In Vitro Synthesis of an Infectious Mutant RNA with a Normal RNA Replicase

Abstract. *When purified Qβ-RNA replicase is presented alternately with two genetically different Qβ-RNA molecules, the RNA synthesized is identical to the initiating template. The results establish that the RNA is the instructive agent in the replicative process and hence that it satisfies the operational definition of a self-duplicating entity. The data also eliminate alternative explanations which do not involve self-propagation of the input RNA. An opportunity is now provided for studying the genetics and evolution of a self-duplicating nucleic acid molecule under conditions permitting detailed control of environmental parameters and chemical components.*

Previous experiments with two serologically distinct (*1, 2*) RNA coliphages (MS-2 and *Qβ*) established (*3, 4*) that each induces in *Escherichia coli* a replicase (RNA replicating enzyme) which exhibits a unique requirement for intact (*5*) homologous RNA as a template. Further studies with purified *Qβ* replicase showed that the RNA synthesized is physically (*6*) and chemically (*7*) indistinguishable from the strands found in the *Qβ* virus.

The ability of the synthetic RNA to program the synthesis of complete virus particles was examined by protoplast infection in the course of a serial transfer (*8*). In these experiments, the products of a reaction initiated with *Qβ*-RNA was serially diluted to prime successive reactions until the original RNA was reduced to less than one strand per reaction mixture. The final tube contained new radioactive RNA which, when assayed in a bacterial protoplast system (*9, 10*), displayed the same ability to generate virus particles as the viral RNA used to start the reaction in the first tube. These experiments indicated that the synthesis of a self-propagating and infectious entity had been achieved in a simple system of known components.

The significance and potential usefulness of the finding encouraged further efforts at purifying the enzyme. A procedure for more extensive purification was developed (*11*) involving equilibrium banding in CsCl followed by zonal centrifugation in linear gradients of sucrose. Here, advantage was taken of expected disparities in size and density between the replicase protein and unwanted impurities. The resulting preparation was effectively free of residual virus particles, permitting direct assay for infectivity and thereby obviating the laborious purification of the RNA product required in the earlier (*8*) study. The concomitant removal of polynucleotide contaminants did not decrease, qualitatively or quantitatively, the ability of the replicase to respond to added *Qβ*-RNA by synthesizing infectious copies. This latter finding makes even more implausible arguments which would explain the increase in infectious units in terms of an "activation" of RNA preexistent in the enzyme by an unknown reaction which requires both added template and new RNA synthesis.

We now come to the central issue of the present communication which stems from the fact that two informed components are present in the reaction mixture, replicase and RNA template. None of the experiments thus far described proved that the RNA synthesized in this system is, in fact, a self-duplicating entity—that is, one which contains the requisite information and directs its own synthesis. What is required is a rigorous demonstration that the RNA, and not the replicase, is the instructive agent in the replicative process. A definitive decision would be provided by an experimental answer to the following question: if the replicase is provided alternatively with two distinguishable RNA molecules, is the product produced always identical with the initiating template?

A positive outcome would establish that the RNA is directing its own synthesis and simultaneously completely eliminate any remaining possibility of "activation" of preexisting RNA.

Our data establish that the RNA synthesized is a self-duplicating entity. The discriminating selectivity of the replicase for its own genome as template makes it impossible to employ heterologous RNA in the test experiments and recourse was, therefore, had to mutants. For ease in isolation and simplicity in distinguishing between mutant and wild type, temperature sensitive (ts) mutants were chosen. Their diagnostic phenotype is poor growth at 41°C as compared with that at 34°C. The wild type grows equally well at both temperatures.

Temperature-sensitive mutants of *Qβ* were isolated by a modification of the method described by Davern (*12*). *Escherichia coli* K-38 (*13*) was grown in a rotary shaker at 34°C in modified 3XD medium (*14*) to an optical density (660 mμ) of 0.15. *Qβ* bacteriophage was added to a multiplicity of 5, and the suspension was mixed and allowed to stand for adsorption of virus at 34°C for 5 minutes. Shaking was reinstituted for 10 minutes, whereupon 20 μg of 5-fluorouracil was added per milliliter of culture, and the incubation was continued for 2 hours. The resulting lysate was cleared by low-speed centrifugation and plated for plaques arising at 34°C. Isolated plaques were stabbed with a needle and suspended in 1 ml of water. A small loopful of the suspension was transferred to each of two plates seeded with *E. coli* K-38, and respective plates were incubated at 34° or 41°C. Plaques arising only at 34°C were picked for further testing, and those which retained the ts phenotype were chosen. Mutant virus particles isolated in this manner are quite stable to passage and

Table 1. Relative efficiency of plating at 34° and 41°C. Dilutions were plated with *E. coli* K-38 as the indicator organism, and duplicate plating series were incubated at 34° and 41°C. The relative efficiency of plating (REOP) of 100 is defined relative to the plaque forming units (PFU) observed at 34°C.

	34°C	41°C
	Virus Qβ	
REOP	100	100
PFU	1.14×10^{13}/ml	1.16×10^{13}/ml
	Virus ts Qβ	
REOP	100	2.5×10^{-2}
PFU	4.4×10^{7}/ml	1.1×10^{4}/ml

Table 2. Efficiency of infection of protoplasts by three RNA preparations. Infectious RNA assays were carried out on Qβ RNA, synthetic Qβ RNA and ts RNA. Duplicate pairs were incubated at 34° and 41°C. Efficiencies at 34° are defined as 100. The synthetic Qβ-RNA was the result of a 20-fold synthesis carried out by Qβ replicase purified through CsCl and sucrose centrifugation; 0.1 μg of Qβ RNA was used to initiate the standard reaction.

	34°C	41°C
	Natural Qβ-RNA	
REOP	100	93
PFU	4.56×10^{5}/ml	4.24×10^{5}/ml
	Synthetic Qβ-RNA	
REOP	100	92
PFU	2.90×10^{6}/ml	2.66×10^{6}/ml
	Natural ts-Qβ-RNA	
REOP	100	1.5
PFU	1.86×10^{6}/ml	2.75×10^{4}/ml

possess low efficiencies of plating at 41°C (Table 1). To provide a supply of mutant RNA, large lysates were prepared from plaque inocula of the ts-Qβ and RNA was isolated from the virus as previously described (*5*).

The ts phenotype is easily recognized by parallel platings of intact virus particles at 34° and 41°C on receptor cells (Table 1). It remained, however, to be seen whether this difference would be retained when the corresponding purified mutant RNA preparations were assayed for infectivity in the protoplast system. This check is particularly necessary since one of the steps requires a 10-minute incubation of the infected protoplasts at 35°C. During this interval, "revertants" could be produced and contribute to the background of plaques developing at 41°C. In addition, it was necessary to establish that the synthetic product of the replicase, primed by a normal $Q\beta$-RNA, behaves like the natural viral RNA in its behavior at 41°C (Table 2). It is evident that the synthetic wild type $Q\beta$-RNA behaves exactly like its natural counterpart at the two temperatures. On the other hand, the ts-$Q\beta$-RNA again shows the lower efficiency at 41°C, although it will be noted that the background at 41°C is higher than in the intact cell assay (Table 1), as expected. The 65-fold difference at the two temperatures is, however, more than adequate for a clear diagnosis.

It is evident that the system available will permit us to determine whether the product produced by a normal replicase primed with ts-$Q\beta$-RNA is mutant or wild type. As in previous investigations, this is best done by a serial transfer experiment to avoid the ambiguity of examining reactions containing significant quantities of the initiating RNA. Accordingly, seven standard reaction mixtures (0.25 ml) were prepared, each containing 60 μg of $Q\beta$ replicase isolated from cells infected with normal virus and purified through the CsCl banding sucrose sedimentation steps (*9*). To the first reaction mixture was added 0.2 μg of RNA, and synthesis was allowed to proceed at 35°C. After a suitable interval, one-tenth of this reaction mixture was used to initiate a second reaction which, in turn, was diluted into a third reaction mixture, and so on for seven transfers. A control series was carried out in a manner identical to that just described, save that no RNA was added to the first tube.

Portions from each reaction mixture

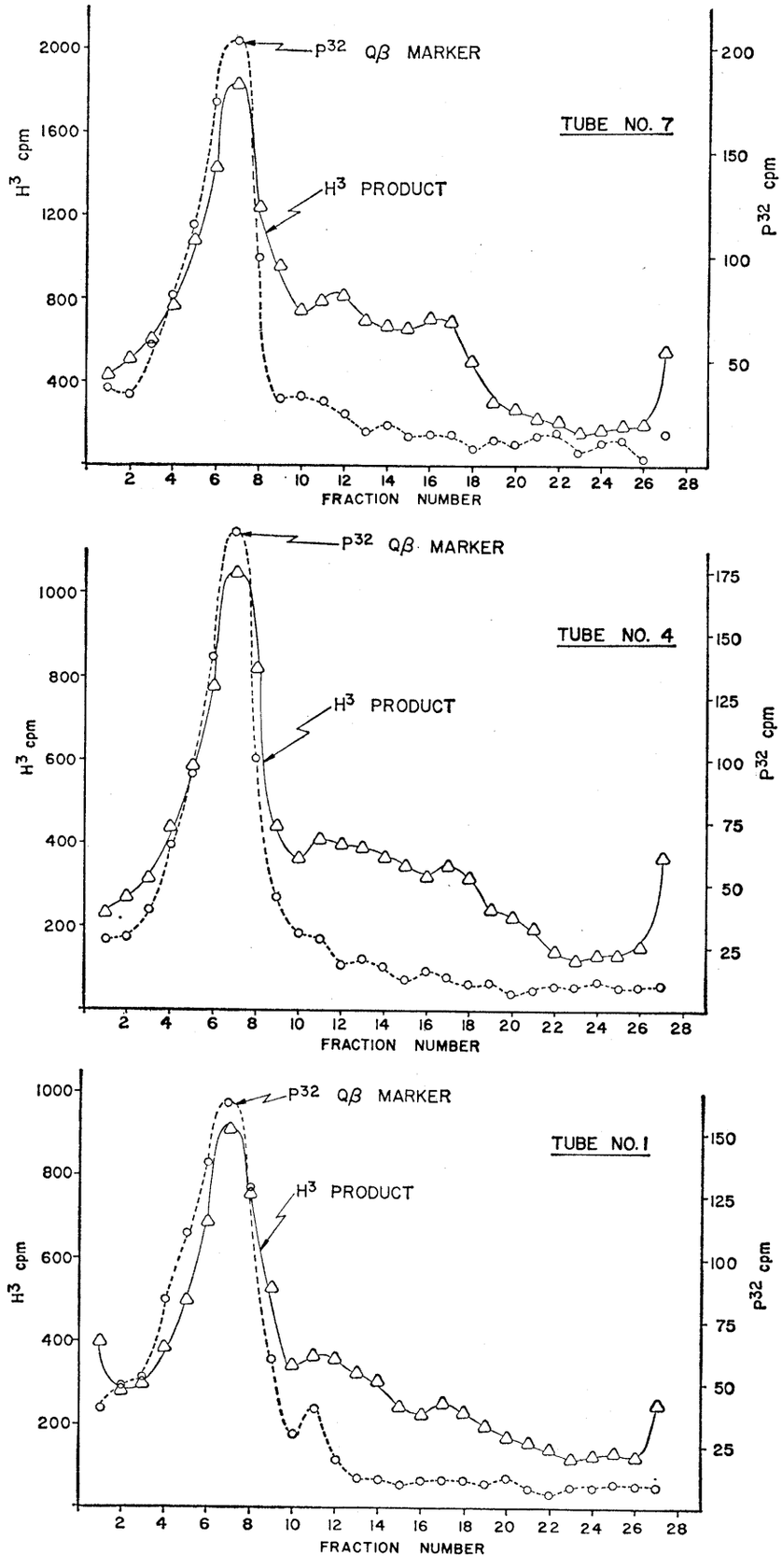

Fig. 1. Sedimentation analysis of products. A portion (0.04 ml) from reaction mixtures 1, 4, and 7 (see Table 3) were each mixed with 0.01 ml of P^{32}-$Q\beta$-RNA, 0.01 ml 20 percent sodium dodecyl sulphate, and 0.20 ml TM (tris magnesium solution), and layered onto linear gradients of 2.5 to 15 percent sucrose in a solution of 0.01M tris, *p*H 7.4; 0.005M $MgCl_2$; and 0.1M NaCl. Gradients were centrifuged at 10°C for 14 hours in the Spinco SW-25 rotor. Fractions were collected and analyzed for radioactivity (count/min) as described previously (*6*).

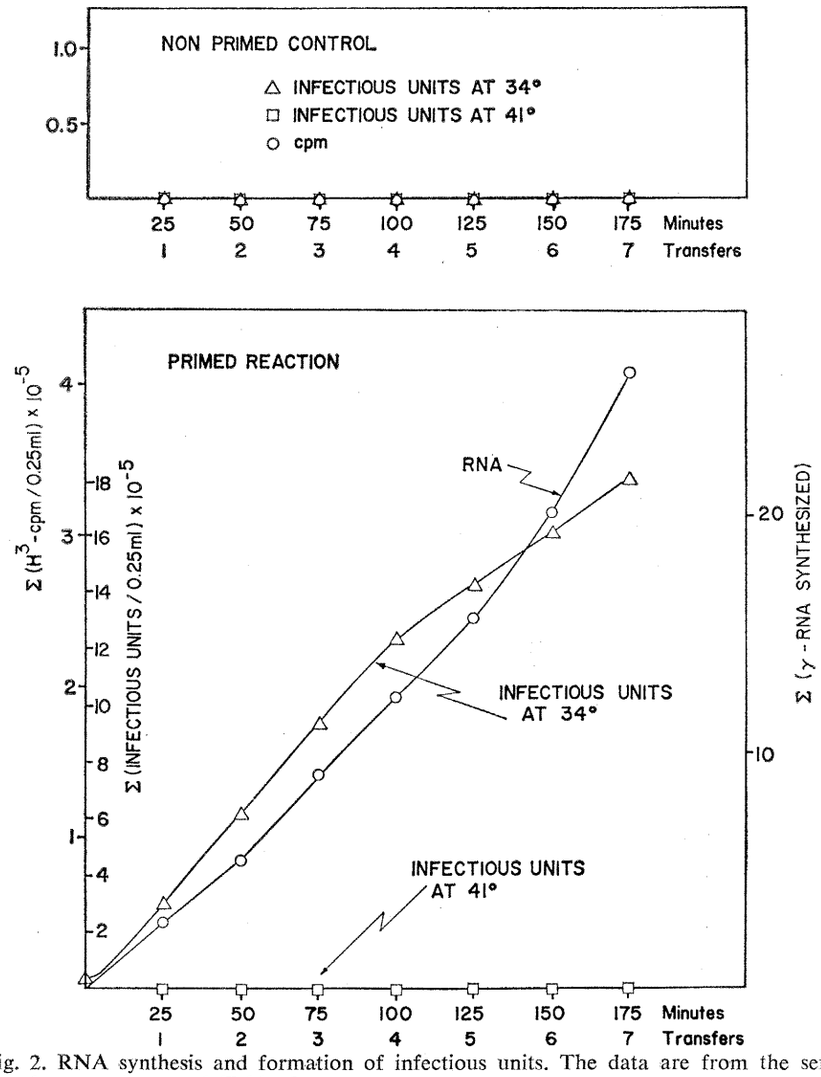

Fig. 2. RNA synthesis and formation of infectious units. The data are from the serial transfer experiment of Table 3.

were examined for radioactivity in material precipitable by trichloroacetic acid (TCA) and assayed for infectious RNA at 34° and 41°C. In addition, samples from reactions 1, 4, and 7 were examined for physical similarity to the input RNA by sedimentation through sucrose gradients. As shown in Fig. 1, the bulk of material synthesized is similar in sedimentation characteristics to ts-$Q\beta$-RNA derived from virus particles.

Table 3 is a record of such a serial transfer experiment. If we first focus attention of the RNA formation in the experimental series (columns 3 to 6), it is evident that ts-$Q\beta$-RNA serves as an excellent initiator for the normal replicase. Included also is the radioactivity (count/min) observed in the nonprimed control series (columns 7 and 8). No detectable synthesis occurs in the first three tubes although a few counts accumulate near the end which are, however, negligible from the point of view of the chemical amounts of RNA synthesized. Though quantitatively insignificant, this "long-term background" is persistently observed with some enzyme preparations.

Columns 9 and 10 of Table 3 give the actual number of plaques counted in the assay for infectious units at each transfer, the numbers representing the average of two duplicate plates. Comparisons of columns 9 and 10 reveal that the relative number of plaque formers at the two temperatures agree with those obtained with the original ts-$Q\beta$-

Table 3. *Transfer Experiment with ts-Qβ-RNA.* Each 0.25 ml of standard reaction mixture (*4*) contained 60 μg of $Q\beta$ replicase purified through CsCl and sucrose centrifugation, and H^3-CTP (cytidine triphosphate) at a specific activity such that 15,600 count/min signifies 1 μg of synthesized RNA. The first reaction was initiated by addition of 0.2 μg of temperature sensitive infectious RNA. Each reaction was carried out at 35°C for 25 minutes, whereupon 0.02 ml was drawn for counting, and 0.025 ml was used to prime the next reaction. All samples were stored frozen at −70°C until infectivity assays were carried out. Dilutions for infectious RNA assays were made into a solution of 0.01*M* tris, *p*H 7.4, and 0.005*M* $MgCl_2$, and used immediately. Columns 1 and 2 give the reaction number and total time elapsed during the experiment. Column 3 lists acid-precipitable radioactivity (count/min) found in each 0.25 ml reaction mixture and column 4 lists the corresponding sum. Similarly, columns 5 and 6 list the RNA formation during each reaction and their cumulative amounts. Columns 7 and 8 present radioactivity incorporated in the control transfer without added RNA. Columns 9 and 10 are the averages of plaques observed on duplicate plates in the assays for infectious RNA, on plates incubated at 34° and 41°C. In all cases, reaction products were diluted 1.6×10^{-3} during the course of the assay. Column 11 presents the actual number of infectious units appearing in each reaction tube, and column 12 is the sum of infectious units appearing at 34°C.

Transfer No.	Time (Min)	Formation of RNA: With RNA (count/min × 10^{-5}) Radioactivity		Formation of RNA: With RNA Amt RNA		Formation of RNA: Without RNA Radioactivity (count/min × 10^{-5})		Formation of infectious units: With RNA PFU observed		Formation of infectious units: With RNA Infectious units × 10^{-5} at 34°C	
		Each	Sum	Each (μg)	Sum (μg)	Each	Sum	34°C	41°C	Each	Sum
1	2	3	4	5	6	7	8	9	10	11	12
1	25	0.446	0.446	2.86	2.86	0	0	487	9	3.04	3.04
2	50	.418	.864	2.68	5.54	0	0	486	10	3.04	6.08
3	75	.560	1.424	3.59	9.13	0	0	500	12	3.12	9.20
4	100	.508	1.932	3.26	12.39	0.002	0.002	464	4	2.90	12.10
5	125	.527	2.459	3.38	15.77	.012	.014	299	6	1.87	13.97
6	150	.685	3.149	4.39	20.16	.0007	.014	295	5	1.85	15.82
7	175	.927	4.071	5.94	26.10	.004	.018	289	2	1.81	17.63

RNA (Table 2) in the protoplast assay. The proportions of plaques seen at 41°C (Table 3, column 10) is not significantly different from the expected 1 to 2 percent of the numbers developing at 34°C. Thus the ts phenotype of the initiating ts-$Q\beta$ is faithfully inherited. Column 11 gives the number of ts-infectious units per reaction mixture calculated from the dilution used; column 12 lists the corresponding cumulative sums. No evidence of the synthesis of infectious RNA which could produce plaques at either 34° or 41° C appeared in the control nonprimed reaction. The corresponding negative columns are therefore omitted from Table 3.

The average infective efficiency of the RNA in the protoplast assay is 2×10^{-7}. The initial input in tube 1 was 0.2 μg corresponding to 1.2×10^{11} strands and 2.4×10^{4} plaque forming units. Since each transfer involves a 1 to 10 dilution, it is clear that less than one of the 1.87×10^{5} plaque formers observed in the 5th tube can be ascribed to the initiating ts-$Q\beta$-RNA. Finally, by tube 7 which contains 3.6×10^{12} new strands, the number of plaque formers (1.8×10^{5}) exceeds in absolute terms the number (1.2×10^{4}) of old strands present. It is clear that the serial dilution experiment has demonstrated the appearance of newly synthesized infectious RNA possessing the temperature-sensitive phenotype.

In the lower portion of Fig. 2 the outcome of the experiment shown in Table 3 is summarized by plotting against time the cumulative sums of the RNA synthesized (column 6) and plaque formers at 34°C (column 12). The fact that the plaque formers at 41°C are not statistically above the background of the assay of ts-$Q\beta$-RNA means that no detectable wild type $Q\beta$-RNA has been produced, a fact indicated by the open squares. For comparison the control reaction of Table 3, in which the initiating RNA was omitted, is similarly plotted on the same scale in the upper part of Fig. 2. No significant synthesis of either RNA or infectious units were observed.

It is apparent from the experiments described that one and the same normal replicase can produce distinguishably different but genetically related RNA molecules. The genetic type produced is completely determined by the RNA used to start the reaction and is always identical to it. The following two conclusions would appear to be inescapable from these findings; (i) the RNA is the instructive agent in the replicating process and therefore satisfies the operational definition of a self-duplicating entity; (ii) it is not some cryptic contaminant of the enzyme but rather the input RNA which multiplies.

The experiments described generate an opportunity for studying the genetics and evolution of a self-replicating nucleic acid molecule in a simple and chemically controllable medium. Of particular interest is the fact that such studies can be carried out under conditions in which the only demand made on the molecules is that they multiply; they can be liberated from all secondary requirements (for example, coding for coat protein, and so forth) which serve only the needs and purposes of the complete organism.

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Copper and the Role of Isopods in Degradation of Organic Matter

Abstract. *On an artificial substrate of filter paper,* Porcellio scaber *cannot extract copper from leaf litter. If one increases the copper content of the food by soaking the leaves in solutions of $CuSO_4$ or in organic extracts, assimilation of copper becomes possible, but only at concentrations higher than 1 microgram of copper per milligram of ash. This is too high a level for primary vegetable matter to be considered a plausible source of copper for isopods. I present evidence that in fecal material the critical level at which assimilation of copper becomes feasible is lower than in primary organic material by nearly an order of magnitude, and that isopods are obliged to switch to coprophagy in order to allow accumulation of copper in their bodies.*

Terrestrial isopods constitute an important link in the feeding chain that connects plant material with the humus of fertile soils. So far they have been considered to be primary consumers, breaking down the original plant material and thus preparing it for attack by other types of consumers such as mites, collemboles, protozoans, and microorganisms (*1*). In keeping with this picture it has been held that isopods consume large amounts of litter and other plant material, but cause only small alteration in the chemical composition of their food.

However, these animals have very considerable quantities of calcium in their integument (*2*) and of copper in their hepatopancreas (*3*). They lose calcium during each molt and copper when they feed on what has been considered to be their staple diet (*4*); thus they must compensate the loss of the two elements. Calcium can be extracted from the food rather efficiently (*2*); moreover, these animals often reingest their exuvia after molting. I now deal with copper.

I investigated several populations of *Porcellio scaber* L. from the outskirts of Vienna, Austria. Their standard food came from a large and thoroughly mixed batch of leaf litter from *Populus nigra*, collected in spring (after outdoor wintering) and stored at room temperature; its copper content was 0.13 to 0.32 ($X = 0.24$) μg Cu per milligram of ash. Before feeding experiments the leaves were dried in a desiccator for 24 hours, weighed, soaked in various extracts (to be specified), dried, weighed again, and then offered to single animals, each in a small petri dish with a disk of moist filter paper on the bottom.

Each experiment, lasting 5 days, was performed at 20°C in natural light.