

AN EXTRACELLULAR DARWINIAN EXPERIMENT WITH A
SELF-DUPLICATING NUCLEIC ACID MOLECULE*

By D. R. MILLS,† R. L. PETERSON, AND S. SPIEGELMAN

DEPARTMENT OF MICROBIOLOGY, UNIVERSITY OF ILLINOIS, URBANA

Communicated May 18, 1967

The replicases (RNA-dependent RNA polymerases) induced by two unrelated^{1, 2} bacteriophages (MS-2 and Q β) have been isolated and shown to require their intact³ homologous RNA as template.^{4, 5} It was further demonstrated that the RNA molecules synthesized could serve as templates for further synthesis⁶ and were fully competent to program the synthesis of complete virus particles in protoplasts.⁷ Finally, when Q β -replicase is presented with either of two genetically distinct Q β -RNA molecules, the RNA synthesized is identical to the initiating template.⁸ This specific response of the same enzyme preparation to the particular template added proved that the RNA is the instructive agent in the replicative process and hence satisfies the operational definition of a self-duplicating entity.

An opportunity is thus provided for studying the evolution of a self-replicating nucleic acid molecule outside of a living cell. It should be noted that this situation mimics an early precellular evolutionary event, when environmental selection presumably operated directly on the genetic material. The comparative simplicity of the system and the accessibility of its known chemical components to manipulation permits the imposition of a variety of selection pressures during growth of the replicating molecules. We wish to report here one example of the experiments thus made possible.

In the universe provided to them in the test tube, the RNA molecules are liberated from many of the restrictions derived from the requirements of a complete viral life cycle. A restraint imposed is that they retain whatever sequences are involved in the recognition mechanism employed by the replicase. Thus, sequences which code for the coat proteins and replicase components *may* now be dispensable. Under these circumstances, it is of great interest to design an experiment which attempts an answer to the following question: "What will happen to the RNA molecules if the only demand made on them is the Biblical injunction, *multiply*, with the biological proviso that they do so as rapidly as possible?" The conditions required are readily attained by a serial transfer experiment⁷ in which the intervals of synthesis between transfers are adjusted to select the first molecules completed.

It is the purpose of the present paper to detail the results of this type of selection. The outcome is what might have been expected on *a priori* grounds. The smaller the polynucleotide chain, the shorter the time required for its completion. Consequently, if the initial Q β -RNA molecules possess sequences which are dispensable under the conditions of the experiment, their elimination could confer a selective advantage. In accordance with this expectation, it was found that as the experiment progressed, the multiplication rate increased and the product became smaller. By the 74th transfer, 83 per cent of the original genome had been eliminated.

Aside from their intrinsic interest, it is evident that such experiments generate molecules potentially useful for the resolution of a variety of problems. These include elucidation of the mechanism of replication and the identification of the recog-

nition device which allows the replicase to select the molecule to be replicated. Finally, these abbreviated molecules open up a novel pathway for a highly selective interference with the replication of the complete viral genome.

Materials and Methods.—(a) *Enzyme, substrates, and assays:* Synthesis of radioactive ribonucleotide triphosphates and liquid scintillation counting of labeled RNA on membrane filters have been detailed previously.⁴ RNA from a temperature-sensitive mutant of Q β (ts-1) was extracted from the virus as described previously.⁸ The first reaction in the series was initiated at a concentration of 0.2 μ g/0.25 ml of a standard⁵ reaction. The same replicase preparation purified through the CsCl and sucrose steps⁹ was used in all the steps of the transfer experiments to be described.

(b) *Sedimentation analysis of products:* Aliquots (0.01–0.10 ml) were withdrawn from various reactions and adjusted to 0.2% (by weight) with respect to sodium dodecyl sulfate (SDS). Each sample was diluted to a final volume of 0.20 ml in TE buffer (0.01 *M* Tris, pH 7.4, and 0.003 *M* EDTA), then layered on a 5-ml linear gradient of sucrose (2–20% in 0.10 *M* Tris, pH 7.4, and 0.003 *M* EDTA). These gradients were centrifuged in a Spinco SW-39 rotor at 39,000 rpm at 4°C for 5 hr. Fractions of 0.25 ml were collected dropwise, precipitated with 10% trichloroacetic acid (TCA), washed onto cellulose nitrate membrane filters, and counted in a Packard liquid scintillation counter.

(c) *Gel electrophoresis:* Unswollen ethylene diacrylate cross-linked polyacrylamide gels (3.6%) and preswollen N,N'-methylene-bis-acrylamide cross-linked polyacrylamide gels (2.4%) were prepared as described previously.¹⁰ Electrophoresis runs were made at room temperature for 90 min, at 5 ma/gel and 50 volts for gels 0.7 cm in diameter and 10 ma/gel for gels 0.9 cm in diameter and 9 cm in length.

Optical density measurements of gels were performed by scanning each gel (transferred to a quartz cell 0.5 cm in depth) with transmitted ultraviolet light in a Joyce high-resolution "chromoscan" equipped with a 266-m μ interference filter. Frozen gels were sectioned in 0.5-mm slices with the use of a carbon dioxide-cooled microtome.¹⁰ Successive pairs of 0.5-mm sections were placed in vials and eluted in TE or SSC (0.015 *M* sodium chloride and 0.015 *M* sodium citrate) buffers with gentle agitation for 12 hr at 5°C. Aliquots were removed from each elution, precipitated with cold 10% TCA, washed onto cellulose nitrate membrane filters, and counted in a Packard liquid scintillation counter.

(d) *Ribonuclease resistance assays:* Samples from each gel were adjusted to 0.15 *M* sodium chloride and 0.015 *M* sodium citrate, 20 μ g/ml pancreatic ribonuclease, and 20 μ g/ml T₁ ribonuclease. After a 2-hr incubation at 35°C, each sample was washed onto a cellulose nitrate membrane filter with cold 10% TCA, and counted in the Packard liquid scintillation counter. Heated (100°C for 1 min) and quick-cooled (in ice) samples were contained in TE buffer which was then adjusted to 0.15 *M* sodium chloride and 0.015 *M* sodium citrate for ribonuclease assay.

(e) *Synthesis of RNA and infectious units:* Samples were withdrawn and set aside for sedimentation analysis or gel electrophoresis from 0.125-ml reaction volume (or half standard replicase reaction). Samples for infectivity assays were diluted into 0.003 *M* EDTA and treated as described by Pace and Spiegelman.⁹

(f) *Base composition analysis:* In addition to the standard components,⁵ reaction solution for base composition analysis contained the four ribonucleotide triphosphates (labeled in the α -phosphorus with P³²) at a specific activity of 7.53×10^7 cpm/0.2 μ M for each triphosphate. The volume was 1.0 ml and contained 160 μ g of replicase. The reaction was initiated with 0.3 μ g of gel purified single-stranded variant RNA obtained from the 74th transfer. After incubation at 35°C for 40 min, the replicase reaction was terminated by rapid chilling to 0° and addition of SDS to a final concentration of 0.2%. The terminated reaction was dialyzed 12 hr at 5°C against 500 ml TE buffer. This dialyzed solution was then reduced in volume to about 0.1 to 0.2 ml with fine grade G-25 Sephadex and subjected to gel electrophoresis. RNA in the peak single-strand region was pooled and repurified by gel electrophoresis. The peak single-strand regions were again pooled. To remove any residue of labeled riboside triphosphate, bulk *E. coli* RNA was added to the major portion of the pool, precipitated with a solution of saturated sodium pyrophosphate, saturated sodium biphosphate, and saturated TCA (1:1:1 by volume), and washed onto a cellulose nitrate membrane filter with cold 10% TCA. The membrane was then cut into

small pieces and eluted with 0.3 *M* aqueous potassium hydroxide. Three 1-ml washes with 0.3 *M* KOH were used. These were pooled and incubated 12 hr at 35°. Chromatographic analysis of the resulting 2'-3'-nucleotides was performed on a Dowex-formate column as detailed by Hayashi and Spiegelman.¹¹

Results.—(a) *Selection during serial transfer:* An account of a transfer experiment involving 75 serial reactions is illustrated in Figure 1. The first reaction (0th) was allowed to proceed 20 minutes at 35°C, whereupon a 20 λ aliquot was used to seed the second, and so on for the first 13 reactions. The incubation periods were then reduced as detailed in the legend of Figure 1. These periodic reductions in the incubation intervals between transfers were instituted in an attempt to maintain the selection pressure for the most rapidly multiplying molecules.

Three outstanding features of Figure 1 may be noted. As may be seen from the inset, the synthesis of biologically competent RNA ceased between the fourth and fifth transfers. Second, a dramatic increase in the rate of incorporation of P³²-UTP into RNA occurred between transfers 8 and 9. Last, an apparent decrease in the rate of RNA synthesis, coinciding with the reduction in the incubation time from 15 minutes to 10 minutes, occurred after transfer 29.

The RNA products from the reactions indicated by arrows in Figure 1 were expanded by using them to initiate new replicase reactions which were continued for 40 minutes at 35°C. The resulting products were then examined in sucrose gradients. The product obtained from the reaction initiated by the 0th transfer

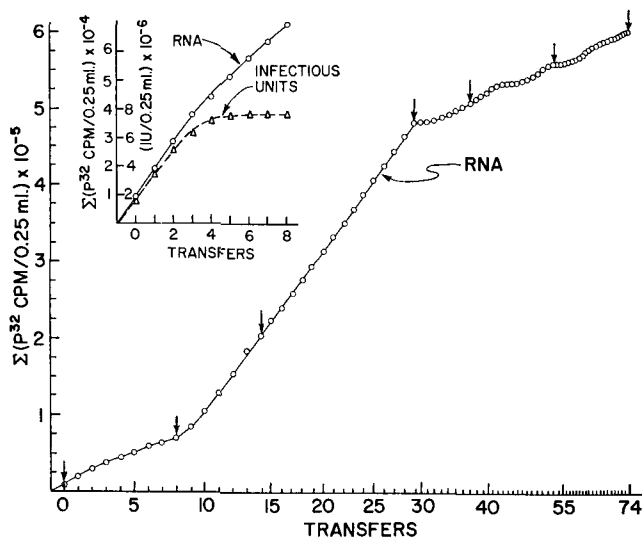
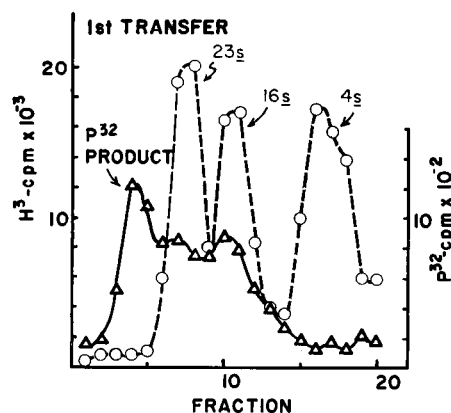
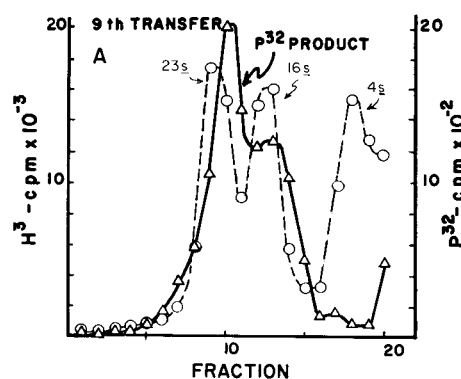


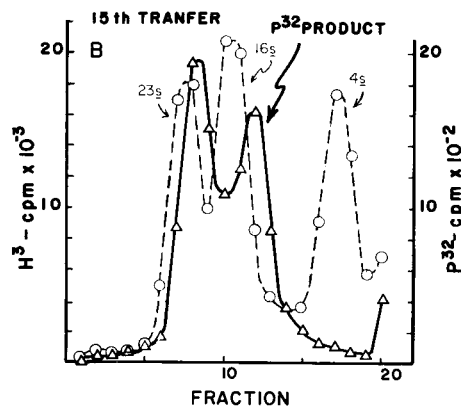
Fig. 1.—Serial transfer experiment. Each 0.25-ml standard reaction mixture⁵ contained 40 μ g of Q β replicase purified through CsCl and sucrose centrifugation, and (P³²) UTP (uridine triphosphate) at a specific activity such that 4,000 cpm corresponds to μ g of synthesized RNA. The first reaction (0 transfer) was initiated by the addition of 0.2 μ g ts-1 (temperature-sensitive RNA) and incubated at 35°C for 20 min, whereupon 0.02 ml was drawn for counting and 0.02 ml was used to prime the second reaction (1st transfer) and so on. After the first 13 reactions, the incubation periods were reduced to 15 min (transfers 14–29). Transfers 30–38 were incubated for 10 min. Transfers 39–52 were incubated for 7 min, and transfers 53–74 were incubated for 5 min. The arrows above transfers (0, 8, 14, 29, 37, 53, and 73) indicate where 0.01–0.1 of product was removed and used to prime reactions for sedimentation analysis on sucrose (see Figs. 2–5). The inset examines both infectious and total RNA. The results show that biologically competent RNA ceases to appear after the 4th transfer.



(Above) FIG. 2.—Sedimentation analysis of 1st transfer reaction. As described in *Methods*, 0.02 ml of the 0 reaction was used to initiate a reaction for a 1st transfer reaction product. After completion, this reaction was adjusted to 0.2% SDS, an aliquot was withdrawn, diluted to 0.2 ml in TE buffer (0.01 *M* Tris pH 7.4, 0.003 *M* EDTA), and then layered onto a 5-ml linear sucrose (2–20% in 0.1 *M* Tris, pH 7.4, and 0.003 *M* EDTA) and run as described in *Methods* (section *b*). H^3 -labeled bulk RNA of *E. coli* was included as internal size markers.



(Right) FIG. 3.—Sedimentation analysis of 9th transfer (*A*) and 15th transfer (*B*) reaction products. Details are as in Fig. 2.



shows (Fig. 2) the 28S peak characteristic of Q β -RNA as well as the peaks corresponding to the usual complexes observed during the *in vitro* synthesis.¹² Comparison with subsequent transfers reveals, however, dramatic changes in the nature of the replicating entity. Thus, by the ninth transfer (Fig. 3A) there is no material synthesized corresponding to the original 28S viral RNA. In its place we see a major component at about 20S and a minor one at about 15S. This pattern is essentially maintained through the 15th transfer (Fig. 3B).

By the 30th transfer (Fig. 4A) the major component has decreased to 15S and the minor one to about 14S. The product of the 38th transfer shows variant RNA which no longer splits into two peaks, a feature retained through subsequent transfers. It will be noted (Fig. 5A and B), however, that the single peak moves more slowly so that by the 74th transfer it is at about 12S.

(b) *Gel electrophoresis of variant RNA*: At this point it was decided to examine the nature of the variant in greater detail. Transfer 75 was expanded with replicase to a total of 120 μ g of RNA and subjected to analysis by polyacrylamide gel electrophoresis (Fig. 6). Clearly, the apparently homogeneous peak of Figure 5B is composed of at least two distinct RNA species. As may be seen from Figure 6, the major component is sensitive to ribonuclease whereas the minor one is resistant. It would appear that the faster component is the single-stranded variant and that the slower minor peak contains a mixture of the Hofschneider¹³ and Frank-

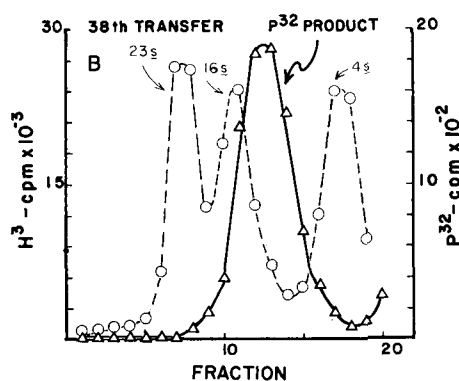
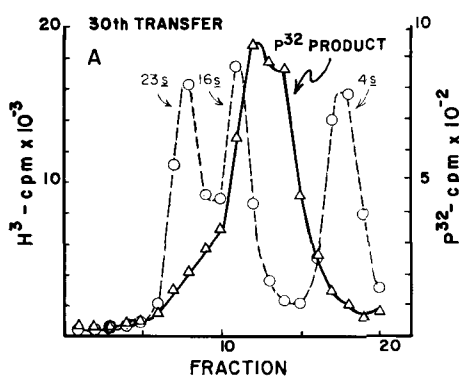


FIG. 4.—Sedimentation analysis of the 30th transfer (A) and the 38th transfer (B) reaction products. Details are as in Fig. 2.

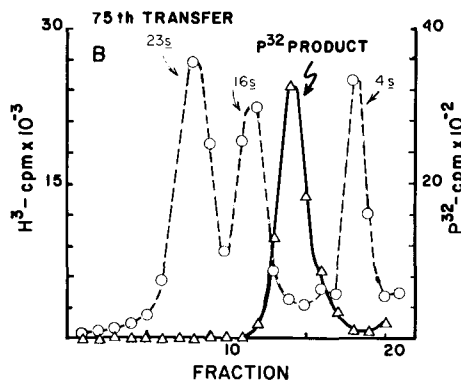
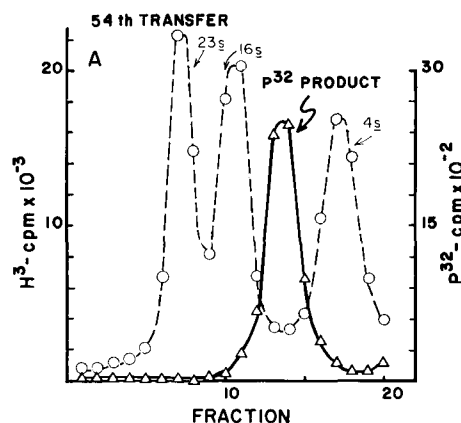


FIG. 5.—Sedimentation analysis of the 54th transfer (A) and the 75th transfer (B) reaction products. Details are as in Fig. 2.

lin¹⁴ structures observed first *in vivo* and seen in *in vitro* synthesis of Q β -RNA with purified replicase.^{12, 15}

(c) *Molecular weight of variant RNA:* We have previously shown¹⁰ that the relative electrophoretic mobility (REM) is linearly related inversely to the molecular weight of single-stranded RNA. Consequently, to determine the molecular weight, the single-stranded variant RNA was subjected to gel electrophoresis with seven internal marker RNA's of known size. The results are shown in Figure 7 and indicate that the variant RNA has a molecular weight of about 1.7×10^5 daltons.

(d) *Base composition of variant RNA:* To determine its base composition, a standard reaction mixture was initiated with the variant isolated by gel electrophoresis. In this reaction, all four ribonucleotide triphosphates were labeled with P³² at the α -position (*Methods*, section f). The RNA product of this reaction was purified twice by gel electrophoresis, hydrolyzed, and analyzed as described in *Methods* (section f). Comparison with the base composition of the original Q β -RNA (Table 1) indicates that there has been a considerable (5 mole %) increase in the G content in the variant RNA. On the other hand, A and C have decreased by 2.4 mole per cent, the uridine content remaining constant.

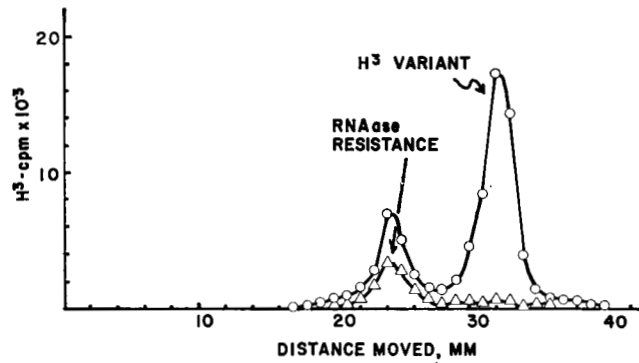


FIG. 6.—Gel electrophoresis of H^3 CTP-labeled 75th transfer reaction product. A preswollen N,N' -methylene-*bis*-acrylamide cross-linked gel (2.4%) was prepared and run as described in *Methods* (section *c*). Samples for electrophoresis were about 0.1 ml in E buffer and sucrose. One-mm sections were eluted in 0.5 ml TE buffer (0.01 *M* Tris, pH 7.4, and 0.003 *M* EDTA) for 12 hr at 4°C with gentle shaking. Aliquots for ribonuclease were withdrawn and treated as in *Methods* (section *d*). All data are represented as cpm/0.05 ml.

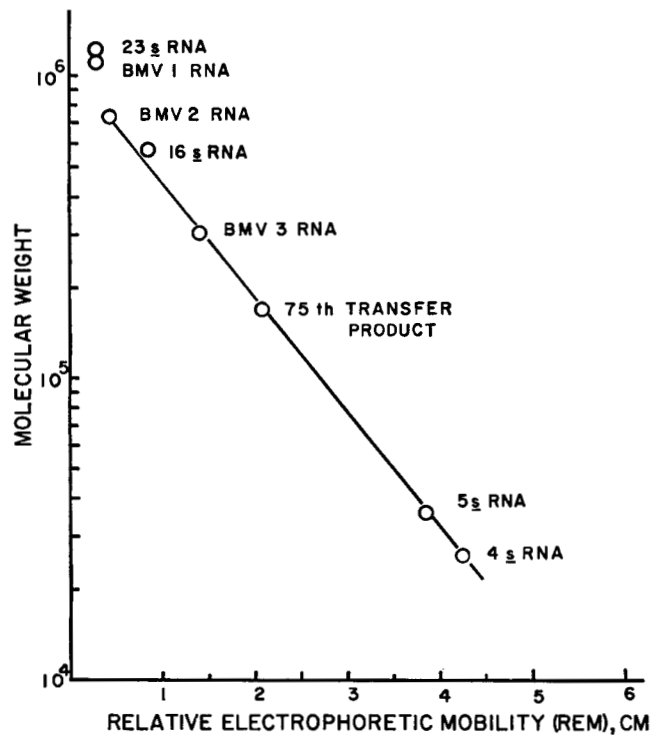


FIG. 7.—Molecular weight of the variant RNA determined by electrophoretic mobility. The relative electrophoretic mobility (REM) is plotted against molecular weight. Unswollen ethylene diacrylate cross-linked polyacrylamide gels (3.6%) as described in *Methods* (section *c*) were used in these determinations. Nucleic acid markers include: *E. coli* (H^3) bulk RNA (23S, 16S, 5S, and 4S) and brome grass mosaic virus RNA (BMV), which gives three distinct size components (BMV-1, BMV-2, BMV-3), kindly donated by Dr. Paul Kaesberg.

TABLE 1
BASE COMPOSITION OF VARIANT RNA

RNA	C	A	U	G
Variant	22.3	19.7	29.3	28.7
Q β -RNA-1	25.0	22.5	29.5	23.0
Q β -RNA-2	24.7	22.1	29.1	23.7

Variant RNA uniformly labeled with P³² was prepared, purified, and analyzed as described in *Methods* (section *f*). The resulting data are given in the first line. To monitor the quantitative adequacy of the analysis a parallel experiment was carried out with a similarly prepared and uniformly labeled 28S Q β -RNA (second line, Q β -RNA-1). The last line (Q β -RNA-2) gives for comparison the base composition of RNA isolated from virus particles.^{1, 2} Numbers represent mole per cent of the corresponding bases.

(c) *Kinetics of Q β and variant RNA*: A comparison of the kinetics of synthesis at saturation of the 75th variant and the original ts-Q β -RNA reveals (Fig. 8) some interesting differences. It will be noted that the Q β -RNA shows the usual six minutes of nonlinear synthesis which precedes the linear phase. The variant has decreased this apparent lag to 1.5 minutes. Further, the slope of the linear portion of the variant synthesis is 2.6 times that of the original Q β -RNA. Since the variant is only 17 per cent of the original size, its growth rate in terms of the production of new individuals is 15 times that of the complete viral RNA molecules.

Discussion.—The primary purpose of the present paper was to demonstrate the potentialities of the replicase system for examining the extracellular evolution of a self-replicating nucleic acid molecule. Further, the experimental situation provides its own paleology; every sample is kept frozen and can be expanded at will to yield the components occurring at that particular evolutionary stage. While only seven such samples are detailed here, they indicate that progress to a small size occurs in a series of steps. It should be noted that we have learned how to modify the enzyme reaction so that this process is greatly accelerated. This involves changing the proportions of the two components¹⁶ of the Q β replicase and will be reported subsequently.

The last product examined in the present study is a molecule which has eliminated 83 per cent of its original length and has experienced a significant change in base composition. The fact that it replicates 15 times faster than the complete viral RNA suggests that in addition to becoming smaller, the variant has increased the efficiency with which it interacts with the replicase. In any event, the findings reported establish that neither the specific recognition nor the replicating mechanism requires the complete original sequence. In this connection, it should be noted that although abbreviated, these variants are not equivalent to random fragments. The latter are unable to complete the replicative act.³

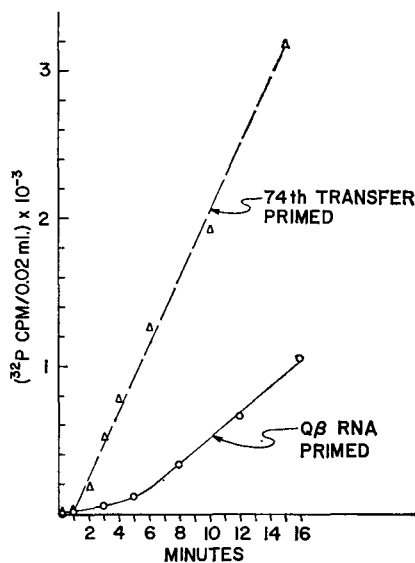


FIG. 8.—A comparison of the kinetics of synthesis of the 74th variant and the original ts-Q β -RNA. Two 0.25-ml standard reactions (as detailed in *Methods*, section *a*), one primed with gel purified single-stranded variant RNA (74th transfer) and the other primed with ts-Q β -RNA (both above saturations), were initiated at 35°C. Aliquots of 0.02 ml were drawn at times indicated and assayed for incorporation of P³²-UTP. Data are represented as cpm/0.02 ml.

The availability of a molecule which has discarded large and unnecessary segments provides an object with obvious experimental advantages for the analysis of many aspects of the replicative process. Finally, these abbreviated RNA molecules have a very high affinity for the replicase but are no longer able to direct the synthesis of virus particles. This feature opens up a novel pathway toward a highly specific device for interfering with viral replication.

It should not escape the attention of the reader that the situation described places at our disposal a completely novel method for the resolution of a variety of interesting problems. Potentially, other selective stresses can be imposed on the system to generate RNA entities which exaggerate other molecular features.

Summary.—Experiments were performed to explore the evolutionary consequences for a self-duplicating nucleic acid molecule put under selection pressure for fast growth. As the experiment progressed, the rate of RNA synthesis increased and the product became smaller. By the 74th transfer the replicating molecule had eliminated 83 per cent of its original genome, becoming the smallest known self-duplicating entity.

Aside from their intrinsic interest, such studies can provide insight into a number of central issues. Thus, they can tell us the smallest self-duplicating entity which can be constructed by such devices and provide much simpler objects for analyzing the replication process. Further, the sequences involved in the recognition mechanism between template and enzyme are enriched in the smaller molecules which evolve. Finally, these abbreviated molecules have a very high affinity for the replicase but are no longer able to direct the synthesis of virus particles. This feature opens up a novel pathway toward a highly specific device for interfering with viral RNA replication.

* This investigation was supported by USPHS research grant CA-01094 from the National Cancer Institute and grant GB-4876 from the National Science Foundation.

† Predoctoral trainee, USPHS grant F-T01-GM-319, in Microbial and Molecular Genetics.

¹ Overby, L., G. Barlow, R. Doi, M. Jacob, and S. Spiegelman, *J. Bacteriol.*, **91**, 442 (1966).

² *Ibid.*, **92**, 739 (1966).

³ Haruna, I., and S. Spiegelman, these PROCEEDINGS, **54**, 1189 (1965).

⁴ Haruna, I., K. Nozu, Y. Ohtaka, and S. Spiegelman, these PROCEEDINGS, **50**, 905 (1963).

⁵ Haruna, I., and S. Spiegelman, these PROCEEDINGS, **54**, 579 (1965).

⁶ Haruna, I., and S. Spiegelman, *Science*, **150**, 884 (1965).

⁷ Spiegelman, S., I. Haruna, I. B. Holland, G. Beaudreau, and D. Mills, these PROCEEDINGS, **54**, 919 (1965).

⁸ Pace, N. R., and S. Spiegelman, *Science*, **153**, 64 (1966).

⁹ Pace, N. R., and S. Spiegelman, these PROCEEDINGS, **55**, 1608 (1966).

¹⁰ Bishop, D. H. L., J. R. Claybrook, and S. Spiegelman, *J. Mol. Biol.*, in press (1967).

¹¹ Hayashi, M., and S. Spiegelman, these PROCEEDINGS, **47**, 1564 (1961).

¹² Mills, D., N. R. Pace, and S. Spiegelman, these PROCEEDINGS, **56**, 1778 (1966).

¹³ Francke, B., and P. H. Hofschneider, *J. Mol. Biol.*, **16**, 544 (1966).

¹⁴ Franklin, R. M., these PROCEEDINGS, **55**, 1504 (1966).

¹⁵ Bishop, D. H. L., J. R. Claybrook, N. R. Pace, and S. Spiegelman, these PROCEEDINGS, **57**, 1474 (1967).

¹⁶ Eikhom, T. S., and S. Spiegelman, these PROCEEDINGS, **57**, 1833 (1967).