sample was mixed with 2 μmoles of unlabeled UTP or GTP and subjected to electrophoresis on preswollen 3.6% polyacrylamide gels for 90 min at 10 ma per gel and 50 v. Pairs of 0.5-mm gel slices were extracted for 5 hr with 10% TCA and plated on membrane filters before being dissolved in 0.5 ml 30% v/v hydrogen peroxide at 70° for 8 hr. Samples were finally counted in Kinard's scintillation fluid and corrected for background and crossover.

Results.—We know[17] that, as is the case with the parental Qβ-RNA, the 5′-terminus of complete minus and plus strands of the variant is GTP. A reaction using β,γ-P^{32}-GTP serves, therefore, as a measure of the adequacy of detecting 5′-termini in the various components at different stages of the reaction. In examining the details of the pulse-chase experiments to be described, the following features must be borne in mind: (*a*) to maintain the specific activity of the β,γ-P^{32}-riboside triphosphates at acceptable levels during the "pulse" and permit an adequate "chase," we were compelled to run the reactions at concentrations of GTP which result in rather slow rates of RNA synthesis. The effect is not so severe when the other ribosidetriphosphates are similarly limited; (*b*) at 45 seconds unlabeled GTP (or UTP) was added (see *Methods*) to monitor the fate of the strands synthesized in the first period of the reaction; (*c*) the negative template employed is labeled with H^3 which permits us to follow its fate in the *early* stages of the reaction *before* the appearance of significant amounts of product. The latter is labeled with P^{32} from the β,γ-label of GTP and H^3 from H^3-ATP which is included to monitor chain elongation. The contributions of H^3 from the original template is corrected for by a control synthesis as described in the legend of Table 1.

The results of the β,γ-P^{32}-GTP synthesis are detailed in the upper portion of Figure 1 and the profiles of Figure 1*A–E*. Comparison with the zero time reveals movement of H^3-template from the single-stranded region (V) first into HS structures, then into FS complexes, and all this before detectable appearance of mature single strands. The behavior of the P^{32}-label is consistent with this sequence of events. In the first place, it will be noted that *all* structures when

TABLE 1. *Incorporation of β,γ-P^{32}-labeled UTP or GTP and H^3-labeled ATP into H^3-labeled variant minus-strand templated reactions.*

	Gel region	Time (sec) 0	45	65	85	300
(A) β,γ-GTP^{32}						
	FS Per cent H^3 template	2	30	37	36	30
	FS $\mu\mu$Mole H^3 product	0	29	33	34	33
	FS cp5Min P^{32} product	619	8,402	10,670	9,879	3,800
	HS Per cent H^3 template	12	27	31	36	47
	HS $\mu\mu$Mole H^3 product	0	20	16	29	49
	HS cp5Min P^{32} product	0	8,447	7,096	4,884	5,642
	V Per cent H^3 template	86	43	32	28	23
	V $\mu\mu$Mole H^3 product	0	0	0	0	73
	V cp5Min P^{32} product	3	436	521	944	6,081
(B) β,γ-UTP^{32}						
	FS Per cent H^3 template	2	27	28	24	16
	FS $\mu\mu$Mole H^3 product	0	198	87	85	94
	FS cp5Min P^{32} product	505	751	386	183	104
	HS Per cent H^3 template	12	33	30	34	40
	HS $\mu\mu$Mole H^3 product	0	96	79	116	311
	HS cp5Min P^{32} product	310	392	273	123	96
	V Per cent H^3 template	86	40	42	42	44
	V $\mu\mu$Mole H^3 product	0	49	87	126	345
	V cp5Min P^{32} product	385	376	232	91	82

Experimental details for the P^{32}-β,γ-labeled UTP or GTP minus-strand templated reactions and separation of the components are given in the *Methods* section and Figs. 1, 2. The approximate contribution of H^3-label from the primer to the total H^3-label was obtained from identical parallel experiments with unlabeled UTP or GTP. The $\mu\mu$mole content of H^3 in the various regions was calculated from the *net* incorporation of summed counts divided by the specific activity of the triphosphate under the counting conditions employed.

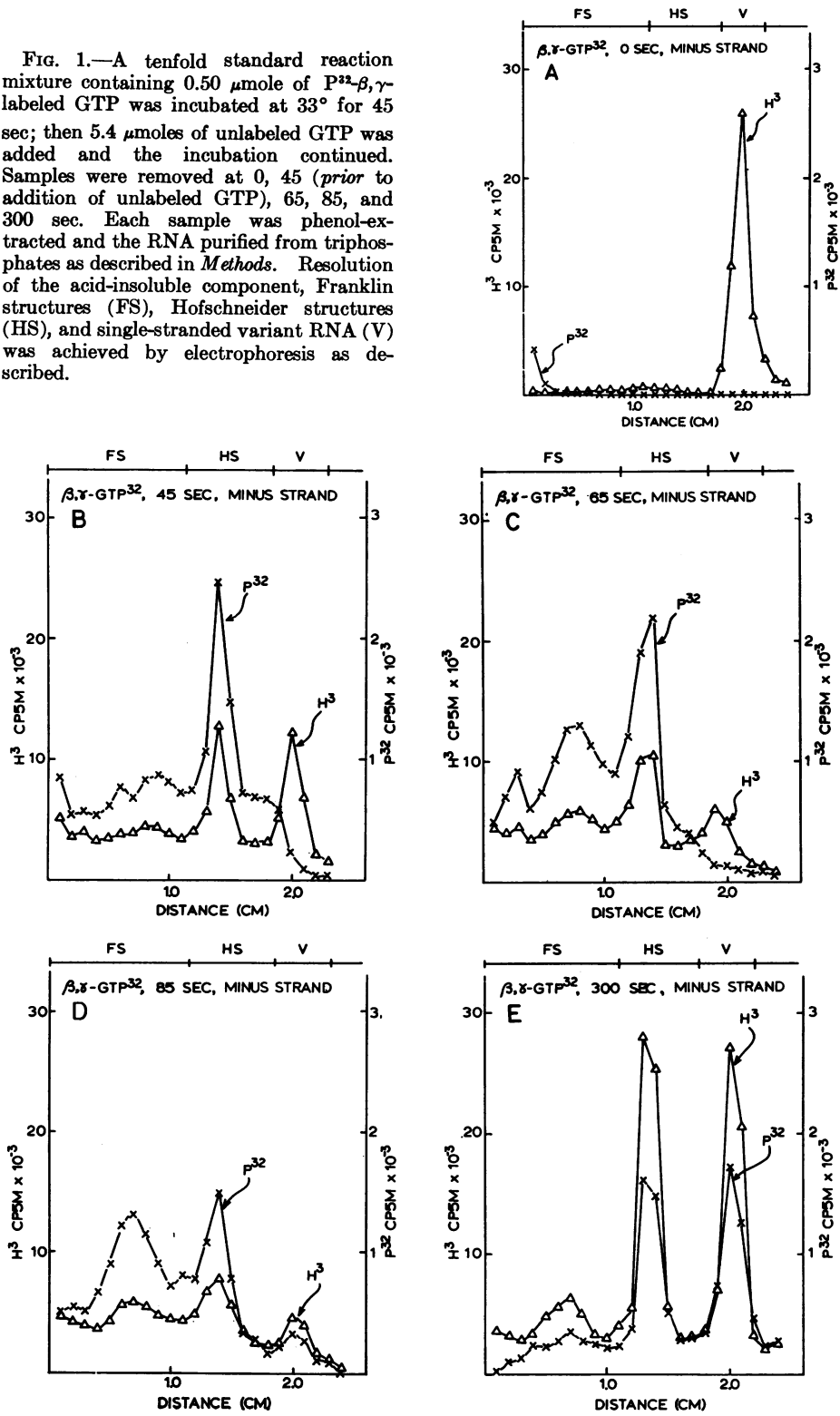
they appear contain a P^{32}-label. This agrees with what we observed[12] earlier with Qβ-RNA and is consistent with the deduction that the "little" variant also contains pppGp as its 5′-terminus on both plus and minus strands.

At the end of the 45-second pulse (Fig. 1*B*) most of the P^{32} is found in the HS region. However, after 20 seconds of chase, the P^{32}-label in the HS falls and that in the FS region rises with no increase in the single-stranded region. Finally, after two minutes of chase (Fig. 1*E*), the net P^{32} in the FS region falls by a factor of 3 and reappears almost quantitatively in the single-stranded region.

It is clear that the behavior of H^3-template and the P^{32}-product during the pulse and chase are consistent with the scheme of events outlined in the introductory paragraph which specify the HS structures as the first intermediates and FS complexes as the immediate physical precursors of the plus strands.

It would appear that the same time sequence and sorts of intermediate complexes are obtained with *negative* variant strands as were seen with positive strands of the original Qβ-RNA. When, however, the β,γ-P^{32}-UTP experiment is examined, a surprisingly different picture is observed. No P^{32}-β,γ-UTP is detected in *any* of the structures. This result is in striking contrast to the unique labeling of FS structures observed in reactions containing plus strands of Qβ-RNA as templates. The fact that the total recovery of P^{32}-β,γ-GTP from the chase was close to 90 per cent argues that the terminal GTP in the HS and FS is not lost by removal of pyrophosphate and hence by inference 5′-termini containing UTP should also be stable.

FIG. 1.—A tenfold standard reaction mixture containing 0.50 μmole of P^{32}-β,γ-labeled GTP was incubated at 33° for 45 sec; then 5.4 μmoles of unlabeled GTP was added and the incubation continued. Samples were removed at 0, 45 (*prior* to addition of unlabeled GTP), 65, 85, and 300 sec. Each sample was phenol-extracted and the RNA purified from triphosphates as described in *Methods*. Resolution of the acid-insoluble component, Franklin structures (FS), Hofschneider structures (HS), and single-stranded variant RNA (V) was achieved by electrophoresis as described.



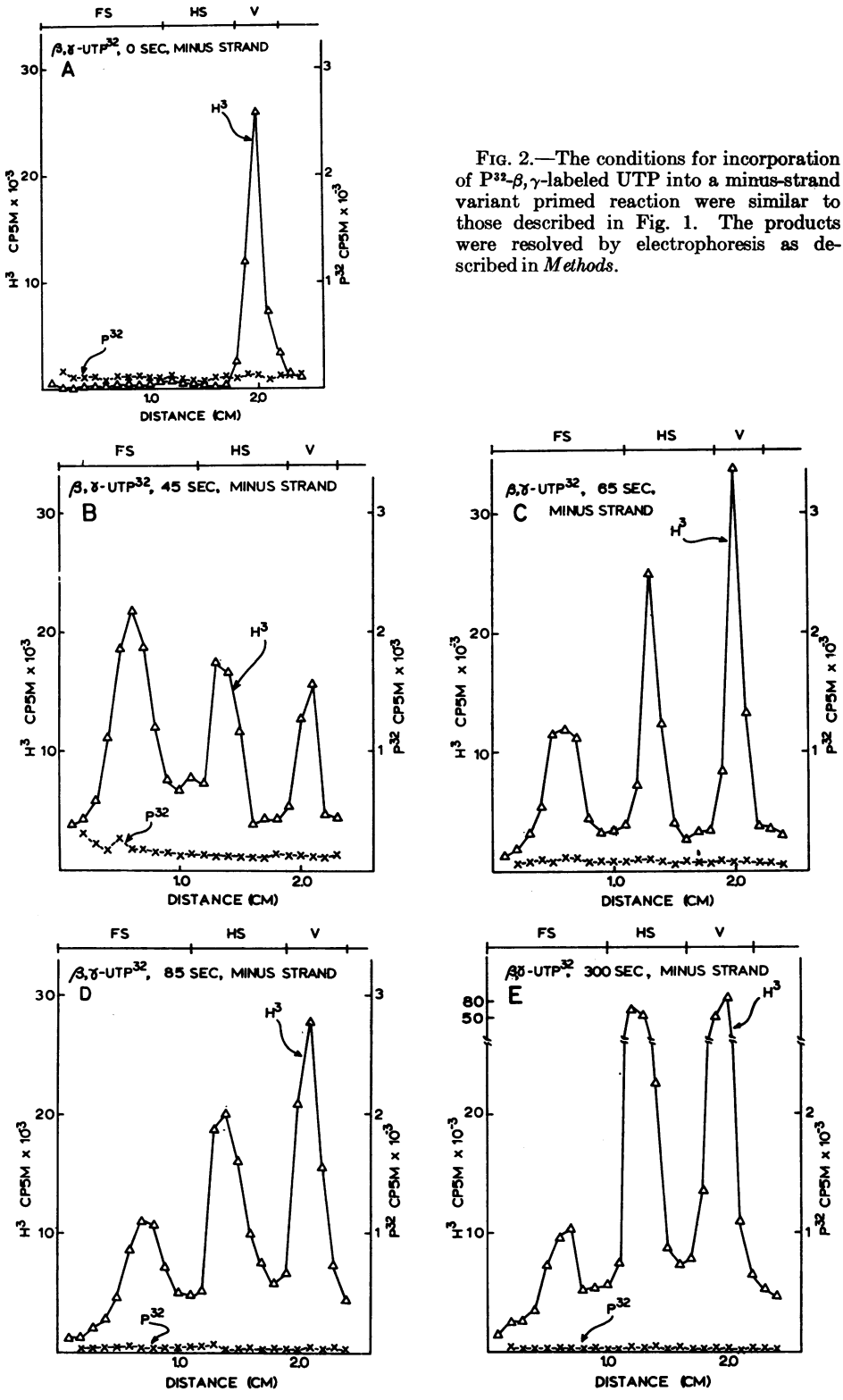


FIG. 2.—The conditions for incorporation of P^{32}-β,γ-labeled UTP into a minus-strand variant primed reaction were similar to those described in Fig. 1. The products were resolved by electrophoresis as described in *Methods*.

In a reaction initiated with a negative strand, the FS, and possibly the HS, should contain nascent positives. The absence of UTP as a 5′-terminus implies that, under these conditions, polymerization of the positive of the "little" variant proceeds in the 5′ to 3′ direction.

Discussion.—Let us consider the weight of the evidence we can place on the negative finding with respect to UTP. The variant plus strands contain about 550 nucleotides of which 110 are A residues.[17] If the direction of synthesis were 3′ to 5′, an incomplete plus strand would theoretically have any one of the four possible nucleotides as its 5′-growing-terminus. Consequently, HS structures which contain a minus template and an incomplete product plus might be expected to contain 0.25 $\mu\mu$mole P^{32}-β,γ-UTP per 110 $\mu\mu$moles of H^3-ATP (i.e., 1 to 440) for almost completed positives. For shorter nascent chains this ratio would be higher. The experimental finding (Table 1) is that the *maximum* observed ratio of P^{32} to H^3 is 1–50,000 at 45 seconds, or less than 1 per cent of that expected. The proportion of incomplete positive strands in the HS structures is uncertain. However, they would be expected to occur predominantly on the right side of the HS peak (see, e.g., Fig. 1*B*). Nevertheless, no evidence of β,γ-labeling is seen anywhere for β,γ-P^{32}-UTP (Fig. 2*B*).

For FS structures the situation is more definitive. These would be expected to have one complete minus and one or more incomplete pluses. Consequently, the ratio of P^{32}-β,γ-UTP to H^3-ATP should be greater than 0.125 $\mu\mu$mole of P^{32} to 110 $\mu\mu$moles of H^3. The observed value (Table 1) is 1–40,000.

Thus, examination of both HS and FS structures formed with minus strand templates of the variant lead to the conclusion that polymerization of the plus strand is the 5′ to 3′ direction.

The conclusion reached here can be explained if either one, or both, of the following statements are true: (*a*) the "little" variant does not employ the 3′ to 5′ polymerization for either plus or minus strands; (*b*) the enzyme always initiates a 5′ to 3′ polymerization on a *single* strand regardless of whether it is plus or minus. The relevant experiments to decide these issues are now in progress.

Summary.—Purified *minus* strands of an *abbreviated variant* of Qβ-RNA were employed as templates in the *in vitro* synthesis of RNA by purified replicase. The behavior of template and product are consistent with those observed when *plus* strands of the *parental* serve as template.[5, 6] Double-stranded complexes (HS) are the first intermediates followed by the appearance of multistranded structures (FS) which are the immediate physical precursors of single-stranded plus chains.

The absence of 5′-termini, other than GTP in nascent positives, suggests that when the enzyme is working on a *single-stranded negative* template, the direction of synthesis is 5′ to 3′.

Abbreviations: RNA, ribonucleic acid; ATP, GTP, UTP, and CTP, the 5′-triphosphates of adenosine, guanosine, uridine, and cytidine, respectively; tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate; TCA, trichloroacetic acid.

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